MECHANISMS OF SHEAR STRESS-INDUCED ATHEROSCLEROTIC PLAQUE REGRESSION

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DEDICATION

I dedicate this work to my Nonno Silvio (Grandfather). He sadly passed away during the writing of my thesis. He was always my #1 fan and was so excited that I would one day be a “Dottore” (Doctor). His love, support and comical nature helped me through my doctoral studies. He will be greatly missed but never forgotten.

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LIST OF ABBREVIATIONS

ABCA1	ATP binding cassette subfamily A member 1
AP1	activator protein 1
APC	antigen presenting cell
ApoA1	apolipoprotein A-I
ApoB	apolipoprotein B
ApoE	apolipoprotein E
ASK1	apoptosis signal-regulating kinase 1
AVF	arteriovenous fistula
BCA	brachiocephalic artery
CCL5	chemokine (C-C motif) ligand 5
CCL7	chemokine (C-C motif) ligand 7
CTRL	control
CVD	cardiovascular disease
CXCL12	C-X-C motif chemokine 12
DC	dendritic cell
DKO	double knockout
ECM	extracellular matrix
eNOS	endothelial nitric oxide synthase
EC	endothelial cell
ER	endoplasmic reticulum
ERK5	extracellular-signal-regulated kinase 5
ET-1	endothelin-1
FACS	fluorescence-activated cell sorting

FAK	focal adhesion kinase
GFP	green fluorescent protein
HDL	high-density lipoprotein
HFD	high fat diet
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HUVEC	human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule 1
IFN	interferon
IL	interleukin
ip	intraperitoneal
IVUS	intravascular ultrasound
JNK	c-Jun N-terminal kinase
KLF2	krüppel-like factor 2
KO	knockout
LDLR	low density lipoprotein receptor
LFA-1	lymphocyte function-associated antigen 1
L-NAME	L-N^G-Nitroarginine methyl ester
MCP-1	monocyte chemotactic protein 1
M-CSF	macrophage colony-stimulating factor
MEF2	myocyte enhancer factor-2
MEK5	MAP extracellular signal-regulated kinase 5
MERTK	MER Proto-Oncogene, Tyrosine Kinase
MFGE8	milk fat globule-EGF factor 8 protein
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging

MTTP	microsomal triglyceride transfer protein
MX1	MX dynamin-like GTPase 1
NADPH	nicotinamide adenine dinucleotide phosphate
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitrogen oxide
NRF2	nuclear factor-like 2
oxLDL	oxidatively modified low-density lipoprotein
p38	mitogen-activated protein kinases
PBS	phosphate buffered saline
PECAM-1	platelet/endothelial cell adhesion molecule 1
PET	positron emission tomography
PGI2	prostaglandin I2
PS	phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand-1
RCCA	right common carotid artery
SSRE	shear stress response element
SRA	scavenger receptor A
TBP	TATA-binding protein
TG	transgenic
TGFβ	transforming growth factor β
Th1	T helper cell type 1
TIMP	tissue inhibitors of metalloproteinases
TNF	tumor necrosis factor
tPA	tissue plasminogen activator
VCAM-1	vascular cell adhesion molecule 1

VSMC	vascular smooth muscle cells
WK	week
WT	wild type

ABSTRACT (ENGLISH)

Background

Differences in shear stress form the basis of the differences in atherosclerosis predilection. Plaques form in regions of low, oscillating flow, whereas vessels exposed to high, steady shear stress tend to remain lesion-free due to the release of protective factors from the endothelial layer, primarily nitric oxide. While progressing plaques are characterized by the persistent entry of immune cells in the vascular wall, regressing plaques are characterized by 2 key features, effective emigration and efferocytosis of these immune cells. We hypothesize that shear stress reverses atherosclerosis by promoting macrophage exit from plaque (emigration) and more efficient uptake of cellular debris (efferocytosis).

Methods

In vivo: The arteriovenous fistula (AVF) is a surgical model established in the laboratory, whereby the right carotid artery is anastomosed into the right jugular vein, creating an arterio-venous shunt. We studied atherosclerotic plaques in the brachiocephalic artery (BCA), the plaque-prone vessel that links the aorta with the right carotid artery. Low density lipoprotein receptor (LDLR) knockout (KO) mice were placed on a high-fat diet. Sham and AVF surgery was performed at week 12 and mice were kept on a high-fat diet for a further 4 weeks (week 16). Control mice were sacrificed at week 12.

In vitro: Endothelial cells (ECs) were co-cultured with macrophages in a shear stress cone system wherein ECs were exposed to high or low shear stress and macrophages were exposed to the EC effluent.

Results

The AVF procedure increases the shear stress in the BCA but does not alter serum lipid levels. Using this AVF model in mice, we found that elevated shear stress can reverse the atherosclerotic process, reducing plaque size. This increased shear stress also leads to decreased necrotic core size, increased matrix metalloproteinase (MMP) activity, increased MMP-9 expression and decreased collagen IV in addition to increased macrophage migration. MMP or endothelial nitric oxide synthase inhibition abolished shear stress-induced plaque regression. MMP inhibition also decreases macrophage migration with specific MMP-9 inhibition having the same effect. Using the *in vitro* shear stress co-culture apparatus to mimic the plaque environment, we found that uptake of apoptotic cells by macrophages was higher in the high shear conditions with MMP inhibition having no effect. However, a cytokine array on the co-culture effluent revealed a potential role for specific cytokines.

Conclusions

Our findings suggest that not only is shear stress protective against plaque development but it can effectively reverse the atherosclerotic process without interfering with plasma lipid levels. Shear stress acting on ECs may influence the cells within the plaque by increasing macrophage mobility and efferocytosis, the combination of which likely leads to plaque regression. These results and future work will help us further identify the molecular mechanisms that cause the atheroprotective effects of shear stress, in hopes of identifying new therapeutic targets for the treatment of atherosclerosis and cardiovascular diseases.

ABSTRACT (FRENCH)

Contexte

Les variations des contraintes de cisaillement sont à l'origine des modulations dans l'athérosclérose. La plaque d'athérome se forme dans des régions de faible flux oscillatoire, tandis que dans les régions vasculaires exposées à un flux sanguin élevé et constant aucune lésion ne se développe. Ceci est due à la libération de facteurs de protection par la couche endothéliale, principalement l'oxyde nitrique. Tandis que les plaques qui progressent se caractérisent par l'entrée constante de cellules immunitaires dans la paroi vasculaire; les plaques qui régressent sont caractérisées par 2 phénomènes, l'émigration et l'efferocytose de ces cellules immunitaires. Nous émettons l'hypothèse que les contraintes de cisaillement diminuent l'athérosclérose en favorisant la sortie des macrophages de la plaque (émigration) et l'élimination plus efficace des débris cellulaires (efferocytose).

Méthodes

In vivo: La fistule artérioveineuse (FAV) est un modèle chirurgical établi dans le laboratoire, où l'artère carotide droite est anastomosée avec la veine jugulaire droite, créant un pont artério-veineux. Nous avons étudié les plaques d'athérosclérose dans l'artère brachiocéphalique (TABC), reliant l'aorte à l'artère carotide droite, qui est une région où les plaques ont tendance à se former. Des souris où le récepteur au LDL est invalidé sont soumises à un régime hypercholestérolémiant. Des chirurgies sham et FAV sont effectuées à 12 semaines de régime et les souris sont conservées sous régime 4 semaines supplémentaires (16 semaines). Les souris contrôles sont sacrifiées à semaine 12.

In vitro: Les cellules endothéliales (CEs) sont mises en culture avec les macrophages dans un système ou les CEs sont exposées à un flux élevé ou faible et les macrophages exposés au surnageant des CEs.

Résultats

La chirurgie FAV augmente les contraintes de cisaillement dans le TABC, sans modifier les taux de lipides plasmatiques. En utilisant le modèle FAV chez la souris, nous avons constaté que le processus d'athérosclérose peut être inversé dans les plaques préétablies lorsqu'elles sont exposées à des contraintes de cisaillement élevées, menant à la réduction de la taille des plaques. Ce processus est associé à une diminution de la taille du noyau nécrotique, à l'augmentation de l'activité des metalloproteinases de la matrice (MMP) et de l'expression de MMP-9 et à la diminution du collagène IV, et de l'augmentation de la migration des macrophages. L'inhibition des MMPs ou de la nitrique oxide synthase endothéliale abolissent la régression des plaques induites par des contraintes de cisaillement élevées. L'inhibition des MMPs ainsi que de MMP-9, plus spécifiquement, diminuent la migration des macrophages. En utilisant le système de co-culture *in vitro*, simulant l'environnement de la plaque, nous avons constaté que l'élimination des cellules apoptotiques par les macrophages est augmentée par des contraintes de cisaillement élevées independamment des MMPs. Cependant, le dosage des cytokines dans le surnageant de co-culture des macrophages et CE a révélé un rôle potentiel de certaines cytokines.

Conclusions

Nos résultats suggèrent que les contraintes de cisaillement sont non seulement protectrices dans le développement de la plaque, mais peuvent également reverser le processus

d'athérosclérose sans affecter les niveaux de lipides plasmatiques. Les contraintes de cisaillement agissant sur les CE peuvent réguler la fonction des cellules de la plaque en augmentant la mobilité des macrophages et l'efferocytose, conduisant probablement à la régression de la plaque. Ces résultats et les travaux futurs vont nous permettre d'identifier d'autres mécanismes moléculaires impliqués dans les effets athéroprotecteurs des contraintes de cisaillement, dans l'espoir d'identifier de nouvelles cibles thérapeutiques pour le traitement de l'athérosclérose et des maladies cardiovasculaires.

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I am grateful, first and foremost, to my supervisor, Dr. Stephanie Lehoux, for giving me the opportunity to join her team, for her expertise and advice during my PhD research and for her editorial help with my thesis. There was never a dull moment in the lab and we always had fun performing unique experiments.

Any great research is done best with a great team. I am very grateful to Dr. Talin Ebrahimian, the Research Associate of the laboratory, for all her guidance and help with my project and experiments. Also, for all the stimulating debates regarding methods and results and allowing me to vent my frustrations during my graduate program which made it all the more fun! She became more of a friend and mentor than a colleague and without her I would not have survived this journey. Thank you to Daniel Rivas and David Simon for excellent technical assistance. Thank you to the animal quarters for looking after the precious mice and specifically the animal technician of the Lady Davis Institute, Veronique Michaud, for her excellent surgical skills. A big thank you to all students from the Dr. Lehoux lab, past and present; and other students and supervisors from the Lady Davis Institute, especially on the 1st and 2nd floor and the Segal Center, for all their helpful input and discussion.

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A special and heartfelt thank you to my family and friends for all their encouragement throughout my PhD studies and the writing of my thesis. Especially to my parents, Giuseppe and Silvana Simeone, who always placed great importance on the value of education and the advancement of knowledge and to my fiancé, Anthony Masella, for his love and support. Without them I would not have successfully completed this degree and I owe them tremendous gratitude!

PREFACE

Contribution of Authors & Originality of Project

I am the author of this thesis and I am the primary scientist of the research projects presented in this thesis. I was involved in all aspects of the project and I have primarily performed all the research experiments and analysis except for certain parts mentioned below.

Dr. Talin Ebrahimian, the Research Associate of the laboratory, assisted me with some of the *in vitro* efferocytosis work and performed the irradiation/transplantation blood FACS.

Veronique Michaud, the animal technician, performed the AVF surgeries and echogradiography. All analysis and all other mouse *in vivo* work was done by me.

Dr. Yves Castier in Paris created the AVF model and performed the preliminary experiments in these mice as well as the L-NAME and GFP experiments.

Daniel Rivas and David Simon provided technical assistance.

Dr. Stephanie Lehoux provided mentorship, guidance and editorial help.

Experimental design was performed by Dr. Stephanie Lehoux, Dr. Yves Castier, Dr. Talin Ebrahimian and I.

All research elements of the thesis are considered original scholarship and distinct contributions to knowledge. The following are our new contributions to the field and we are the first to study these effects:

- Novel surgical mouse model of increased shear stress.

- Increased shear stress and its effect on plaque regression and plaque composition in the absence of lipid level changes *in vivo*.
- Effect of inhibition of eNOS and MMPs in shear stress-induced plaque regression *in vivo*.
- Effect of shear stress, with or without MMP inhibition, on macrophage migration and efferocytosis *in vitro*.
- Preliminary work on the specific inflammatory changes due to shear stress *in vivo* and *in vitro.*

This dissertation was written in accordance with the guidelines for thesis preparation from the faculty of graduate studies and research at McGill University. It is written as a manuscript-based thesis.

PhD Abstracts, Conference Presentations & Awards

I attended multiple conferences and presented many poster and oral presentations. Below are the ones in which awards were received.

- Arteriosclerosis, Thrombosis and Vascular Biology (ATVB) Scientific Sessions
    - Poster: **ATVB Travel Award for Young Investigators,** Chicago, April 2011
    - Poster: **Experimental Medicine Dr. Gerald B. Price Memorial Travel Award and Great Travel Award,** Chicago, April 2012
    - Poster: **Lady Davis Institute Travel Award,** Orlando, May 2013
- American Heart Association Scientific Sessions
    - Poster**: Best of Specialty Conferences Session,** Orlando, November 2011
    - Poster**: Experimental Medicine Dr. Gerald B. Price Memorial Travel Award and Great Travel Award,** Orlando, November 2011
- Canadian Cardiovascular Congress
    - Oral: **Finalist for the Trainee Research Award - Basic Science Session,** Montreal, October 2013

    - Oral: **Canadian Society of Arteriosclerosis, Thrombosis and Vascular Biology (CSATVB) Travel Award,** Montreal, October 2013
    - Oral: **CSATVB Young Investigator Award,** Montreal, October 2013
    - Oral: **Finalist for the Trainee Research Award - Basic Science Session,** Vancouver, October 2014
    - Oral: **CSATVB Young Investigator Award,** Vancouver, October 2014
    - Oral: **Experimental Medicine Dr. Gerald B. Price Memorial Travel Award and Great Travel Award,** Montreal, October 2015
    - Oral: **Finalist for the Trainee Research Award - Basic Science Session,** Montreal, October 2015
    - Oral: **CSATVB Young Investigator Award,** Montreal, October 2015
- Canadian Institutes of Health Research (CIHR) Canadian Student Health Research Forum (CSHRF) Competition
    - Poster: **Silver Award for poster,** Winnipeg, June 2011
    - Poster: **Lady Davis Institute Travel Award,** Winnipeg, June 2011
    - Poster: **CIHR CSHRF Travel Award,** Winnipeg, June 2011
- International Symposium on Biomechanics in Vascular Biology and Cardiovascular Disease
    - Oral: **2nd place oral presentation prize,** Montreal, April 2014
- La Société Québécoise d'Hypertension Artérielle Réunion scientifique annuelle
    - Oral: **1st place oral presentation prize,** Quebec City, January 2014 (presentation in French)
- McGill Cardiovascular Research Day
    - Oral: **3rd place oral presentation,** Montreal, May 2012
    - Poster: **3rd place poster presentation,** Montreal, May 2014
    - Poster: **1st place poster presentation,** Montreal, May 2015
- Lady Davis Institute for Medical Research Scientific Retreat
    - Oral: **2nd place oral presentation**, Montreal, May 2012
    - Poster: **1st place poster presentation,** Montreal, May 2013
    - Oral: **Best of elevator talk oral presentation**, Montreal, May 2014
- McGill Biomedical Graduate Conference
    - Poster: **1st place poster prize,** Montreal, February 2012

- **McGill Invited Speaker:** Atherosclerosis seminar, McGill Medical Grand Rounds for Undergraduate Students, January 2013

PhD Papers

- **Simeone S**, Ebrahimian T, Michaud V, Lehoux S. Shear Stress-Induced Atherosclerotic Plaque Regression is Reversed by Regulation of Macrophage Mobility via Matrix Matalloproteinase Inhibition. ***Manuscript submitted to Circ Research.***

- Castier Y, **Simeone S**, Ebrahimian T, Michaud V, Tedgui A, Lehoux S. Shear Stress-Induced Atherosclerotic Plaque Regression in the Absence of Lipid Modulation. ***Manuscript submitted to PNAS.***
- Ebrahimian T, Simon D, Lemarié CA, **Simeone S**, Heidari M, Mann KK, Wassmann S, Lehoux S. Absence of Four-and-a-Half LIM Domain Protein 2 Decreases Atherosclerosis in ApoE-/- Mice. ***Arteriosclerosis, Thrombosis, and Vascular Biology.*** 2015 May; 35(5):1190-7.
- Ebrahimian T, Arfa O**, Simeone SM**, Lehoux S, Wassmann S. Inhibition of Four-and-a-Half LIM domain protein-2 increases survival, migratory capacity and paracrine function of human early outgrowth endothelial progenitor cells through activation of the sphingosine kinase-1 pathway: Implications for endothelial regeneration. ***Circulation Research.*** 2014 Jan 3; 114 (1): 114-23.
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- Ebrahimian T, Li MW, Lemarie CA, **Simeone SM**, Pagano PJ, Gaestel M, Paradis P, Wassmann S, Schiffrin EL. MAP Kinase-activated protein kinase 2 in angiotensin II-induced inflammation and hypertension: regulation of oxidative stress. ***Hypertension.*** 2011 Feb; 57(2):245-54.
- **MSc paper**
  Simeone SM, Li MW, Paradis P, Schiffrin EL. Vascular gene expression in mice overexpressing human endothelin-1 targeted to the endothelium. ***Physiological Genomics***. 2011 Feb 11; 43(3):148-60.
- **MSc collaboration paper**
  Lemarie CA, **Simeone SM**, Nikonova A, Ebrahimian T, Deschenes ME, Coffman TM, Paradis P, Schiffrin EL. Aldosterone-Induced Activation of Signaling Pathways Requires Activity of Angiotensin Type 1a Receptors. ***Circulation Research.*** 2009 Oct 23; 105(9):852-9.

PhD Scholarships

- Experimental Medicine Graduate Excellence Fellowship, March 2012
- Fonds de recherche du Quebec – Santé (FRQS) Doctoral Award ($20,000 for 3 years), April 2011 – April 2014
- Experimental Medicine Principal's Fellowship, Aug 2010

INTRODUCTION

Rationale and Objectives of the Research

As blood flows in the vascular tree, it generates a frictional force on ECs called shear stress. Shear stress is beneficial, favouring EC survival and the release of anti-inflammatory and anti-thrombotic factors from the endothelium. The levels of shear stress vary greatly according to arterial geometry. They tend to be greatest in straight arterial segments and lowest at branch points and bifurcations. These differences in shear stress form the basis of the differences in atherosclerosis predilection. Plaques form in regions of low, oscillating flow, whereas vessels exposed to high shear stress tend to remain lesion-free. Pro-inflammatory stimuli and entry of immune cells underlie the development and continuous growth of the plaque.

Much is known about how plaques progress however, less is known about the more clinically relevant, regression of a plaque. Most studies looking at plaque regression involve altering lipid levels. The rationale for the project was to answer the following main question: Does increased shear stress, besides being atheroprotective, also have the ability to cause an already established plaque to reduce in size and if so by what mechanisms? This is the first study looking at the role of shear stress in plaque regression and one of the first studies looking at regression in the absence of lipid changes. We used an original model of mouse AVF to investigate the effects of increased blood flow on established plaques. We hypothesized that high shear stress would reverse atherosclerosis by promoting more effective egress and efferocytosis which are required for plaque regression.

Objective 1) To characterize and compare the plaques from sham and fistulated arteries (created by an AVF surgical procedure which connects the carotid artery to the jugular vein). LDLR KO mice were placed on a high-fat diet for 12 weeks, then divided in three groups: control, sham, and AVF. Controls (CTRL) were sacrificed at 12 weeks, and all other animals were maintained on high fat for 4 weeks post-surgery. Body weight, blood pressure, and plasma cholesterol were measured in all groups. Plaque size was evaluated in the BCA after fixing vessels at physiological pressure. We have shown that carotid artery blood flow is increased more than six-fold in AVF compared with shams. Plaque size and composition was evaluated. The specific role of endothelial nitric oxide synthase (eNOS) in transducing the protective effects of shear stress was evaluated by treating a separate set of sham and AVF mice with a non-hypertensive dose of the eNOS inhibitor, L-NAME, in the 12-16 week interval. In addition, the specific role of MMPs was also evaluated by inhibition using doxycycline and tissue inhibitors of metalloproteinases (TIMPs).

Objective 2) To investigate how ECs exposed to shear stress affect macrophages. Murine EC were obtained by magnetic bead selection from the lung and macrophages harvested from the bone marrow. ECs were exposed to protective shear stress (15 dynes/cm^2) or low flow (6 dynes/cm^2) during 24 hours in a cone plate apparatus, and the macrophages exposed to the EC effluent concurrently. Macrophage cell migration was quantified by scratch assay. Phagocytosis of cells by macrophages was assessed by immunofluorescent efferocytosis assay.

Cardiovascular diseases are by far the most prevalent cause of death in developed countries. Heart attacks and strokes, both major causes of death, often result from

atherosclerosis. Treatment with lipid-lowering statins provides an exceptional health benefit; however, many patients do not respond adequately to these drugs[14].

The knowledge from this thesis has the potential to identify the molecular mechanisms that cause the atheroprotective effects of shear stress, in hopes of identifying new therapeutic targets for the treatment of atherosclerosis and cardiovascular diseases. This outcome will definitely impact the health of people with heart disease and stroke by bringing us one step closer to the elimination of these ailments.

CHAPTER I: REVIEW OF THE LITERATURE

1.1 The Vasculature

1.1.1 Cardiovascular Disease

As the global population ages and grows in number, we have seen a rise in deaths attributable to cardiovascular diseases (CVDs) including cerebrovascular disease (strokes). About one third of all deaths in 2013 were due to CVDs[1]. This translates to about 17.3 million deaths, up from 12.3 million in 1990 (World Health Organization Global Atlas on cardiovascular disease prevention and control). According to the American Heart Association, 1 out of every 2.8 deaths in America in 2004 was due to CVD[2]. About 2300 Americans die of CVD each day which accounts for 1 death each 38 seconds[3]. About 151,000 Americans who died of CVD were under the age of 65[3]. Moreover, more than 5 million individuals are diagnosed every year as having coronary disease[4]. It is therefore the leading cause of death worldwide even exceeding all cancers combined. Surprisingly, CVD is no longer a disease seen only in developed countries but is now also a growing problem in developing countries. CVD places a huge burden on the healthcare system not only medically but also economically. Ten percent of the global disease burden is due to CVDs (World Health Organization Global Atlas on cardiovascular disease prevention and control).

There are many risk factors for CVD some of which are not controllable (age, gender and genetics) and others which we can modify (smoking, high cholesterol, hypertension, sedentary lifestyle, obesity and diabetes). Almost three quarters of all deaths from CVD involve heart attacks and strokes and this is caused mainly by atherosclerosis[5]. Atherosclerosis is the main contributor of CVDs and is the main topic of this thesis. Understanding this disease will have a huge impact on our healthcare system and the lives of many individuals.

1.1.2 The Arteries

"You are as old as your blood vessel" is a telling statement since our blood vessels reveal many clues about our health.

Atherosclerotic plaques form in the large arteries which are composed of multiple layers as seen in Figure 1.2. The adventitia is the outermost layer and is composed of connective tissue and cells such as fibroblasts and some inflammatory cells. It also contains some vessels known as the vasa vasorum. The media, composed of vascular smooth muscle cells (VSMCs), is the middle layer. The VSMCs constrict and dilate to produce tone based on signals from the ECs. VSMCs also synthesize extracellular matrix (ECM) and cytokines which affects their proliferation and migration.

The endothelium is 1cell layer thick that lines the vessels and is part of the innermost layer known as the intima. It is these cells that are in contact with the flowing blood and sense mechanical and hormonal stimuli and release factors that regulate angiogenesis, inflammation, thrombogenesis, permeability as well as vascular tone[6]. The total volume of these cells in the body is similar to that of the liver and is one of the largest organs of the body[6]. ECs are semi-permeable and non-thrombogenic and play a role in vascular tone and regulate VSMC growth. Within the layers of the vessel are the ECM proteins, the elastic lamina and the basal lamina giving the vessel structure and stability.

The aortic arch is a common location for plaque growth and study. It is composed of the aortic sinus which is found just above the aortic valve in the heart and is part of the ascending aorta. The 3 leafed valve can be seen in the aortic sinus. The first branch off of the aortic arch is

the BCA where plaques tend to form due to the geometrical structure, which will be discussed later. This artery divides into the right subclavian artery and the right common carotid artery.

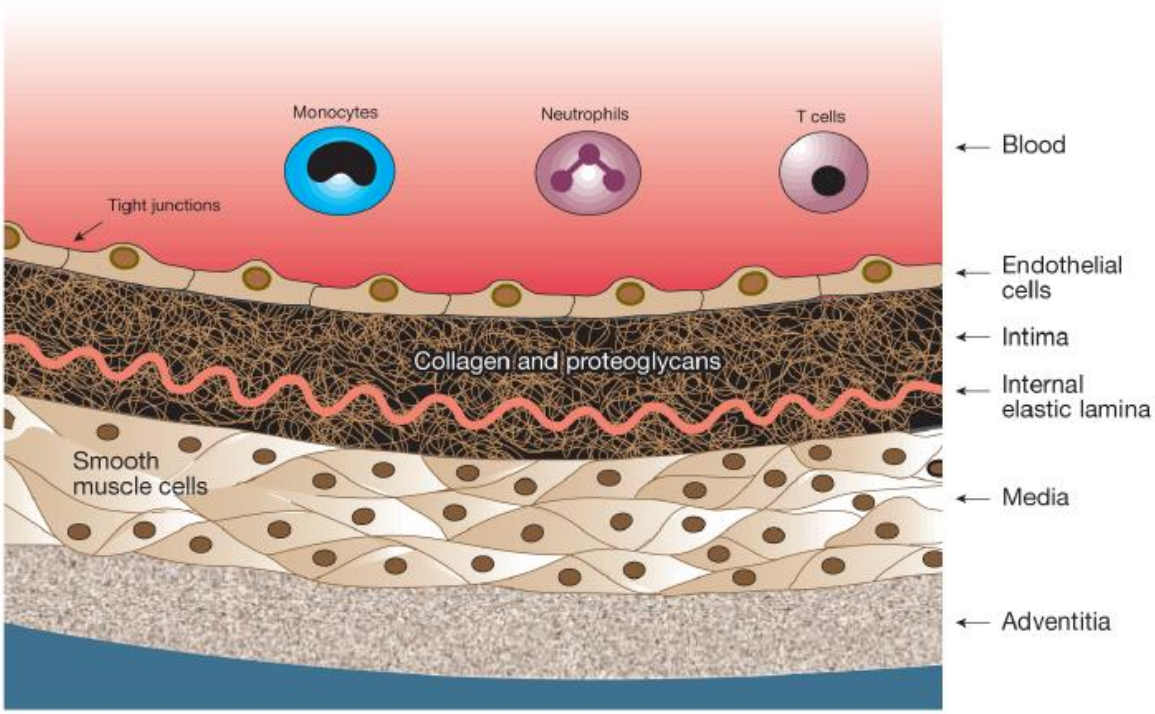

Figure 1.1: The layers of the artery[7]. An artery is composed of the outermost adventitia layer, the middle media layer of smooth muscle cells and the innermost intima where the endothelial cells and extracellular matrix is found. Healthy endothelial cells have tight junctions with very little space between cells. Blood flows in the vessel lumen as the immune cells look for areas of damage.

1.1.3 Atherosclerosis

Atherosclerosis is defined by a fatty cholesterol deposit in the arterial wall between the endothelium and VSMCs. This causes lesions also known as plaques and narrowing of the arterial lumen which causes a decrease in blood flow. This decreased flow leads to less blood supply to the heart, brain and other tissues known as ischemia. The rupture of these plaques leads to the devastating cardiovascular issues such as heart attacks and strokes. Plaques have been shown to form as early as 15 years of age and progresses throughout life[8].

For many years, it was believed that atherosclerosis was simply due to increased cholesterol in the blood leading to increased lipid accumulation in the artery wall[9]. However, new ideas about other causes of the disease began to emerge. In the 1980s, leukocytes were discovered in atherosclerotic arteries exposed to years of damaging effects from common CVD risk factors[10]. This damage is recognized by the body as a wound which signals immune cells to migrate to that site. The initial goal of these cells is to clear the damage. However, due to the continuous presence of risk factors, there is a continued recruitment and accumulation of inflammatory cells and the lesions continue to grow [11]. This led to atherosclerosis being defined as a maladaptive chronic inflammatory disease[9]. It is now well accepted that the immune system, both innate and adaptive, plays a major role in all phases of plaque formation from initiation to progression to possible rupture[12]. All cells of the immune system have been found both within the plaques as well as the different layers of the vessels, including T cells, B cells, dendritic cells (DCs) and macrophages (Figure 1.2). Atherosclerosis is a progressive disease consisting of many stages with specific cellular composition and morphology[12] which will be further discussed.

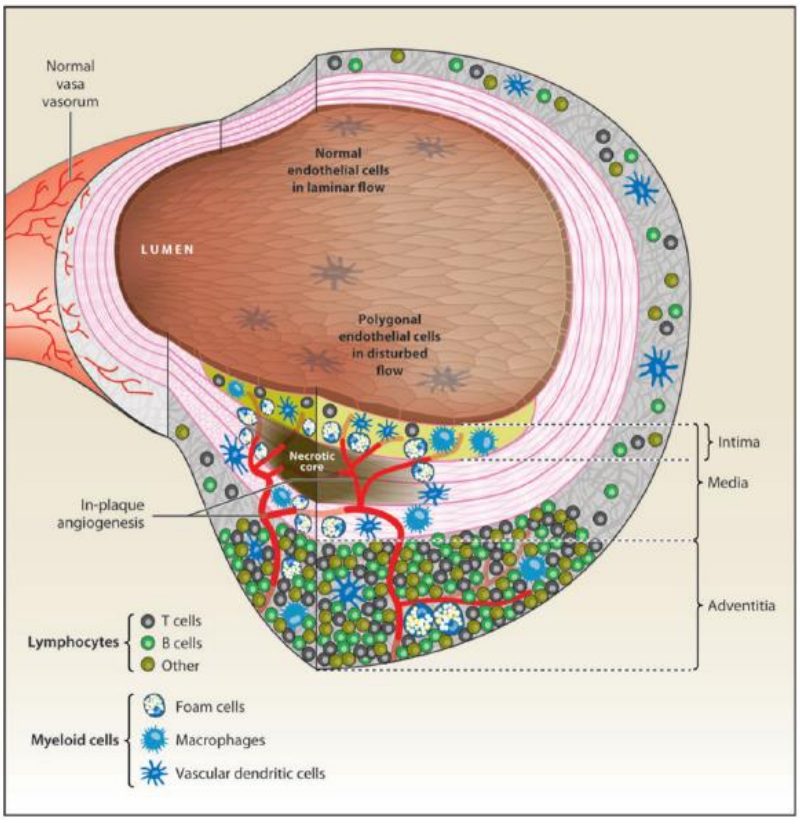

Figure 1.2: Atherosclerosis is an inflammatory disease[13]. Both lymphocytes and myeloid cells can be found in all 3 layers of the vessel specifically in plaque prone regions.

Cell	Progenitor	Function
Monocyte	Myeloid	migrate from circulation to plaque in response to damage
Ly6C hi monocyte	Myeloid	pro-inflammatory monocyte
Ly6C low monocyte	Myeloid	anti-inflammatory monocyte, immune surveillance and wound healing
Macrophage	Myeloid	differentiate from monocytes in lesions, become foam cells, phagocytosis, most abundant cell in the plaque
M1 macrophage	Myeloid	classical macrophage, pro-inflammatory, plaque vulnerability, plaque progression
M2 macrophage	Myeloid	alternative macrophage, anti-inflammatory, wound healing, plaque stability, plaque regression
Dendritic cell	Myeloid	differentiate from monocytes in lesion, antigen presenting cells, migrate to lymphatic vessels
T cell	Lymphoid	invades the vascular wall in response to antigens
T helper cell	Lymphoid	CD4 cell, release cytokines to attract other helpers of the immune response to the plaque
Th1 cell	Lymphoid	pro-inflammatory T helper cell
Th2 cell	Lymphoid	anti-inflammatory T helper cell
Regulatory T cell	Lymphoid	CD25 cell, anti-inflammatory Th2 cell, athero-protective
Cytotoxic T cell	Lymphoid	CD8 cell, release proteins that kill infected cells
B cell	Lymphoid	antibody producing cells, role in atherosclerosis remains controversial

Table 1.1: Immune cells in atherosclerosis and their function

1.1.3.1 Detection and Treatment

Atherosclerosis is detected by echocardiography, computed tomography (CT) scan, magnetic resonance imaging (MRI), positron emission tomography (PET) and most commonly, angiography and intravascular ultrasound (IVUS). Angiography uses a contrast agent which can be visualized by X-Ray in order to locate locations of vessel narrowing. IVUS uses a catheter with a probe and is visualized by ultrasound in order to view the inside of the vessel and measure plaque size. Detection is not always accurate since some small occlusions, which still allow blood to flow through, may in fact be vulnerable depending on the plaque composition which will be further discussed. Another issue in plaque detection is that atherosclerosis is mostly asymptomatic until it is too late, when the plaque has reached a large and advanced state leading to cardiovascular events and death. Allowing the plaque to reduce in size and regress back to an early state without any risk of re-growth would be the most ideal treatment.

The current treatment for atherosclerosis includes angioplasty and statins. Angioplasty widens the vessel via a balloon or stent inserted into the vessel. Statins are HMG-CoA reductase inhibitors and act to lower cholesterol by targeting its metabolic pathway. There is currently no atherosclerotic treatment which specifically targets the inflammatory response or other cellular targets. Interestingly, statin treatment has been shown to have anti-inflammatory and ant-thrombotic effects beyond its lipid lowering effects. Cholesterol is present in the cell membrane and many immune signaling molecules are found in lipid rafts in the membrane, therefore disrupting lipid rafts will alter inflammation[14]. Simvastatin (a statin) reduces the inflammatory effects of low shear stress[15]. Statins also have others effects, such as increasing nitric oxide (NO) availability, increasing migration and inhibiting MMPs[15, 16]. Furthermore, stents coated with

anti-proliferative and immunosuppresive agents are effective in reducing plaque re-growth[12]. These treatments are limited by the fact that many patients do not respond to statin treatment (only 30% do) and re-growth of the plaque is often observed with stents[11]. In addition, statins reduce plaque size, however, only by 20-40% leaving much of the disease untreated[17]. Therefore, a more effective treatment is needed for atherosclerosis and targeting inflammatory cells may be ideal.

1.1.3.2 Mouse models of atherosclerosis

In order to study atherosclerosis, different mouse models were created to simulate plaques found in humans. Atherosclerosis does not occur naturally in mice due to low levels of detrimental LDL and high protective high density lipoprotein (HDL) levels in the liver of mice compared to humans. Therefore, a genetic KO is required for plaque formation and is further exacerbated when fed a high fat diet. The LDLR KO mouse is defined by the absence of the LDLR causing an increase in free LDL cholesterol leading to lesion formation[18]. This receptor allows for the endocytosis of LDL into liver tissue by recognizing apolipoprotein B (apoB) which is found on the outer layer of LDL particles. The apolipoprotein E (apoE) KO mouse lacks this protein found on lipoprotein particles essential for the breakdown of triglyceride-rich lipoproteins. These mice therefore have elevated plasma lipid levels causing plaques[18]. These KO animals have tremendously increased the amount of knowledge regarding atherosclerosis.

1.2 Monocytes and Macrophages

Monocytes and macrophages are very important cells of the body since they play a key role in the immune system. Monocytes are produced in the bone marrow from hematopoetic stem cells, the cells that gives rise to all blood cells. More specifically, monocytes are derived from the myeloid progenitor cells. Monoyctes circulate in the blood and in response to inflammatory signals due to wounds or infection, they migrate quickly to the tissue site of injury or infection where they then differentiate into either DCs or macrophages[19]. Macrophages are one of the main phagocytic cells of the body and exist in nearly all tissues. They engulf foreign particles and debris, making them part of the innate immune system. Despite DCs being the main antigen presenting cells (APCs), macrophages can also secrete cytokines and act as APCs. They present foreign particles to the T cells and so they are also involved in activating the adaptive arm of the immune system [11, 12]. Macrophages are the most abundant cell found in the plaque and they play a role in plaque progression. In fact, depletion of macrophages using clodronate in rabbits reduces atherosclerotic development[20]. In addition, less monocyte recruitment due to monocyte chemoattractant protein-1 (MCP-1) KO also leads to reduced atherosclerosis[21].

Although monocytes and macrophages play a role in plaque progression, some studies depleting these cells did not in fact cause reduced plaque size[22]. Our understanding of the role of monocytes/macrophages in atherosclerosis has increased dramatically over the years. It was recently discovered that monocytes/macrophages form a heterogeneous population. This heterogeneity allows for macrophages to be either beneficial or detrimental depending on the environment. They consist of 2 subtypes which are defined by their membrane bound protein surface markers and therefore distinguishable by flow cytometry and have diverse functions[10]. In

mice, these subtypes are defined as Ly-6C^{hi} (Gr1^{+}) monocytes (known in humans as CD14^{hi}CD16^{-}) and Ly-6C^{lo} (GR1^{-}) monocytes (known in humans as CD14^{+}CD16^{-})[10]. However, the specific role that these monocytes play in atherosclerosis is not well understood. These monocytes can differentiate into 2 major macrophage subtypes, with others still being discovered, depending on the environment.

1.2.1 The Classical Macrophage

The classically activated macrophage is known as M1and it tends to differentiate from mice monocytes that have high levels of the surface marker, Ly6C known as the Ly6C^{hi} monocyte, in addition to the chemokine receptor, CCR2 and the adhesion molecule, L-selectin on their surface[19].

M1 macrophages have the typical pro-inflammatory function. They are involved in phagocytosis, bacterial clearance and antigen presentation and because of these functions they are ultimately involved in tissue destruction[23]. These macrophages produce high levels of the pro-inflammatory interleukin cytokine, IL-12, low levels of the anti-inflammatory interleukin, IL-10 and they also produce toxic reactive oxygen species (ROS) hence the cytotoxic effect of these cells[23]. These cells are linked to the helper T cell subset known as Th1, hence the M1 nomenclature[24]. Th1 cells like M1 cells produce pro-inflammatory responses. The presence of interferon γ (IFNγ) skews macrophages towards an M1 profile[25].

Much research has shown that this group of monocytes/macrophages are the ones linked to inflammation. Bacterial stimulated inflammation shows an increase in the number of Ly6C^{hi}

monocytes at the site of inflammation[26]. Furthermore, infection with *Listeria monocytogenes* and *Leishmania major* induces an increase in these cells[26]. Using an adoptive transfer technique, when mice Ly6C^{hi} monocytes are injected into recipient mice with inflamed peritoneum, these cells quickly migrate and invade the site of inflammation[27].

Swirski *et al.,* in 2007, explored the presence of Ly-6C^{hi} monocytes in mice with atherosclerotic plaques and hypothesized that the presence of plaques alters the class of monocytes[28]. It was found that apoE KO mice on a high fat diet had a 4 fold increase in total circulating monocytes when compared to apoE KO mice on a regular diet[28]. Furthermore, when dividing the population into the Ly-6C^{hi} and the Ly-6C^{lo} population, the high fat fed apoE KO mice had a 14 fold increase in Ly-6C^{hi} monocytes whereas the Ly-6C^{lo} subset showed no change in any of the groups[28]. Studies using apoE KO mice which also lack CCR2, which is associated with Ly-6C^{hi} monocytes, show decreased lesion formation without having an effect on plasma lipid levels[29]. It is now understood that these Ly6C^{hi} monocytes enter into atherosclerotic plaques and differentiate into the M1-type classical macrophage which mediates inflammation and phagocytosis[10]. These studies clearly indicate that atherosclerotic plaques have increased Ly6C^{hi} monocytes in their circulation which enter the plaque and become M1 macrophages causing plaque growth.

Interestingly, studies of myocardial infarction and obesity also show subtype preference for M1 and both are highly linked with atherosclerosis. When myocardial infarctions are induced in apoE KO mice, the healing of the heart tissue post heart attack is diminished perhaps due to an increased presence of Ly6C^{hi} and reduced Ly6C^{lo} monocytes[30]. In obese mice, macrophages

express many of the M1 macrophage type genes with decreased levels of M2 macrophage related genes; meanwhile, lean mice had the opposite results[31].

1.2.2 The Alternate Macrophage

The alternately activated macrophage, also known as the M2 macrophage, tends to differentiate from the Ly6C^{lo} monocyte[10, 32]. These monocytes also have high expression of the CX3CR1 chemokine receptor and LFA-1 integrin[10, 32]. M2 cells form a very interesting population and are distinct both in structure and in function compared to the M1 macrophages.

M2 macrophages are involved in wound repair and tissue remodeling[10]. These macrophages are also pro-angiogenic and immunosuppressive[33]. They produce high amounts of anti-inflammatory IL-10 and low amounts of inflammatory IL-12[23]. In addition, they can also be characterized by their secretion of arginase. These cells are linked to the T helper subtype known as a Th2 immune response which is anti-inflammatory[24]. The presence of IL-4 induces the differentiation of M2 macrophages[25].

The group of Auffrey *et al.* examined the trafficking of the Ly6C^{lo} cells *in vivo* and in real time by confocal microscopy in either steady state or inflammatory conditions[32]. Using mice with fluorescent Ly6C^{lo} monocytes, they discovered that these cells follow a crawling path along the vasculature in all directions independent of blood flow and remain close to the EC layer as if surveilling it[32]. They then proceeded to expose the tissues to irritants and discovered that these cells enter the inflamed tissue compared to the tissue exposed to PBS which had no cell entry[32]. Ly6C^{lo} monocytes have been shown to promote healing in mice with myocardial infarction by

inducing myofibroblast accumulation, angiogenesis and collagen accumulation[30]. Therefore, Ly6C^{lo} cells patrol the vessels looking for signs of damage where they then enter the tissue and differentiate into M2-type macrophages, a remarkable new function for monocytes/macrophages.

Interestingly, the Ly6C^{lo} population express the DC association marker, CD11c [34]. It was shown that DCs in the plaque are mobile and emigrate to lymph nodes and this cell emigration is a characteristic of regressive plaques as discussed later[35]. Perhaps Ly6C^{lo} cells entering the plaque, differentiating into DCs and emigrating out of the plaque is characteristic of plaque regression. Moreover, apoE KO mice deficient in certain components of the CD40 signaling axis have a smaller plaque size due to reduced blood Ly6C^{hi} monocytes, less recruitment of these cells to the arterial wall and a polarization towards an M2 profile[36]. In addition, IL-4 and IL-13, which polarizes macrophages to M2, slow down plaque progression while their absence leads to accelerated atherosclerosis[37]. Therefore, it seems that Ly6C^{lo} monocytes and M2 macrophages are linked to smaller plaques and perhaps are playing a protective role in resolving the inflammatory state of the plaque and healing the damage.

1.3 Steps of Atherosclerosis

1.3.1 Atherosclerotic Progression

1.3.1.1 Endothelial Cell Dysfunction and Adhesion

Atherosclerotic lesions begin with the dysfunction of the endothelium due to damage or injury from CVD risk factors such as LDL accumulation, free radicals from cigarettes, high

blood pressure and genetics causing the ECs to change their normal behavior[9]. It is thought that the main culprit in initiating atherosclerosis is an accumulation of apolipoprotein B (apoB) containing lipoproteins which get converted to LDL[38]. The endothelium becomes more permeable and becomes activated from the injury. ECs then begin to express adhesion factors such as selectins, VCAM-1 and ICAM-1 making the vessel more adherent[12, 39]. These adhesion molecules bind integrins on inflammatory cells. This facilitates the entry of monocytes and T cells from the circulation into the intima. Cells roll over the endothelium and tether via selectins followed by adhesion via chemokines and their receptors followed by integrin dependent adhesion and finally transmigration know as diapedesis through the endothelial layer[39].

Selectins are carbohydrate ligands. P-selectin is stored in Weibel-Palade bodies of ECs which are released to the plasma membrane once the endothelium is activated[39]. P-selectin binds PSGL-1 found on immune cells. LDL induces P-selectin release and is found on sites with plaques and absent in normal vessels[39]. The absence of P-selectin leads to less macrophages migrating into the plaque and consequently reduced plaque size[39]. Integrins are a family of 24 cell surface receptors composed of α and β units forming heterodimers mediating cell-cell and cell-ECM binding and therefore are involved in leukocyte adhesion and migration[39]. Specifically, β2 integrin mediates adhesion and is known as a pro-atherogenic integrin. Transplantation of β2 overexpressing cells in mice led to larger plaques[40]. VCAM-1 and ICAM-1 are immunoglobulin domains found on ECs and bind the integrins. VCAM-1/LDLR double knockout (DKO) mice showed 40% reduced plaque size[41]. Migration into the tissue is also aided by chemoattractants such as MCP-1[11]. Blocking chemokines or their receptors in mice inhibits monocyte recruitment and slows down atherosclerosis[38].

1.3.1.2 Fatty Streak Formation

The next step in the atherosclerotic process is fatty streak formation which is considered as early lesions. These lesions begin as early as infancy and are composed primarily of macrophages and T cells[9]. The increased LDL is able to diffuse from the blood into the intima where it becomes modified by oxygen radicals to form oxidized LDL (oxLDL)[11]. In addition, macrophages can also secrete trapping proteoglycans which keeps these atherogenic particles within the lesion allowing no inflammatory resolution[38]. In the lesion, monocytes that entered will differentiate into macrophages which will uptake oxLDL and become foam cells full of lipids[12]. Macrophages uptake the oxLDL via scavenger receptors such as CD36 and SRA. Mice lacking these receptors show smaller plaques[42]. The oxLDL is converted to free cholesterol which then goes to the endoplasmic reticulum (ER) and leads to the foamy appearance of the macrophages[43]. These macrophages will then secrete pro-inflammatory cytokines as well as proteases such as MMPs, pro-coagulants and pro-apoptotic factors[11]. Macrophages and especially DCs, will also present antigens such as oxLDL to T cells causing them to become activated to a mostly Th1 response producing pro-inflammatory cytokines such as IL-1, IFN-γ and tumor necrosis factor (TNF)[11]. T cells can also cause macrophages to further secrete tissue factor, MMPs and cytokines which demonstrate a crosstalk between different arms of the immune system[11]. DCs and interestingly even VSMC from the intima can also form foam cells and proliferate and migrate to the lesion[44, 45]. This all leads to a reduced ability to resolve the inflammation due to the vicious circle of inflammatory products.

1.3.1.3 Advanced Plaques

As the lesion matures and advances it becomes known as an atheroma and contains many dead cells and debris forming the necrotic core all of which is contained within an extensive ECM acting as a trap for all these cells[12]. Macrophage foam cells apoptose due to ER stress from the lipids. Plaque VSMC number is reduced due to death from apoptosis causing less collagen secretion[38]. The large accumulation of dead cells remains in the plaque and are not cleared due to defective efferocytosis which leads to the necrotic core. Large cores are characteristic of an advanced plaque covering over 25% of the lesion area[46]. Advanced plaques can become quite complex with calcification and vessel growth into the lesion as well[7].

The lesion has a fibrous cap composed of collagen and VSMCs which separates the inner necrotic core from the lumen[12]. The fibrous cap is most prominent and important in the advanced plaque since it protects the pro-thrombotic inside of the lesion from coming in contact with the lumen contents. If the atherosclerotic process is not prevented or reversed, it can continue until the fibrous cap thins due to increased inflammatory proteases which attack and eat away at the cap and it becomes known as unstable[12]. Leakage of the inner contents of the plaque into the lumen due to plaque rupture causes the pro-thrombogenic factors to come in contact with blood platelets causing the thrombosis cascade which could lead to complete occlusion of the vessel and subsequent cardiovascular events[12]. Rupture sites tend to be located at the shoulder region of the lesion. Mouse vessels are significantly smaller than human vessels and therefore plaque rupture is difficult to study. In humans, plaque rupture is easy to identify. In mice, rupture is defined instead by the presence of haemorrhage in the plaque[47]. MMPs may be involved;

however, the exact timing and which MMPs remains controversial. Figure 1.3 demonstrates the steps leading up to plaque rupture.

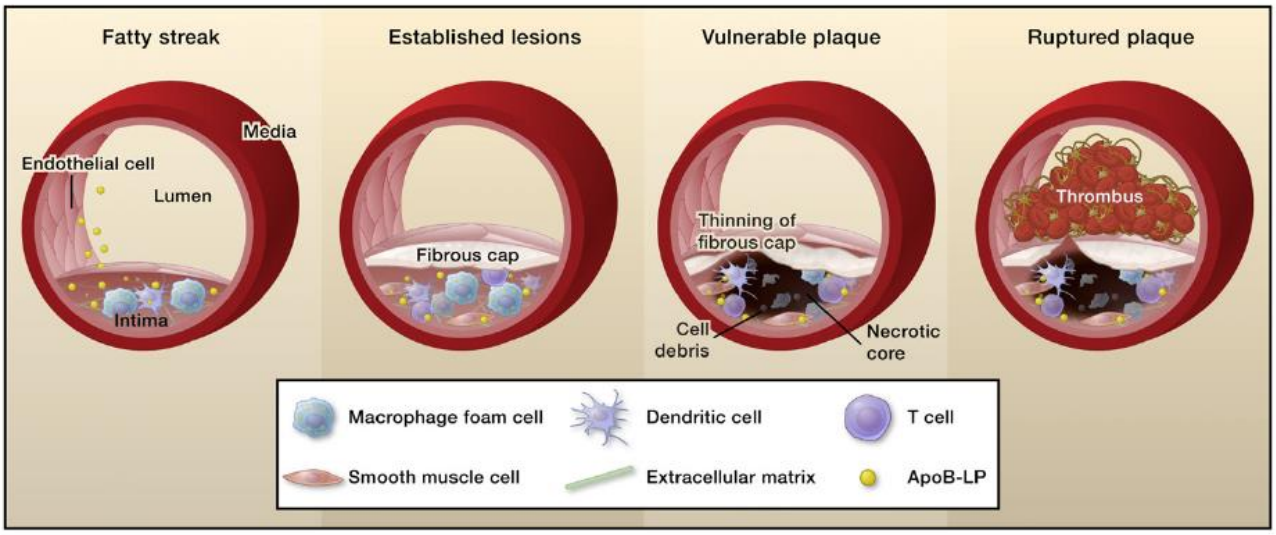

Figure 1.3: Progression of an atherosclerotic plaque[38]. Plaques begin as benign fatty streaks which then progress to established lesions with a fibrous cap protecting the lesion from rupture. As the plaque continues to grow due to a maladaptive immune response, the necrotic core forms as the fibrous cap thins making it a vulnerable plaque. If the cap becomes thin enough, the lesion can rupture and the contents of the lesion can leak out causing a thrombus formation and occluding the vessel.

1.3.2 Plaque regression

Plaque regression is more clinically relevant than plaque progression since understanding this process could lead to treatment of atherosclerosis and reduced mortality. In an ideal situation, physicians would be able to identify vulnerable plaques and cause them to become smaller and more stable, reducing the risk of a cardiovascular event. It was initially believed that the plaque process was irreversible. However, plaque regression has been shown to be feasible both in humans and animals. Much more research is required before fully understanding this process and new drugs targeting regression go on the market.

1.3.2.1 Regression in Humans

In humans with coronary disease, lipid lowering caused not only slower plaque progression but also increased plaque regression[48]. Targeting lipids seemed to be an appropriate strategy in order to cause regression.

Nissen's group did much work looking at human plaque regression with modulating cholesterol levels. The REVERSALL (Reversal of Atherosclerosis with Aggressive Lipid Lowering) trial, plaques regressed with high dose atorvastatin treated groups after 18 months compared to low dose prevastatin[49]. The ASTEROID (A Study to Evaluate the Effect of ROsuvastatin on Intravascular Ultrasound Derived Coronary Atheroma Burden) trial showed that after 24 months of treatment with rosuvastatin, 63.6% of patients showed a 7% regression[50]. Figure 1.4 demonstrates the smaller plaques after treatment. Statin treatment for 24 months showed plaque regression by IVUS examination[50]. Intravenous administration in patients with

acute coronary syndrome for 5 weeks of apoA1 Milano showed a 4.1% regression in plaque volume[51]. This is a variant of apoA1 which mimics protective HDL which rapidly metabolizes cholesterol and is found in an Italian population that is protected from atherosclerosis[51]. Other studies such as the ESTABLISH (Early Statin Treatment in Patients With Acute Coronary Syndrome) trial showed that statin treatment resulted in regression after 6 months due to LDL lowering[52].

Therefore, targeting lipids is a good strategy for plaque regression since excess LDL is the first step in plaque progression. However, the outcome of this research is over several months to years and regression is less than 10%. Therefore, clinical outcomes are not as ideal as they could be if perhaps other pathways would be targeted instead. To further understand regression, work in animal models is vital.

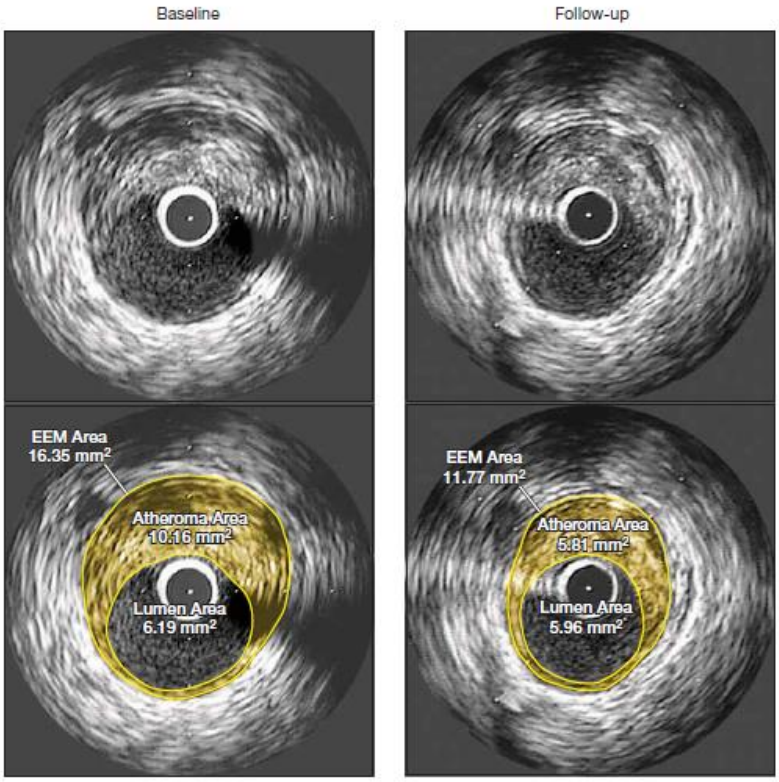

Figure 1.4: IVUS of patient in the ASTEROID trial [50]. Atheroma and lumen area as seen in an IVUS of the same patient at baseline and at follow-up after 24 months of statin treatment. Plaque size regressed. EEM= external elastic membrane area.

1.3.2.2 Regression in Mice

Few mouse models of plaque regression exist. One model uses a transplant of the thoracic aortic arch of a high fat fed apoE mouse into the abdominal aorta of a wild type (WT) mouse with normal lipid levels[53, 54]. This new environment of decreased lipid levels causes plaque regression.

ApoE KO mice on a high fat diet, when switched to a regular chow diet showed 40% regression. Expressing ApoE in these mice had an additive effect and regression rose to 70%[55]. ApoE mice infected with adenovirus encoding apoE cDNA decreases total cholesterol and after 6 months causes a regression in plaque size. ApoE KO animals injected with apoA1 causes regression due to fewer macrophages[56]. ApoA1 is the major HDL apolipoprotein needed for HDL metabolism[57]. LDLR KO animals injected with human apoA1 expressing adenovirus showed plaque regression compared to the control and compared to baseline[58].

The REVERSA mouse model also shows plaque regression in LDLR KO mice[59]. REVERSA mice are homozygous for LDLR deficiency and have extremely high lipid levels. This increase in lipids can be reversed by inducing a transgene which inhibits the secretion of apoB lipoproteins[59]. After 2 weeks of reduced lipids, there were less macrophages and smaller plaques[59]. Looking at the gene expression patterns in these regressing plaques revealed that arginase I, which is a marker of M2 macrophages, was more highly expressed[60]. Contractile genes were also upregulated and cell adhesion was decreased possibly leading to increased migration which is important for plaque regression as discussed later[60].

Cholesterol efflux involves ATP-binding cassette transporter (ABCA1) transporter receptors and induces plaque regression by allowing foam cells to eliminate their cholesterol to apoA1 and apoE which then form HDL. The lack of these receptors causes increased plaque formation[17]. MicroRNA, Mir33 suppresses ABCA1 and lowers HDL. Therefore, it was shown that anti-mir33 causes plaque regression due to efficient cholesterol efflux[61]. The liver X receptor (LXR) induces ABCA1 receptors and therefore, has the ability to also cause regression. In fact, various LXR agonists cause plaque regression[62]. Deletion of LXR in macrophages leads to increased

atherosclerosis[63]. LXR agonists delivered in nanoparticles, in order to prevent liver toxicity, reduce plaques by inhibiting inflammation[64]. In another study, in regressing plaques, there is high expression of LXR and ABCA1[65].

HDL is protective by activating eNOS, inhibiting LDL oxidation and by having anti-inflammatory and anti-thrombotic effects and is important in plaque regression[43]. Theranostics is the combining of components to both diagnose and treat a disease. HDL particles can be paired with gadolinium which not only will improve imaging by magnetic resonance but also causes regression in plaques[43]. Moreover, HDL combined with statins significantly reduces plaque size by delivering statins directly to the plaque and reducing inflammation. Using HDL as a carrier protects the statin from breakdown in the serum so it increases its bioavailability by delivering it to be uptaken by plaque macrophages.

Besides reduced cholesterol models of regression, other factors may also be involved. Atherosclerosis regression is similar to wound healing and may be induced by M2 macrophages. When M2 cells are blocked, regression is impaired[66]. The question that remains unanswered is whether M1 macrophages convert to M2 in changing plaque environments or if M2 macrophages proliferate more in regressing plaques or if more M2 cells are recruited[66]. Like wound healing, two efficient functions are required for resolution and regression to occur; efficient emigration and efficient efferocytosis. The defect in these 2 processes is seen in progressing plaques. Therefore, targeting factors required in these 2 processes may be another solution in causing plaque regression.

1.3.2.3 Emigration

The growth of plaques is due to a constant influx of inflammatory cells. However, it also occurs due to less emigration of immune cells out of the plaque leading to accumulation of plaque cells. Pro-atherogenic conditions, such as hypercholesterolemia, alter this migratory balance. It is known that macrophages in plaques have reduced capacity to migrate[67]. Emigration of monocyte-derived cells from atherosclerotic lesions characterizes regressive, but not progressive, plaques[35].

Once monocytes enter the tissue, such as the plaque, they take up residence there and differentiate into macrophages or DCs. Both of these cells have been shown to have migratory capabilities in atherosclerotic plaques with DCs being more mobile[35]. In atherosclerosis, the conversion of monocytes into DCs is blunted and they therefore remain in the lesion[35]. Studies showed that trafficking of migratory DCs into lymph nodes are found in regressing plaques and absent in progressing plaques[35]. Increase in lipid accumulation impairs DC migration to the lymph nodes by inhibitory signals from oxLDL, while HDL reverses this effect[68].

Emigration is heavily dependent on CCR7. Messenger RNA from foam cells isolated from either progressing or regressing plaques using laser capture microdissection, showed an increase in CCR7 expression in regressing plaques[65]. When CCR7 function was blocked using antibodies against its ligands, CCL19 and CCL21, regression was lost and there was higher macrophage content compared to controls[65]. Interestingly, NO enables DCs to migrate towards CCL19 by increasing formation of focal adhesions required for migration[69]. Moreover, oxLDL causes decreased CCL21 and CCR7 in plaques indicating that reduced migration is a hallmark of plaques[70]. CCR7 KO animals treated with an LXR agonist resulted in increased emigration

decreasing macrophage content and inducing regression. In addition, LXR deficient mice have impaired regression due to reduced emigration of macrophages and less CCR7[71].

Emigration is also linked to neuronal cues. Netrin-1, a neuroimmune cue, is released by macrophages in atherosclerosis and inhibits CCL19 induced migration. Deletion of this cue promotes macrophage emigration and leads to reduced atherosclerosis[72]. Moreover, CD36 scavenger receptor-dependent uptake of oxLDL leads to inhibited migration of macrophages due to altered focal adhesion kinase (FAK) signalling and actin filaments, allowing them to be trapped in the plaque leading to reduced emigration[73].

Some argue however, that recruitment of wound healing cells instead of egress is responsible for regression. Injecting adenoviral apoE to cause regression showed very little egress by using the bead tracking method whereby macrophages phagocytose fluorescent beads and can be tracked[74]. Therefore, the exact mechanism is still not fully understood. Furthermore, there may be other inducers of macrophage migration out of the plaque leading to regression which will be discussed further.

1.3.2.4 Efferocytosis

Macrophages are important cells when it comes to phagocytosis of apoptotic cells, also known as efferocytosis, and is an important function in lesions. Efferocytosis is an extremely efficient process and its defect will lead to apoptotic cell accumulation[75]. Macrophages represent 40% of plaque dead cells[76]. Early lesions do not have much apoptotic cells since macrophages are effective in clearing this debris[77]. This clearance prevents the release of damaging and

inflammatory molecules. In fact, efferocytosis releases anti-inflammatory molecules such as transforming growth factor β (TGF-β) and IL-10[75]. High numbers of apoptotic cells are seen in advanced plaques due to impaired phagocytosis, perhaps due to oxLDL competing with the apoptotic cells[77] or due to cleavage or reduced expression of apoptotic receptors in inflammatory conditions[75]. In advanced plaques, apoptosis is detrimental since it adds to the burden of the necrotic core and the defective efferocytosis cannot clear the debris. The beneficial or detrimental effects of apoptosis are dependent on the plaque stage. Increase in macrophage apoptosis at 5 weeks causes a decrease in plaque size however, an increase at week 15 increases plaque size[78]. Therefore, macrophage apoptosis is protective against lesions in early stages but in advanced stages, defective clearance will lead to inflammation and larger plaques[78].

Apoptotic cells have find me, eat me and don't eat me signals. Apoptotic cells contain phosphatidylserine (PS) which is recognized by tyrosine kinase, MERTK receptors on efferocytes with bridging molecule such as milk fat globule-epidermal growth factor 8 (MFGE8)[76]. MFGE8 is expressed in normal and diseased arteries albeit to a lower degree in plaques[79]. Lack of MFGE8 in mouse models of atherosclerosis show increased apoptotic cells in the lesion, in addition to reduced IL-10 and increased IFN expression, all contributing to increased atherosclerosis[79]. MERTK defect also leads to increased apoptosis, increased inflammation and increased plaques[80]. These receptors are important in efferocytosis; however, could other factors, such as cytokines and chemokines, also contribute to increased efferocytosis.

There are still many question marks in the field of plaque regression and it is still a fairly new and very exciting area of research. Figure 1.5 shows the steps required for plaque regression compared to plaque progression.

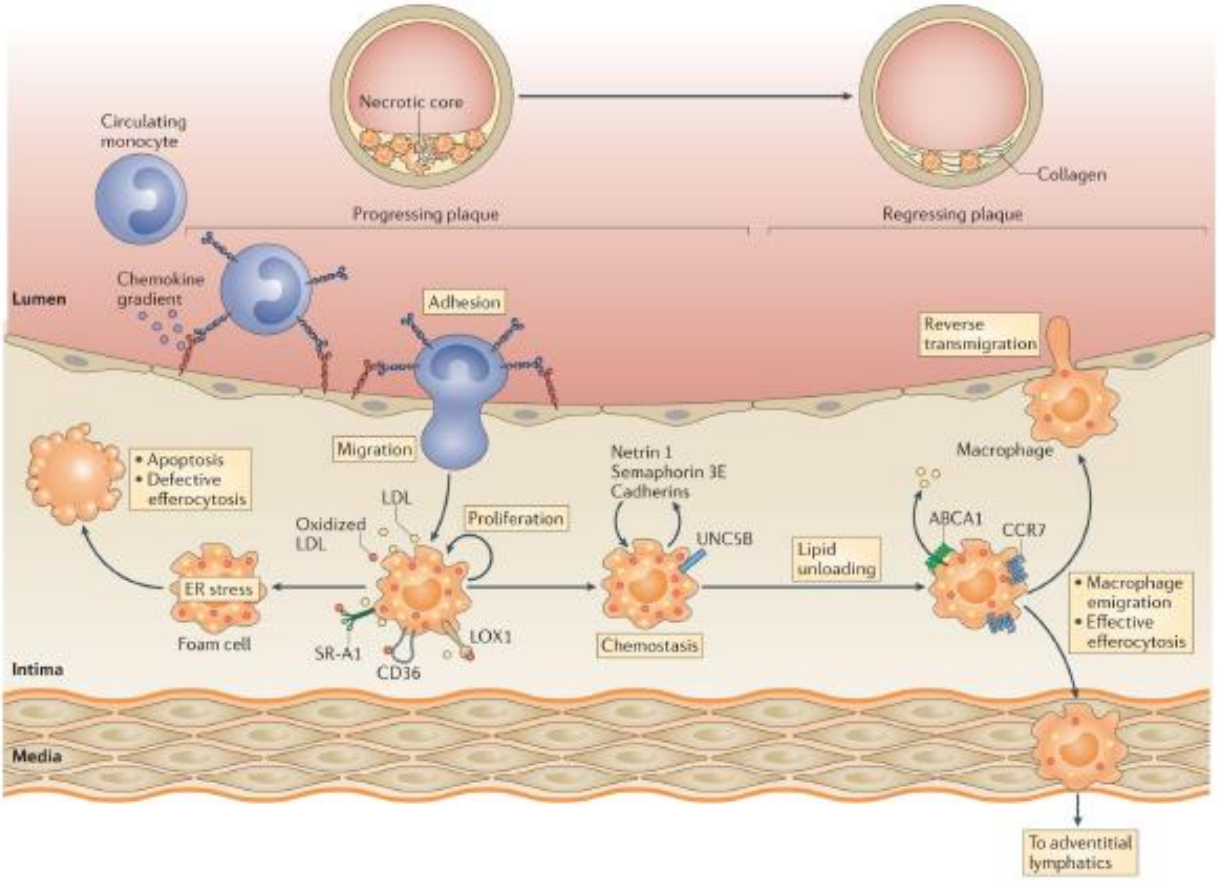

Figure 1.5: Characteristics of a regressing plaque[67]. Progressing plaques are characterized by a persistent influx of inflammatory cells attracted by chemokines and adhesion molecules. Macrophages uptake oxidized LDL and become foam cells. The plaque grows due to defective efferocytosis leading to apoptosis of foam cells and defective egress. Plaque regression occurs when cholesterol efflux (lipid unloading) in the foam cells occurs via ABCA1. CCR7 aids in macrophage emigration out of the vessel (reverse transmigration) or to lymphatics. Emigration combined with effective efferocytosis leads to plaque regression.

1.4 Shear Stress

1.4.1 Definition

As soon as the heart starts to beat *in utero*, the ECs sense the shear stress from the flow of blood. Shear stress plays a role in blood vessel development and angiogenesis[81]. Shear stress is a force due to the flow of blood sensed by the EC layer. This force is parallel to the vessel and allows the cells to line up in the direction of flow. Shear stress is measured by Poiseuille's law (Figure 1.6) which states that it is proportional to velocity of blood flow and viscosity and indirectly proportional to the third power of the radius[82]. Blood viscosity is a property of blood based on its internal friction which resists flow, with blood hematocrit being a major determinant[83]. Human shear levels over 15 dynes/cm^2 is considered high while low shear stress is less than 4 dynes/cm^2. In the human vein, values range from 1-6 dynes/cm^2 and 10-70 dynes/cm^2 in the arterial system[82]. Values over 70 dynes/cm^2 can begin to have detrimental effects such as thrombosis. The body always wants to maintain homeostasis so when the shear stress increases, the diameter of the vessel will also increase in order to decrease shear stress to a level of 15-20 dynes/cm^2 [82]. Shear stress can be either laminar which is a smooth, streamlined flow or oscillatory which is bidirectional[83].

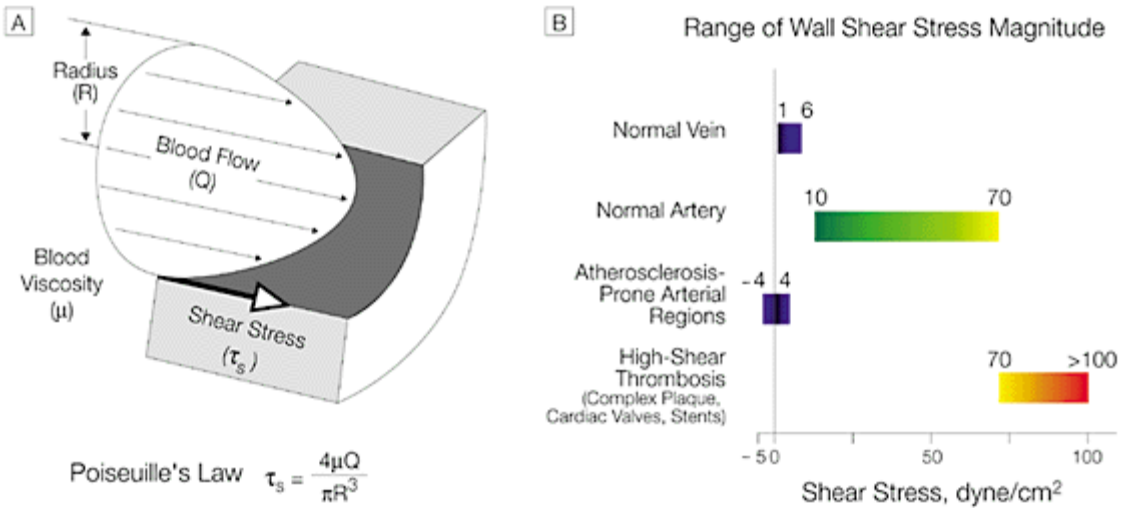

Figure 1.6: Shear stress definition[82]. Shear stress is defined by Poiseuille's law and is dependent on blood viscosity, blood flow and radius. Veins have low levels of shear stress as do atherosclerosis prone regions in the artery. Normal arteries have high levels of shear stress which is protective. Exceptionally high levels of shear stress can lead to thrombosis and is no longer protective.

1.4.2 Shear Stress Signalling

Shear stress acting over the cells is sensed by mechanotransducer receptors such as ion channels, tyrosine kinase receptors, G proteins, caveolae, platelet/endothelial cell adhesion molecule 1 (PECAM-1) and integrins[82, 84, 85]. These sensors will produce signalling molecules which will ultimately phosphorylate and activate different transcription factors that act at gene promoters such as shear stress response elements (SSREs) either inducing or repressing gene

transcription[83]. The SSRE is a 6 base pair motif in the proximal promoter region of shear stress genes[86]. VCAM-2 and NFκB have SSREs in their promoter and are therefore, directly influenced by shear stress[4]. Low shear stress signals ECs to produce pro-thrombotic, pro-migration, pro-apoptotic, pro-inflammatory and pro-growth factors such as MCP-1, VCAM-1, ROS, MMPs and endothelin 1 (ET-1)[83]. This will lead to increased endothelial permeability to LDL and monocytes and increased oxidation of LDL by ROS, all of which are atherogenic. On the contrary, high shear stress signals release of anti-thrombotic, anti-migration, pro-survival and anti-growth factors such as TGF-β, tPA, thombomodulin, PGI_2, and of most importance NO. There are 40-125 genes in ECs modulated by shear stress *in vitro* from different vessels at various time points[87].

Laminar flow activates the MEK5/ERK5/MEF2 cascade which allows NRF2 translocation to the nucleus which together with the Krüppel-like factor 2 (KLF2) transcription factor causes inhibition of inflammation[88]. KLF2 deficiency *in vivo* increases atherosclerosis[89]. Disturbed flow activates the ASK1/p38 and JNK pathway leading to NFκB (p65/relA) translocation to the nucleus and together with AP1 leads to inflammation[88]. Laminar flow and disturbed flow both leads to ROS production. However, in laminar flow the increase in NO in parallel reduces the ROS levels whereas with disturbed flow, ROS production is prolonged leading to inflammation[88]. Shear stress has also been shown to regulate LXR in ECs thus linking shear stress to plaque regression[90]. Figure 1.7 summarizes the receptors and signaling in ECs due to shear stress.

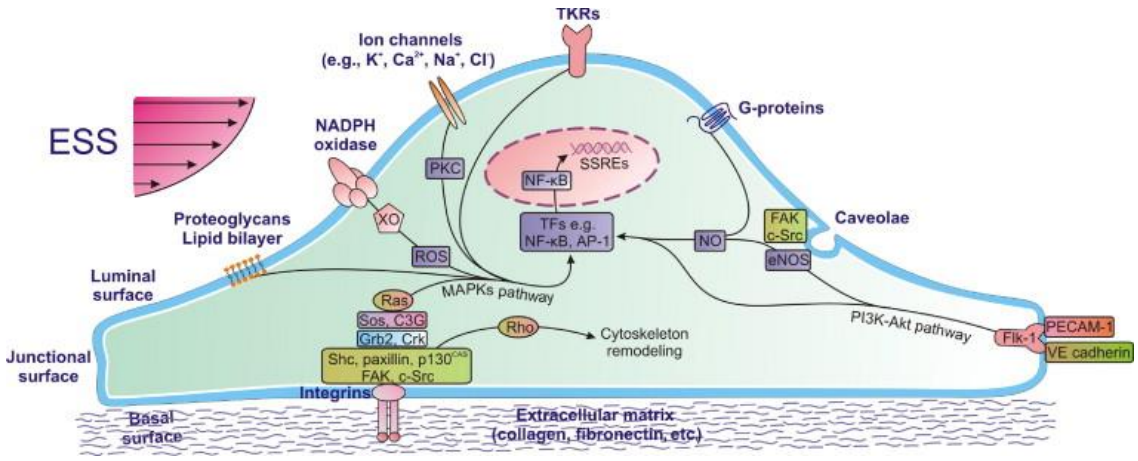

Figure 1.7: Shear stress signaling[83]. Shear stress can act on various receptors leading to a variety of signaling cascades. Transcription factors (TFs) become activated and act on shear stress response elements (SSREs) at the promoter of various genes.

1.4.3 Shear Stress is Atheroprotective

The entire vasculature is exposed to cardiovascular risk factors. However; some areas are more prone to lesions than others. Caro *et al.*, was the first to implicate shear stress with plaque localization in 1969[91]. Atherosclerotic plaques do not form uniformly throughout the vessel. Plaques tend to form depending on vessel geometry dictated by shear stress patterns. Areas of the vessel with bifurcations and branch points tend to be the preferential site for plaque development due to low or oscillatory shear stress.

The role of shear stress in plaque development has been studied in human coronary arteries post-mortem. Areas where flow is disturbed or slow has the highest level of wall

thickening[92]. In a mouse model of varying degrees of shear stress, lesion location was dependent on the value of shear stress at that location[86]. This varying degree of shear stress was achieved by taking a straight vessel segment and applying a cast with a conical lumen. In the area of the vessel where shear stress is uniform and laminar, the shape of the ECs is ellipsoidal and in the direction of blood flow. However, in atherosclerotic prone areas the cells are polygonal with no orientation which increases cell permeability[86]. As the plaques grow, the shear stress becomes lower downstream from the plaque further inducing more plaque growth[4]. Another study showed that areas of low shear have larger plaques and these plaques have fewer VSMCs, more lipids, more inflammation and lead to plaque hemorrhage in the mouse[93]. Figure 1.8 demonstrates the location of plaques in the vessel which is clearly correlated to shear stress patterns.

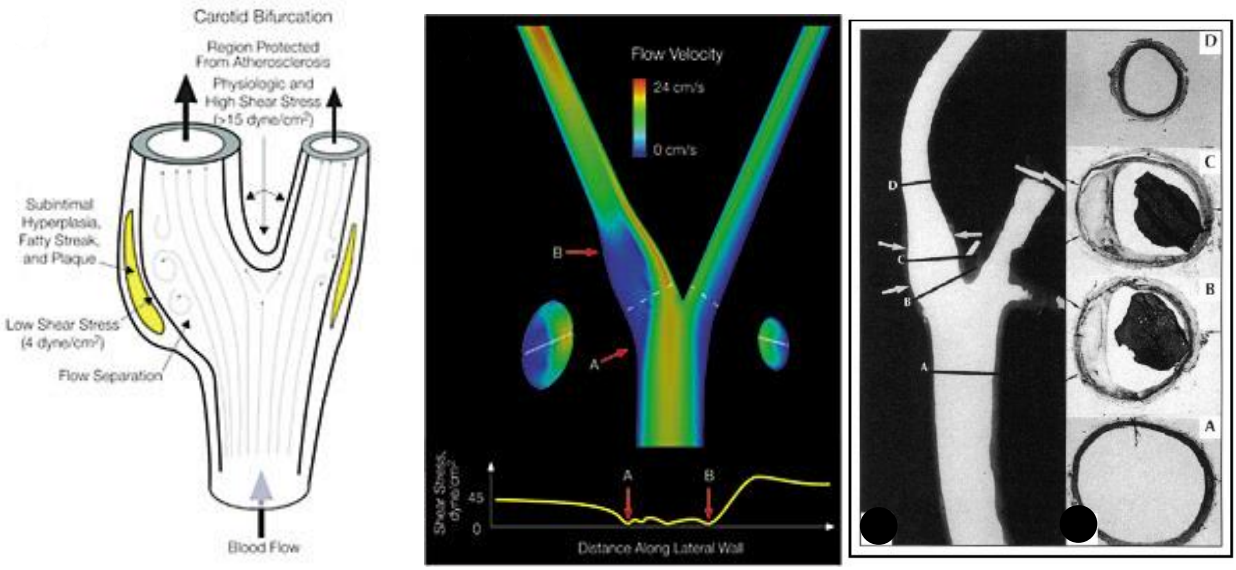

Figure 1.8: Location of atherosclerotic lesions [4, 82]. At bifurcations, areas of the vessel that are exposed to high shear stress are protected from atherosclerosis (inner wall) whereas areas with low and disturbed flow (outer wall) have large plaques (yellow). Velocity map in the middle shows low flow (blue) at the outer wall versus high flow on the inner wall (yellow and green) which correlates with the presence or absence of plaque. High shear stress is found in panel A & D which have no plaque and low shear stress in panel B & C which contain large plaques.

1.4.4 Nitric Oxide

NO has been shown to be extremely beneficial in terms of plaque progression and is induced by shear stress. Endothelial NOS is located in caveoli and remains inactive in its complex with calmodulin. When calmodulin becomes displaced, eNOS is activated which will in turn produce NO. Staining for eNOS at the outside curvature of the vessel where plaques tend

not to form show significant staining, whereas plaque prone areas have reduced eNOS staining[94]. ApoE KO mice given L-NAME to inhibit NO produced significantly more plaques than controls[95]. The same is true with ApoE/eNOS DKO mice[96]. Furthermore, ApoE/eNOS transgenic animals showed less plaques compared to controls[97, 98]. Patients with eNOS mutations have increased risk of cardiac events. Having a genetic polymorphism where eNOS is absent predisposes patients to coronary heart disease due to the increase in atherosclerosis[99]. Injection of adenovirus encoding eNOS into a balloon injury and high fat diet-induced atherosclerotic rabbit causes plaque regression[100]. When these rabbits are fed L-arginine, which increases aortic eNOS, it caused lesion regression[101, 102].

One study of chronic overexpression of eNOS in a transgenic mouse caused increased plaque size due to eNOS dysfunction leading to higher ROS from increased peroxinitrites and less NO. Consequently, eNOS deficiency developed smaller fatty streaks due to less oxidation of LDL compared to controls[103]. However, if these mice were administered an enzyme which leads to increased NO instead of superoxide, plaque size decreases[104]. Therefore, efficiency of eNOS is vital for the protective effect of NO and increased defective eNOS will lead to ROS accumulation.

NO inhibits the recruitment of monocytes, inhibits thrombogenic factors, inhibits vasoconstriction and proliferation of VSMCs and stabilizes plaques. Moreover, NO has been shown to increase MMP activity specifically MMP-2 and MMP-9 via peroxynitrite[105]. NO may also interact with the metal catalytic site of the MMP and increase its activity[106]. Endothelial NOS KO animals have no MMP-9 activity from the ECs[105]. Nitroglycerine, a commonly used

drug for angina, has been shown to increase specifically MMP-9 in human macrophages due to increased NO[107].

Therefore, there is a clear link between NO, plaque regression, migration and MMP activity.

1.4.5 Arteriovenous Fistula

In order to fully understand the effects of shear stress, a good mouse model is required such as the AVF model connecting the right common carotid artery to the right jugular vein. The carotid artery is a higher pressure system compared to the jugular vein. Therefore, the flow of blood being redirected to the jugular vein against a lower pressure allows the shear stress to increase. AVFs exist in medicine primarily in the forearm artery of kidney dialysis patients. The higher pressure of blood in the artery pushes blood into the vein through the fistula. The vessel then becomes larger and thicker allowing for better access to the vasculature for dialysis[82]. These AVFs however do not last more than 1 year with a patency rate of 60% due to proliferation of VSMC leading to neointima hyperplasia[108].

The mouse carotid artery AVF model was used as an increased shear stress model by Castier *et al.* (Figure 1.9). Prior to this, fistulas were described in rats and rabbits but this is the first AVF used in mice which are more easily manipulated and studied. Other fistula studies in mice used an aortocaval shunt which leads to cardiac failure[108]. Using the same AVF model as this thesis, hyperplasia at the fistula site is seen as early as 1 week after surgery and progresses to 4 weeks after which the fistula is no longer patent[108]. Vessel remodeling is not 100% complete

since shear stress does not reduce down to basal levels[105]. This remodelling is observed at the site of anastomosis which is not the best location to study the effect of shear stress on plaques since other factors come into play such as trauma from the surgery causing disturbed flow and this region is also not prone to plaques. So the effect of shear stress can be studied upstream in the BCA where just flow is altered.

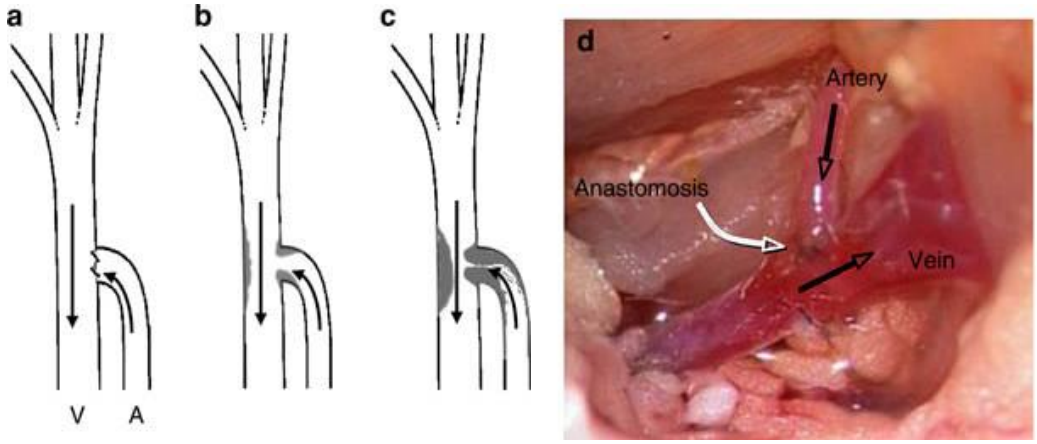

Figure 1.9: The ateriovenous fistula[108]. The carotid artery (A) is anastomosed to the jugular vein (V) leading to remodeling and eventual failure of the fistula after 4 weeks. Panel D is an image of the AVF surgical procedure in a mouse showing the anastomosis site.

1.5 Matrix Metalloproteinases

1.5.1 Structure and Function

MMPs are a family of zinc metalo-endopeptidases secreted by cells. They act extracellularly to degrade ECM proteins such as collagen, proteoglycan, fibronectin and elastin. They consist of 26 members separated into substrate specificity; collagenases (MMP-1, -8, -13), stromelysin (MMP-3, -7), elastases (MMP-12), gelatinases (MMP-2, -9) and membrane bound MMPs (MMP-14, -15, -16, -24). They share the catalytic zinc core domain in common. They are synthesized as pre-proenzymes with the signal peptide being removed during translation. They are secreted as zymogens with a signal sequence and pro-peptidase segment that needs to be cleaved in order for the MMP to be activated. They differ by the active site and substrate binding occurs at the S1 pocket which will vary in depth among MMPs[109].

MMPs function in growth, migration, wound healing and angiogenesis in normal development. MMP activity is usually low in normal healthy tissue and increases with injury or illness[109]. In disease, MMPs are involved in tumor growth and metastasis, arthritis and aortic aneurysms among others[110]. Early on, MMPs were mostly associated with cancer metastasis. However, in the early 90s, MMP-3 was found in the human coronary arteries[111] and MMPs were linked to CVD and more specifically atherosclerosis.

MMP expression is transcriptionally controlled by cytokines, growth factors, hormones and cell-cell or cell-matrix interactions[109, 112]. In general, inflammation upregulates MMPs[113]. In addition, MMPs are increased with increased shear stress specially MMP-2 and -9[114]. This increase is regulated by NO since inhibition of NO decreases MMP activity[114]. MMPs are responsible for shear stress induced vascular remodeling.

MMPs are inhibited by TIMPs of which there are 4, TIMP-1, -2, -3, -4. TIMP-1 is the most widely distributed. TIMPs have different specificity for MMPs. However, there is much overlap. TIMP-1 and TIMP-2 overlap significantly in their substrates. TIMP-1 is known to have a preference for the MMP-9 substrate. TIMP-3 targets mostly the membrane bound MMPs but it too inhibits MMP-9. TIMP-1, -2 and -3 are all found in plaques primarily in shoulder regions and in macrophages around the necrotic core[112]. The balance between MMPs and TIMPs dictate the degradation of ECM proteins.

1.5.2 Matrix Metalloproteinases and the Plaque

Advanced plaques are composed of an advanced matrix composed mostly of collagen I and III found in the lesion and collagen IV found in the basement membrane[113, 115]. Atherosclerotic arteries have more collagen compared to normal arteries mostly in the form of collagen I[116] . Collagen accounts for 60% of plaque protein and is important for stability, structure and acts as an anchor for cells[117]. As mentioned, during plaque progression, ECM proteins such as proteoglycans trap antigenic agents leading to plaque growth.

Normal arteries stain uniformly for MMPs while atherosclerotic plaques have higher levels of MMPs focused on the shoulder and necrotic core areas, where most macrophages are found and where the plaque is most unstable[113]. MMPs are required for ECM remodeling in the vessel and throughout the whole life of the plaque[113]. As the plaque forms, the vessel must remodel to compensate for the decreased lumen space. MMPs also play a role in cell migration[112]. ECM degradation is required for cell migration through tissue[118]. All cells in the

plaques secrete MMPs, from VSMCs to ECs, with macrophages providing the bulk of the production[119, 120].

MMPs are a large family of proteins and to say they all have the same role in atherosclerosis is not accurate. Different MMPs can be beneficial or detrimental depending not only on the type of MMP but also on the time-point and location. Furthermore, the right balance between MMPs and their inhibitors will dictate their beneficial or detrimental actions. Much research has been done looking at the role of specific MMPs on plaque progression using specific MMP KO mice crossed with atherosclerotic mice. No work has been done linking MMPs and plaque regression, however.

MMP-9 is implicated in human atherosclerosis especially pertaining to aneurysms and rupture. MMP-9 is increased in the blood of patients with coronary artery disease compared to healthy adults[10]. The same is true in atherosclerotic plaques in these patients. More specifically, MMP-9 expression is increased in unstable plaques compared to fibrous, thick plaques[10]. MMP-9 is overepxressed in the shoulder of plaques, a location highly susceptible to rupture. The possibility of using MMP-9 as a marker of plaque vulnerability in humans is being studied. Although the role of MMPs in plaque stability is well understood, the role in progression and more importantly regression is not known.

1.5.2.1 The Bad Guys: MMP-2, -8 & -12 in the plaque

MMP-2 null mice show decreased plaque size with a decrease in stability compared to control mice[121]. ApoE/MMP-12 DKO mice have a smaller more stable plaque due to increased

VSMC and decreased macrophages compared to apoE mice[122]. Another study with MMP-12 KO mice showed no difference in plaque size compared to controls[123]. MMP-12 overexpressing mice showed increased plaque size versus controls[124]. MMP-8 is known as a neutrophil collagenase and was not thought to be present in plaques but was later shown to be secreted by EC, VSMCs and phagocytes[125]. MMP-8 KO mice show a decrease in plaque size with increased stability versus controls[126]. MMP-13 is highly expressed in vulnerable plaques[127]. MMP-13 is even more predominant than MMP-8 as the main collagenase in plaques leading to rupture. MMP-13 KO mice have just as much an increase in collagen as MMP-13 and -8 deficiency combined[128]. MMP-13 null mice have no difference in plaque size compared to controls[129].

1.5.2.2 The Good Guys: MMP-3 & -9 in the plaque

ApoE/MMP-3 DKO mice have larger plaques compared to apoE alone indicating their beneficial role[122]. Another study also showed that DKO apoE and MMP-3 have larger plaques with more collagen compared to single apoE KO[130]. In humans, polymorphisms exists which contains an adenine insertion known as 5A or 6A. The 5A promoter has higher transcription of MMP-3 compared to the 6A and the 6A allele patients have more atherosclerosis and increased vessel thickness[131].

MMP-9 is highly implicated in aortic aneurysms but its role in plaque progression is controversial[132]. MMP-9 is highly secreted by macrophages and is one of the first MMPs to be activated in atherosclerosis[119]. MMP-9 has been shown to be both pro-inflammatory and anti-inflammatory[109]. ApoE/MMP-9 DKO mice develop larger plaques versus apoE alone (Figure

1.10)[122]. In addition, the number of buried fibrous layers was also significantly higher indicating plaque rupture and instability in apoE/MMP-9 DKO mice. The authors suggested that when plaque rupture occurs, MMP-9 is activated in order to begin healing and not to promote rupture[122]. Another study however, showed smaller plaques and reduced macrophage infiltration with MMP-9 KO versus controls[123]. MMP-9 overexpressing mice showed no difference in plaque size compared to controls[133, 134].

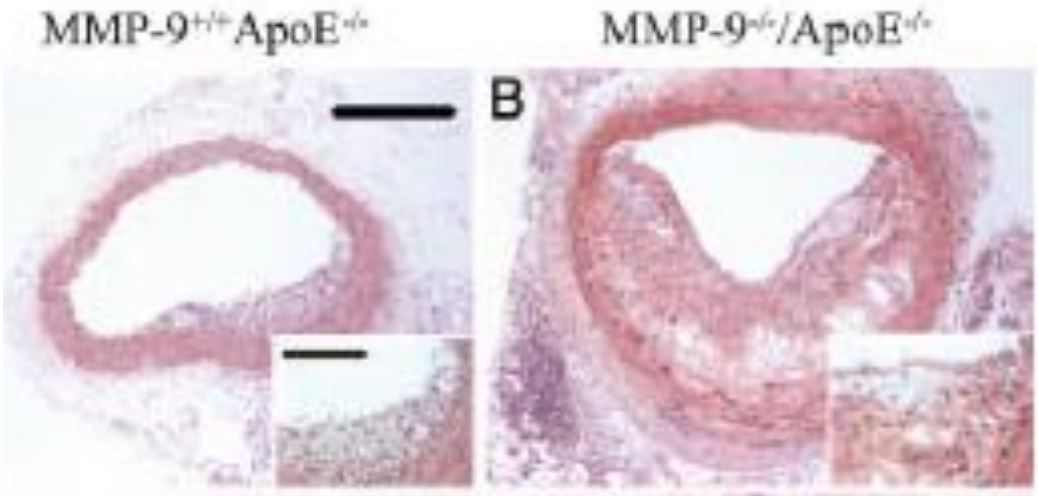

Figure 1.10: MMP-9 is atheroprotective[122]. Images of the cross section of the BCA in control and double knockout mice indicating the presence of a larger plaque in the DKO compared to the control.

1.5.2.3 Tissue Inhibitors of Metalloproteinases

Studies with TIMP KO or overexpressing animals have shown varying results. DKO apoE and TIMP-1 showed larger plaques compared to apoE KO alone[135]. TIMP-1 null mice[135, 136] and TIMP-1 ovexpressing mice[137, 138] showed no difference in plaque size in one study and a decrease in plaque size in another. TIMP-2 overexpresssing mice showed a decrease in plaque size[137]. Plaques have 5 times higher levels of TIMP-3 than in normal ateries[139]. LDLR KO mice with overexpression of TIMP-3 showed reduced atherosclerosis, increased collagen and decreased necrotic core[140]. Comparing foam cells and regular macrophages showed that foam cells have less TIMP-3 and these cells invaded basement membrane, were more proliferative, were found in shoulder regions and had increased MMP-14[141].

1.5.3 Matrix Metalloproteinases and Migration

MMPs facilitate cell migration of many cells through various tissues. MMPs cause migration by freeing cells to migrate via ECM remodeling. They also cleave collagen to create new integrin binding sites and signal via FAK which regulates the cytoskeleton to move. Finally, they can cleave cadherin removing migration constraints[142]. MMPs also free growth factors by cleaving their repressors which can aid in proliferation and migration[142].

After carotid ligation, MMP-3 KO animals have reduced neointima formation compared to controls. Moreover, there is reduced migration of VSMCs into a scratch assay and reduced MMP-9 inhibition in the MMP-3 KO cells. MMP-9 KO mice also have reduced migration into a

scratch by VSMC. Increasing MMP-3 had no effect on migration of MMP-9 KO animals therefore MMP-9 is the key player in migration[143].

MMP-9 is extremely important for VSMC migration into the intima. MMP-9 KO animals combined with injury to the endothelium had reduced neointima formation due to impaired VSMC migration and proliferation[144]. MMP-9 deficiency causes 65% decrease in VSMC migration *in vitro* and reduces hyperplasia *in vivo*[145]. MMP-9 overexpression leads to increased migration in VSMC leading to remodelling in injured rat carotid artery[146]. MMP-9 may achieve increased migration by cleaving type III collagen revealing a pro-migratory signal[146].

MMP-9 is also important in immune cell migration. MMP-9 is required for migration of dermal DCs in skin[147].Using the matrigel transwell migration assay, DCs with TIMP inhibition show less migration and the main MMP involved in the process is MMP-9[148]. TIMP overexpression in rat aortic VSMC causes reduced migration through the basement membrane[149]. Migration of DCs is achieved by a fine balance between MMPs and TIMPs, with TIMP-1 secretion causing reduced DC migration through the ECM[150]. Mice with diminished macrophage ECM migration, showed reduced MMP-9 activation with can be rescued by simply injecting the mice with MMP-9, indicating a role for MMP-9 in macrophage migration[151]. Also, CCL19/CCR7 can upregulate expression of MMP-9, possibly explaining the increased migration seen with CCR7 activation[152]. Given that MMPs are important in migration, perhaps they also play a role of immune cell emigration out of the plaque contributing to regression.

1.5.4 Matrix Metalloproteinase Inhibitors

Besides their physiological inhibitors, MMPs can also be inhibited by drugs. Their inhibition is achieved by preventing their secretion, preventing their activation or blocking their activity. Specific inhibition is very difficult to achieve since MMPs are very similar in structure and as such, off target effects occur[38]. Furthermore, inhibiting the beneficial functions of MMPs is an unwanted side effect.

MMP inhibitors initially seemed extremely promising in cancer treatment. MMPs are highly expressed in malignant cells and pre-clinical work using inhibitors showed promise. However, once these inhibitors entered clinical trials they were terminated due to serious side effects[112]. Clinical trials with MMPs saw patients who developed musculoskeletal syndrome with pain at the joints affecting their quality of life[110]. No clinical trials exist on the use of MMP inhibition in CVD.

Doxycycline is a member of the tetracycline antibiotic family that, apart from its antibacterial action, is also a potent, non selective MMP inhibitor at doses lower than that needed for it antibacterial effects[153]. Doxycycline inhibits both MMP enzymatic activity and global gene transcription[154]. Doxycycline has been shown to be effective in Marfan syndrome in preventing aortic aneurysms by inhibiting MMP-9 and MMP-2 even better than the current treatment[155]. Doxycycline has been used to reduce MMP levels in atherosclerotic specimens[154]. Patients with carotid plaques were given doxycycline for 2-8 weeks, after which plaques were analyzed and MMP-1 was reduced with no difference in any other MMP or TIMPs therefore this showed that doxycycline does enter plaque tissue[154]. Subantimicrobial doses (20mg twice daily) administered

to patients caused reduced inflammation and reduced MMP-9[156]. Doxycycline showed no effect however on lesion size[157].

CHAPTER II: MATERIALS & METHODS

2.1 Animal housing, diet and surgery

The McGill animal use committee approved the experimental protocols and animals were handled in accordance with institutional guidelines. LDLR-/- male mice were obtained from Jackson laboratory (strain B6.129S7-Ldlr^{tm1Her}/J) and mated in house. At 8 weeks of age LDLR-/- mice were placed on a HFD for 12 weeks containing 15% fat (from cocoa butter) and 0.5% cholesterol (Harlan Laboratories, Indianapolis, IN). All mice received water and food *ad libitum*. At 20 weeks of age, mice were separated into 3 groups; control mice, which were sacrificed at 20 weeks, representing the baseline for plaque size, and sham and AVF surgical groups which were kept on the HFD for a further 4 weeks post-surgery and then sacrificed. Mice were operated aseptically under general anesthesia with isofluorane using a Leica dissecting microscope as previously described[105]. Briefly, animals were kept warm until complete awakening under a heating lamp. Each mouse received a bolus of heparin (1/10 dilution in saline, 0.1ml/10g intraperitoneal (ip)) and carprofen (1/50 dilution with sterile water, 100µl/20g subcutaneous) at the onset of the operation. A transversal cervical incision was made, and the right jugular vein and right common carotid artery (RCCA) were dissected free. Flow through the vein was interrupted by means of microvascular clamps and the RCCA was clamped proximally and ligated distally. The RCCA was transected just proximal to the carotid bifurcation, and an end-to-side anastomosis was performed between vein and artery by making 2-3 interrupted 10-0 nylon sutures. The operative field was irrigated with a saline solution. Flow was then re-established through the fistula by releasing the clamps. The incisions were closed with 5-0 sofsilk sutures and the animals were allowed to recover after which they were given saline (1ml ip). Operative time averaged 90 minutes and surgical success rate was 80%. Mice were again given carpofen (subcutaneous) and saline (ip) 24 hours and 48 hours after surgery. Sham animals

were opened and their vessels clamped for an equivalent amount of time. Animals were euthanized at 0 (control) and 28 (sham and AVF) days postoperatively. Later times were not investigated because mice developed stenosis at the site of anastomosis and vessel patency was reduced in many animals after 28 days. Furthermore, no data were collected from animals with fistulas that were not patent. Figure 2.1 shows the blood flow and surgery in the Sham and AVF mice. Additional experiments were later performed using the eNOS inhibitor, L-NAME. L-NAME was administered in the drinking water (450mg/500ml) and given for 4 weeks post-surgically in sham and AVF mice. Additional experiments were also performed using MMP inhibitors. Doxycycline (Sigma-Aldrich, St. Louis, MO) was added to the drinking water of the LDLR-/- mice (3 mg/kg/day/ml of drinking water) and given for 4 weeks post-surgically in sham and AVF mice. TIMP-1, TIMP-3 and empty (pcDNA3.1-luciferase) plasmids were obtained from a group in Netherlands and designed as previously described[158]. One day prior to surgery, sham and AVF animals were injected with hyaluronidase, to increase electroporation efficiency by 50% (1μl in 90μl of 0.9% NaCl) (Calbiochem, Darmstadt, Germany), in each thigh muscle of the mouse (30μl/thigh) while awake. One hour later, the plasmids were injected into each shaved thigh muscle (50μg/thigh dissolved in 30μl of TE buffer and 140mM NaCl) under anaesthesia. Thigh muscles were then lightly massaged and immediately electroporated using a previously described protocol[158]. Briefly, eight 10 millisecond electrical pulses at 200 V/cm with a frequency of 1 Hz were applied. The pulses were generated using an ECM 830 Square Wave Electroporator and administered using electrodes (BTX Harvard Apparatus, Holliston, MA). MMP-9-/- male mice were mated in house and used for experiments at 8 weeks of age. C57BL/6 mice (Harlan Laboratories, Indianapolis, IN) were mated in house and used for experiments at 8 weeks of age.

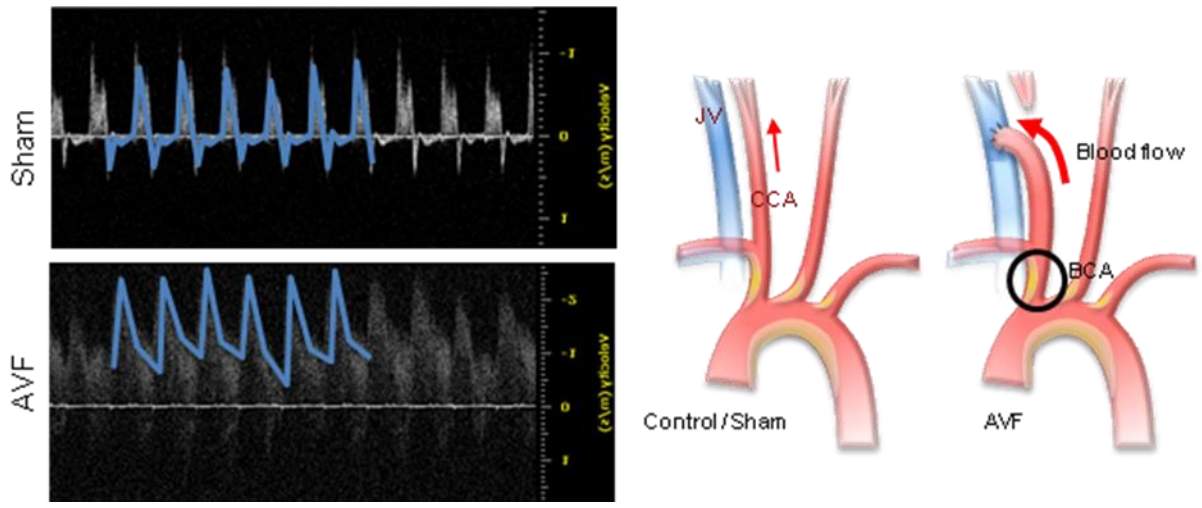

Figure 2.1: AVF surgery. BCA ultrasound of Sham versus AVF blood flow velocity indicating higher velocity in the AVF with values always above 0 and an oscillatory flow in the Sham above and below the 0. Images of AVF surgery showing the common carotid artery (CCA) anastomosed to the jugular vein (JV) in the AVF mice with the control and Sham mice having no surgical procedure.

2.2 Physiological parameter measurement

LDLR-/- body weight was measured at the initiation of the high fat diet, at surgery and at sacrifice. Physiological and hematological parameters were measured in the control at 20 weeks and the sham and AVF mice at 24 weeks. Blood flow velocity was measured by using a 20 MHz pulsed Doppler system (Milar) as previously described (Figure 2.2)[105]. Using the measured velocity, shear stress was calculated indirectly using a formula. Therefore, in this thesis the shear stress measurements are based on geometry assumptions and wherever shear stress is mentioned

it is referring to an indirect measurement based on increased blood flow. In order to more accurately measure the exact alteration in shear stress, the exact geometry of the vessel would need to be mapped in 3D and exact wall shear stress measured based on the specific geometry. Mean arterial blood pressure was measured by tail cuff method using the MC4000 Blood Pressure Analysis System (Hatteras Instruments, Cary, NC). Blood was removed via a catheter through the abdominal aorta at the time of sacrifice and was collected in heparin-coated tubes (Sarstedt, Nümbrecht, Germany) or EDTA coated tubes (Sarstedt, Nümbrecht, Germany). Blood cell enumeration (white blood cells, lymphocytes, monocytes, granulocytes, red blood cells, % hematocrit, hemoglobin and platelets) was performed with a hematology analyzer (scil vet abc animal blood counter, Vet Novations, Barrie, Canada). Blood was centrifuged to collect plasma and total cholesterol, HDL and triglyceride content was determined by either a kit according to manufacturer's protocol (Sigma-Aldrich, St. Louis, MO) or by the Institut de recherches cliniques de Montreal.

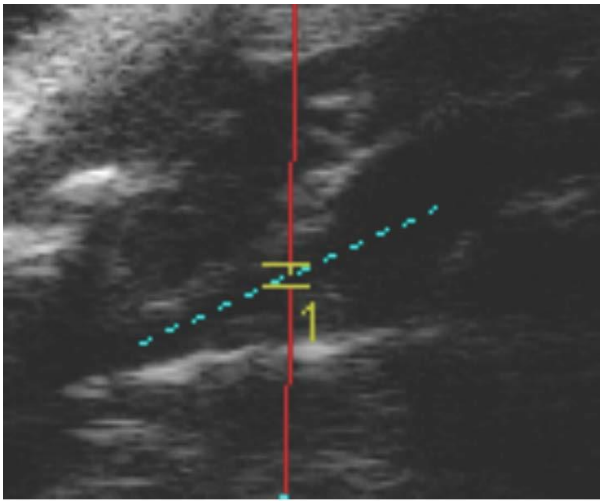

Figure 2.2: Echocardiography image of the BCA. The blue dotted lined is passing through the vessel where blood flow velocity was measured.

2.3 Atherosclerotic lesion measurement and characterization

At sacrifice, under isofluorane anesthesia, blood was washed out of the vasculature by perfusion at 100 mmHg with normal saline solution for 5 minutes through a cannula inserted in the abdominal artery. The vasculature was then perfused and fixed with 4% paraformaldehyde, also at 100mmHg, for 20 minutes. The heart and aortic branch were placed in 30% sucrose (made in PBS) overnight at 4^{o}C on a shaker and then rinsed with 1X PBS. Periadventitial tissue was removed and aortic arches were photographed using a Leica dissecting microscope under bright light. Aortic sinus and BCA segments were embedded vertically in Tissue-tek (Sakura, Torrance, CA), and serial 7um thick sections were cut using a cryostat (Leica CM 3050 S, Leica Microsystems, Concord, Canada). Sections of aortic sinus and BCA were stained with oil red O (Electron Microscopy Sciences, Hatfield, PA) for 45 minutes and mounted using Immu-Mount (ThermoFisher Scientific, Waltham, MA) on superfrost plus slides (VWR, Radnor, PA). Images were acquired using a Leica microscope. The mean lesion areas in mm^2 for the aortic sinus and the BCA were calculated using ImageJ software (National Institute of Health). Echocardiography was performed the day before surgery and each week at the same time and day until sacrifice 4 weeks later. A rodent VEVO 770 ultrasonograph system was used and analyzed for plaque volume using VisualSonics software.

Gelatinase plaque activity was evaluated using *in situ* zymography, in which vessel sections were incubated at 37°C for 8 hours with a fluorogenic gelatin substrate (DQ gelatin, Invitrogen, Eugene, OR) diluted to 1/20 in zymography buffer (50 mmol/L Tris-HCl pH 7.4 and 15 mmol/L $CaCl_2$). Proteolytic activity was detected as green fluorescence. Smooth muscle cell content of the plaque was evaluated by immunohistochemistry staining for smooth muscle cell

actin (5% BSA block, 1/100 monoclonal anti-actin, α-smooth muscle alkaline phosphatase antibody, Sigma-Aldrich, St. Louis, MO) for 1 hour and mounted using Immu-Mount. Plaque elastin content was characterized by Van Giesen staining, apoptosis by red caspase-3 staining and reactive oxygen species by red dihydroethidium staining. Macrophage content, NFκB expression and eNOS expression was quantified by immunofluorescence using the anti-MOMA-2 antibody (1/50, Abcam, Cambridge, UK), anti-p65 (Rel A) pS529 antibody (1/50, Rockland Immunochemicals, Limerick, PA) and anti-eNOS (Ser1177) (1/50, Cell Signaling Technology, Danvers, MA) respectively. MMP plaque expression was measured by immunofluorescence using anti-MMP-3 (1/50, Santa Cruz Biotechnologies, Dallas, TX), anti-MMP-9 (1/50, Santa Cruz Biotechnologies, Dallas, TX), anti-MMP-2 (1/50, Santa Cruz Biotechnologies, Dallas, TX), anti-MMP-14 (1/100, Abcam, Cambridge, England) and anti-MMP-8 (1/100, Abcam, Cambridge, England) antibodies. Collagen I and IV content was measured by immunofluorescence using anti-collagen I (1/40, EMD Millipore, Darmstadt, Germany) and anti-collagen IV (1/40, EMD Millipore, Darmstadt, Germany). Total collagen content of the plaque was evaluated using the picrosirius red stain kit according to manufacturer's protocol (Polysciences, Warrington, PA) and mounted using Eukitt mounting medium (Electron Microscopy Sciences, Hatfield, PA). Necrotic core size was evaluated by immunohistochemistry hematoxylin and eosin staining. Percent BCA plaque necrotic core was quantified using ImageJ software where the white empty space was measured. Plaque cell number was evaluated by staining with Dapi and counting the cells in the plaque using Image J software. Proliferating cells were evaluated by immunofluorescence using Ki67 (1/50, Abcam, Cambridge, England). The immunofluorescence sections were rinsed and blocked with10% serum then incubated with

primary antibody, rinsed, and further incubated with fluorescently labeled secondary antibodies (1/200, Invitrogen, Carlsbad, CA). Percent plaque staining was quantified using ImageJ software.

2.4 Cellular influx

To examine cell influx in the plaque, we performed irradiation-transplantation experiments. Sham and AVF surgeries were performed as described above and two days later, mice were lethally irradiated with a single dose of 9 Gy (0.19 Gy/min, 200 kV, 4 mA) total body irradiation using an Andrex Smart 225 Röntgen source (YXLON International) 1 day before the transplantation. Bone marrow cells were isolated by flushing the femurs and tibias of male green fluorescent protein (GFP) mice with RPMI 10% serum. Irradiated recipients received 10×10^6 GFP bone marrow cells by tail vein injection. Four weeks later, mice were sacrificed, BCAs were taken as described above and immunofluorescence was performed to identify GFP positive cells. At week 1-4, mice were sacrificed and peripheral blood was taken and flow cytometry was performed to identify GFP positive cells. Briefly, 100µl of whole blood was incubated with red blood cell lysis buffer for 10 minutes, centrifuged for 5 minutes at 1500 rpm after washing and resuspended in 2% normal mouse serum in PBS and stained with a viability dye (eBiosciences). Flow cytometry was performed on the BD LSR Fortessa (BD Biosciences). Data analysis was performed using Flow Jo software (Tree Star Inc., OR). Gating was first performed on forward versus side scatter to remove cellular debris and doublets before selection of live cells based on exclusion of a viability dye. GFP positive cells were then gated.

2.5 Confirmation of MMP inhibition

To confirm that the plasmid intramuscular electroporation functioned, protein expression of TIMP-1 was measured in the aortic arches and plasma in all groups of mice 28 days after electroporation. Arches and plasma were collected as above and total protein (20 ug) was extracted, separated by SDS-PAGE, transferred to nitrocellulose membranes and incubated overnight at 4^{o}C with primary antibody against TIMP-1 (1/1000, Santa Cruz Biotechnologies, Dallas, TX). After incubation with secondary antibodies, signals were revealed by chemiluminescence (Western Lightning Plus ECL, Perkin Elmer, Waltham, MA) with the Molecular Imager Chemidoc XRS system (Bio-Rad, Hercules, CA) and quantified by densitometry using Quantity One software (Bio-Rad, Hercules, CA). Membranes were subsequently stripped and re-probed with a β-actin antibody (1/200, Sigma-Aldrich, St. Louis, MO) or stained with ponceau S to verify equal loading.

2.6 *In vitro* co-culture shear stress system

In order to recreate the plaque environment, we developed a co-culture system exposed to shear stress. Macrophages were obtained by flushing the bone marrow of both femurs and tibias of C57BL/6 mice or MMP-9 -/- mice at 2 months of age. Mice were killed by CO_2. Bones were flushed with RPMI 1640 and centrifuged, after which cells were resuspended in RPMI with serum (10% FBS) (macrophage media), plated on a 10 cm petri dish and incubated at 37^{o}C. After 2 hours, cells were washed and differentiated in the presence of macrophage media with mouse macrophage colony-stimulating factor (M-CSF) (50ng/ml, PeproTech, Rocky Hill, NJ) for 7

days. Macrophages were then scraped and added to a well of a 6 well dish to grow overnight. ECs were obtained from the lungs of C57BL/6 mice or MMP-9 -/- mice at 2 months of age. The lungs were rapidly excised and diced into 1-mm-sized fragments with sterile scissors in RPMI 1640. The lung fragments were digested with 0.1% collagenase A (Roche Diagnostics, Mannheim, Germany), and the resulting cell suspension was plated on a gelatin-coated flask and grown in DMEM/F12 + 20% FBS + 1% ECGS (EC media). For 2 successive passages, ECs were detached with trypsin and isolated using a CD102 antibody (BD Bioscience, Mississauga, Canada) coupled to Dynal beads (Invitrogen, Carlsbad, CA). ECs were then plated on a 6 well permeable transwell (Corning, Corning, NY) and allowed to grow overnight. The transwell was added over the well of macrophages in macrophage media with or without the MMP inhibitor GM-6001 (1/1000, Enzo Life Sciences, Farmingdale, NY). A plastic cone plate apparatus which spins using a magnet (designed in house, Figure 2.3) exposes the ECs to high shear stress (15 dynes/cm^2) or low flow (0.5 dynes/cm^2) or no flow (static) allowing only the media to pass and contact the macrophages below.

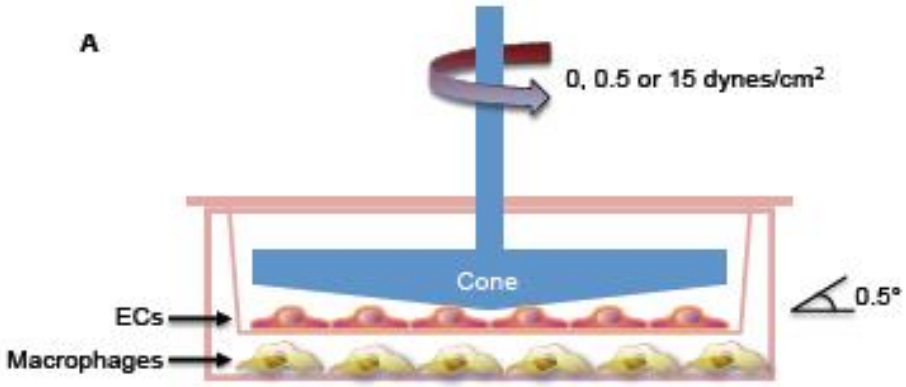

Figure 2.3: Cone plate apparatus. This apparatus was designed in-house to replicate the plaque environment. Macrophages were plated on the petri dish and ECs plated on the transwell above. The transwell is permeable to fluids and impermeable to cells. The cone spins using a magnet rotating at a specific RPM to generate the shear stress.

2.7 *In vitro* macrophage function

Gelatin zymography was performed as described previously[159] using 20µg of macrophage cell lysates or media which was visualized as clear bands of lysis among a blue background of gelatin-containing gels. Cells were lysed, laemmli buffer added and loaded on a SDS gel containing gelatin (0.1mg/ml), after electrophoresis gels were washed using 2.5% triton X-100 and incubated in zymography buffer (50 mmol/L Tris-HCl, pH 7.4, and 15 mmol/L $CaCl_2$) at 37°C for 72 hours then stained with Coomassie brilliant blue. Gels were quantified by densitometry using Quantity One software (Bio-Rad, Hercules, CA).

Macrophage migratory capacity was evaluated by using the scratch assay. Cells from C57BL/6 or MMP-9 -/- mice, obtained as above, were seeded into 6-well plates and grown to 100% confluence. The cells were then rendered quiescent by changing the medium to basal RPMI without serum or M-CSF for 8 hours and were wounded once with a small tip by scratching across the maximum diameter of each well. The cells were then washed twice and macrophage media with or without GM-6001 was added. Cells were then placed in the cone plate apparatus with the endothelial cells in the transwell on top and exposed to high or low shear stress for 24 hours. For experiments with the inhibitor, cells were pretreated 30 minutes before starting the shear stress. Pictures were taken using a Leica digital camera of an inverted microscope. Pictures were taken immediately after scratching, as well as 6 and 24 hours after. Images were analyzed using Image J software by measuring the size of the denuded area.

Macrophage phagocytic capacity was evaluated using an efferocytosis assay. RAW macrophages were kept in culture for 5 days before the assay start point. The cells were then scraped and resuspended in macrophage media containing orange cell tracker to fluorescently label macrophages (10 μM, Invitrogen, Eugene, OR), incubated at 37 ºC for 30 minutes and then washed and resuspended in fresh macrophage media and placed under the UV light for 30 minutes and incubated at 37 ºC overnight to induce apoptosis. Macrophages were then taken from the co-culture system exposed to no or high shear stress and apoptotic cells (ratio 5:1) were added and incubated again at 37 ºC for 30 minutes. The cells were washed and fixed with 2% paraformaldehyde for 15 minutes at 37 ºC and stained with dapi. Fluorescent pictures were taken using Leica microscope and the number of phagocytic cells was counted.

2.8 *In vitro* cytokine expression

To assess the effect of shear stress on cytokine production, we used the same *in vitro* co-culture shear stress system as previously described. After 24 hours exposed to no or high shear stress conditions, the media was used on a mouse cytokine array panel (R&D System, Minneapolis, MN) following instructed protocol. Spots of expression were revealed on the membrane and compared between conditions.

To confirm the expression of cytokines of interest from the cytokine array, total RNA of macrophages and ECs after 24 hours of the co-culture shear stress system in either static or high shear conditions was extracted using a total RNA mini kit (Geneaid Biotech, New Taipei City, Taiwan) following instructed protocol. RNA was quantified using a spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific, Waltham, MA). cDNA was synthesized from 0.4 μg RNA by using qScript cDNA Supermix kit (Quanta Biosciences, Gaithersburg, MD). The expression level of CCL5 and CXCL12 was assessed by quantitative real time polymerase chain reaction (RT-qPCR) using 7500 Fast PCR (Applied Biosystems, Foster City, CA) under standard conditions of 60°C annealing temperature for 40 cycles. All primers were designed using Primer 3 Plus software and SYBR green chemistry (SensiFAST SYBR Lo-ROX kit, Bioline, Humber Road, London) was used with specific primers for all genes. Results were analyzed using the ΔΔCt method as calibrator samples. The analyzed genes were expressed relative to the murine TATA-binding protein (TBP) housekeeping gene.

2.9 *In vitro* activated integrins

Presence of activated β1 and β2 integrins on the macrophages were assessed using human cells obtained as follows. Human peripheral blood mononuclear cells were isolated from blood of healthy donors (age 20-45 years, male and female, no known disease or medication) using a Ficoll density gradient (Sigma-Aldrich, St. Louis, MO). Cells were plated in macrophage media in a 37^{o}C incubator for 2 hours, washed and differentiated to macrophages over 7 days using human M-CSF (50ng/ml, R&D Systems, Minneapolis, MN). Human macrophages were used along with human umbilical vein endothelial cells (HUVECs) after 24 hours in the shear stress co-culture system, with or without MMP inhibition, in low or high shear stress conditions. Then the cells were scraped, collected, centrifuged, and resuspended in FACS buffer (2% FBS in PBS) and stained for the surface markers, activated β1 integrin (1/400, Millipore, Darmstadt, Germany) and activated β2 integrin (1/100, Hycult Biotech, Uden, Netherlands). The activation of integrins was quantified using flow cytometry (BD LSR Fortessa, BD Biosciences, Mississauga, Canada) and analyzed using Flow Jo software (Tree Star, Ashland, OR). Fluorescence minus one control was used to remove the fluorescent background.

2.10 *In vitro* activated T cells

Using a different shear stress apparatus which involves cells in suspension and a pump to create the shear stress, we looked at immune cells other than macrophages. Whole spleen cells were extracted and added to the system for 24 hours in static, low or high shear conditions. Cells were collected from the shear stress apparatus and stained for the surface markers CD4 and

CD69 (eBioscience, San Diego, CA). Flow cytometry was performed on the BD LSR Fortessa (BD Biosciences, Mississauga, Canada). Fluorescence minus one control was used to remove the fluorescent background. Data analysis was performed using Flow Jo software (Tree Star, Ashland, OR.).

2.11 Statistical analysis

Results are expressed as mean±SEM. Data was evaluated by one-way ANOVA for multiple groups followed by the Newman-Keuls post test or a 2-tailed Student *t* test for 2 groups. A probability value of $P<0.05$ was considered to be statistically significant. GraphPad Prism software was used.

CHAPTER III: FIRST PAPER

3.1 Hypothesis and Objectives

While detection methods of atherosclerosis have been improving, also having the ability to cause a plaque to regress even more than statins are able to achieve and more safely than stents would be ideal. Many investigations on this subject examined lipid lowering techniques very similarly to how statins cause regression. However, using these drugs is a slow process and does not achieve overly significant reduction. Thus, would targeting another plaque component have an even bigger effect on plaque reduction remains an unanswered question?

The first question this thesis tries to answer is whether increased shear stress, being atheroprotective, also has the ability to cause plaque regression in an already established plaque. The effect of shear stress on plaque regression is very novel as well as the increased shear stress mouse model we use to answer this question. In addition, the plaque composition during regression is an extremely important question in order to investigate the mechanisms at play to further understand this vital clinical process. Given that NO is regulated by shear stress and is also involved in atheroprotection, assuming that NO plays a role is highly possible.

In this chapter, we look at these important questions by measuring plaque size in our shear stress model and comparing it to our baseline control mice 4 weeks prior and by examining more closely the plaque composition and the role of NO.

We hypothesize that: Shear stress has the ability to cause plaque regression in an already established advanced plaque via a NO dependent mechanism.

Specific objectives:

- To determine the effect of increased shear stress on plaque size.

- To measure plaque components in increased shear stress plaques, specifically plaque stability, inflammatory content and advancement in order to determine a potential mechanism of action.

- To determine the effect of NO inhibition on plaque size in an increased shear stress mouse model as a potential mechanism.

SHEAR STRESS-INDUCED ATHEROSCLEROTIC PLAQUE REGRESSION IN THE ABSENCE OF LIPID MODULATION

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Atherosclerotic plaques form in regions of low blood flow, whereas vessels exposed to protective high shear stress remain lesion-free. Release of nitric oxide (NO), among other protective factors, in areas of high shear stress appears to be key to atheroprotection. We hypothesized that exposing established atherosclerotic plaques to elevated shear stress will lead to lesion regression. We developed a high fat fed, LDLR-/-, shear stress model of arteriovenous fistula in mice, where the right carotid artery is anastomosed into the jugular vein. This procedure increases the shear stress in the brachiocephalic artery (BCA) and leads to a drastic 50% plaque regression in arteriovenous fistula (AVF) mice after only 4 weeks ($p<0.05$) and of most importance, in the absence of lipid level changes. We observed a smaller necrotic core, reduced macrophage content, less NFκB and increased endothelial nitric oxide synthase (eNOS) expression in plaques of AVF mice ($P<0.05$). Furthermore, treatment with L-NAME, an eNOS inhibitor, abrogated plaque regression in the AVF mice. Our findings suggest that not only is shear stress protective against plaque development but it can effectively reverse the atherosclerotic process without interfering with plasma lipid levels, through an NO dependant mechanism.

3.2 Introduction

Cardiovascular diseases are by far the most prevalent cause of death in developed countries with the major culprit being atherosclerosis (1). Atherosclerosis is a maladaptive, chronic inflammatory disease characterized by a persistent influx of inflammatory cells (2). Treatment with lipid-lowering statins provides an exceptional health benefit; however, the majority of patients do not respond adequately to these drugs. While much is known about plaque progression, less is known about the more clinically relevant regression of an already established plaque. Almost all human and animal studies investigating plaque regression involve alterations in lipid levels (3). As blood flows in the vascular tree, it generates a frictional force on endothelial cells (ECs) called shear stress. Shear stress is beneficial, favouring the release of protective factors from the endothelium such as NO (4). The levels of shear stress vary greatly according to arterial geometry. They tend to be greatest in straight arterial segments and lowest at branch points and bifurcations. Plaques form in regions of low, oscillating flow (less than 4 dyne/cm^2) whereas vessels exposed to high shear stress (greater than 15 dyne/cm^2) tend to remain lesion-free (5). This study is the first to show, *in vivo*, a direct effect of high shear stress on established plaque regression, with no change in lipid levels.

3.3 Material & Methods

3.3.1 Animal housing, diet and surgery

The McGill animal use committee approved the experimental protocols and animals were handled in accordance with institutional guidelines. LDLR-/- male mice were obtained from

Jackson laboratory (strain B6.129S7-Ldlr^{tm1Her}/J) and mated in house. At 8 weeks of age, LDLR-/- mice were placed on a high fat diet (HFD) for 12 weeks containing 15% fat (from cocoa butter) and 0.5% cholesterol (Harlan Laboratories). All mice received water and food *ad libitum*. At the 12 week time point, mice were separated into 3 groups. Control mice were sacrificed at this time, which represents the baseline for plaque size; Sham and AVF surgical groups were kept on the HFD for a further 4 weeks post-surgery and then sacrificed. Sham and AVF mice were operated aseptically under general anesthesia with isofluorane using a Leica dissecting microscope as previously described (6). Briefly, a transversal cervical incision was made, and the right jugular vein and right common carotid artery (RCCA) were dissected free. Flow through the vein was interrupted by means of microvascular clamps and the RCCA was clamped proximally and ligated distally. The RCCA was transected just proximal to the carotid bifurcation, and an end-to-side anastomosis was performed between vein and artery by making 2-3 interrupted 10-0 nylon sutures. Flow was then re-established through the fistula by releasing the clamps. Sham animals were opened and their vessels clamped for an equivalent amount of time. The incisions were closed with 5-0 sofsilk sutures. No data was collected from animals with fistulas that were not patent. Additional experiments were later performed using the eNOS inhibitor, L-NAME. L-NAME was administered in the drinking water (450mg/500ml) and given for 4 weeks post-surgically in Sham and AVF mice.

3.3.2 Physiological parameter measurement

LDLR-/- body weight was measured at the initiation of the HFD, at surgery and at sacrifice. Blood flow velocity was measured at time of surgery and sacrifice using a 20 MHz

pulsed Doppler system (Milar) as previously described (6). Mean arterial blood pressure was measured by tail cuff using the MC4000 Blood Pressure Analysis System (Hatteras Instruments). At sacrifice, blood was collected through the abdominal aorta for total cholesterol and HDL cholesterol analysis was determined via a kit according to manufacturer's protocol (Sigma-Aldrich)

3.3.3 Atherosclerotic lesion measurement and characterization

The vasculature was perfused and fixed with 4% paraformaldehyde, at 100mmHg, for 20 minutes. The heart and aortic branch were dissected and placed in 30% sucrose overnight at 4^{o}C. BCA segments were embedded vertically in Tissue-tek (Sakura), and serial 7um thick cryo-sections were cut (Leica CM 3050 S, Leica Microsystems). Sections of BCA were stained with oil red O to assess plaque size. Echocardiography was performed the day before surgery and each week at the same time and day until sacrifice 4 weeks later. A rodent VEVO 770 ultrasonograph system was used and analyzed for plaque volume using VisualSonics software. Total collagen content of the plaque was evaluated using the picrosirius red stain kit according to manufacturer's protocol (Polysciences). Plaque elastin content was characterized by Van Giesen staining, apoptosis by red caspase-3 staining and reactive oxygen species by red dihydroethidium staining. Proliferating cells were evaluated by immunofluorescence using Ki67 (1/50, Abcam, Cambridge, England). Plaque vascular smooth muscle cell (VSMC) content was evaluated by immunohistochemistry using anti-smc α actin alkaline phosphatase antibody (Sigma-Aldrich). Macrophage content, NFκB expression and eNOS expression was quantified by immunofluorescence using the anti-MOMA-2 antibody (1/50, Abcam), anti-p65 (Rel A) pS529

antibody (1/50, Rockland Immunochemicals) and anti-eNOS (Ser1177) (1/50, Cell Signaling Technology) respectively. Percent BCA plaque necrotic core was quantified using ImageJ software where the white empty space was measured. Plaque cell number was evaluated by staining with dapi and counting the cells in the plaque using Image J software. The mean lesion areas in mm^2 for the BCA and percent plaque staining was quantified using ImageJ software (National Institute of Health).

3.3.4 Cellular influx

To examine cell influx in the plaque, we performed irradiation-transplantation experiments. Sham and AVF surgeries were performed as described above and two days later mice were lethally irradiated with a single dose of 9 Gy (0.19 Gy/min, 200 kV, 4 mA) total body irradiation using an Andrex Smart 225 Röntgen source (YXLON International) 1 day before the transplantation. Bone marrow cells were isolated by flushing the femurs and tibias of male green fluorescent protein (GFP) mice with RPMI 10% serum. Irradiated recipients received 10×10^6 GFP bone marrow cells by tail vein injection. Four weeks later, mice were sacrificed, BCAs were taken as described above and immunofluorescence was performed to identify GFP positive cells. At week 1-4, mice were sacrificed and peripheral blood was taken, cells were counted, and flow cytometry was performed to identify GFP positive cells. Briefly, 100µl of whole blood was incubated with red blood cell lysis buffer for 10 minutes, centrifuged for 5 minutes at 1500 rpm after washing and resuspended in 2% normal mouse serum in PBS and stained with a viability dye (eBiosciences). Flow cytometry was performed on the BD LSR Fortessa (BD Biosciences). Data analysis was performed using Flow Jo software (Tree Star Inc.). Gating was first performed

on forward versus side scatter to remove cellular debris and doublets, before selection of live cells, based on exclusion of a viability dye. GFP positive cells were then gated.

3.3.5 Statistical Analysis

Results are expressed as mean±SEM. Data was evaluated by one-way ANOVA for multiple groups followed by the Newman-Keuls post test or a 2-tailed Student *t* test for 2 groups. A probability value of $P<0.05$ was considered to be statistically significant. GraphPad Prism software was used.

3.4 Results & Discussion

Our study demonstrates for the first time, the possibility of causing plaque regression in the absence of lipid levels changes. Table 1 reveals that mouse body weight, blood pressure, total cholesterol and HDL cholesterol is comparable between the 3 groups. The only parameter that changes is that of increased RCCA flow (5.9±0.9 ml/min in AVF vs. 0.6±0.5 ml/min in Sham), which in consequence increases the shear stress 4 fold (Table 1). Flow is oscillatory in Sham animals whereas the AVF surgery produces a pulsatile, non reversing flow with the shear stress increasing specifically at the BCA. Our lab has previously used the AVF model in the context of vessel remodeling to show that p47phox-dependent NADPH oxidase and NFκB regulates flow-induced vascular remodeling (6,7), whereas this study focuses on the possibility of plaque regression. AVFs exist in medicine primarily in the forearm artery of kidney dialysis patients in

order to better access the vasculature (5). Other fistula studies in mice use an aortocaval shunt which leads to cardiac failure (6). Therefore, the AVF fistula is, in our opinion, the best model of increased shear stress in mice.

The size of the Sham plaques progress slightly over the 4 week period, as expected, compared to the control mice. However, the size of the AVF plaques (56.8±9.8 μm^2 x 10^3) is not only smaller compared to the Sham plaques (134.9±14.3 μm^2 x 10^3) but also compared to the baseline control 4 weeks prior (117.0±11.7 μm^2 x 10^3) and shows about a 50% reduction in only 4 weeks (Figure 1A & B). This regression is due to a gradual decrease in size and not to plaque disruption from the shear differential, as proven by the lack of size differences after 3 days (data not shown) and from the echocardiography images. The AVF plaque volume diminishes over time and becomes significant only at 3 weeks (0.67±0.18 mm^3 relative to 1 at week 0) and 4 weeks (0.63±0.18 mm^3 relative to 1 at week 0) compared to Sham (1.72±0.36 mm^3 and 1.68±0.23 mm^3 respectively, relative to 1 at week 0) (Figure 1C). Plaque macrophage area was significantly reduced in the AVF (12.7±4.3 μm^2 x 10^3) compared to the Sham (30.1±7.5 μm^2 x 10^3), indicating potentially less inflammation or increased stability (Figure 1D). Necrotic core was also reduced in the AVF (9.1±2.6%) compared to both the Sham (18.1±2.0%) and control (17.1±2.2%) (Figure 1D) with no change in plaque cell number (data not shown). Fewer macrophages and a smaller core are indicative of a less advanced plaque with the small AVF plaque being cellularly dense. Despite the AVF plaque being small, some may argue that it may be more unstable. However, plaque collagen, VSMC and elastin content were comparable between the 3 groups indicating similar stability (Figure 1D). Therefore, the AVF plaque is less inflammatory, less advanced and smaller, all while maintaining its stability.

Plaque regression is more clinically relevant than plaque progression and even more so in the absence of lipid changes. Patients enter the clinic with already large established plaque, therefore understanding how plaques regress, which was initially believed to be impossible, is critical. In humans with coronary disease, lipid lowering causes not only slower plaque progression but also increased plaque regression (8). Human plaque regression due to statin treatment was observed in the REVERSALL, ASTEROID and ESTABLISH trial as well as the apoA1 Milano administration study (9-12). These human studies all had lipid modulation as the main contributor to plaque regression. High dose statins were necessary for a prolonged period of time of 18-24 months and only a 4-7% reduction in plaque size was observed (9-12). Studies in mice have these same issues. Few mouse models of plaque regression exist. One such model uses a plaque-laden, aortic arch transplant from a hyperlipidemic apoE knockout mouse into the abdominal aorta of a wild type (WT) mouse with normal lipid levels (13,14). It is the decreased lipid environment that drives the regression. Other mouse models of regression all use lipid modulation in both LDLR and apoE knockout animals either via switching to a regular chow diet, adenoviral injection of apoE or apoA1 cDNA, increasing cholesterol efflux via anti-mir33 or inhibiting apoB lipoproteins (15-19). Methods of plaque regression, other than via lipid changes, have not been fully studied.

The plaque regression model that we propose uses variations in shear stress levels as a potential mechanism. Shear stress is the flow of blood over the endothelial cell layer sensed by mechanotransducers (4, 5, 20). High laminar shear stress (over 15 dynes/cm^2) is atheroprotective and signals release of anti-thrombotic, anti-migration, pro-survival and anti-growth factors, with NO being of most importance (21,22). Low oscillatory shear stress (under 4 dynes/cm^2) signals ECs to produce pro-thrombotic, pro-migration, pro-apoptotic, pro-inflammatory and pro-growth

factors with NFκB being a key player in the detrimental effects (21,23). Shear stress alterations due to vessel geometry is the reason plaques do not form uniformly. In comparison, areas of the vessel with bifurcations and branch points tend to be the preferential site for plaque development due to low shear stress, whereas straight vessel segments with high shear stress remain lesion free (24,25).

To investigate the molecular mechanism of his regression, we investigated the role of NO. Activation of eNOS appears to be a key player in the atheroprotective effects of shear stress. NO acts by inhibiting the recruitment of monocytes, inhibiting thrombogenic factors and stabilizing plaques. Staining for eNOS at the outside curvature of the vessel which is free of lesions, show significant staining, whereas plaque prone areas have reduced eNOS staining (26). Much work has been done regarding the role of NO in plaque progression. ApoE knockout (KO) mice with NO inhibition produced significantly more plaques (27), similarly with ApoE/eNOS DKO mice (28). Conversely, ApoE/eNOS transgenic (TG) animals have less plaques (29,30). Furthermore, patients with eNOS mutations have increased risk of cardiac events (31). Atherosclerosis in rabbits, when injected with adenovirus encoding eNOS, leads to plaque regression (32), similarly when fed L-arginine which increases NO synthase (33,34). Our study expands on this notion of NO but in the context of regression. In our AVF plaque, we saw increased eNOS staining at 3 days (0.33±0.09% in Sham vs. 0.86±0.09% in AVF) and 4 weeks (0.33±0.07% in Sham vs. 0.58±0.02% in AVF) indicating a potential role in the regression mechanism (Figure 2A). The eNOS quantification was measured as a ratio of the quantity in the vessel to solely measure plaque levels. Inhibiting NO production by L-NAME abrogates the regression (132.1±13.3 μm^2 x 10^3 in Ctrl vs. 207.4±16.3 μm^2 x 10^3 in Sham vs. 209.8.±24.9 μm^2 x 10^3 in AVF) (Figure 2D). Plaque eNOS is no longer more activated in the AVF (0.50±0.05% in

Sham vs. 0.38±0.10% in AVF) further confirming the role of NO (Figure 2E). Moreover, NFκB, which is pro-inflammatory and regulated by shear stress, was reduced in the AVF plaques at 3 days (20.3±1.1% in Sham vs. 14.9±1.0% in AVF) and 4 weeks (20.2±1.8% in Sham vs. 12.4±3.6% in AVF) post-surgery (Figure 2B). This difference is lost upon NO inhibition (19.0±0.8% in Sham vs. 19.3±2.7% in AVF) (Figure 2E), indicating less inflammation and a role of NFκB in plaque progression. Reactive oxygen levels (ROS) was also quantified and there was no difference between groups (Figure 2B&C). The ROS does not co-locolize with NFκB. Therefore, NFκB is not related to the ROS production in this case.

As the AVF plaque appears to be less inflammatory and advanced, we examined the influx of cells which is characteristic of progressing plaques. We used transplantation with green fluorescent protein (GFP+) bone marrow cells into irradiated Sham and AVF mice. After irradiation and transplantation, almost all cells in the blood were GFP+ (87.3±6.0% in Sham vs. 89.9±6.5 in AVF) and these numbers remained high throughout the 4 weeks (Figure 3D). Furthermore, monocyte (1.1±0.1 x 10^3/mm^2 at week 0 to 0.4±0.1 x 10^3/mm^2 in both Sham and AVF at week 1) and lymphocyte number (5.45±0.005 x 10^3/mm^2 at week 0 to 1.6±0.3 x 10^3/mm^2 in Sham and 1.9±0.8 x 10^3/mm^2 in AVF at week 1) did not differ between groups and showed a reduction at week 1 with a steady incline throughout the 4 weeks reaching levels similar to week 0 (Figure 3D). These results indicate that the irradiation and transplantation was successful. The GFP cells do not hinder plaque regression in the AVF plaques (165.6±9.9 μm^2 x 10^3 in Sham vs. 58.1±12.0 μm^2 x 10^3 in AVF irradiated and transplanted) (Figure 3C). Although GFP percentage in the plaque was altered, given the difference in size when examining total content there was no difference in GFP area staining in the AVF plaque (Figure 3E) nor a difference in GFP+ cells in the blood (Figure 3D). These findings cannot be explained by changes in apoptosis or

proliferation since values were consistent between groups (Figure 3A & B). Therefore, there is continuous influx of cells, even as the plaque is regressing. However, we observed a reduced macrophage number in the AVF plaque (refer to Figure 1D) indicating that perhaps there may be an increase in cellular efflux, which is a hallmark of plaque regression (35) and is currently being studied by our group.

In summary, this is the first study using a mouse carotid fistula model to investigate the molecular mechanisms of shear stress-induced atherosclerotic plaque regression. We reveal, for the first time, that plaque regression is possible without any alteration in lipid levels and may be dependent on NO. These results and future work will help us further identify the molecular mechanisms that cause the atheroprotective effects of shear stress, in hopes of identifying new therapeutic targets for the treatment of atherosclerosis and cardiovascular diseases.

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Disclosures

None

	Ctrl	AVF	Sham
n =	11	15	13
Weight - wk 12 (g)	34 ± 3	34 ± 1	41 ± 5
Weight - wk 16 (g)	-	38 ± 2	43 ± 6
Total cholesterol (g/L)	11.5 ± 0.4	10.2 ± 0.2	9.3 ± 0.9
HDL cholesterol (g/L)	1.4 ± 0.4	1.0 ± 0.2	1.3 ± 0.5
Blood pressure (mmHg)	96 ± 4	100 ± 5	100 ± 5
Post-op RCC flow (ml/min)	-	5.9 ± 0.9***	0.6 ± 0.5

Table 3.1: AVF surgery does not cause a change in lipid levels. Blood parameters of control (Ctrl), AVF and Sham mice. Wk = week, Ctrl = control, RCCA = right common carotid artery. Data are mean±SEM of n=11-15, ***P<0.001 vs. Sham.

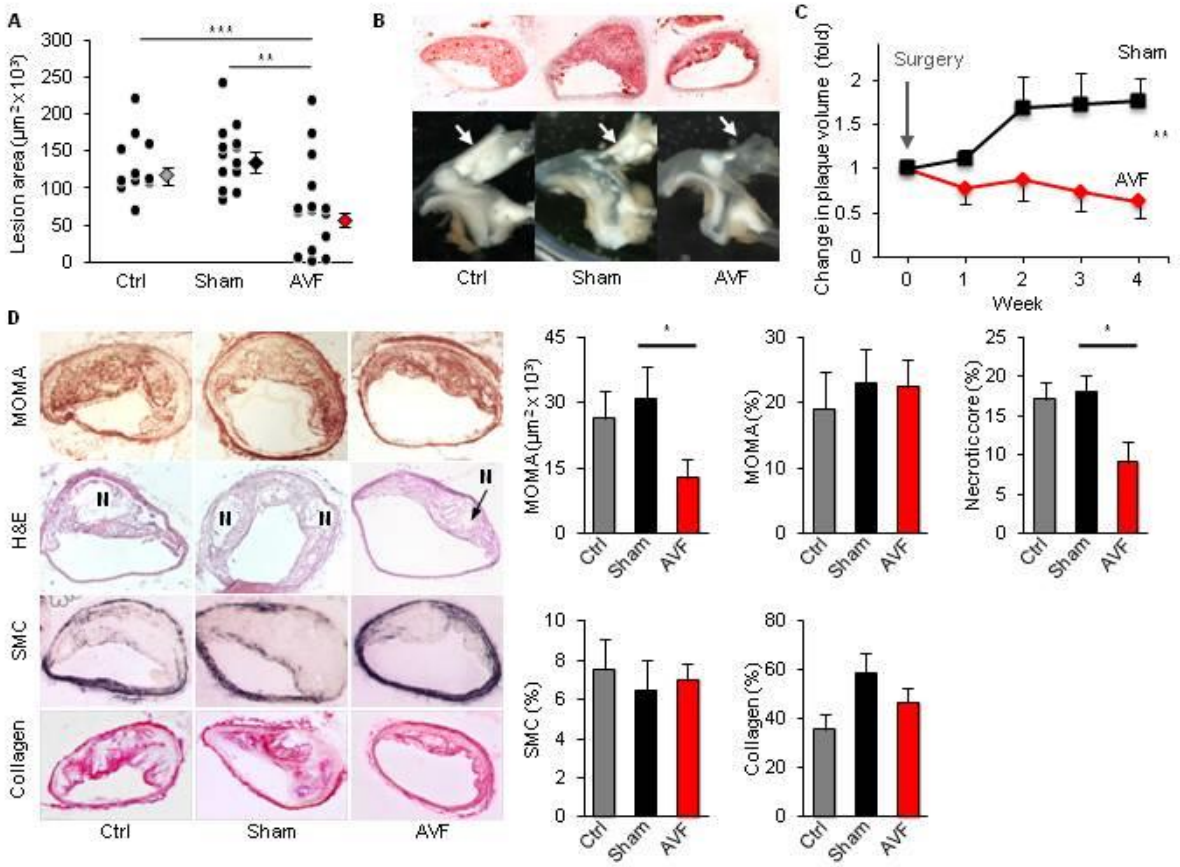

Figure 3.1: AVF induces plaque regression to a less advanced plaque A) Quantification of BCA lesion area in control (Ctrl), Sham and AVF mice. Data are mean±SEM of n=11-15, **P<0.01 and ***P<0.0001. **B)** Oil red O plaque staining in the cross section of the BCA and whole artery preparations with the BCA plaque in white, visible by transparency. **C)** Quantification of changes in BCA plaque volume via echocardiography at various weekly time points. Data are mean±SEM of n=5-8, **P<0.01 vs. AVF week 4. **D)** Brown monocyte/macrophage (MOMA) staining in cross section of BCA plaques with quantification of MOMA area and MOMA percentage in the plaque. Hematoxylin and eosin (H&E) staining

showing necrotic core as empty space (N) with quantification of percent plaque area. Lesion smooth muscle cell (SMC) content (alpha-actin immunohistochemistry purple staining) and collagen content (Sirius red staining) with percent plaque area. Data are mean±SEM of n=5-8, *P<0.05.

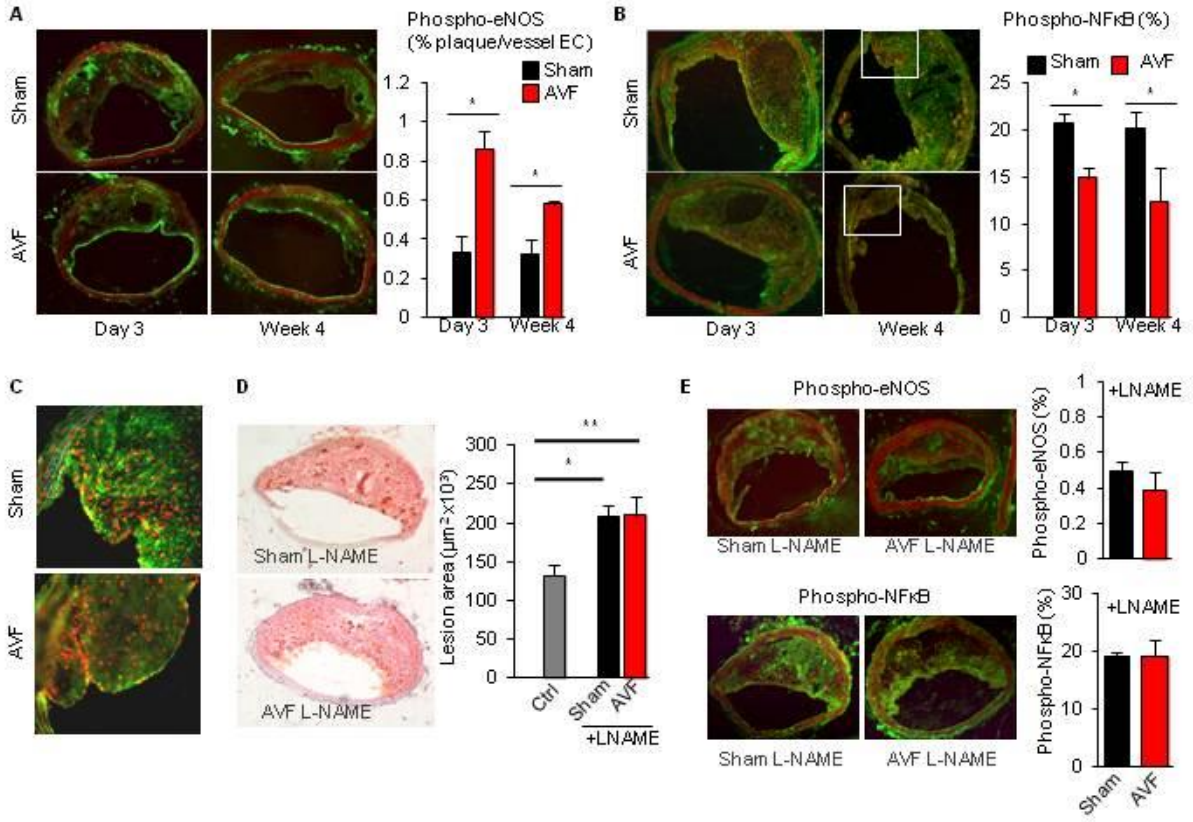

Figure 3.2: Increased eNOS activity is involved in AVF plaque regression A) Green phosphorylated eNOS (serine 1177) immunofluorescence in BCA plaque of Sham and AVF mice at 3 days and 4 weeks post surgery. Quantification of percent plaque staining over EC staining. **B)** Red dihydroethidine and green phospho-NFκB staining in the Sham and AVF plaque at day 3 and week 4 post-surgery. Quantification of percent NFκB plaque staining. **C)** Higher magnification of box in panel B. **D)** Oil red O plaque staining in the cross section of the BCA in Sham and AVF mice treated with L-NAME. Quantification of BCA lesion area in treated mice. **E)** Green phosphorylated eNOS (serine 1177) and phospho-NFκB immunofluorescence in BCA plaque of treated mice. Quantification of percent plaque staining. Data are mean±SEM of n=5-8, *P<0.05 and **P<0.01.

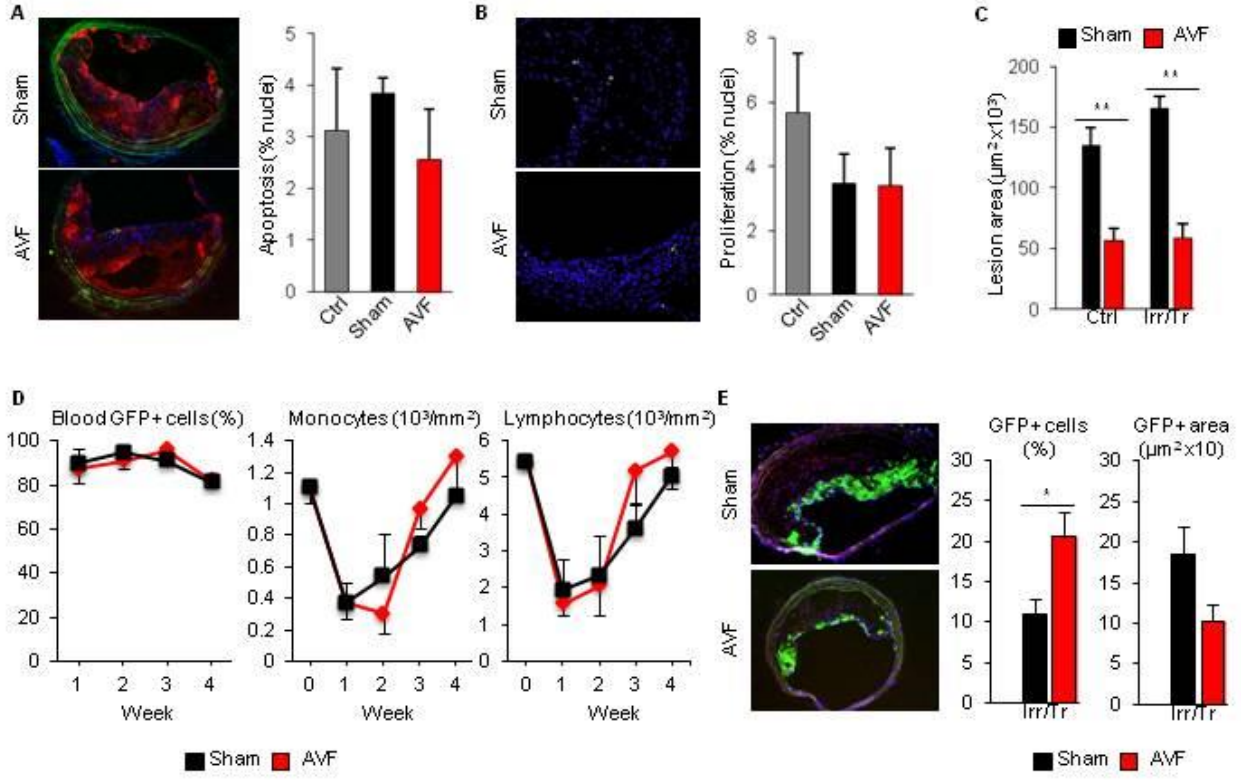

Figure 3.3: Cellular influx into the plaque is similar in Sham and AVF mice A) Red caspase-3 staining for apoptosis in Sham and AVF BCA plaques. Quantification of percent apoptosis in control (Ctrl), Sham and AVF mice. **B)** Green Ki67 staining for proliferation with cell nuclei in blue. Quantification of percent plaque proliferation. **C)** BCA lesion area in mice with Irradiation/Transplantation (Irr/Tr) of GFP+ cells in Sham and AVF mice compared to non-Irr/Tr mice (Ctrl). **D)** Percent GFP+ cells and monocyte and lymphocyte number in peripheral blood after Irr/Tr of Sham and AVF mice over 4 weeks. **E)** Green GFP+ staining in BCA plaques.

Quantification of plaque GFP+ cells and GFP+ area. Data are mean±SEM of n=5-8, *P<0.05 and **P<0.01.

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CHAPTER IV: SECOND PAPER

4.1 Hypothesis and Objectives

We have shown that, in fact, shear stress causes plaque regression in established plaques by about 50% and potentially acts via NO. The plaque is less advanced due to decreased necrotic core with no change in plaque stability. There is a similar influx of cells within the plaque, however; there is less macrophage area in the high shear stress plaque. Shear stress-induced regression involves NO since NO is not only increased in high shear stress plaques but inhibition abrogates the regression seen. Key questions still remain to be investigated. How is NO causing the regression? Given that NO, MMPs and shear stress are linked as explained in the introduction, perhaps MMPs are also playing a role in regression.

Furthermore, there seems to be a consistent influx of cells within the regressing plaque, however, fewer macrophages, therefore where are these macrophages going? Since efflux of these cells out of the plaque is a key component of regression, whether increased shear stress causes increased macrophage mobility is yet to be answered. MMPs especially MMP-9 is important in macrophage migration and could possibly be the link between shear stress and regression.

In this chapter, we look at these important questions by measuring MMP activity in the plaque of our shear stress model and by examining the effect of MMP inhibition on regression. Moreover, we will examine the effect of increased shear stress on migration of macrophages. This will further explain the mechanism of action for shear stress-induced plaque regression in order to find a specific target.

We hypothesize that: Shear stress-induced regression acts via induction of MMP activity which leads to increased macrophage mobility by untrapping macrophages in plaque ECM.

Specific objectives:

- To measure MMP activity in the regressing plaque.
- To determine the effect of MMP inhibition on plaque size in an increased shear stress mouse model as a potential mechanism.
- To examine the effect of macrophage migration in an *in vitro* shear stress system with and without MMP inhibition.
- To examine the specific effect of MMP-9 on *in vitro* migration.

SHEAR STRESS-INDUCED ATHEROSCLEROTIC PLAQUE REGRESSION IS REVERSED BY REGULATION OF MACROPHAGE MOBILITY VIA MATRIX METALLOPROTEINASE INHIBITION

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Submitted to Circulation Research

Background: Atherosclerotic plaques form in regions of low blood flow, whereas vessels exposed to high shear stress remain lesion-free. We hypothesized that exposing established atherosclerotic plaques to elevated shear stress leads to lesion regression by facilitating inflammatory cell movement within the plaque.

Methods: We developed a model of arteriovenous fistula (AVF) in mice, where the right carotid artery is anastomosed into the jugular vein. LDLR-/- mice were placed on a high-fat diet. Control mice were sacrificed at week 12, which coincided with Sham and AVF surgery. Sham and AVF mice were kept on a high-fat diet for a further 4 weeks.

Results: This procedure increases the shear stress in the brachiocephalic artery (BCA) and leads to plaque regression in AVF ($P<0.05$). All groups had comparable lipid levels. We observed greater gelatinase activity in plaques of AVF mice ($P<0.05$), suggesting a role for matrix metalloproteinases (MMPs) in plaque regression. A separate group of mice was therefore treated post-surgery with an MMP inhibitor, doxycycline, or with a more specific TIMP-1 or TIMP-3

over-expressing plasmid. All three prevented the reduction in plaque size in the AVF group. Moreover, MMP-9 expression was increased in AVF plaques and collagen IV was decreased ($P<0.05$). To better define the mechanism of plaque regression in the AVF, we devised an endothelial cell (EC)-macrophage co-culture system where the ECs were exposed to high or low shear stress, and macrophages exposed to the EC effluent. MMP-9 activity was increased in the macrophages in the high shear stress setup ($P<0.05$). There was a 2.5 fold increase in the migration of macrophages exposed to high shear effluent vs. low shear stress ($P<0.05$). When repeated using the MMP inhibitor, GM6001, the high shear increase in migration was blocked. In addition, this same trend was also present when using specific MMP-9 knockout macrophages and ECs.

Conclusion: Our findings suggest that shear stress acting on ECs may influence the cells within the plaque by increasing MMP-9 activity, allowing for better macrophage motility, an important feature of regressing plaques.

Keywords: shear stress, arteriovenous fistula, atherosclerotic plaque regression, matrix metalloproteinase, MMP-9

4.2 Introduction

Cardiovascular disease (CVD) is by far the most prevalent cause of death worldwide with atherosclerosis being the main contributor[163]. Atherosclerosis is a chronic inflammatory disease characterized by a persistent influx of inflammatory cells[164]. A key event in atherosclerosis, allowing for the continuous growth and progression of atherosclerotic plaques, is a maladaptive inflammatory response leading to poor inflammatory resolution which is characterized by defective egress of inflammatory cells and defective efferocytosis (clearance of apoptotic cells by phagocytosis)[165]. While much is known about plaque progression, less is known about the more clinically relevant regression of an already established plaque. However, what is understood is that effective clearance of apoptotic cells and promotion of inflammatory cell egress is needed in order for a plaque to regress in size[166]. The most effective, current treatment for atherosclerosis is the lipid-lowering statins; however, many patients do not respond well to these drugs[14]. Therefore, there is a need for alternate therapies which will be made possible by better understanding the molecular mechanisms that influence plaque regression.

An important concept in plaque development and lesion location is that of shear stress, the frictional force created by blood flow on endothelial cells (ECs). Atherosclerotic plaques are not found uniformly in the vessels; their distribution depends on the local flow of blood. Vessel segments exposed to laminar or high flow (greater than 15 dyne/cm^2) are usually devoid of plaques [82] due to the release of protective factors from the EC lining[85]. Vessels exposed to oscillatory or low flow (less than 4 dyne/cm^2), commonly found at bifurcations, are prone to plaque formation [82] due to adhesion molecules and chemokine release from the damaged ECs[85, 167, 168]. While it is known that shear stress is atheroprotective, whether it also has the ability to

cause plaque regression in an already established plaque has been recently shown by our group indicating that shear stress has the potential to cause already established plaques to regress.

Furthermore, members of the matrix metalloproteinase (MMP) family have been implicated in atherogenesis. MMPs are a large family of about 20 secreted or membrane bound proteases acting on extracellular matrix (ECM) substrates and also involved in many other cellular functions such as proliferation, migration and apoptosis[119]. Tissue inhibitors of matrix metalloproteinases (TIMP) are their biological inhibitors of which there are 3. TIMP-1 inhibits many of the secreted MMPs but has a high affinity for MMP-2, -3 and -9 whereas TIMP-3 targets membrane bound MMPs[119]. MMPs are produced by many of the cells found within a plaque especially the macrophages[169] and MMP-2, -3, -9. -14 as well as TIMP-1 have all been expressed in atherosclerotic plaques[119]. While it is widely accepted that MMPs contribute to plaque instability and aneurysm, when it comes to their role in atherosclerosis, results have been conflicting. MMPs are important in the plaque environment since lesions contain many ECM proteins and to an even higher extent in more advanced plaques. Collagen, primarily types I, III, IV and V, is the most prevalent ECM found in the plaque[170] comprising 60% of total plaque protein[117]. Mouse studies have shown that some MMPs have beneficial roles in plaque development by decreasing growth and promoting stabilization (MMP-3, -9) whereas others have opposite effects (MMP-2, -14)[171]. Besides providing stability, the ECM also provides a structure for cells to anchor to and migrate through. The question of whether MMPs could contribute to more efficient cell egress from the plaque, a hallmark of plaque regression, remains unanswered.

We propose to use an original model of mouse arteriovenous fistula (AVF) to investigate the effects of increased blood flow on established plaque size and composition and to study how

high shear stress, acting on endothelial cells, can impact on the functions of immune cells that are typically found in the plaque. We hypothesize that shear stress is able to reverse atherosclerotic plaque formation by facilitating inflammatory cell mobility within the plaque via activation of MMPs, specifically MMP-9, leading to increased macrophage migration.

4.3 Material & Methods

4.3.1 Animal housing, diet and surgery

The McGill animal use committee approved the experimental protocols and animals were handled in accordance with institutional guidelines. LDLR-/- male mice were obtained from Jackson laboratory (strain B6.129S7-Ldlr^{tm1Her}/J) and mated in house. At 8 weeks of age, LDLR-/- mice were placed on a high fat diet (HFD) for 12 weeks containing 15% fat (from cocoa butter) and 0.5% cholesterol (Harlan Laboratories). All mice received water and food *ad libitum*. At the 12 week time point, mice were separated into 3 groups. Control mice were sacrificed at this time, which represents the baseline for plaque size; Sham and AVF (arteriovenous fistula) surgical groups were kept on the HFD for a further 4 weeks post-surgery and then sacrificed. Sham and AVF mice were operated aseptically under general anesthesia with isofluorane using a Leica dissecting microscope as previously described[105]. Briefly, a transversal cervical incision was made, and the right jugular vein and right common carotid artery (RCCA) were dissected free. Flow through the vein was interrupted by means of microvascular clamps and the RCCA was clamped proximally and ligated distally. The RCCA was transected just proximal to the carotid bifurcation, and an end-to-side anastomosis was performed between vein and artery by

making 2-3 interrupted 10-0 nylon sutures. Flow was then re-established through the fistula by releasing the clamps. Sham animals were opened and their vessels clamped for an equivalent amount of time. The incisions were closed with 5-0 sofsilk sutures. No data were collected from animals with fistulas that were not patent. Additional experiments were later performed using MMP inhibitors. Doxycycline (Sigma-Aldrich) was added to the drinking water of the LDLR-/- mice (3 mg/kg/day/ml of drinking water) and given for 4 weeks post-surgically in Sham and AVF mice. TIMP-1, TIMP-3 and empty (pcDNA3.1-luciferase) plasmids were designed as previously described[158]. One day prior to surgery, Sham and AVF animals were injected in the quadriceps with hyaluronidase (1µl in 90µl of 0.9% NaCl) (Calbiochem) followed 1 hour later by plasmid injection (50µg/leg) and electroporation[158].

4.3.2 Physiological parameter measurement

LDLR-/- body weight was measured at the initiation of the HFD, at surgery and at sacrifice. Blood flow velocity was measured at time of surgery and sacrifice using a 20 MHz pulsed Doppler system (Milar) as previously described[105]. Mean arterial blood pressure was measured by tail cuff using the MC4000 Blood Pressure Analysis System (Hatteras Instruments).

4.3.3 Atherosclerotic lesion measurement and characterization

At sacrifice, blood was collected through the abdominal aorta for biochemical analysis and cell count. The vasculature was then perfused and fixed with 4% paraformaldehyde, also at

100mmHg, for 20 minutes. The heart and aortic branch were dissected and placed in 30% sucrose overnight at 4°C. Aortic sinus and BCA segments were embedded vertically in Tissue-tek (Sakura), and serial 7um thick cryo-sections were cut (Leica CM 3050 S, Leica Microsystems). Sections of aortic sinus and BCA were stained with oil red O to assess plaque size. Total collagen content of the plaque was evaluated using the picrosirius red stain kit according to manufacturer's protocol (Polysciences). Smooth muscle cell content of the plaque was evaluated by immunohistochemistry using anti-smc α actin alkaline phosphatase antibody (Sigma-Aldrich) for 1 hour and mounted using Immu-Mount. Plaques were further characterized by immunofluorescence using the following antibodies: anti-collagen I, anti-collagen IV (EMD Millipore), anti-MMP-2, anti-MMP-3, anti-MMP-9 (Santa Cruz), anti-MMP-8 and anti-MMP-14 (Abcam). The sections were incubated with fluorescently labeled secondary antibodies (Invitrogen). Gelatinase plaque activity was evaluated using *in situ* zymography as described previously[105]. Vessel sections were incubated at 37°C for 8 hours with a fluorogenic gelatin substrate (DQ gelatin, Invitrogen) diluted in zymography buffer (50 mmol/L Tris-HCl pH 7.4 and 15 mmol/L $CaCl_2$). Proteolytic activity was detected as green fluorescence. Images were acquired using a Leica microscope. The mean lesion areas in mm^2 for the aortic sinus and the BCA and percent plaque staining were quantified using ImageJ software (National Institute of Health).

4.3.4 Western blotting

Aortas and plasma were collected as above. Total protein (20 ug) was extracted in lysis buffer (50mM NaF, 5mM EGTA, 0.5% Triton 100, 2mM orthovanadate, anti-proteases),

separated by SDS-PAGE, transferred to nitrocellulose membranes and incubated overnight at 4^{o}C with primary antibody against TIMP-1 (Santa Cruz Biotechnologies). After incubation with secondary antibodies, signals were revealed by chemiluminescence (Western Lightning Plus ECL, Perkin Elmer) with the Molecular Imager Chemidoc XRS system (Bio-Rad) and quantified by densitometry. Membranes were subsequently stripped and re-probed with a β-actin antibody (Sigma-Aldrich) or stained with ponceau S to verify equal loading.

4.3.5 *In vitro* co-culture shear stress system

In order to recreate the plaque environment, we developed a co-culture system exposed to shear stress. Macrophages were obtained by flushing the bone marrow of both femurs and tibias of C57BL/6 mice or MMP-9 -/- mice at 2 months of age. Bones were flushed with RPMI 1640 containing 10% fetal bovine serum and cells were differentiated in the presence of mouse M-CSF (50ng/ml, PeproTech) for 7 days. ECs were obtained from the lungs of C57BL/6 mice or MMP-9 -/- mice at 2 months of age. The lungs were excised and diced into 1-mm-sized fragments and lung fragments were digested with 0.1% collagenase A (Roche Diagnostics). The resulting cell suspension was grown in DMEM/F12 + 20% FBS + 1% ECGS (EC media). For 2 successive passages, ECs were isolated using a CD102 antibody (BD Bioscience) coupled to Dynal beads (Invitrogen). ECs were then plated on permeable transwell filters (Corning) and paired with lower transwell dishes containing macrophages. ECs were exposed to a shear stress of 0.5 or 15 dynes/cm^2 using a rotating cone device.

4.3.6 Macrophage function

Macrophage MMP production was assessed by gelatin zymography, as described previously[159]. Twenty μg of macrophage cell lysates were obtained after 24 hour co-culture with ECs at high or low shear stress and loaded on an SDS gel containing gelatin (0.1mg/ml). After electrophoresis, gels were washed in 2.5% triton X-100 and incubated in zymography buffer (50 mmol/L Tris-HCl, pH 7.4, and 15 mmol/L $CaCl_2$) at 37°C for 72 hours. Gels were then stained with Coomassie brilliant blue and bands of lysis quantified by densitometry using Quantity One software (Bio-Rad). Macrophage migratory capacity was evaluated by using the scratch assay. Confluent macrophages in the lower transwell were wounded once with a small tip by scratching across the maximum diameter of each well. Cells were then placed in the cone plate apparatus with the ECs in the upper transwell exposed to high or low shear stress for 24 hours. Pictures were taken immediately after scratching as well as 6 and 24 hours after. Images were analyzed using Image J software by measuring the size of the denuded area. For experiments performed with the MMP inhibitor, GM-6001 (1/1000, Enzo Life Sciences) was added to the co-culture system 30 minutes before application of the shear stress stimulus. In addition, ECs, obtained as above, were grown in culture for 24 hours after which the effluent from the cells or regular macrophage media was placed on macrophages followed by the scratch assay. Media was collected from the ECs and macrophages for zymography studies.

4.3.7 Statistical analysis

Results are expressed as mean±SEM. Data was evaluated by one-way ANOVA for multiple groups followed by the Newman-Keuls post test or a 2-tailed Student *t* test for 2 groups. A probability value of $P<0.05$ was considered to be statistically significant. GraphPad Prism software was used.

4.4 Results

4.4.1 Increased shear stress causes plaque regression

The physiological and hematological parameters in the control, sham and AVF mice were comparable (Supplemental Table 1); likewise there was no difference in HDL, triglycerides and total cholesterol. The only parameter that did change significantly was the increased blood flow (0.6±0.5ml/min in Sham vs. 5.9±0.9ml/min in AVF mice) and consequently shear stress measured in the right common carotid artery of animals (Supplemental Table 1). Plaque size in the aortic sinus increased from 12 weeks (control) to 16 weeks (sham and AVF) of high fat diet (Figure 1B). However, there was a significant reduction of plaque size in the BCA of AVF mice compared not only to the sham, but also to the baseline control (Figure 1A). When quantified relative to aortic sinus lesion area, to account for any variability in lesion progression in the mice, we found an almost 50% plaque reduction in the BCA of AVF mice (0.25±0.03mm^2 in AVF vs. 0.48±0.03 mm^2 in Sham vs. 0.41±0.03 mm^2 in ctrl mice) (Figure 1B). Hence, increased local blood flow leads to plaque regression in atherosclerotic mice.

4.4.2 Increased shear stress does not alter plaque stability but increases gelatinase activity

We have previously shown that creation of an AVF leads to a local increase in vascular MMP activity[105]. We verified whether the same phenomenon occurred in plaques, and indeed we found that gelatinase activity was enhanced in AVF (27.4±1.5%) plaques compared with control (not shown) or Sham (19.9±1.4%) (Figure 2A). In order to assess whether increased MMPs could lead to reduced plaque stability, we compared the plaque content of collagen and smooth muscle cell (Supplemental 1) between the 3 groups of mice. We found no difference in these plaque components. Moreover, we observed a distinct smooth muscle cell cap, an indication of stability, in all groups (Supplemental 1). Therefore, this indicates that perhaps one of the mechanisms in shear stress-induced plaque regression could be increased MMP activity. Despite that the plaque in the AVF mice is smaller; it is just as stable as the control and Sham mice plaques.

4.4.3 Increased shear stress-induced plaque regression is reversed by MMP inhibition

To verify whether MMPs actually contribute to plaque reduction, mice were treated with the general MMP inhibitor doxycycline, added to the drinking water. Plaque gelatinase activity was lowered with doxycycline treatment, as expected (15.3±0.9% in sham mice and 12.1±1.1% in AVF mice) (Figure 2A). Strikingly, doxycycline also prevented plaque regression in the AVF mice (0.39±0.03mm^2 in sham vs. 0.38±0.04mm^2 in AVF) (Figure 2B). We then used a more specific approach to MMP inhibition using TIMP-1 plasmids. A plasmid expressing TIMP-1 was

administered by electroporation in Sham and AVF mice at the time of surgery. At the time of sacrifice, increased TIMP-1 (3.16±0.58 units in sham and 5.42±1.48 units in AVF) was detected in aortas of mice administered the TIMP-1 plasmid, compared with the empty plasmids (1.2±0.12 units in sham and 0.47±0.26 units in AVF) (Figure 3A). As well as increased levels in the plasma (data not shown). TIMP-1 plasmid effectively decreased plaque MMP activity in the AVF mice (11.4±1.0%) compared to the empty plasmid treated animals (17.6±1.6%) (Figure 3B). Similar to the doxycycline treated group, prevention of AVF-associated BCA plaque regression was also evident in the mice treated with the TIMP-1 plasmid (0.31±0.02mm^2 in sham and 0.30±0.01mm^2 in AVF) (Figure 3C). When treated with the empty plasmid, regression occurred in the AVF, as expected (0.32±0.02mm^2 in sham vs. 0.18±0.02mm^2 in AVF) (Figure 3C). No difference in plaque size was observed at the aortic sinus (data not shown). TIMP-3 injection showed a similar trend to TIMP-1, with no decrease in regression in the AVF compared to the sham (0.29±0.02mm^2 in sham and 0.34±0.02mm^2 in AVF) (Supplemental 2).

4.4.4 MMP-9 increased with high shear stress

Increased gelatinase activity in the AVF plaques, and prevention of AVF plaque regression in doxycycline and TIMP-treated animals strongly suggested a major role for MMPs in this context. We therefore investigated which MMPs were expressed in mouse BCA plaques by immunofluorescence. We observed an increase in MMP-9 expression in lesions of the AVF mice (Table 1 & Figure 4). From the MMP-9 expression image, we can see that in the control and sham plaques, the MMP expression is concentrated at the cap and shoulders which is where the plaques are most unstable whereas in the AVF, the expression is located all throughout the

plaque (Figure 4). Similarly, expression of MMP-3, which activates MMP-9[172] was enhanced (Table 1). Protein levels of MMP-2 which is activated by MMP-14 tended to be lower in BCA plaques of AVF mice compared to sham (Table 1). MMP-8 did not differ between groups. Hence, increased MMP-9 in the BCA plaques of AVF mice is likely accountable for the enhanced gelatinase activity and decreased plaque size.

4.4.5 Decreased collagen IV with increased shear stress

Different members of the MMP family have diverse substrates; consequently measuring specific plaque collagen expression was important. Total collagen was not altered (Supplemental 1A) nor was collagen I which is degraded by multiple MMPs (Figure 5B). However, collagen IV, which is degraded by MMP-9 and MMP-3[119], was less expressed in AVF plaques (8.9±1.0%) compared to control (12.5±1.1%) and sham (13.4±1.4%) (Figure 5A).

4.4.6 Increased macrophage migration with increased shear stress

In order to examine, more closely, the role of shear stress on macrophage function, we performed *in vitro* studies. We recreated the plaque environment using a co-culture shear stress system with ECs exposed to the shear stress and the macrophages exposed to the EC effluent. There is more MMP-9 activity in the macrophages in high shear conditions compared to low shear (Figure 6A). When we measured macrophage migration using the scratch assay compared to low shear stress (5 dynes/cm2), we observed that when ECs were exposed to high levels of

shear stress (15 dynes/cm2) the underlying macrophages were more mobile and they had a higher migrated area after 24 hours (0.70±0.13 in high conditions vs. 0.43±0.11 in low conditions) (Figure 6B). This effect was lost when MMPs were inhibited using the general inhibitor, GM6001 (0.30±0.06 in the high conditions and 0.33±0.13 in the low conditions) (Figure 6B). Given that our mouse AVF data point to a predominant role of MMP-9, we performed the same experiments using MMP-9 knockout macrophages and ECs. Much like general MMP inhibition, MMP-9 knockout in the macrophages and ECs was enough to lose the increased migration seen in high shear conditions (Figure 6C). Using either control ECs with knockout macrophages or the inverse, caused an intermediate phenotype (Figure 6C) indicating that MMP-9 coming from both cells are important. The macrophages alone, without the presence of the effluent from the ECs exposed to shear stress, were less migratory than in the co-culture (data not shown) indicating that the shear stress on the ECs is causing the release of a factor contributing to increased mobility. In addition, the presence of EC effluent on the macrophages leads to not only increased mobility but also increased MMP-9 activity in the media compared to regular macrophage media (Supplemental 3) further indicating that MMP-9 is playing a key role in macrophage migration and the importance of the ECs in MMP-9 activity.

4.5 Discussion

The present study demonstrates that shear stress can effectively reverse the atherosclerotic process. Moreover, this regression is possible even in the context of high plasma lipid levels. Our data suggests a mechanism of plaque regression via increased MMP activation.

The key protective MMP involved appears to be MMP-9, targeting collagen IV. Presumably, increased MMP-9 leads to increased macrophage migration and subsequently plaque regression.

Body weight, lipid levels, global inflammatory cell count and other physiological parameters were comparable between groups. This revealed that the surgery did not have a systemic health effect on the AVF animals. In fact, the increased shear stress in our model is a local effect, acting at the site of the BCA only. This was confirmed by measuring the aortic sinus plaques which showed no regression. The sham and AVF mice had a larger sinus plaque compared to the control since the mice were on a HFD for a further 4 weeks. For the same reasons, the shams also had larger BCA plaques than controls. However, BCA plaques in the AVF animals regressed considerably in the 4 week interval following surgery, despite maintenance of a high fat diet. Taking all this into account, we can be confident that the regression seen at the BCA in the AVF is due strictly to the increase in blood flow and not other factors. Inhibition of MMPs using doxycycline, TIMP-1 and TIMP-3 abrogated this effect and regression was lost. TIMP-1 and -3 were used in order to target more specific MMPs leading to the effect seen. TIMP-1 more specifically targets MMP-9 and TIMP-3 targets membrane bound MMPs in addition to MMP-9. Given the similar effect seen by using both overexpressing plasmids further supports the idea of MMP-9 being a key player in plaque regression.

Regression has been shown to be a reasonable goal in both humans and in animal models. The ASTEROID trial showed plaque regression in patients using rosuvastatin[50]. Similarly, the REVERSAL trial using atorvastatin showed reduced plaque size in patients at follow up compared to baseline[49]. In rhesus monkeys, switching from a high fat diet to a normal diet caused smaller lesions[173]. LDLR -/- mice fed a high fat diet injection with apoA-1 significantly reduced plaque size compared to baseline controls[58]. Transplantation of plaque rich aortic arches

from apoE-/- mice fed a HFD, into the abdominal aorta of wild type mice fed a normal chow demonstrated lesion regression[54]. All these studies, however, involved an alteration of lipid levels. Whether plaque regression is feasible without lipid changes is investigated in very few studies. In one such study, rabbits on a HFD, which were administered L-arginine, slowed the progression of plaques compared to control groups[174]. In humans, patients with coronary artery disease showed regression in the presence of high wall shear stress compared to low, however the smaller plaques were less stable[175].

One may argue that although the plaque is smaller in the AVF, it could also be less stable. It is well understood that MMPs are involved in plaque destabilization and rupture especially MMP-1, MMP-8 and MMP-13[128]. Collagen I is not only the main protein found in a plaque, it is also the protein most important for maintaining plaque stability and can be found throughout the plaque with MMP-8 being the most important MMP to degrade collagen I more so than MMP-13 and MMP-1[125]. In addition, MMP-8 has not been shown to affect the development of atherosclerosis[128]. In our mice, plaque total collagen, collagen I and MMP-8 does not differ between our 3 groups indicating that plaque stability is consistent between groups. Furthermore, stability is also assessed by the presence of smooth muscle cells, especially at the cap. Staining for smooth muscle cells, in both the general plaque area and more specifically in the cap, was equivalent in the BCA plaques of control, sham and AVF animals. Thus, in our model, shear stress causes plaque regression with no loss of plaque stability.

Collagen IV, which is found primarily in the basement membrane[115] and in the fibrous cap region[117], was decreased in the AVF group compared to the control and sham groups. This is a very interesting finding since it is known that collagen I is not a substrate of MMP-9 whereas collagen IV is. This further confirms the idea that MMP-9 is in fact upregulated in high shear

conditions which is in turn degrading collagen IV. Furthermore, the location of the MMP-9 expression in the plaque differed between groups. In the control and sham animals, the expression lies in areas that are known to be susceptible to plaque rupture whereas in the AVF it is evenly distributed throughout the plaque perhaps leading to better macrophage motility.

It is known that migration of inflammatory cells is important in plaque regression[65, 74]. Numerous studies have demonstrated a link between MMP activation and cell migration. MMP-9 was shown to be critical for dendritic cell migration from the skin[147]. Moreover, human dendritic cells passing through a matrigel matrix showed less migration with MMP inhibition with MMP-9 being very important[148]. Increased TIMP-1 was shown to be correlated with reduced dendritic cell migration through the ECM[150]. MMP-3 and subsequent MMP-9 inhibition reduced vascular smooth muscle cell (VSMC) migration with the addition of either MMP restoring the migration[176]. In our *in vitro* model that replicates the plaque environment, the effluent of ECs exposed to high shear stress enhanced the migration of macrophages in culture. Once plated, macrophages begin secreting a matrix through which they can move. MMP inhibition abolished macrophage migration in the presence of EC effluent. More specifically, MMP-9 is an important contributor since inhibiting this MMP alone can reverse the increased migration in high shear conditions. Furthermore, MMPs from both the ECs and macrophages are both important for increased macrophage mobility in high shear conditions. Inhibiting MMP-9 from either the EC or the macrophage alone had an intermediate phenotype with each not being enough to completely reverse the increased migration with high shear stress. Macrophages alone showed reduced migration than in co-culture and macrophages with regular media instead of EC effluent had reduced MMP-9 activity in addition to reduced migration. Therefore, ECs and the factors they release are important for migration.

What is the link between increased shear stress and MMP activation? It is known that shear stress induces the release of NO and increases vascular MMP activity[105, 114]. Rabbits treated with a NO inhibitor showed reduced MMP activity indicating that NO has the ability to increase MMP activity[114]. Similarly, eNOS knockout mice prevented vascular increase in MMP activity in the AVF model[160]. Therefore, we propose that increased shear stress induced increase in MMP activity is an indirect effect from the increased NO. In fact, we already showed that inhibiting eNOS in our AVF mouse model reverses the regression, similarly to MMP inhibition.

MMPs are composed of a large family with members performing redundant but also different functions. In the development and progression of a plaque, the type of MMP, the time of activity and the location of activity will all dictate whether the MMP is acting in a protective or detrimental fashion and both situations have been reported in the literature. MMP-3 and apoE double knockout mice developed larger plaques compared to the control mice indicating a protective role for MMP-3[130, 171]. The same observation was seen with MMP-9 and apoE double knockout studies[123, 171] whereas TIMP-1 deficient mice showed reduced plaque size[135]. Double LDLR and TIMP-3 knockout mice reduces atherosclerotic lesion size[140]. Interestingly, in the population, a MMP-3 polymorphism exists with lower promoter activity. Coronary atherosclerosis patients with this polymorphism show increased disease progression[131, 177]. MMP-2 and -14 expression was reduced in the AVF mouse. Rabbit foam cell macrophages with increased MMP-14 protein represent an interesting population which have properties similar to the pro-inflammatory, damaging macrophages that are involved in plaque progression[141]. MMP-2 and apoE double knockout mice demonstrated reduced plaque size revealing that this MMP leads to plaque development[178]. However, results in MMP studies have been controversial. Other studies have shown MMP-9 to be detrimental in plaque growth with knockout animals having

significantly smaller plaques[123]. These studies all look at plaque development whereas our study looks at plaque regression where the effect of MMP inhibition has not been studied. Thus, the combined contribution of increased protective MMPs (MMP-3 and -9) and the reduced detrimental MMPs (MMP-2 and -14) may be implicated in the observed regression. This indicates specific potential targets in order to find an alternative therapy for atherosclerotic regression in addition to the current statins. MMP inhibitors have failed in clinical trials given their diverse roles in healing and disease[110]. A better strategy of inhibiting only specific MMPs has not been utilized given that MMPs have a similar structure making specific targeting difficult.

In summary, this is the first study using a mouse carotid fistula model to investigate the molecular mechanisms of shear stress-induced atherosclerotic plaque regression. Shear stress acting on ECs may influence the cells within the plaque by increasing MMP expression allowing for better macrophage mobility, a hallmark of plaque regression. These results and future work will help us further identify the molecular mechanisms that cause the atheroprotective effects of shear stress, in hopes of identifying new therapeutic targets for the treatment of atherosclerosis and cardiovascular diseases.

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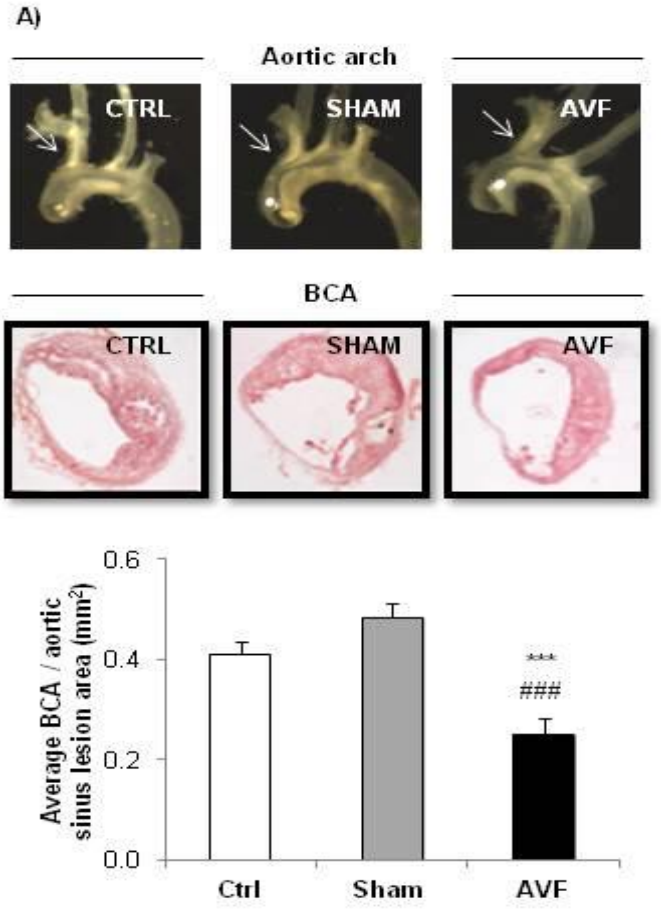
Disclosures

None


A)
Aortic arch
CTRL
SHAM
AVF
BCA
CTRL
SHAM
AVF
Average BCA / aortic sinus lesion area (mm²)
0.6
0.4
0.2
0.0
***
###
Ctrl
Sham
AVF

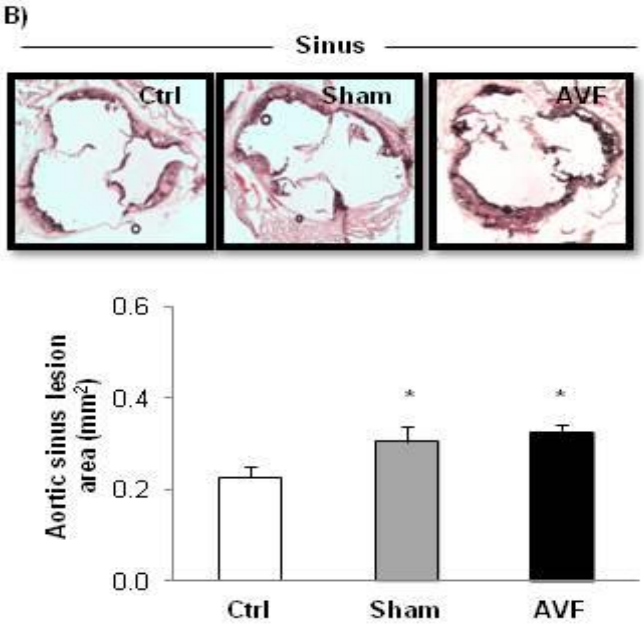
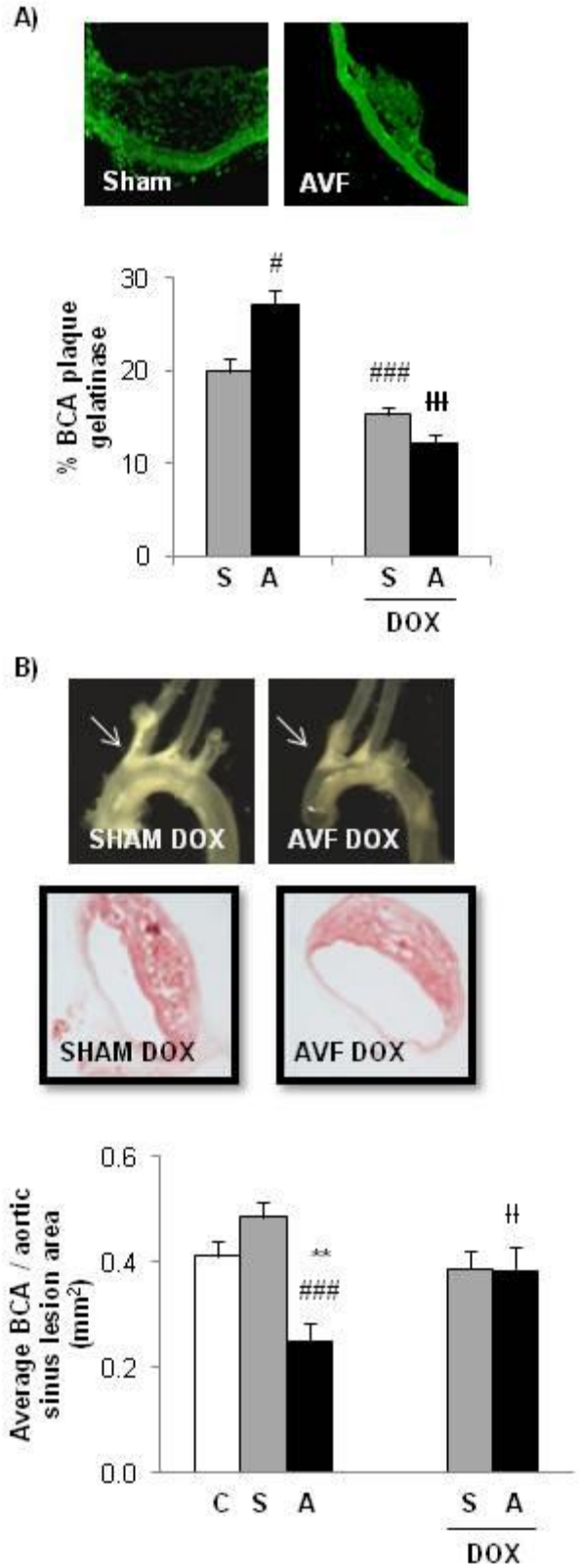

B)
Sinus
Ctrl
Sham
AVF
Aortic sinus lesion area (mm²)
0.6
0.4
0.2
0.0
*
*
Ctrl
Sham
AVF

Figure 4.1: Increased shear stress in the BCA causes plaque regression A) Whole artery preparations with arrow pointing to BCA plaque in white, visible by transparency in control (Ctrl), Sham and AVF mice. Oil red O staining in cross section of the BCA. Quantification of average BCA lesion area relative to sinus. **B)** . Oil red O staining in cross section of the aortic sinus. Quantification of average aortic sinus lesion area. Data are mean± SEM of n=7-8, *P<0.05 vs. Ctrl, ***P<0.001 vs. Ctrl and ###P<0.001 vs. Sham.


A)
Sham
AVF
% BCA plaque gelatinase
30
20
10
0
#
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S A
S A
DOX
B)
SHAM DOX
AVF DOX
SHAM DOX
AVF DOX
Average BCA / aortic sinus lesion area (mm²)
0.6
0.4
0.2
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###
ǂ
C S A
S A
DOX

Figure 4.2: Increased shear stress-induced plaque regression is reversed by MMP inhibition by doxycycline A) Green immunofluorescence of plaque gelatinase activity in BCA of Sham and AVF mice. Quantification of % BCA plaque gelatinase activity with or without MMP inhibition using doxycycline (DOX) in Sham (S) vs. AVF (A) mice. **B)** Doxycycline treated mice whole artery preparations with arrow pointing to BCA plaque in white, visible by transparency. Oil red O staining in cross section of the BCA. Quantification of average BCA / aortic sinus lesion area in Sham vs. AVF mice treated with DOX. Data are mean± SEM of n=7-8, #P<0.05 vs. Sham, ###P<0.001 vs. Sham, ǂǂP<0.001 vs. AVF, **P<0.01 vs. Ctrl and ǂP<0.01 vs. AVF.

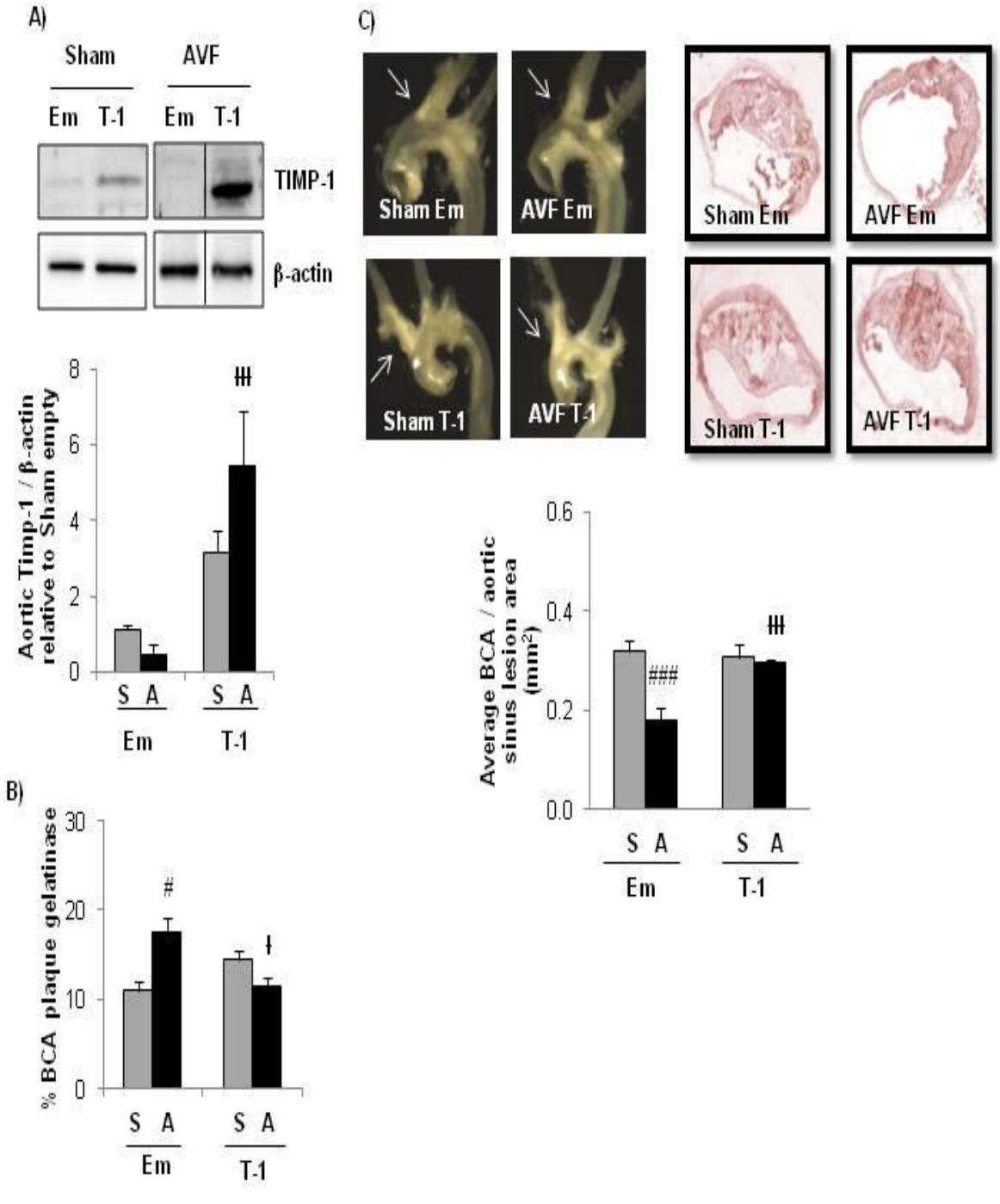

A)
Sham
AVF
Em
T-1
Em
T-1
TIMP-1
β-actin
Aortic Timp-1 / β-actin relative to Sham empty
8
6
4
2
0
###
S
A
S
A
Em
T-1
B)
% BCA plaque gelatinase
30
20
10
0
#
ǂ
S
A
S
A
Em
T-1
C)
Sham Em
AVF Em
Sham T-1
AVF T-1
Sham Em
AVF Em
Sham T-1
AVF T-1
Average BCA / aortic sinus lesion area (mm²)
0.6
0.4
0.2
0.0
###
###
S
A
S
A
Em
T-1

Figure 4.3: Increased shear stress-induced plaque regression is reversed by MMP inhibition with TIMP-1 plasmids. **A)** Increased TIMP-1 protein with plasmid injection. Representative western blot of aortic TIMP-1 and β-actin expression in Sham (S) and AVF (A) injected with either the empty (Em) or the TIMP-1 (T-1) plasmid. Quantification of TIMP-1/β-actin expression relative to sham injected with empty plasmid. Data are mean± SEM of n=3-5, ǂǂP<0.001 vs. AVF empty. **B)** Quantification of % BCA plaque gelatinase activity with MMP inhibition using plasmid injection (Empty or TIMP-1). Data are mean± SEM of n=3-5, #P<0.05 vs. Sham empty and ǂP<0.05 vs. AVF empty. **C)** Plasmid injected mice whole artery preparations with arrow pointing to BCA plaque in white, visible by transparency. Oil red O staining in cross section of the BCA. Quantification of BCA /aortic sinus lesion area in Sham vs. AVF mice injected with empty or TIMP-1 plasmid. Data are mean± SEM of n=7-8, ###P<0.001 vs. Sham empty and ǂǂP<0.001 vs. AVF empty.

MMP	Ctrl	Sham	AVF
3	5.0 ± 0.8	6.0 ± 0.6	9.2 ± 1.2 *#
9	3.9 ± 0.7	4.6 ± 1.1	10.8 ± 1.4 *** ###
14	9.5 ± 1.6	13.9 ± 1.4 *†	7.8 ± 1.1
2	6.7 ± 1.3	11.3 ± 1.6 *	5.7 ± 0.5
8	6.5 ± 0.8	8.2 ± 0.8	6.1 ± 1.2

Table 4.1: MMP-3 and -9 expression is increased with increased shear stress. Percent MMP-3, -9, -14, -2 and -8 protein expression levels in the BCA plaque of Control (Ctrl), Sham and AVF mice. Data are mean± SEM of n=5-7, *P<0.05 and ***P<0.001 vs. Ctrl, #P<0.05 and ###P<0.001 vs. Sham, and ŀP<0.05 vs. AVF.

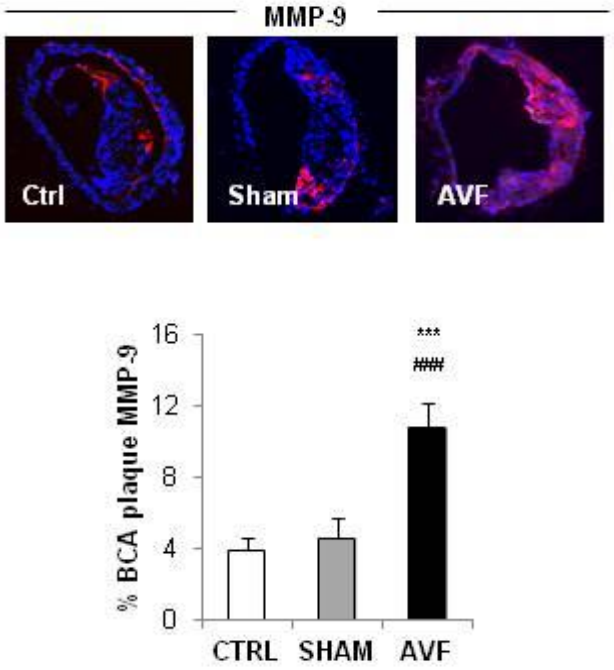


Figure 4.4: MMP-9 expression is increased with increased shear stress. Red immunofluorescence of plaque MMP-9 protein expression in BCA in Ctrl (C), Sham (S) and AVF (A) mice. Cell nuclei in blue. Data are mean± SEM of n=5, ***P<0.001 vs. Ctrl, ###P<0.001 vs. Sham.

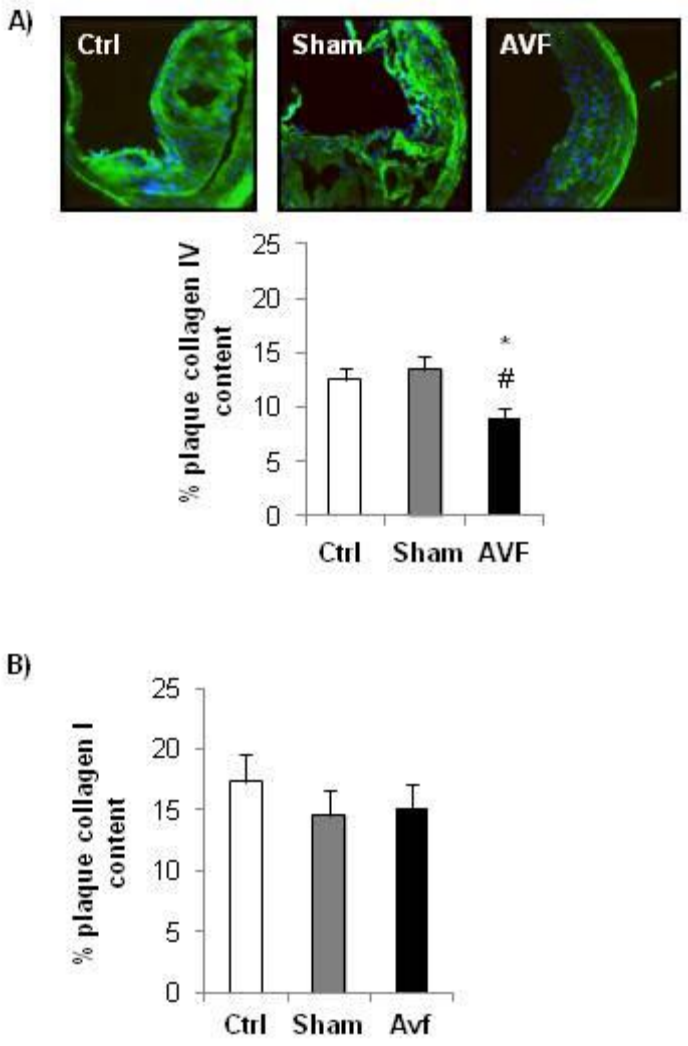


Figure 4.5: Collagen IV expression is decreased with increased shear stress. Lesion collagen IV content (green immunostaining) with cell nuclei in blue **(A)** and collagen I **(B)** were quantified in the BCA plaque of control (Ctrl), Sham and AVF mice. Data are mean± SEM of n=5-9, *P<0.05 vs. Ctrl and #P<0.05 vs. Sham.

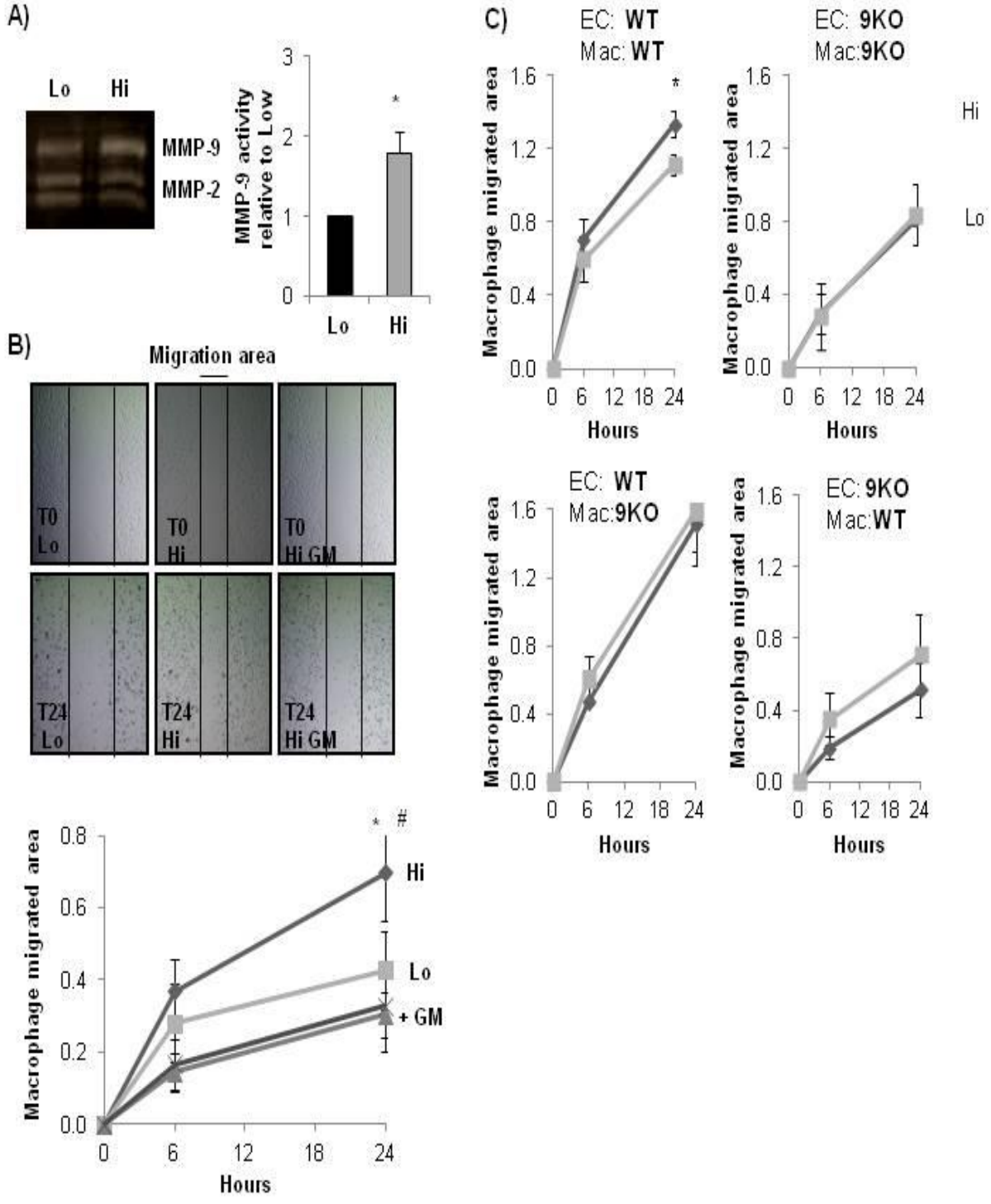
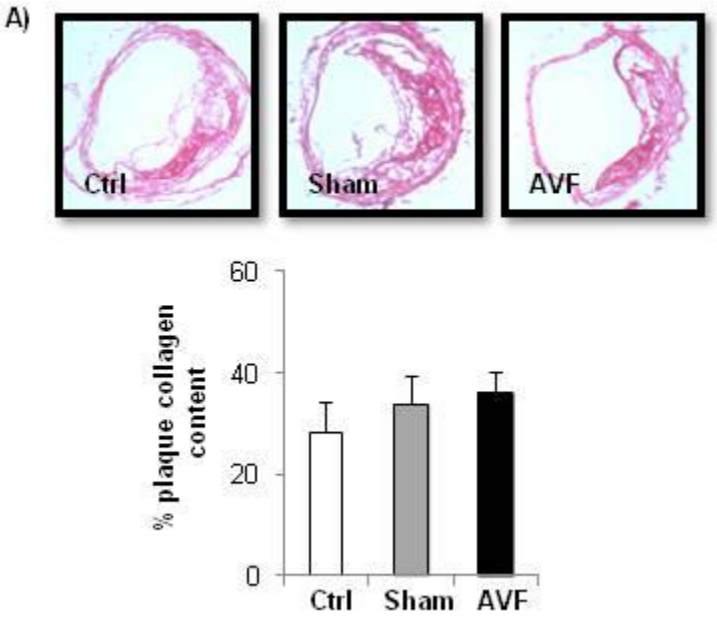

A)
Lo
Hi
MMP-9
MMP-2
MMP-9 activity relative to Low
B)
Migration area
T0 Lo
T0 Hi
T0 Hi GM
T24 Lo
T24 Hi
T24 Hi GM
Macrophage migrated area
Hours
Hi
Lo
+ GM
C)
EC: WT
Mac: WT
EC: 9KO
Mac:9KO
EC: WT
Mac:9KO
EC: 9KO
Mac:WT

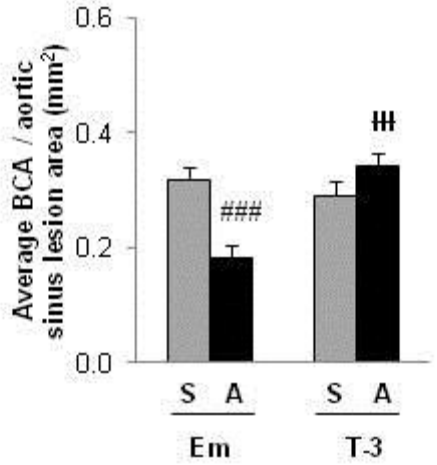
Figure 4.6: Macrophage migration is increased with increased shear stress through MMP-9. **A)** Representative zymography gel measuring MMP-9 and MMP-2 activity in macrophage cell lysates in low (Lo) vs. high (Hi) shear conditions after 24 hours. Quantification graph of MMP-9 activity in high shear stress relative to low shear stress. **B)** Scratch assay showing macrophage migration into the empty space (migration area) at time point 0 (T0) and after 24 hours (T24) in low vs. high shear conditions in the presence or absence of MMP inhibition using GM6001 (GM). Quantification of macrophage migrated area in high vs. low conditions with or without GM. **C)** Quantification of macrophage migrated area after scratch assay in high vs. low conditions in control conditions and using MMP-9 knockout (9KO) endothelial cells (EC) and macrophages (Mac) or wild type (WT) cells. Data are mean± SEM of n=4-8, *P<0.05 vs. Lo, #P<0.05 vs. Hi GM.

Parameter	Ctrl	Sham	AVF
body weight (g) - wk 12	39.3 ± 0.8	30.6 ± 1.8	32.4 ± 0.4
body weight (g) - wk 16	-	33.1 ± 2.2	32.5 ± 3.7
blood pressure (mmHg)	96 ± 4	100 ± 5	100 ± 5
RCC flow (ml/min)	-	**0.6 ± 0.5**	**5.9 ± 0.9 ###**
total cholesterol (mmol/L)	27.5 ± 6.0	28.6 ± 9.7	35.1 ± 5.6
HDL cholesterol (mmol/L)	6.9 ± 0.6	6.1 ± 1.6	6.2 ± 1.5
triglycerides (mmol/L)	2.3 ± 0.7	2.5 ± 0.8	3.4 ± 0.7
WBC (10^3/mm^3)	1.2 ± 0.3	1.5 ± 0.5	1.7 ± 0.3
LYM (10^3/mm^3)	0.8 ± 0.1	0.7 ± 0.2	1.1 ± 0.1
MON (10^3/mm^3)	0.10 ± 0.06	0.13 ± 0.03	0.15 ± 0.03
GRA (10^3/mm^3)	0.6 ± 0.2	1.3 ± 0.6	0.8 ± 0.2
RBC (10^6/mm^3)	8.3 ± 0.4	7.4 ± 0.8	8.2 ± 0.8
HCT (%)	38.2 ± 1.9	33.6 ± 3.6	38.6 ± 3.8
HGB (g/dl)	12.8 ± 0.7	12.0 ± 1.3	13.2 ± 1.4
PLT (10^3/mm^3)	738 ± 132	812 ± 252	851 ± 85

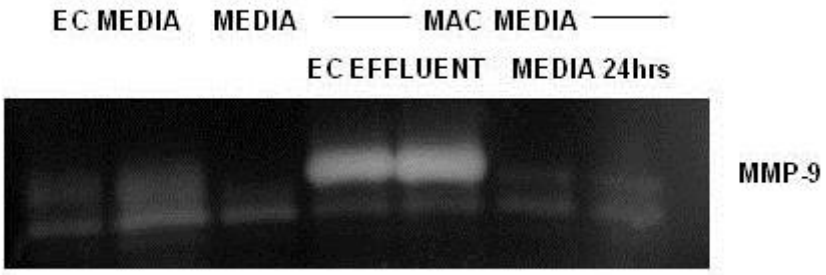
Supplemental Table 4.1: Shear stress is the only changed parameter between groups. Blood parameters of control (Ctrl), Sham and AVF mice. R = right common carotid flow, WBC = white blood cells, LYM = lymphocytes, MON = monocytes, GRA = granulocytes, HCT = hematocrit, HGB = hemoglobin, PLT = platelets. n=4-11, ###P<0.001 vs. Sham.


A)
Ctrl
Sham
AVF
% plaque collagen content
60
40
20
0
Ctrl
Sham
AVF
B)
Ctrl
Sham
AVF
% plaque SMC actin area
8
6
4
2
0
Ctrl
Sham
AVF

Supplemental 4.1: Increased shear stress does not alter plaque stability. Lesion collagen content (Sirius red staining) **(A)** and smooth muscle cell (SMC) content (alpha-actin immunohistochemistry purple staining) **(B)** in BCA plaque of control (Ctrl), Sham and AVF mice. n=5-6.



Supplemental 4.2: Increased shear stress-induced plaque regression is reversed by TIMP-3 overexpression. Quantification of average BCA/aortic sinus lesion area in Sham vs. AVF mice injected with TIMP-3 (T-3) plasmid. n=7-9, ###P<0.001 vs. Sham empty and ǂǂP<0.001 vs. AVF empty.



Supplemental 4.3: Increased MMP-9 activity in the presence of endothelial cells.

Representative zymography gel measuring MMP-9 activity in the media of various conditions. EC MEDIA = media from endothelial cells after 24 hours in culture. MEDIA = cell media from bottle. EC EFFLUENT = media from macrophages after 24hrs of the scratch assay exposed to endothelial cell media. MEDIA 24hrs = media from macrophages after 24hrs of the scratch assay exposed to regular cell media.

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CHAPTER V: EFFEROCYTOSIS & INFLAMMATION

5.1 Hypothesis, Objectives and Introduction

We have shown that shear stress induced plaque regression is caused by directly increasing NO activity which in turn increases MMP activity, specifically MMP-9, which degrades collagen IV and allows for better macrophage mobility. Therefore, targeting MMP-9 can be a new strategy in decreasing atherosclerosis burden.

Key questions still remain to be investigated, however. Besides emigration, effective efferocytosis is also vital in plaque regression. Given that necrotic core size decreases with increased shear stress, it is possible that this effect occurs via increased phagocytosis. MMPs, perhaps through degradation of plaque ECM matrix, allow macrophages to become untrapped, reach cellular debris target and phagocytose it.

Moreover, the role of specific immune cells in plaque regression is an area yet to be fully explored. The exact subtype of inflammatory cells found in a regressing plaque and the cytokine environment is not fully known. Studying the inflammatory composition of a plaque is difficult given the small amount of tissue to work with and the lack of proper specific immunofluorescence markers in regards to M1 and M2 macrophages. Newer methods of analysis are required.

In this chapter, we look at these important questions by measuring efferocytosis with increased shear stress with or without MMP inhibition and by beginning a preliminary analysis of the inflammatory milieu as a future focus of study to further explain the mechanism of regression.

We hypothesize that: Shear stress causes increased efferocytosis and that shear stress patterns will alter the inflammatory environment.

Specific objectives:

- To examine the effect of macrophage efferocytosis in an *in vitro* shear stress system.
- To examine the inflammatory environment *in vitro* with increased shear stress.

5.2 Methods

5.2.1 *In vitro* macrophage function

Macrophage phagocytic capacity was evaluated using an efferocytosis assay. RAW macrophages were kept in culture for 5 days before the assay start point. The cells were then scraped and resuspended in macrophage media containing orange cell tracker to fluorescently label macrophages (10 μM, Invitrogen, Eugene, OR), incubated at 37 ºC for 30 minutes and then washed and resuspended in fresh macrophage media and placed under the UV light for 30 minutes and incubated at 37 ºC overnight to induce apoptosis. Macrophages were then taken from the co-culture system exposed to no or high shear stress and apoptotic cells (ratio 5:1) were added and incubated again at 37 ºC for 30 minutes. The cells were washed and fixed with 2% paraformaldehyde for 15 minutes at 37 ºC and stained with dapi. Fluorescent pictures were taken using Leica microscope and the number of phagocytic cells was counted.

5.2.2 *In vitro* cytokine expression

To assess the effect of shear stress on cytokine production, we used the same *in vitro* co-culture shear stress system as previously described. After 24 hours exposed to no or high shear stress conditions, the media was used on a mouse cytokine array panel (R&D System, Minneapolis, MN) following instructed protocol. Spots of expression were revealed on the membrane and compared between conditions.

To confirm the expression of cytokines of interest from the cytokine array, total RNA of macrophages and ECs after 24 hours of the co-culture shear stress system in either static or high shear conditions was extracted using a total RNA mini kit (Geneaid Biotech, New Taipei City, Taiwan) following instructed protocol. RNA was quantified using a spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific, Waltham, MA). cDNA was synthesized from 0.4 μg RNA by using qScript cDNA Supermix kit (Quanta Biosciences, Gaithersburg, MD). The expression level of CCL5 and CXCL12 was assessed by quantitative real time polymerase chain reaction (RT-qPCR) using 7500 Fast PCR (Applied Biosystems, Foster City, CA) under standard conditions of 60°C annealing temperature for 40 cycles. All primers were designed using Primer 3 Plus software and SYBR green chemistry (SensiFAST SYBR Lo-ROX kit, Bioline, Humber Road, London) was used with specific primers for all genes. Results were analyzed using the ΔΔCt method as calibrator samples. The analyzed genes were expressed relative to the murine TATA-binding protein (TBP) housekeeping gene.

5.2.3 *In vitro* activated integrins

Presence of activated β1 and β2 integrins on the macrophages were assessed using human cells obtained as follows. Human peripheral blood mononuclear cells were isolated from blood of healthy donors (age 20-45 years, male and female, no known disease or medication) using a Ficoll density gradient (Sigma-Aldrich, St. Louis, MO). Cells were plated in macrophage media in a 37^{o}C incubator for 2 hours, washed and differentiated to macrophages over 7 days using human M-CSF (50ng/ml, R&D Systems, Minneapolis, MN). Human macrophages were used along with human umbilical vein endothelial cells (HUVECs) after 24 hours in the shear stress

co-culture system, with or without MMP inhibition, in low or high shear stress conditions. Then the cells were scraped, collected, centrifuged, and resuspended in FACS buffer (2% FBS in PBS) and stained for the surface markers, activated β1 integrin (1/400, Millipore, Darmstadt, Germany) and activated β2 integrin (1/100, Hycult Biotech, Uden, Netherlands). The activation of integrins was quantified using flow cytometry (BD LSR Fortessa, BD Biosciences, Mississauga, Canada) and analyzed using Flow Jo software (Tree Star, Ashland, OR). Fluorescence minus one control was used to remove the fluorescent background.

5.2.4 *In vitro* activated T cells

Using a different shear stress apparatus which involves cells in suspension and a pump to create the shear stress, we looked at immune cells other than macrophages. Whole spleen cells were extracted and added to the system for 24 hours in static, low or high shear conditions. Cells were collected from the shear stress apparatus and stained for the surface markers CD4 and CD69 (eBioscience, San Diego, CA). Flow cytometry was performed on the BD LSR Fortessa (BD Biosciences, Mississauga, Canada). Fluorescence minus one control was used to remove the fluorescent background. Data analysis was performed using Flow Jo software (Tree Star, Ashland, OR.).

5.2.5 Statistical analysis

Results are expressed as mean±SEM. Data was evaluated by one-way ANOVA for multiple groups followed by the Newman-Keuls post test or a 2-tailed Student *t* test for 2 groups. A probability value of $P<0.05$ was considered to be statistically significant. GraphPad Prism software was used.

5.3 Results

5.3.1 Increased shear stress causes increased efferocytosis

In order to examine, more closely, the role of shear stress on macrophage function, we performed *in vitro* studies. We recreated the plaque environment using a co-culture shear stress system with ECs exposed to the shear stress and the macrophages exposed to the EC effluent. Labelled macrophages were rendered apoptotic and incubated together with healthy macrophages that were exposed to high or low shear stress. Phagocytosis is seen as the red labelled apoptotic macrophage within the blue healthy macrophage. Macrophages exposed to high shear stress are more phagocytic relative to static conditions set at 1 (1.57±0.17%) (Figure 4.1). In order to understand the mechanism, we performed the same experiment using MMP inhibition and this showed the same effect on efferocytosis as compared to no MMP inhibition (data not shown). Therefore, while shear stress is playing a role in efferocytosis, the mechanism is unknown and requires further investigation. We hypothesized that the effect of shear stress on efferocytosis may be occurring via a specific cytokine environment inducing the efferocytosis.

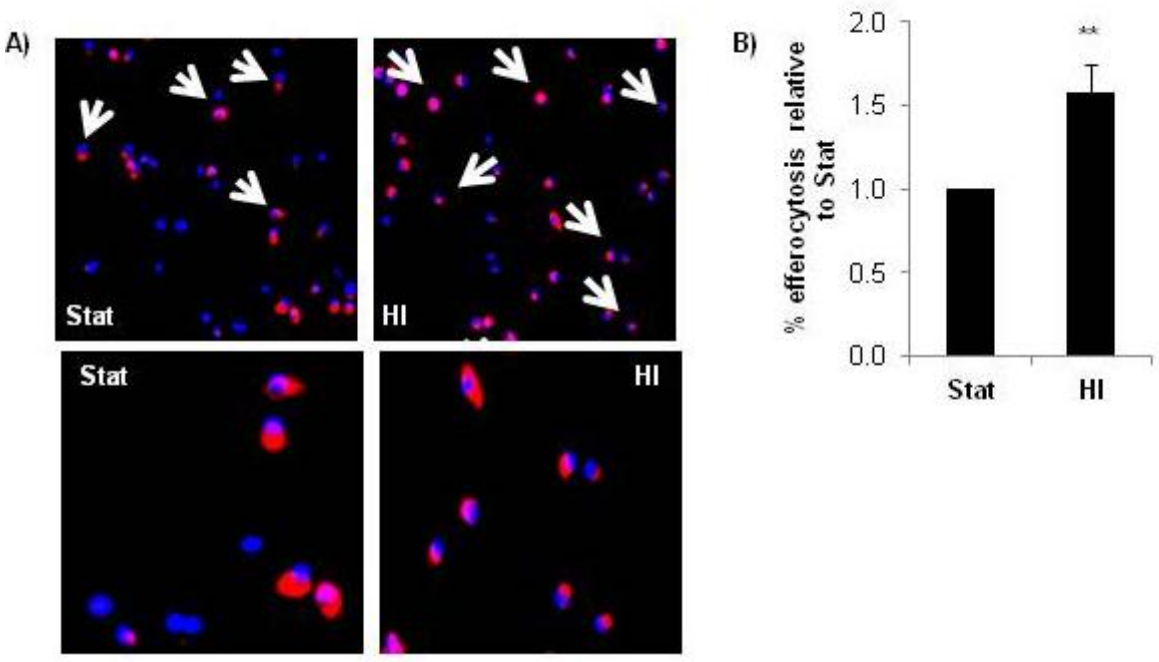

Figure 5.1: Macrophage efferocytosis is increased with increased shear stress A) Representative image of an efferocytosis assay after 24 hours in the shear stress system showing red apoptotic cells merged with blue phagocytic macrophages. Arrows point to positive efferocytosis. Bottom panels are a zoomed in view of the top panels. **B)** Quantification of percent efferocytosis in high shear conditions (HI) relative to static conditions (Stat). Data are mean± SEM of n=7, **P<0.01 vs. Stat.

5.3.2 Increased shear stress causes increased CCL5 expression

In order to get insight on the potential mechanism of increased efferocytosis, we performed a cytokine array of the media after 24 hours of shear stress. The array measures the

density of expression of multiple cytokines from the EC effluent in static versus high shear stress conditions. Some cytokines were either increased or decreased compared to our static conditions which were set as a density of 1 (Figure 4.2 A & B). Of interest to us was CCL5 due to its potential role in efferocytosis[179] and due to its change of expression between static and high shear conditions. The expression of this gene was confirmed by qPCR. In fact, CCL5 was increased in high shear stress relative to static conditions set at 1, only in macrophages (1.7±0.2) and not in ECs (Figure 4.2 C). CXCL12 expression was shown to be increased in the array. However, when expression was confirmed by qPCR, it was in fact decreased in high shear conditions in both macrophages (0.14±0.04) and ECs (0.5±0.2) relative to static conditions set at 1 (Figure 4.2 C). Only those cytokines that had the highest change in expression were confirmed by qPCR.

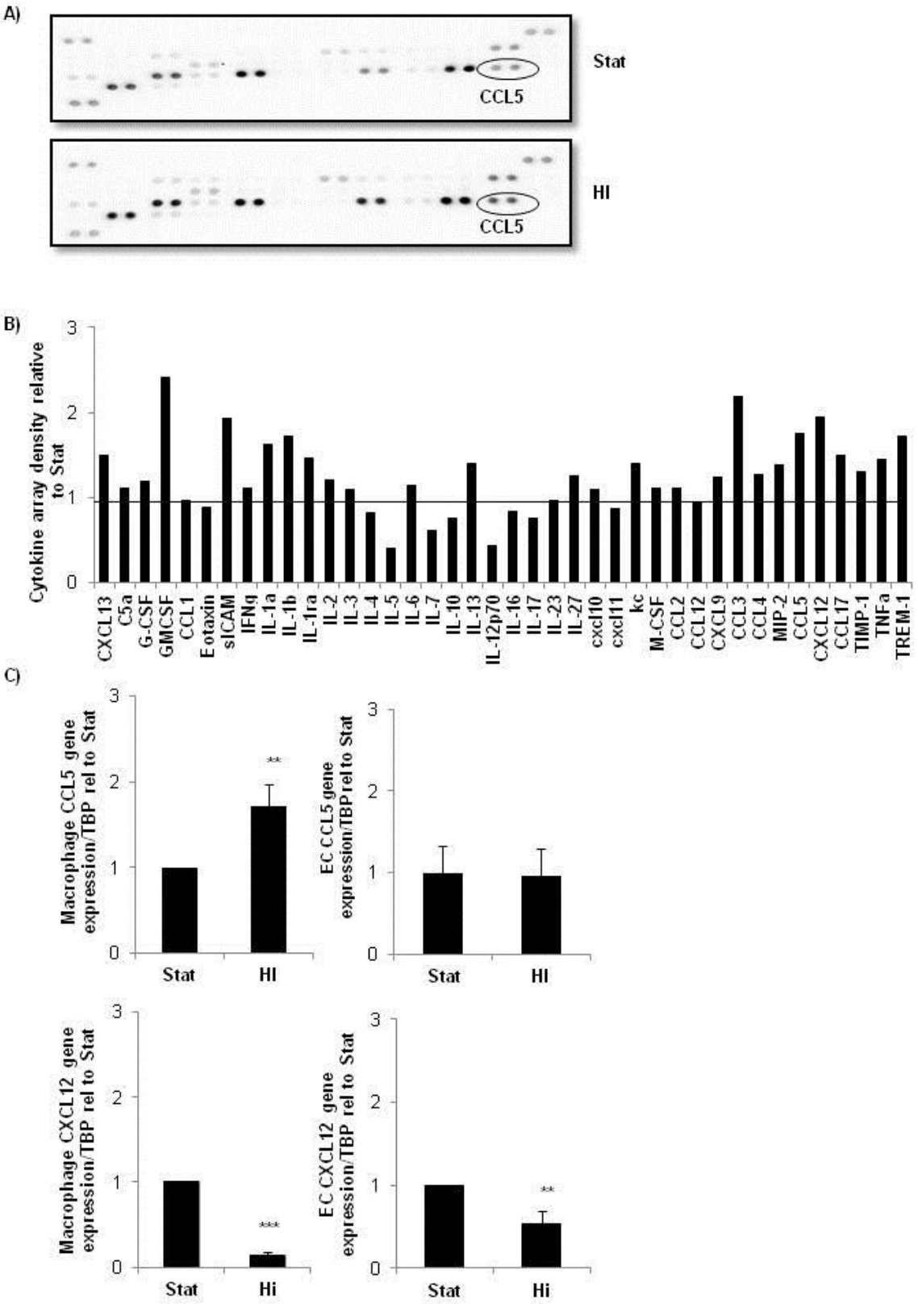
A)
Stat
CCL5
HI
CCL5
B)
Cytokine array density relative to Stat
0
1
2
3
CXCL13
C5a
G-CSF
GMCSF
CCL1
Eotaxin
sICAM
IFNg
IL-1a
IL-1b
IL-1ra
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-10
IL-13
IL-12p70
IL-16
IL-17
IL-23
IL-27
cxcl10
cxcl11
kc
M-CSF
CCL2
CCL12
CXCL9
CCL3
CCL4
MIP-2
CCL5
CXCL12
CCL17
TIMP-1
TNFa
TREM-1
C)
Macrophage CCL5 gene expression/TBP rel to Stat
**
Stat
HI
EC CCL5 gene expression/TBP rel to Stat
Stat
HI
Macrophage CXCL12 gene expression/TBP rel to Stat
***
Stat
Hi
EC CXCL12 gene expression/TBP rel to Stat
**
Stat
Hi

Figure 5.2: Increased CCL5 and decreased CXCL12 expression with increased shear stress

A) Representative image of a cytokine array after 24 hours in shear stress system showing increased CCL5 expression dot in static conditions (Stat) vs. high shear stress (HI). **B)** Quantification of cytokine array in HI vs. Stat at a value of 1. **C)** Expression of CCL5 and CXCL12 in Stat and HI in both macrophages and ECs. Data are mean± SEM of n=5-7, **P<0.01 and ***P<0.0001 vs. Stat.

5.3.3 Increased shear stress causes decreased activity of β2 integrins

Integrins play an important role in migration. Human mononuclear cells were obtained from healthy volunteers, differentiated into macrophages and co-cultured with HUVECs. Analysis via FACS was used to measure the macrophage percentage with the activated form of the integrin. We show that shear stress has the ability to reduce β2 integrins relative to low shear conditions set at 1 (0.49±0.15%) while having no effect on β1 integrins (Figure 4.3 A & B). β2 integrin is known to be pro-atherogenic due to its adhesion properties[40]. The low numbers are normal as this experiment is measuring the activated form of the integrin. When we inhibited MMPs using GM-6001, we saw the same results as with no MMP inhibition (data not shown). Thus, MMPs do not play a role in shear-induced β2 integrin differences.

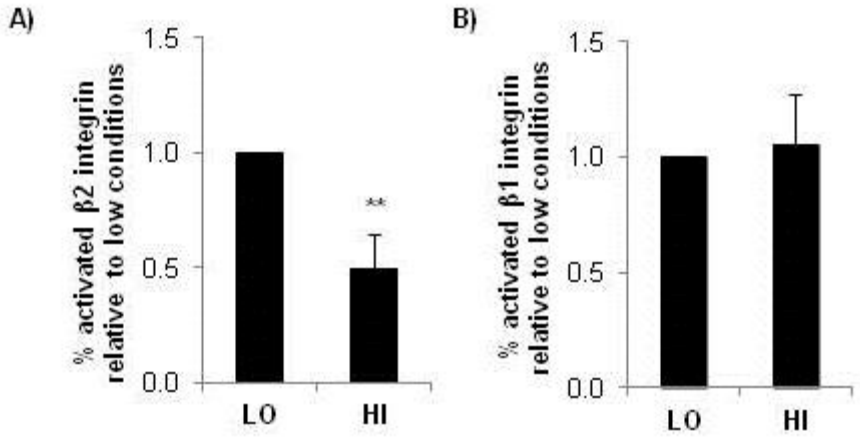

Figure 5.3: Decreased activated β2 integrin with increased shear stress A) Quantification of percent activated β2 integrin and **B)** Quantification of percent activated β1 integrin in high (HI) shear conditions relative to low (LO) shear**.** Data are mean± SEM of n=8-9, **P<0.01 vs. LO.

5.3.4 Increased shear stress increases activated CD4+ cells

Much of our focus has been on the macrophages, yet the effects of shear stress on other immune cells is not known. Besides macrophages, T cells are another important cell in plaque development. These 2 cells work closely together linking the adaptive and innate immune system. Understanding the entire immune cell composition of a regressing plaque would further evolve the field of atherosclerosis. We used a different shear stress, *in vitro* system allowing us to use all cells of the spleen. Spleen cells were isolated and placed in a compartment which allowed the flow of media to pass through allowing the cells to be exposed to different levels of

shear stress. Percentage of various types of immune cells were analyzed via FACS. We show that high shear condition not only increased CD4+ cell percentage but also increased activated CD4+ cells (40.3±5.0% and 5.8±0.8% respectively) compared to the conditions before shear stress (18.9±0.9% and 1.5±0.2% respectively), static (26.8±6.8% and 1.3±0.2% respectively) and low shear (26.6±2.5% and 3.1±0.7% respectively) conditions (Figure 4.4 A & B). These activated CD4 cells are required for M2 activation[180], indicating a potential link between high shear stress and this wound healing macrophage subtype.

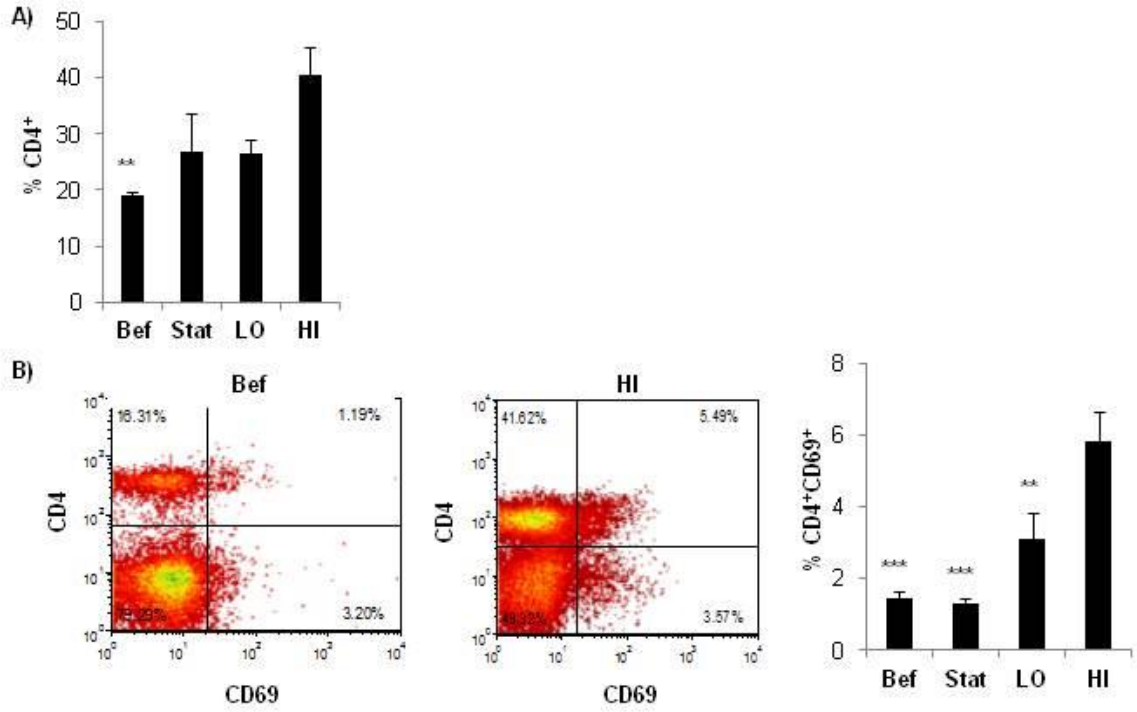

Figure 5.4: Increased activated CD4+ cells with increased shear stress A) Quantification of % CD4+ cells and **B)** Representative FACS image and quantification of % CD4+CD69+ cell from spleen cells before being put in the shear stress system (Bef), in static conditions (Stat), in low shear stress conditions (LO) and in high shear stress conditions (HI). Data are mean± SEM of n=4-8, **P<0.01 and ***P<0.001 vs. HI.

5.4 Discussion

We showed that increased shear stress causes increased efferocytosis through a MMP independent mechanism. This increased efferocytosis can explain the reduced necrotic core seen in the AVF mice. The exact link between shear stress and increased efferocytosis is unknown and requires further investigation. We hypothesized that perhaps shear stress may lead to a specific cytokine environment which may explain the more efficient efferocytosis. Using a cytokine array we demonstrated that CCL5 is increased specifically in the macrophages and not the ECs. These results indicate that the high shear stress is causing the release of a factor from the ECs leading to increased CCL5 expression from the macrophage.

Chemokines are divided into 4 families based on the position of the conserved cysteine residue (C, CC, CXC, CX3C) with CC chemokines mostly regulating leukocyte trafficking and CXC attracting granulocytes[181]. Chemokines have been shown to mainly be involved in plaque growth by attracting monocytes[182]. Mice with bone marrow deficient in CCR5 (the receptor for CCL5), transplanted into lethally irradiated mice, had 30% less macrophage number in the plaque and reduced lesion size[183]. However, it was also shown that atypical chemokine receptor D6 plays an important role in macrophage inflammatory clearance[179]. D6 is a chemokine receptor that binds inflammatory chemokines and degrades them. D6 deficient mice have increased macrophages in a peritonitis mouse model, have higher phagocytic ability and secrete more CCL5. Therefore, CCL5 may be involved in increased efferocytosis. There are still unanswered questions regarding efferocytosis. Does NO play a role in efferocytosis? Will targeting CCL5 affect efferocytosis in our system? Does the inflammatory process play an

important role in efferocytosis? This will all be further explored as better ways to detect immune components in the plaque are developed.

CXCL12 expression was increased in high shear conditions in the cytokine array. However, it is not the most sensitive measurement and had to be confirmed. This cytokine is in fact decreased in high shear conditions. This chemokine is not affected by MMP inhibition. CXCL12 is inflammatory due to it being highly chemotactic for monocytes. Given that high shear stress is known to secrete protective, anti-inflammatory factors, it is not surprising that this inflammatory chemokine would be decreased in both the ECs and macrophages.

β2 integrin is known to be pro-atherogenic due to it being an adhesive molecule for blood monocytes. Studies in mice revealed that overexpression of this integrin leads to atherosclerotic progression compared to KO mice[40]. The decreased activation of this integrin with high shear stress further points to the anti-atherosclerotic nature of high shear stress. In addition, the reduced adhesive integrin in high shear conditions could help explain the increased migration seen with high shear stress. Although this increased migration via β2 cannot be explained via MMP production.

We also showed that high shear stress leads to increased CD69+CD4+ activated T cells. CD4+ T cells are important in regulating macrophage polarization. Macrophages from CD4-/- mice display an exaggerated M1 profile[180] suggesting that activated CD4+ T cells play a dominant role in providing the cytokine environment for regulating macrophages towards an M2 profile. This further leads to more questions regarding the link between shear stress and macrophage polarization. Does increased shear stress change the M1/M2 ratio? Does high shear stress lead to more M2 macrophage infiltration, more M1 macrophage exit or a switch from M1

to M2 cells in the plaque? Further exploring the plaque immune environment *in vivo* will answer these questions. M2 macrophages are more effective wound healing cells and are able to egress quickly from the lesion leading to regression.

We show that high shear stress leads to increased efferocytosis, increased CCL5, decreased CXCL12, decreased activated β2 integrin and increased activated CD4+ cells. The exact mechanism of these changes and the link to shear stress is unknown and may be answered by more closely examining the inflammatory environmental changes during regression.

CHAPTER VI: DISCUSSION & CONCLUSIONS

6.1 Discussion and Conclusions

The purpose of this thesis was to understand the protective effect of shear stress in the context of atherosclerotic plaque regression and to determine a potential mechanism. To this end, we developed an *in vivo* mouse model of increased shear stress specifically in the BCA. This allowed us to study the local effect of increased shear stress in an area where plaques tend to form due to the geometry of the vessel. Plaque changes were not expected to occur elsewhere given that the shear stress remains consistent. Local plaque changes were strictly associated with increased shear stress. In fact, measurements of the plaque at the aortic sinus showed no change in size between groups since the shear stress was similar. The sinus plaque measurements were also used to eliminate the variability between individual mice by reporting BCA plaque size over sinus plaque size. Moreover, no changes in physiological parameters were observed. These finding indicate that any changes seen in the plaque composition and size were strictly due to shear stress.

Most atherosclerotic regression studies examined plaque reduction due to changes in lipid levels[66]. This is one of the first studies that examine plaque reduction due to an alternative mechanism. Finding a substitute treatment for atherosclerosis instead of the commonly used statins is important. Finding new methods to reduce plaque size without targeting lipid levels could lead to fewer side effects, a more substantial reduction in size and a quicker mode of action. We show clearly that shear stress does in fact have the ability to cause a drastic 50% reduction in lesion size in a 4 week period. One may argue that perhaps plaque size was reduced due to the increased shear stress damaging the plaque right after surgery. However, not only was

there no change in plaque size after 3 days but the echocardiography indicated significant changes in size only at week 3, indicating a gradual change in size over time.

The fact that the plaque is smaller does not always indicate that it is stable and rupture free. Small plaques which are unstable and have a thin cap are at greater risk of rupture than larger plaques with thick fibrous caps[12]. The reduced plaque with increased shear stress, showed the presence of a fibrous cap and had no changes in relative content of smooth muscle cells, elastin or collagen. These components provide structure and stability to the plaque[117]. Thus, the AVF plaque is smaller is size with no loss in stability.

In terms of the advancement and stage of the plaque, this is measured by the size of the necrotic core[46]. The necrotic core is present in advanced plaques and is filled with cellular debris and dead cells and is therefore an acellular space[12]. Although smaller, the AVF plaque had the same plaque cell count as the larger Sham and control plaques. Larger plaques have larger necrotic cores with void space and although large, have low cell number. The smaller AVF plaques are dense and compact cellular space with little to no necrotic cores, indicating that not only did the plaque become smaller but it also regressed towards a less advanced and vulnerable plaque. Smaller necrotic cores are also suggestive of increased efferocytosis, which is known to be associated with regressing plaques[67].

Decreased absolute macrophage content observed in the AVF lesions perhaps contributes to less inflammation. This result signifies either less influx of inflammatory cells or increased efflux out of the plaque. The AVF plaque compared to the Sham contains similar influx of transplanted GFP labelled cells, indicating constant influx even during regression. Thus, the decreased macrophage content is likely due to more efflux. Macrophage efflux is a sign of

wound healing and is required for plaque regression. The general stain for all macrophages used gives us no clue as to the subtype of macrophage present, whether it be M1 or M2. The lack of very specific immunofluorescent markers makes this difficult to study. NFκB is also reduced in AVF plaques further pointing to less inflammation in the regressing plaques. Thus, the AVF plaque is small, stable, less advanced and less inflammatory.

Increasing the shear stress indefinitely in humans in order to get the beneficial effect of plaque regression is not possible. Therefore, understanding a potential mechanism is imperative in order to find new therapeutic targets in the process. It is known that shear stress directly increases NO production from the ECs and NO has been linked to protection from atherosclerosis[83, 94]. Increased eNOS activity is seen in the AVF plaques and may be contributing to the plaque regression. In fact, inhibition of eNOS leads to a loss in plaque regression indicating NO as an important player in the protective mechanism.

Studies thus far have solely examined MMPs in the context of plaque progression which has conflicting and controversial conclusions. Ours is the first study to examine MMPs in the context of regression. We further explored the plaque composition by measuring MMP activity. Given that MMP-9 is linked to atherosclerosis and shear stress[122,160], we measured gelatinase activity measuring both MMP-9 and MMP-2. In fact, like NO, MMP activity was increased in AVF plaques which led us to hypothesize that MMPs may too be involved in the regression mechanism. MMPs were inhibited in the mice using two methods in order to confirm our hypothesis. Doxycycline is a general MMP inhibitor and was administered in the drinking water of the mice. Doxycycline inhibits MMP activity as well as MMP transcription[154]. The overexpression of MMP inhibitors, TIMP-1 and TIMP-3, was also used to inhibit MMPs via

electroporation into the thigh muscle. The TIMPs are slightly more specific than doxycycline, although they also have much overlap. What is interesting is that TIMP-1 has a preference for MMP-9 inhibition and TIMP-3, besides inhibiting mostly the membrane bound MMPs, also inhibits MMP-9[112]. All methods of MMP inhibition abolished the regression, indicating the involvement of MMPs in this process. The TIMP plasmids, although injected in the thigh muscle, are secreted proteins and are able to reach the aorta. We observed TIMP expression in the aortic arch and plasma and therefore the plasmid presumably reaches the plaque. Specific MMP expression was measured in the AVF plaque in order to determine a precise MMP involved in the mechanism of plaque regression. While expression does not always indicate activity, the lack of specific MMP activity assays makes expression the only alternative. MMP-8 expression was not different between our 3 groups. This is important; since MMP-8 is an important MMP causing unstable plaques[126]. This further suggests that the AVF plaque, although smaller, is not less stable. MMP-14 activates MMP-2, and both were decreased in the AVF plaque. These MMPs are known to have detrimental effects by promoting plaque progression[121,141]. Furthermore, MMP-14 is linked to M1 type foam cells[141]. MMP-3 activates MMP-9, and both were increased in the AVF plaque. Both these MMPs have been associated with beneficial effects by slowing down plaque progression[122]. Interestingly, the pattern of MMP-9 plaque expression is quite different between groups. MMP-9 in the control and Sham plaques is expressed in the plaque shoulder where plaques are most unstable, whereas in the AVF the expression is diffuse covering the whole plaque. Therefore, placing MMPs into specific categories of either beneficial or detrimental is misleading. Instead, the location and timing of activity play an important role. MMP-2 expression decreases while MMP-9 increases, and if this translates to MMP activity, we can assume that the main contributor to the elevated gelatinase

activity in the AVF plaque is due to a large increase in MMP-9 activity to compensate for the reduced MMP-2 activity. It seems that MMP-9 activity in an advanced plaque and located throughout the lesion is protective and leads to regression. Collagen I which is not cleaved by MMP-9[113] is consistent between groups. However, collagen IV which is cleaved by MMP-9[115] decreases in the AVF plaque. This further confirms that MMP-9 activity is increased with increased shear stress. The diffuse nature of MMP-9 staining in AVF plaques compared to our control and Sham groups could be contributing to the release of macrophages via collagen IV degradation, allowing them to mobilize.

We investigated, *in vitro*, macrophage function. We showed that high shear stress leads to increased MMP-9 activity in macrophages. High shear stress leads to increased macrophage migration when co-cultured with ECs. This increased migration is higher compared to macrophages cultured alone with no ECs or in the absence of shear stress. General MMP inhibition abolished this increased migration. Since MMP-9 is highly implicated in migration of cells[151] and is important in our shear stress model, we looked at the direct effect of MMP-9 inhibition using MMP-9 KO cells. MMP-9 KO in the ECs and macrophages in the co-culture system abolished the effect of increased migration. When the KO was present in only the ECs or macrophages, the increased migration was also lost but to a lesser extent than the KO in both cells, indicating that MMP-9 is important in both cells to have the highest migratory effect. Furthermore, the presence of EC effluent must be present for MMP-9 activity and ultimately migration. Macrophage migration in the presence of regular cell media instead of EC effluent, showed no MMP-9 activity and little to no migration. Macrophage migration in the presence of media from ECs exposed to shear stress, showed significantly increased MMP-9 activity and migration. These results indicate that the ECs are releasing a factor that is making the

macrophages more mobile. The link between shear stress and increased MMP activity can be due to increased NO since NO has been shown to increase MMP activity, specifically MMP-9[140]. Therefore, this leads to the conclusion that increased shear stress leads to increased migration via increased MMP-9 activity. Whether this translates to our *in vivo* model as increased cellular efflux has yet to be studied and will require further analysis.

We showed that in addition to increased migration, shear stress also increases macrophage efferocytosis *in vitro*. However, inhibiting MMPs did not alter this effect. Therefore, shear stress induces efferocytosis by a yet unknown mechanism. This increased efferocytosis may explain the decreased necrotic core in the AVF plaques. Looking at the protein expression in the media after shear stress indicates that certain cytokines and chemokines are altered with shear stress. Of particular interest was the increase in CCL5 with high shear stress which was confirmed at the mRNA level. CCL5 was over expressed only in the macrophages but levels in the ECs were comparable between static and high conditions. While CCL5 is linked to inflammation, it has more recently been linked to efferocytosis[179], which could point to a potential mechanism. The link between shear stress and CCL5 requires further study. CXCL12 was shown to be decreased in both macrophages and ECs with high shear stress. This is an inflammatory chemokine and its decrease could lead to reduced inflammation in high shear conditions. Activated β2 was also decreased with high shear. This integrin is a pro-atherogenic adhesion molecule[40], and the decrease in high shear stress could explain the increased migration. However, this integrin is not affected by MMP inhibition.

Finally, using a different shear stress apparatus which allowed us to have all spleen cells in the system, we demonstrated that activated CD4+CD69+ cells were increased with high shear.

These cells are required for M2 activation[180]. Thus, perhaps high shear stress polarizes macrophages to the wound healing and anti-inflammatory macrophage subtype. More research is needed to further investigate the role of the immune system and efferocytosis in our regression shear stress model.

Our proposed mechanism of shear stress protection is seen in Figure 6.1. Low shear stress contributes to advanced plaques and release of damaging factors from the ECs. The advanced plaque contains an extensive collagen matrix network trapping the macrophages rendering them immobile and unable to efflux from the plaque. The presence of damaging MMPs is increased and degrades the shoulder and cap area of the plaque making it unstable. There is a large necrotic core due to inefficient efferocytosis. The presence of inflammatory cytokines and chemokines cause continuous influx of macrophages. This cycle continues and the plaques continue to grow larger. When shear stress is elevated, it causes an increase in NO which increases MMP activity, specifically the beneficial MMP-9. This leads to a degradation of the collagen matrix which allows the macrophages to be more mobile and efflux out of the plaque. This process is also accompanied by an increase in efferocytosis, leading to smaller necrotic cores. All these steps cause the plaques to regress in size, while maintaining their stability.

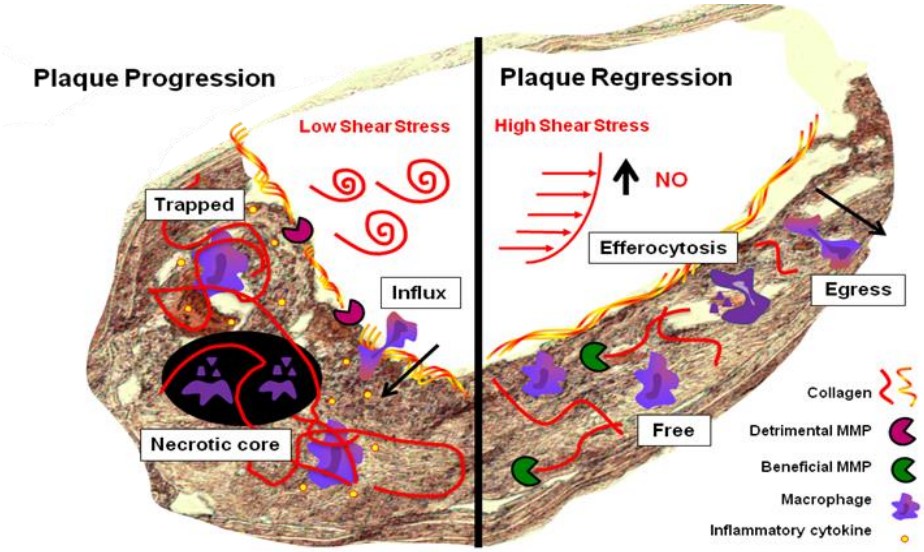

Figure 6.1: Proposed mechanism of increased shear stress

6.2 Limitations and Future Directions

The research from this thesis does come with some limitations. When examining cellular protein markers or GFP expression, either immunofluorescence or FACS analysis can be used. FACS analysis is superior given that it is a more quantitative approach. It would also provide better insight into what the GFP influx cells differentiate into since the cell markers can be specifically measured. The specific plaque immune cells and subtypes can also be measured comparing our groups of mice. Our study of plaque composition mostly used immunofluorescence, since to our knowledge FACS analysis in the plaque alone without the surrounding vessel has never been performed. The amount of plaque material is so small that it is extremely difficult to retrieve many live cells. Very recently, our lab optimized FACS analysis of plaque tissue alone and it is now a possibility. This will allow future work in measuring exact

immune cell subtypes at various weeks through the regression of the AVF plaque. This will give us a clear picture as to the immune cell changes over time, further understanding the mechanism involved.

Studying specific MMPs is difficult given the lack of specific MMP activity assays and inhibitors. We had to rely on gelatinase activity which measures both MMP-9 and MMP-2 to give us a clue about MMP-9 activity. We also had to rely on specific MMP expression levels which do not always translate to activity. As of yet, no specific MMP activity assays exist. Furthermore, our study only uses general MMP inhibitors to study the effect on plaque regression. However, specifically inhibiting MMP-9 would further confirm if it is the key player in this mechanism. Studies injecting MMP-9 KO cells in our AVF animals would answer this question and is currently in progress in our laboratory. Using KO cells from these mice and injecting them into our AVF group is more physiologically relevant than performing the surgery directly on MMP-9 KO animals. These mice lack MMP-9 from birth and would have adapted to the lack of this MMP by compensating and overexpressing other MMPs, therefore not giving us an accurate role of MMP-9 inhibition alone. Instead, injecting MMP-9 KO cells at the point of the surgery will allow us to determine the single effect of MMP-9 on plaque regression and determine if MMP-9 injection is a feasible therapeutic option. Irradiation kills cells that are constantly multiplying while non-proliferating cells survive. These non-proliferating cells are found in an advanced plaque. Therefore, we may not see any effect with transplanted MMP-9 KO cells given that the cells in the plaque that survived the irradiation will still be producing MMP-9.

For the in vitro macrophage function assays, specifically measuring migration, a matrigel matrix surface was not used. A matrigel matrix mimics an ECM environment, yet the matrigel failed in our shear set-up. However, macrophages are known to secrete a matrix when plated on a surface such as a petri dish which eliminated the need for the matrigel. Boyden chambers would have been a more ideal migration assay. However; due to our co-culture conditions, this was not possible unless we examined the indirect effect of shear stress. This indirect effect is not as relevant as the direct effect we were able to achieve using the scratch assay. This thesis studies migration and efferocytosis mostly *in vitro*. Ideally, future work will look at these mechanisms directly in our AVF animals. Now that our group has established a protocol for FACS in the plaque, examination of these processes in *vivo* can be done. Macrophages can phagocytose labelled microspheres and their efflux can be tracked. Also, markers such as MERTK can be used to quantify efferocytosis. This work focuses mostly on macrophages; however, there are many other cell types present in the heterogeneous plaque. More recently, it was shown that VSMCs also play an important role in plaque progression since they too can uptake LDL and become foam like cells. Future work studying the role of shear stress in VSMC function would be interesting.

In spite of these limitations and the need for future studies, this thesis provides interesting, novel insight into the protective mechanism of shear stress-induced atherosclerotic plaque regression and offers potential new targets in the treatment of atherosclerosis.

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