FROM FIBROCYTE MIGRATION TO RESIDENT CARDIAC MACROPHAGE PHENOTYPES: THE EVOLUTION IN UNDERSTANDING OF MACROPHAGES IN NON-ISCHEMIC MYOCARDIAL INJURY AND FIBROSIS

by

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DEDICATION PAGE

Few things in life, if any, are individual accomplishments. This thesis is dedicated to all the people that helped me get here.

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ABSTRACT

Heart failure is a growing societal and healthcare issue for which the only long-term therapy is transplantation. Heart failure therapies have been limited by our understanding of a key process involved in healing termed myocardial fibrosis. Myocardial fibrosis is a primarily a reparative response for replacing and reinforcing injured/stressed tissue with extracellular matrix (ECM) proteins. Unfortunately, the ECM proteins which comprise fibrosis are non-contractile. In turn, as fibrosis accumulates, the heart loses its normal contraction and heart failure can ensue.

This thesis focuses on characterizing the evolution of thought on an ambiguous, elusive cell type in myocardial fibrosis. Work in the AngII model of myocardial fibrosis initially suggested a process driven by a single cell type, termed fibrocyte, that accumulates in the heart prior to the deposition of ECM proteins. Fibrocytes express hematopoietic and mesenchymal markers – a unique, hybrid phenotype that suggested they may be a novel source of ECM-producing fibroblasts. As such, my initial aim was to characterize fibrocyte migration to the heart in order to identify therapeutic opportunities to reduce myocardial fibrosis. As contrary evidence accumulated, my aim shifted toward targeting a more "monocyte-like" fibrocyte in circulation with cytotoxic liposomes. Through happenstance, I arrived at the realization that fibrocytes were likely a blip on an infinite scale of macrophage (MΦ) phenotypes. The focus of this thesis shifted once again: I was characterizing MΦ phenotypes in early AngII-mediated myocardial injury. Finally, as a culmination of misdirections and realizations, I found myself characterizing a truly novel cell type: cardiac resident MΦ (rCMΦ).

Specifically, the work I present in this thesis demonstrates that fibrocytes do not behave/migrate like progenitor cells. Rather, using the AngII model in tandem with chemokine receptor antagonists, receptor knockouts, and cytotoxic liposomes, I provide evidence that fibrocytes are more like an atypical monocyte/MΦ. Lastly, my work in $CX3CR1^{-/-}$ and $CCR2^{-/-}$ supported growing evidence for the importance of "anti-inflammatory" rCMΦ phenotypes in myocardial injury. Together, this body of work has helped erode the fibrocyte misdirection and drawn attention to the importance of circulation-derived and rCMΦ in non-ischemic myocardial injury, such as that which occurs in the AngII model.

LIST OF ABBREVIATIONS AND SYMBOLS

∴	Therefore
αSMA	Alpha smooth muscle cell actin
β-MHC	β-myosin heavy chain
µL	Microliter
µm	Micrometer
ACE	Angiotensin converting enzyme
ACK	Ammonium-Chloride-Potassium
ADH	Antidiuretic Hormone
AMD3100	CXCR4 antagonist
AngI	Angiotensin II
AngII	Angiotensin II
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
ARB	Angiotensin receptor blocker
AT1R	Angiotensin type 1 receptor
AT2R	Angiotensin type 2 receptor
AT4R	Angiotensin type 4 receptor
BM	Bone marrow
BMDMΦ	Bone marrow derived macrophages
BP	Base pair
BSA	Bovine Serum Albumin
BW	Body weight

C57BL/6	C57 Black 6
cDNA	Complementary DNA
CO	Cardiac output
Col-1	Collagen type 1
COL1A1	Collagen type 1, A1 subunit
CTGF	Connective tissue growth factor
CVD	Cardiovascular Disease
Cy2	Cyanine 2
Cy3	Cyanine 3
Cy5	Cyanine 5
d	Day
DAB	3,3’-diaminobenzidine
DAMP	Damage associated molecular pattern
DMEM	Dulbecco's modified eagle medium
DMEM-C	DMEM complete
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
ECHO	Echocardiogram
ECM	Extracellular Matrix
E9	Embryo, day 9
EF	Ejection Fraction
FBS	Fetal bovine serum
FcγR	Fc gamma receptor

FG	Fast green
FSC	Forward scatter
g	Gram
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony stimulating factor
H&E	Haematoxylin and eosin
hr	Hour
HW	Heart weight
ICAM	Intercellular adhesion molecule
IFN-γ	Interferon gamma
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-1β	Interleukin 1 beta
IL-35	Interleukin 35
iNOS	Inducible nitrous oxide
IVSW	Interventricular septal wall
kBP	Kilobase pair
L	Litre

LV	Left ventricle
LVIDd	Left ventricular internal diameter (diastolic)
LVIDs	Left ventricular internal diameter (systolic)
LVPWd	Left ventricular posterior wall (diastolic)
LVPWs	Left ventricular posterior wall (systolic)
Ly6C	Lymphocyte antigen 6 complex
M1	Classically activated macrophage
M2	Non-classically activated macrophage
MABP	Mean arterial blood pressure
MHC II	Major histocompatibility complex II
min	Minute
mg	Milligram
mL	Millilitre
mM	Millimolar
mmol	Millimole
MMP	Matrix metalloproteinases
mol	Mole
mRNA	Messenger RNA
MΦ	Macrophage
NIH	National Institutes of Health
nm	Nanometer
NO	Nitrous oxide
PAMP	Pathogen associated molecular pattern

PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PECAM	Platelet endothelial cell adhesion molecule
PFA	Paraformaldehyde
PPARγ	Peroxisome proliferator-activated receptor gamma
PS	Phosphatidylserine
qPCR	quantitative real-time polymerase chain reaction
RAAS	Renin Angiotensin Aldosterone system
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RV	Right ventricle
SAP	Serum amyloid P
SEM	Standard error of the mean
SR	Sirius/picosirius red
SSC	Side scatter
t	Time
TGF-β	Transforming growth factor beta
TH-1	Type 1 helper T-cell
TIMP	Tissue inhibitor of matrix metalloproteinase
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha

U	Enzyme unit
VCAM	Vascular cell adhesion molecule
VLA-4	Very late antigen 4
VR	Vascular resistance
VSMC	Vascular smooth muscle cell
wk	Week
WT	Wild type
yr	Year

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In life, there are easy lessons and difficult lessons, and I have had my fair share of both. Regardless, I owe people credit for the lessons I have learned over the course of my degree:

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My family (both immediate and extended) – I'm still not sure if they exactly know what I study in the lab, but they have always been proud of my hard work and success. And for that, I am grateful.

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Building, where we interacted with students in and outside our department. Much like my experience in the lab, the students in the Pathology Department often felt like an extended family.

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Dr. Jean Francois Légaré – Dr. Légaré – or as I know him, JF – is a remarkable mentor and friend. He has been immense support throughout my degree. He provided an invaluable clinical perspective to our lab's research endeavors and gave me the freedom to explore my interests. Through JF, I learned that a disease is more than just cells, molecules, tissues, and organs: there's a human or animal on the other end and there are emotions tied to that disease. This was disease from a big picture perspective: from the smallest changes in mRNA through to the emotions that come when someone succumbs or triumphs in the face of a disease. Disease is complex. As I look at the Pathology Department, I cannot help but think that others may be narrowing their sights on the

mechanisms of disease. For example, in the General Pathology course, there was never any discussion about who is suffering from the disease and why. Sure, cigarette smokers and obese people are more likely to develop a range of conditions including cardiovascular disease and cancer, but why do some people smoke cigarettes and others not? These were the questions that rounded off my degree. I was fortunate to have JF around to discuss the answers to these questions. He saw disease the same way.

JF was also a support outside work. When dealing with personal or family health issues, JF was always there to help. His wisdom got me through some very difficult times. I am happy that JF is moving onto what seems like a better fit for his life and career, but I am also sad to see my mentor move away. Fortunately, as people go their separate ways, we can always keep and share the lessons we have learned from others. I am who I am today because of JF as a supervisor and mentor.

CHAPTER 1 INTRODUCTION

In my acknowledgments, I aimed to highlight the fact that my education is more than just research – it is a complex blend of science, experiences, attitudes, and people. This is a concept that I wanted to come across throughout my thesis. All the relevant information has been included, but it is written in my own style. In addition, I set out to write an 'accessible' thesis – one that can be read without having previously researched macrophages (MΦ) or cardiovascular disease (CVD). In doing so, I hope reading about my work is less of a chore.

To open the Introduction and set the stage for my thesis, I have included the figure below (Fig. 1.1) that draws parallels between the general biphasic response in many forms of sterile myocardial injury (e.g. infarction and hypertension) and the evolution of my own research. In the heart, the initial stage of healing is characterized by a primarily pro-inflammatory, hostile, destructive environment. The latter phase is one of resolution and healing. When I began my graduate degree, I was in the 'hostile environment' of fibrocyte research. Fibrocytes were a contested cell type and observations regarding their phenotype and function seemed irreconcilable with their proposed role in injury, healing, and fibrosis. The advent of MΦ subsets in my research helped bring clarity to my observations in a myocardial fibrosis model. The MΦ activation spectrum could more accurately describe how the heart responds to injury. Finally, the discovery of resident cardiac MΦ (rCMΦ) seemed to fill in some remaining knowledge gaps, despite also raising more questions for future students.

Thus, my research focus over the course of my degree was biphasic like the heart's response to injury; it began with fibrocytes and ended with rCMΦ. And like the healing phase (2), I had found a sense of resolution from the early fibrocyte turmoil.

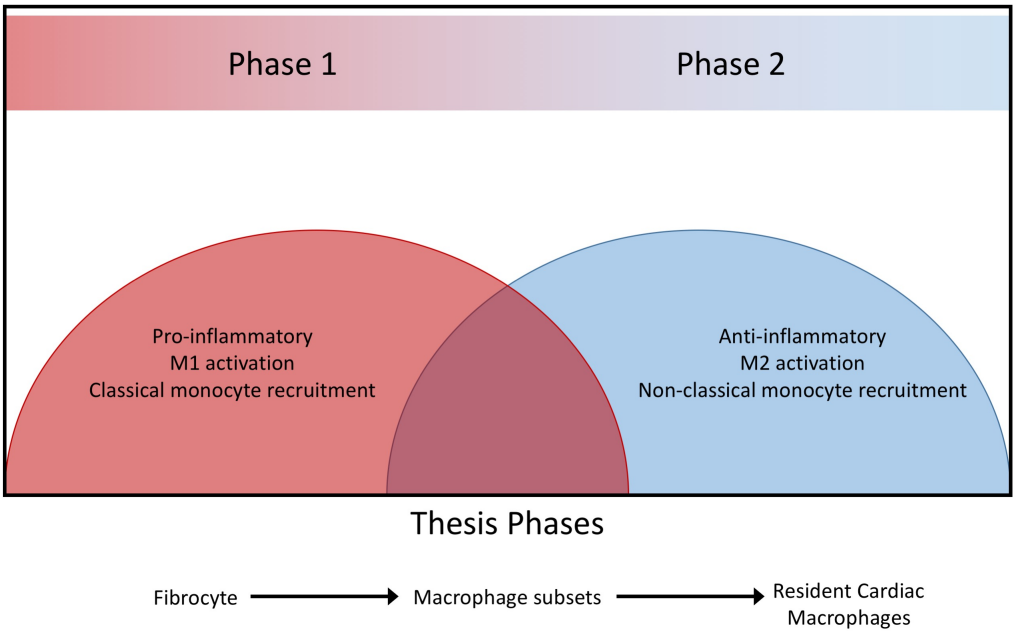


Figure 1.1 - An analogy for the story arc in my thesis. This figure is an adaptation from Nahrendorf et al. on the phases of myocardial healing[1]*. In general, Phase 1 is characterized as a pro-inflammatory environment, while Phase 2 is anti-inflammatory and pro-fibrotic (resolution of injury and inflammation). The details of myocardial healing will be discussed in greater detail throughout my thesis. For me, Phase 1 of my degree primarily revolved around fibrocytes. In contrast, Phase 2 of my degree focused on macrophages. The transition from fibrocytes to macrophages was bridged by the advent of macrophage subsets.*

1.1 Heart Failure, Hypertension, and Myocardial Fibrosis

1.1.1 Impact and Demographics

If I were giving my elevator speech about my research, I would probably start with the broadest topic: heart failure. The broadest topic also happens to be the

culmination of a tragic demise of an otherwise incredible organ. The heartbeat is detectable as early as 6-7wks and continues until the end – sometimes even taking a detour into another individual's body (i.e. transplantation)[2]. Throughout its life, the heart grows stronger in the face of adversity, pushing its cells to their limits until the walls close in on the chambers or the force of blood stretches the chambers thin. As the heart bends beyond the point of compensation, it loses its native structure and with it, its normal function: heart failure ensues.

Simply put, *heart failure* describes the inability for the heart to adequately circulate blood to peripheral tissues[3,4]. As such, any number of cardiovascular complications can be upstream of heart failure. Importantly and unfortunately, it is the final pathway for many cardiovascular pathologies[5]. For the estimated 600 000 Canadians currently living with heart failure, it carries a 3-year mortality that approaches 50%[6].

Heart failure is a growing global health issue with no obvious solution in sight[7,8]. On one hand, common risk factors such as obesity and hypertension are on the rise[9–11]. For example, 2/3 of Americans are either pre-hypertensive (at or above 120/80mmHG, but below 140/90mmHg) or clinically hypertensive (at or above >140/90mmHg)[12]. Some point to genetic differences as a primary cause, but the magnitude of hypertension in countries such as the United States of America and Canada suggests the underlying factors are more widespread[13]. Indeed, other risk factors for hypertension include tobacco, alcohol, salt-intake, obesity, and lack of physical activity, but epidemiological studies suggest many of these factors can be a consequence of complex socioeconomic differences in populations[7,14–17]. And while many suffer from risk factors such as hypertension, the detrimental effects are often not realized until later in life – such is the

insidiousness of CVD[18]. Moreover, our life-saving and -extending therapeutic approaches for CVD can have unintended consequences. For example, the more people that survive acute cardiac events, such as heart attacks, the more go on to develop heart failure: 50 000 new patients are diagnosed in Canada each year[6].

Patients suffering from heart failure may experience symptoms that lower their quality of life such as pain, anxiety, fatigue, shortness of breath, coughing, confusion, swelling in extremities, and depression[19–22]. One of the sadder realizations I came to while writing this thesis was that, like hypertension, we do not all suffer from heart failure equally: some are more likely to suffer than others. Yes, some of this group include those with congenital heart defects and rare heart infections, but more so, those with a lower socioeconomic status and a stressful environment (i.e. social determinants of health) suffer disproportionately[4,7,22]. Thus, as socioeconomic disparity increases both within and between countries, rates of heart failure can be expected to rise.

Together, these facts and statistics support an enormous demand for researching and addressing the prevalence and impact of heart failure, its risk factors, and underlying causes.

1.1.2 Mechanics of Cardiac Dysfunction

Thus far, my aim has been to describe the types and number of people who suffer from heart failure and the upstream risk factor, hypertension. In this section, my objective is to deconstruct heart failure and hypertension into the mechanics of cardiac function and dysfunction. In addition, I will introduce myocardial fibrosis – the excess deposition of ECM proteins – as an important culprit in cardiac dysfunction.

In order to understand the physiological impact of heart failure, one must first understand the normal mechanics of cardiac function and their importance in providing adequate circulation. As previously mentioned, the heart relies on coordinated, fluid contraction to efficiently pump blood to peripheral tissues – each part of the heart has a role in the different stages of contraction. As such, cardiac dysfunction takes many forms depending on the type and location of injury, but can be broadly separated into the 2 phases of the cardiac cycle: (i) diastole and (ii) systole[5]. These 2 phases represent the 'fill' and 'pump' of the heart cycle, respectively.

Returning to basics: the mammalian heart has 4 chambers that coordinate to accept and eject blood to and from systemic and pulmonary circulation[23]. Each side of the heart has an atrium and a ventricle. Mechanically, the atria – sitting atop the ventricles – are weak pumps, primarily serving as collecting reservoirs for blood[i]. In contrast, the ventricles are the powerful, ejecting chambers, drawing blood from the atria to recirculate through pulmonary and systemic vasculature. During diastole, the heart is in a stage of relaxation. Oxygen-poor blood returns to the heart via the right atrium. As systole (i.e. contraction) sets in, the heart begins to contract in a top-down motion. In quick succession, this sends the returning blood from the right atrium to the right ventricle and off to the lungs for re-oxygenation. On the left side of the heart, diastole allows the left atrium to fill with blood returning from the lungs. Like the right side of the heart, during systole, there is a quick succession of contraction from the left atrium to left ventricle that ultimately ejects blood to circulation. While not belittling the role of the atria, the cardiac cycle highlights the functional importance of the ventricles. Without properly functioning ventricles, blood can neither be pumped to the lungs for re-oxygenation nor, circulated to

peripheral tissues[23–27]. Thus, one can begin to imagine how maladaptive changes in the ventricles could not just impair normal cardiac function, but also entire bodily systems.

In the face of adversity, the heart has 2 primary compensation mechanisms - (i) physiological and (ii) structural – to address changing demands and challenges. In essence, the heart attempts to return itself to a homeostatic state – one in which it meets the demand of peripheral tissues and maintains its structural integrity[5,24,28–32]. Neither of these are mutually exclusive – rather, prolonged changes in the physiological state affect the structure of the heart and vice versa. Relevant to this thesis, in hypertension, the heart also faces 2 types of challenges in order to maintain homeostasis: (i) reduced blood vessel diameter and (ii) increased blood volume[33]. These 2 general factors that influence blood pressure modulation are downstream of complex signalling pathways that include renin-angiotensin-aldosterone system (RAAS) activation and sympathetic nervous system activation.

Physiologically, blood pressure is constantly modulated to influence the stress on the heart and the ability to perfuse peripheral tissues[23,34]. Simply, blood pressure is the sum of the control of blood flow to tissues in proportion to metabolic demand. Physically, *blood pressure* describes the force circulating blood exerts on the endothelial lining of blood vessels[35]. Our bodies regulate blood pressure by adjusting the volume of blood being circulated, termed *cardiac output* (CO), and/or contracting or relaxing blood vessels, reflected as *vascular resistance* (VR). CO and VR are primarily modulated through neuroendocrine systems and salt homeostasis – the former includes the RAAS, which will be discussed in greater detail in the Angiotensin signalling section of the introduction[35]. Increases in CO and/or VR lead to the development of high blood

pressure, or *hypertension.* Hypertension is a condition in which said pressure is greater than is necessary for tissue perfusion[23,33,35]. And while many people with transient or even chronic hypertension may not feel any overt symptoms, the excess pressure can cause injury to the vessels and requires the heart to contract with more force to circulate blood. In turn, the vessels and heart can suffer the long-term consequences of hypertension. Fortunately, many of the physiological adaptations that modulate stress on the heart are reversible, which is why a short period of moderate hypertension may not cause significant long-term injury. In contrast, the maladaptive structural changes the heart undergoes in response to stress can be more permanent.

Structurally, the way the heart compensates is dependent on the cause of hypertension – though the causes are not mutually exclusive. In blood pressure-hypertrophied (i.e. ↑VR or narrowing vasculature) hearts, the heart needs to increase the force of contraction and in turn, fundamentally change its functional units: cardiomyocytes. As terminally differentiated, non-dividing cells, cardiomyocytes can only compensate for increased demand by amplifying cell growth, termed *hypertrophy*[33,36]. The cardiomyocytes increase contractile protein synthesis, laying down new sarcomeres in parallel. Thus, as the cardiomyocytes increase their ability to contract with greater force, they are able to compensate against resistance, such as that which occurs with hypertension[36]. There are limits and consequences to this compensation though. The cell growth thickens the ventricle wall(s), invading on the available chamber volume and reducing the potential volume of blood ejected[5]. As such, the ventricle forms greater resistance for normal blood flow and the upstream pressure increases the stress on

the left atrium, pulmonary circulation, and the right side of the heart. The combination of the intensity and duration of this stress can culminate in right-sided heart failure as well.

Volume overload hypertrophy can increase cardiac mass without increasing wall thickness. Volume overload hypertrophy differs from pressure hypertrophy in that the former leaves the heart with high ventricular pressure post-contraction[36]. As a consequence of volume overload, the chamber dilates to provide more space for the increased volume of blood. In turn, heart mass can still increase in volume overload due to the increased size of the chamber, but not necessarily with a concurrent increase in wall thickness. Thus, both pressure and volume overload push the heart to its limits and disrupt its normal shape and structure – these changes are reflected by adaptations and changes at the cellular level. As these compensation mechanisms fail and cardiomyocytes become apoptotic, the heart reinforces by increasing ECM deposition, or myocardial fibrosis[5,36].

Focusing on left heart failure, the primary difference between systolic and diastolic heart failure is the preservation of ejection fraction (EF) in the latter, for which the formula can be found below[ii23]:

$$\frac{Left\ ventricular\ diastolic\ volume - Left\ ventricular\ systolic\ volume}{Left\ ventricular\ diastolic\ volume} \times 100 = EF\ (\%)$$

The volumes in the above equation refer to blood within the heart's left ventricle (LV) at different times during contraction. As previously mentioned, blood enters the LV during diastole (i.e. relaxation) and leaves the LV during systole (i.e. contraction). Conceptually, one can imagine a case of systolic heart failure as blood filling the LV, but because the

heart has a weak contraction, the blood remains in the LV, thus reducing EF. Impaired systolic function is typical of patients suffering from coronary artery disease, previous myocardial infarctions, and infections of the heart[37]. These are largely conditions that result in a loss of cardiomyocytes over a short duration and in turn, require a quick, large-scale repair with less regard for maintaining *normal* function. These large-scale repairs, such as that which occurs in an infarct zone, are primarily composed of immune cells, fibroblasts, and the ECM proteins synthesized by the latter[29,38–40]. In summary, systolic heart failure is often a result of significant cardiomyocyte death in the left ventricle and is characterized by the impaired ability for the heart to eject blood to systemic circulation.

Dissimilarly, the concept of diastolic heart failure grew from the observation that some patients exhibited heart failure symptoms with minimal change to systolic function (i.e. preserved ejection fraction)[41–43]. Diastolic heart failure is often observed in (i) elderly patients who typically have (ii) not suffered significant coronary artery disease, but (iii) have a history of hypertension or restrictive cardiomyopathy[41,43]. As such, diastolic heart failure is more a consequence of an insidious disease process – one that slowly stiffens and hypertrophies the ventricle without large-scale cardiomyocyte loss. These patients also typically exhibit (iv) concentric dysfunction due to extensive interstitial fibrosis, such as that observed with hypertension[43]. The interstitial fibrosis observed with diastolic heart failure may not contain an abundance of immune cells, but still likely features fibroblast proliferation and excessive ECM production. Physiologically, diastolic heart failure patients present with reduced CO as a result of left ventricular hypertrophy (i.e. thickening ventricle wall ∴ reduced chamber volume)[41,44]. Conceptually, one can imagine diastolic heart failure as the left ventricle filling with a

reduced volume of blood, but said blood is still able to eject efficiently from the chamber. Taken together, the types of patients suffering from diastolic heart failure may not initially appear to be as morbid as their systolic heart failure counterparts and the preserved ejection fraction masks the former's clinical presentation.

Importantly, while heart failure is most commonly associated with left ventricular systolic dysfunction, up to 40% of heart failure patients present with a normal or slightly reduced ejection fraction[45]. Moreover, complex combinations of comorbidities (e.g. hypertension, coronary artery disease, and cardiomyopathies) translates to patients that may not exclusively fall into a category of systolic or diastolic heart failure[41–43]. Further uniting heart failure patients is the importance of myocardial fibrosis in cardiac dysfunction.

1.1.3 Role of Myocardial Fibrosis in Cardiac Dysfunction

Beginning this thesis with heart failure was a bit like giving away the ending of a story. Rather, heart failure is a series of symptoms reflective of pathological changes in the myocardial structure and function, as described above. These changes are, in large part, a by-product of myocardial injury and the subsequent healing response that involves scar formation.

The development of scar tissue in the heart, termed myocardial fibrosis, is a common pathological feature in CVD and the beginning of the end for a heart entering heart failure[24–27]. Myocardial fibrosis is characterized by the excessive deposition of ECM proteins in response to injury and/or disorder[46]. There are 2 overarching types of myocardial fibrosis: (i) reactive interstitial fibrosis and (ii) replacement fibrosis. Reactive

interstitial and replacement fibrosis can occur throughout the heart and are common in models of hypertension, including the exogenous Angiotensin II (AngII)-mediated model used in our laboratory[47–50].

Regardless of the etiology of myocardial fibrosis, there are 2 hallmarks of the pathology: (i) an upset in the balance of ECM protein deposition and degradation and (ii) the inability for these proteins to contract[39,51,52]. ECM proteins serve as the scaffolding for tissue organization and structure – they provide the 'skeleton' for organs like the heart[53]. In addition, they act as a reservoir for growth factors and cytokines. As such, the deposition of ECM proteins alone is neither reflective of a pathology, nor necessarily cause for concern. ECM proteins are constantly turned over by a tandem of the enzymes matrix metalloproteinases (MMP) and their inhibitors, termed tissue inhibitors of metalloproteinases (TIMP). While MMP degrade the existing ECM, TIMP inhibit said degradation[39,40].

The other half of the fibrosis equation is the cell type producing the majority of ECM proteins: the fibroblast[40]. Fibroblasts and their activated/differentiated form, myofibroblasts, are derived from mesenchymal origins and represent the most abundant population of cells in the myocardium, constituting 40-60% of the cells in a normal heart[30,39,51,54]. These cells are responsible for building the ECM, producing a significant portion of the collagen type I (~85% of myocardial collagen) and collagen type III (~11% of myocardial collagen) found in the heart[55]. The remaining ECM components include other collagens (e.g. types IV and V), fibronectin, laminin, and elastin[24,56]. Early anatomists suggested fibroblasts formed the glue that held the myocardium together during contraction. While this was and continues to be an oversimplification, the types I

and III collagen that fibroblasts produce provide the scaffolding for cardiomyocyte contraction. My favourite analogy for the fibroblast-cardiomyocyte relationship is that of a weightlifter and a spotter[38]. The fibroblast sits idly by as the spotter, watching to make sure that the weightlifting cardiomyocytes are able to do their job. When the cardiomyocyte can no longer perform its job, then the fibroblast steps in to fill the void – figuratively and literally. Figuratively, the fibroblast fills the void by quickly responding to myocardial stressors, such as hypertension, by promoting protein synthesis in cardiomyocytes[29]. As such, fibroblasts can help cardiomyocytes fill the "functional void" caused by hypertension. Literally speaking, the fibroblast synthesizes more ECM proteins to either replace lost cardiomyocytes (i.e. apoptotic/necrotic) or structurally reinforce the interstitial spaces between cardiomyocytes.

The unfortunate consequence to this response is the increased deposition of non-contractile proteins in an organ that relies on contractile proteins to function properly. Not only does the heart require strong contraction to eject the blood and perfuse peripheral tissues, the contraction must also be beautifully synchronized to encourage ideal function[45,57,58]. As Dr. Légaré's previous student – Nicole Rosin – analogized, increasing the interstitial myocardial ECM proteins is a bit like wrapping a belt around an inflating balloon. To take that analogy one step further, it is also a bit like adding a rigid band inside the balloon too: ultimately, the heart needs to contract and relax in a coordinated fashion that is disrupted by myocardial fibrosis.

In defense of myocardial fibrosis – yes, I'm defending the pathological feature that seemingly impairs cardiac function and can lead to heart failure – over and over again, research has demonstrated that it is a necessary process to stabilize and reinforce

the heart under acute and chronic stress[51,59]. In many respects, cardiac healing operates much like an emergency room – "stem the bleeding and death at all costs, regardless of the long term consequences[iii]. After all, the heart does not stop beating while it is being repaired – it is not like others muscles that can fully rest during recovery. Rather, myocardial fibrosis is a delicate dance between excessive and insufficient scar formation. Experimentally inhibiting fibrosis after myocardial infarction, for example, results in maladaptive structural changes, such as dilatation, that can culminate in rupture[59,60]. In contrast, excessive scarring – the hallmark of myocardial fibrosis – can unnecessarily stiffen the ventricles, causing cardiac dysfunction[25,29,46,61,62]. But how much fibrosis is too much? And as we move toward therapies that modulate the fibrotic response, how much is too little?

For now, the take home message with myocardial healing is that a certain amount of fibrosis, regardless of the resulting cardiac dysfunction, may be a necessary evil. I say 'for now' because targeting fibrosis is only one approach for reducing cardiac dysfunction. Other experimental therapeutic approaches focus on another shortfall of myocardial healing: cardiomyocytes do not replenish[iv] [63,64]. In addition, downstream vasculature lost to injuries such as infarcts are also not readily or easily replaced. As such, even as new cells could be laid down, there may be insufficient blood supply to encourage or maintain novel cardiomyocytes[64–67]. Thus, the delicate dance between too much and too little fibrosis does not just involve one cell type (i.e. the fibroblast). Rather, all the myocardial cells play a role in appropriate healing, including endothelial cells, fibroblasts, progenitor cells, cardiomyocytes, and the yet to be mentioned white blood cell populations that can shape the tissue environment[29]. Despite the body's best efforts,

the consequence of what continues to be 'normal' cardiac healing is ever increasing ECM proteins that lead to dysfunction that can culminate in heart failure. One of the key factors involved in promoting ECM deposition, particular in hypertension, is AngII.

1.1.4 Role of AngII in myocardial fibrosis

AngII signaling is immensely complex, involving practically every organ of which one can think including the brain, heart, liver, and kidneys. When I first set out to describe the role of AngII, I was almost 20 pages into my thesis and the amount of detail was becoming exponential. I was captivated by its role in blood pressure regulation because it was such an intricately linked system that seemed to have a response to any possible deviation from homeostasis. On the other hand, AngII can have profound destructive effects that culminate in inflammatory injury and a resulting fibrotic response[47,68–72]. The focus of this section is to further bridge the gap from the symptoms and mechanics of heart failure and hypertension to mediators of myocardial injury and fibrosis.

The hormone AngII is the main effector molecule in the RAAS[73]. Indeed, the RAAS is one of the key long-term regulatory systems for blood pressure and the upregulation of its constituents and activation are associated with a variety of CVD processes including hypertension and heart failure[74,75]. In brief, AngII is generated through a series of enzymatic reactions that proceed as follows[73,76–78]:

(1) The enzyme renin is produced in the kidneys, but is also produced locally in the myocardium.

(2) Renin cleaves the angiotensin precursor, angiotensinogen, which is

produced in the liver and also locally in the myocardium. Angiotensinogen is cleaved to Angiotensin I (AngI), which inhibits angiotensinogen production in a feedback loop.

(3) AngI is cleaved to AngII by angiotensin converting enzyme, or ACE. ACE is primarily produced in the pulmonary vascular endothelial cells, where it remains membrane bound. ACE is also able to degrade the anti-hypertensive bradkynin, further enhancing the pro-hypertensive properties of the RAAS.

AngII and its other metabolites exert their effects via the 3 primary AngII G-protein coupled receptors: Type 1 (AT1R), Type 2 (AT2R), and Type 4 (AT4R)[v]. The 3 receptors exhibit distinct functions and expression profiles that reflect their differing roles in CVD. AT1R is the primary receptor for AngII in adults and mediates many of the cardiovascular effects observed with AngII stimulation[77]. In contrast, AT2R is expressed at its highest levels shortly after birth and is normally expressed at low levels in adult tissue[79,80]. Highlighting the importance of AT1R in CVD, 25% of hypertensive patients worldwide take angiotensin receptor blockers (ATRB), which exhibit significantly higher selectivity (i.e. 10,000-30,000x) for AT1R relative to the other Angiotensin receptors[81]. Moreover, the efficacy of ARBs in reducing CVD risk, treating hypertension, and slowing the progression of chronic kidney disease reflects the importance of this receptor in a variety of disease processes[82–85].

The vasoactive properties of AngII are well-established[31,73,77]. The intense vasoconstriction elicited by AngII is primarily mediated by direct effects of the peptide on AT1R expressed on vascular smooth muscle cells (VSMC)[86]. AngII also acts directly

on the kidneys to increase tubular sodium resorption, which, increases blood volume and in turn, increases blood pressure. In addition, AngII is also able to indirectly increase blood pressure via the production of other hormones. For example, AngII acts on (i) the brain's hypothalamus and posterior-pituitary to increase the production of anti-diuretic hormone (ADH, or vasopressin) and (ii) the adrenal cortex to promote the production of aldosterone[35,87,88]. Modulation of these pathways in the brain alters thirst and appetite for salt, thus also influencing the kidneys' blood pressure regulation[80].

AT2R also appears to have a minor role in blood pressure regulation through the regulation of drinking behavior[87,88]. In contrast to AT1R signaling though, AT2R is believed to serve as a counterbalance to the pro-hypertensive effects of AngII; while less understood, AT2R appears to mediate a minor role in reducing blood pressure[89]. Together, these pathways and systems represent an oversimplification of how AngII influences blood pressure. Importantly, the response to RAAS over-activation, such as that which occurs in hypertension, can lead to cardiac hypertrophy and myocardial fibrosis through mechanisms that include:

(i) Oxidative stress in cardiomyocytes, endothelial cells, and vascular smooth muscle cells[90–92],

(ii) the production of chemokines such as CCL2 and CXCL12[vi], which are involved in the recruitment of pro-inflammatory leukocyte populations (e.g. classical monocytes and T-cells) and the migration of myofibroblasts[47,70,71,93],

(iii) the increased expression of adhesion molecules and integrins, which allow leukocyte populations to bind to the vessel wall and undergo diapedesis[94–99],

(iv) the initial pro-inflammatory state of infiltrating cells (e.g. production of pro-inflammatory cytokine production and reactive oxygen species) favours further tissue injury in the perivascular[72,74,100–102],

(v) and finally, through the expansion and activation of fibroblasts in tandem with a shift toward pro-fibrotic leukocyte populations, the injured tissue is repaired/replaced through fibrotic deposition.

These mechanisms are largely conserved between hypertensive models of myocardial fibrosis, which include (i) aortic banding, (ii) exogenous aldosterone infusion, (iii) spontaneously hypertensive rodents, (iv) nephrectomy, and (v) stress[31,39,94,103–108]. The detrimental effects of AngII extend beyond its hypertensive role though.

While short durations of AngII infusion seemingly favour hypertension-dependent pathways to myocardial fibrosis, chronic AngII signaling in the absence of hypertension appears to be a pro-fibrotic factor in itself. In age-related fibrosis studies using spontaneously hypertensive rats, inhibition of AngII signaling using ACE inhibitors or ARBs ameliorated cardiac hypertrophy and myocardial fibrosis. However, when age-matched controls were also controlled for blood pressure using the anti-hypertensive hydralazine, animals with preserved AngII signaling still exhibited greater hypertrophy and fibrosis. Highlighting the role of AngII signaling in this response:

(i) Aged myocardia exhibit increased AT1R mRNA relative to younger controls and[79]

(ii) Increased AT1R signaling is associated with a breadth of pro-inflammatory and pro-fibrotic cytokine production[40,109,110].

This is in light of growing evidence that AngII is a potent pro-inflammatory mediator in the pathway to tissue fibrosis [108,111–118]. Similar to hypertension-mediated injury and in support of the role for AngII in inflammation:

(i) AT1R is expressed on a variety of leukocyte populations including – and most relevant to this thesis – monocytes and MΦ[119,120],

(ii) AngII promotes monocyte and MΦ production of pro-inflammatory cytokines including TNF-α and IL-6[120,121],

(iii) AngII increases the expression of CCR2 on monocytes, thus enhancing the chemoattractant effect of CCL2[122,123],

(iv) AngII promotes the release of pro-inflammatory monocytes from a splenic reservoir[74],

(v) AngII promotes $CD4^+$ T-cell IFN-γ and TNF-α production[124,125],

(vi) increased AngII signaling promotes vascular endothelial dysfunction[vii] which involves decreased nitrous oxide (NO) production and increased production of pro-inflammatory cytokines/chemokines (e.g. CCL2) and reactive oxygen species (ROS)[viii][124,126],

(vii) AngII also promotes chemokine production in VSMC including CCL2[127],

(viii) AngII promotes monocyte adhesion to endothelial cells in processes dependent and independent of adhesion molecules and selectins[95,128–130],

(ix) And local renin-angiotensin system activation, including AngII synthesis, increases vascular permeability[131,132].

While this is by no means an exhaustive list, the aforementioned mechanisms likely serve a primary role in the destructive force of AngII. Moreover, a commonality amongst these mechanisms is the direct and indirect effects AngII has on the vasculature.

In addition to its role in inflammation, AngII has a complementary role in the subsequent healing process, but not necessarily in a way that benefits the host. In the process of activating various inflammatory pathways, tissue injury is almost inevitable. As such, tissue lost to inflammatory injury needs remodelling. AngII can act on the primary cells involved in reparative, remodelling processes, namely fibroblasts and their mature, activated form, myofibroblasts[62,133,134]. Myofibroblasts are much more potent producers of ECM proteins, such as collagen. The appearance of myofibroblasts in the myocardium is largely indicative of a pathological process; normal myocardial tissue does not typically contain many myofibroblasts, though they can accumulate with age[29,30,52,134–138]. Fibroblasts and myofibroblasts express the Angiotensin receptors, AT1R and AT2R, and when stimulated with AngII, increase their production of collagen[139–141]. The increased ECM deposition is primarily downstream of pro-fibrotic growth factor intermediaries that include connective tissue growth factor (CTGF) and transforming growth factor-β (TGF-β)[28,29,39,133,135,142]. These growth factors can act in both an autocrine and paracrine manor; TGF-β is able to (i) stimulate the differentiation of fibroblasts to myofibroblasts, while also promoting (ii) cardiomyocyte hypertrophy, (iii) VSMC proliferation, and (iv) epithelial to mesenchymal transition – all important features in AngII- and hypertension mediated cardiac remodeling[62,94,133–135,143]. In support of a direct role for AngII-mediated remodeling, blocking the AngII receptors in cardiac fibroblasts using the ARB losartan attenuates pro-fibrotic TGF-β production[133,134].

The production and effects of TGF-β are neither AngII dependent, nor limited to fibroblasts and myofibroblasts. Indeed, TGF-β-mediated fibrogenesis is largely conserved amongst fibrosis models including those with a hypertension component. For example, inhibiting early TGF-β signaling in pressure overloaded rat hearts with neutralizing antibody limits fibroblast proliferation and in turn, fibrosis[29,143]. In contrast, overexpression of myocardial TGF-β exacerbates hypertrophy and fibrosis[135]. Leukocyte lineages such as MΦ subsets can also produce and respond to TGF-β in ways that can both modulate inflammation and promote fibrosis[29,60,62,135,142,144,145]. Relevant to this thesis, fibroblasts are often downstream targets of MΦ TGF-β production[29,52,60,135,142,146–148]. Together, the abundance of fibroblasts in the myocardium in combination with their activation through both (i) hypertension-dependent and (ii) AngII-dependent mechanisms make them an important mediator in AngII-mediated myocardial fibrosis.

Importantly, inflammatory injury and fibrosis do not represent distinct phases of cardiac healing, but rather a continuum: while the injury is occurring, the processes that mediate the fibrotic response are simultaneously developing[52,142]. Growth factors such as TGF-β may be better known for their role in promoting fibrosis, but even the pro-inflammatory cytokines including TNF-α and IL-6 have profound effects on fibrogenesis[121,149–151]. For example, in different environments, IL-6 can activate both pro-inflammatory or anti-inflammatory/pro fibrotic states in MΦ. And bridging the gap between inflammation and healing, co-culturing MΦ and fibroblasts stimulates IL-6 production. Consequently, this IL-6 production increased TGF-β production and in turn, increased fibroblast production of ECM proteins such as collagen[121]. Moreover, IL-6 is an important factor in promoting cardiomyocyte hypertrophy[152,153]. As such, while one

can imagine this process as beginning in a very pro-inflammatory state and shifting toward an anti-inflammatory/pro fibrotic state, the period in between is characterized by a diverse milieu of cells and signals[1,100,154,155]. It is this transition period from inflammation to healing that may offer the greatest therapeutic opportunity because it provides researchers and clinicians alike the ability to prevent unnecessary inflammatory tissue injury[147]. In turn, shortening this phase may permit the inflammation to mediate the necessary changes for adequate, but not excessive, remodeling.

Together, these processes and interactions highlight the complex relationship between different cell types – MΦ, fibroblasts, cardiomyocytes, endothelial cells, and VSMC – and the pleiotropic signaling molecules involved in cardiac healing[29,156]. In addition, it also demonstrates the complexity in separating the direct effects of AngII from the effects mediated by hypertension.

Thus far, I have introduced some of the key players in myocardial fibrosis, including the hormone AngII and its effects on hypertension, fibroblasts and myofibroblasts, as well as hinted at the main character in this story: MΦ. My thesis has largely focused on the role of this evolving cell type and as I move to the next section of the introduction, I will elaborate on the role of MΦ in myocardial injury and fibrosis.

1.1.5 Cardiac MΦ as Mediators of Myocardial Injury and Repair

1.1.5.1 Etiology

When I began my graduate work, there was only 1 source of MΦ in the heart: circulation[1,157]. MΦ were the tissue-bound, mature form of their monocyte precursors that patrolled circulation. Moreover, MΦ were assumed to be absent in healthy myocardial tissue and a by-product of tissue injury and repair[1,154]. In 2012, Pinto et al. turned that

idea on its head: they discovered an abundant resident MΦ population in the heart during steady-state[157] . In this section of the introduction, I will discuss the role of MΦ in myocardial injury and fibrosis by examining their origins, migration, and phenotypic and functional subsets.

I will begin with a bold statement: MΦ are virtually essential to the development of myocardial fibrosis. No, they may not be the cells producing the collagens and other ECM proteins that comprise fibrosis, but repeatedly, researchers have demonstrated that a significant reduction in infiltrating MΦ in the heart significantly reduces fibrosis[59,72,158,159]. As previously mentioned, this can be both beneficial and detrimental: too much fibrosis and the heart permanently loses function, but too little and the heart can succumb to physiological stress. The story of MΦ in myocardial fibrosis is more complex than just their numbers though. In order to explain the complexities of MΦ and monocytes in myocardial injury and fibrosis, I have divided this section into 2 revelations:

(i) Monocytes and MΦ are on an infinite spectrum of differentiation and activation states

(ii) The presence of a resident MΦ population in the myocardium.

1.1.5.2 Revelation 1 – MΦ Spectrum

MΦ and their circulating precursors, monocytes, are not a uniform cell type and this has important ramifications for their role in disease[145,160,161]. Before I continue though, I need to provide some clarification on the nomenclature:

(i) The nomenclature is an oversimplification of an infinite spectrum of monocyte and MΦ phenotypes. Rather, monocytes and MΦ are often discussed in terms of the spectral poles of activation (i.e. classical vs. non-classical and M1 vs. M2, respectively).

(ii) Monocytes exist on a spectrum of differentiation from an early classical phenotype to a later, more mature, non-classical phenotype[162–166].

(iii) In tissue, the MΦ spectrum extends from the pro-inflammatory M1 subsets to an array of M2 subsets with functions that include regulating inflammation to promoting angiogenesis and fibrosis[1,161,167–174].

(iv) Within literature – and to add unnecessary confusion – M1 and M2 differentiation can be referred to as classical and non-classical activation, respectively.[175,176]

(v) The tissue environment dictates MΦ differentiation towards M1 and M2 activation (i.e. the cytokine milieu will activate MΦ in a particular way that alters their phenotype and function)[160,175,177],

(i) There is no strong consensus on the differentiation of one monocyte subset toward a particular MΦ subset, but evidence supports classical to non-classical monocyte differentiation. This has given rise to non-classical monocytes being referred to as 'MΦ of circulation'[154,165].

(ii) Adding further complexity, while some researchers have suggested all monocytes are inflammatory, evidence from myocardial infarct models contradicts such a theory[154]. For example, disrupting the CX3CL1-

CX3CR1 chemotactic axis impairs the recruitment of non-classical monocytes, which in turn, prevents inflammation resolution[72].

(iii) Lastly, I argue that the terminology has unnecessarily contributed to overlapping research and confusion by assigning monocyte and MΦ subsets with names such as fibrocytes[ix178,179]. If one assumes that monocytes or MΦ are a single phenotype cell, then inevitably, researchers will assume they are studying a novel cell type. Underlying this trend may be the fact that classical monocytes represent a naïve myeloid lineage with vast differentiation potential[180].

The phenotypic characterization of the broad monocyte and MΦ subsets in humans and mice is described below in Table 1. Hopefully, the points above and table below will help provide some clarity as I move forward in describing the roles of monocytes and MΦ in myocardial injury and fibrosis.

Table 1.1 - Phenotypical characterization of monocyte and macrophage subsets in humans and mice

	Classically (inflammatory, M1)	**Intermediate**	**Non-classical (patrolling, M2)**
Human	$CD14^{++}CD16^{-}$	$CD14^{++}CD16^{+}$	$CD14^{+}CD16^{++}$ (or $CD14^{dim}CD16^{++}$)
Mice	$CD11b^{+}/CD115^{+}/Ly6C^{+++}$ (or $Ly6C^{high}$)	$CD11b^{+}/CD115^{+}/Ly6C^{++}$ (or $Ly6C^{mid}$) ?	$CD11b^{+}/CD115^{+}/Ly6C^{+}$ (or $Ly6C^{low}$)
Primary Chemokine Receptors	$CCR2^{++}$ CX_3CR1^{+} (or $CCR2^{high}$ CX_3CR1^{low})	$CCR2^{+}$ CX_3CR1^{+} (or $CCR2^{mid}$ CX_3CR1^{mid})	$CCR2^{+/-}$ CX_3CR1^{++} (or $CCR2^{low/neg}$ CX_3CR1^{high})

Traditionally, MΦ were assumed to be entirely of the M1 phenotype, characterized a population of pro-inflammatory leukocytes and rightfully so: many classical monocytes migrate en masse to areas of inflamed tissue, such as the perivascular

and vascular regions of the hypertensive, AngII-exposed heart[72,181,182]. This mass migration is associated with the production of classical monocyte chemokines including CCL2, CCL3, and CCL5[182,183]. Importantly, classical monocytes strongly express the corresponding chemokine receptors, such as CCR2, which favours their selective recruitment to inflamed tissues[184]. In addition, stressed and injured vasculature upregulates the expression of selectins and adhesion molecules[29,31,95,110,128]. These selectins and adhesion molecules are common features in inflammation, but it is important to note that AngII can also increase the binding of monocytes to the endothelium independent of selectins and adhesion molecules[99,128]. Thus, in the context of in vivo AngII signaling, it is difficult to separate the influence of selectins and adhesion molecules from the direct influence of AngII. Regardless of how monocytes enter the heart, if MΦ were assumed to be strictly of a pro-inflammatory type, then why would they persist during the transition from cardiac inflammation to fibrosis[185]?

The question above can be answered with a novel set of MΦ phenotypes termed non-classical, alternative, and M2[60,72,102,145,147,157,160]. These terms are used to describe a MΦ that does not behave conventionally. Instead, alternative MΦ take on roles in suppressing inflammation and favouring the healing processes that include fibrosis[52,60,145,157,160,161]. There is still debate on the origin of infiltrating M2 MΦ in the myocardium: (i) they are at least in part recruited via the CX3CL1-CX3CR1 chemotactic axis and (ii) may also transition from M1 MΦ already present in the tissue, though this remains to be proven[154,186]. In support of the former, $CX3CR1^{-/-}$ mice exhibit impaired recruitment of $CX3CR1^{high}$ MΦ in models of skin and kidney fibrosis[187–190]. This recruitment appears to be aided through the integrin VLA-4 and VCAM-1[191]. In addition,

significant CX3CL1 upregulation is observed in the later stages (>7d) of healing following myocardial infarction[154,192]. This phase of healing in infarcts likely represents a a somewhat analogous but greater process to that which is observed with AngII infusion or other hypertensive models of myocardial fibrosis.

Unfortunately, there has still been less research on monocyte migration in non-ischemic cardiac models than ischemic and infarct cardiac models. However, where non-ischemic models are lacking, ischemic and infarct models have helped fill the gaps[74,154,186]. In an elegant experiment using an infarct model, Nahrendorf et al. mapped the temporal and chemotactic migration of the 2 monocyte subsets in an infarct model using a combination of $CCR2^{-/-}$ and $CX3CR1^{-/-}$ mice[154]. In the first phase of healing (~1d post infarct), $CCR2^{-/-}$ mice exhibited a significant reduction in the number of M1 MΦ in the heart, while in $CX3CR1^{-/-}$ mice, this population was unaffected. In contrast, $CX3CR1^{-/-}$ mice were unable to accumulate M2 MΦ during the healing phase, while again, this population was unaffected in $CCR2^{-/-}$ mice. I have stopped short of explicitly stating this is a by-product of modulated migration because, as the same group demonstrated, CX3CR1 has roles beyond chemotaxis[193–196]. For example, another mechanism by which CX3CR1 may stop runaway inflammation in the heart is via the retention of monocytes in the bone marrow[193]. In turn, this would discourage further monocyte recruitment to the myocardium and the consequential inflammation. Nevertheless, this body of research supports a biphasic mechanism by which MΦ of different phenotypes and functions accumulate from monocytes via distinct chemotactic pathways following injury[1,102,154,197].

At this point, I will begin to shift the attention from monocytes to MΦ; however, one cannot entirely separate one from the other. MΦ represent the tissue-bound form of

the monocyte lineage and as indicated above, monocyte recruitment can directly impact MΦ accumulation. Moreover, the current evidence in infarct models suggests that the cardiac environment influences monocyte subset recruitment, which may, in turn, influence which MΦ activation occurs[154,198]. Thus, the ability to recruit monocytes with differing functional predispositions may allow the injured organ to direct its repair[154,165,184].

The biphasic recruitment of monocyte subsets and, in turn, MΦ subset accumulation is one of those beautifully organized immune processes that highlights the crosstalk between the tissue environment and leukocytes[100,154]. As hinted at above, each monocyte and MΦ subset serves a vital role in mediating cardiac repair[1,154,197]. Much of what we know about monocyte and MΦ subsets is due to researchers being able to selectively deplete monocyte and MΦ populations[72,154,165,199,200]. The experimental techniques for depleting monocytes and MΦ include (i) administration of cytotoxic clodronate liposomes and (ii) administration of diphtheria toxin to mice with the diphtheria toxin receptor under the CD169 or CD11b promoter[x72,154,200–203]. And rather than simply inhibiting monocyte migration using chemokine receptor knockouts (e.g. CCR2 and CX3CR1), these methods are able to temporarily deplete monocyte and MΦ populations[71,72,154]. Moreover, the depletion can be timed according to which stage of healing one wants to modulate[154,165]. Unfortunately, much of the experimentation to date has again focused on infarct and ischemia models[154,165]. These models have still proven useful in delineating the role of M1 versus M2 MΦ in the healing myocardium. In addition, in vitro models that aim to replicate tissue environments, which in turn

influence MΦ differentiation, have helped support general functional states for the subsets[204–207].

In general, the tissue environments that favour M1 activation often include factors such as IFN-γ, TNF-α, specific TLR activation (e.g. TLR4) through damage- and/or pathogen-associated molecular patterns (DAMPs and PAMPs), and GM-CSF[174,175,180,208]. Indeed, even AngII has been shown to promote M1 MΦ activation via AT1R, further supporting a pro-inflammatory role for the hormone[209,210]. Many of these signaling pathways and their downstream effects support an environmental demand to amplify the pro-inflammatory response[xi100,154,211]. The pathways most relevant to this thesis include AngII, DAMP/TLR, IFN-γ, and TNF-α signaling, which are upregulated early (i.e. 1d) in the AngII-model, as well as during early hypertensive cardiac injury[100].

The M1 subset performs the functions *classically* associated with MΦ such as strong phagocytic activity and promoting inflammation[175,177]. While there is strong evidence to suggest that these functions are important in ischemic and infarct models, the evidence is less clear in hypertensive and AngII-mediated models[59,72,154]. In infarcts for example, the loss of cardiomyocytes due to ischemia requires M1 MΦ to aid in the phagocytosis of apoptotic cells and to promote the recruitment of other leukocytes such as neutrophils to phagocytize necrotic cells[59,154]. In the absence of M1 MΦ, dead tissue is not sufficiently removed to catalyze the next phase of healing: granulation and remodeling[59]. Moreover, injured and necrotic cells can produce DAMP, which are recognized by MΦ (preferentially the M1 subset)[xii212,213]. In turn, MΦ can further the pro-inflammatory response by producing a cascade of cytokines and chemokines that include TNF-α, IL-1β, CCL2, and IL-8[204,206]. As such, one can understand why impaired clearing

of cellular debris and necrotic tissue could dysregulate normal resolution of inflammation.

AngII and hypertensive cardiac models also exhibit an early inflammatory response, but with distinct differences such as a lack of IL-8 signaling and in turn, fewer polymorphonuclear cells[47,69,71,72,100]. This can likely be attributed to a relative lack of apoptotic and necrotic cardiomyocytes, which are not subject to extreme oxygen and nutrient deprivation during hypertension. In addition, unlike ischemic injury, the early inflammation that promotes M1 activation in hypertension may be an overreaction to the injury such that the inflammation does more harm than good. In support of a theory of inflammatory overreaction, blocking various inflammatory pathways upstream and downstream of M1 activation significantly reduces myocardial fibrosis in AngII and hypertensive models:

(i) Upstream: impaired IFN-γ and TLR4 signaling reduced pro-inflammatory (e.g. iNOS and Mac-2) and remodeling (e.g. αSMA, collagen, TGF-β) markers,[xiii101,214]

(ii) Downstream: impaired TNF-α, IL-1β, CCL2, and IL-6 signaling also reduces markers of leukocyte recruitment (e.g. ICAM-1 and VCAM-1, CCL2), inflammation (e.g. iNOS, Mac-2, IFN-γ, IL-12, IL-6, TNF-α) and remodeling (e.g. αSMA, collagen, TGF-β)[70,71,93,215].

Placing MΦ in the center of this process, studies employing non-specific monocyte and MΦ depletion (i.e. classical and non-classical monocytes and M1 and M2 MΦ) have also shown significant reductions in markers of remodeling (e.g. αSMA, collagen, TGF-β)[72,154]. Moreover, the fact that none of these non-ischemic studies report adverse events

in response to impaired inflammation suggests that, unlike M1 MΦ in infarcts, their pro-inflammatory role may be largely unnecessary and maladaptive[72,158]. Thus, this body of research supports the concepts that, in AngII and hypertensive models, (i) inflammation generally begets more inflammation (e.g. IFN-γ → M1 activation → TNF-α), (ii) MΦ activation has a key role in promoting this inflammation, and (iii) this, in turn, drives the subsequent fibrotic response.

Further highlighting their role as orchestrators in cardiac healing, M1 MΦ also appear to serve an important role in initiating the subsequent remodeling that is required in an injured heart. While often conceptualized as distinct phases, the inflammatory component of myocardial repair influences the remodeling phase through direct and indirect interactions between M1 MΦ and both fibroblasts and myofibroblasts[52,216]. For example, MΦ-produced TNF-α has been shown to activate pathways involved in the upregulation of pro-fibrotic factors including TGF-β[150]. Moreover, there are abundant pro-fibrotic factors, including latent TGF-β bound to the ECM, which can be liberated through the production of MMPs by both MΦ subsets[217–219]. As previously mentioned, these factors go on to promote fibroblast differentiation to myofibroblasts, as well as stimulating the production of ECM proteins including collagens.

As previously mentioned and most relevant to this thesis is the role of IL-6 in MΦ and fibroblast communication because of evidence in the AngII model[121,153]. In an elegant set of experiments, Ma et al. demonstrated that AngII infusion upregulates the production of IL-6 in myocardial myofibroblasts[121]. In addition, mice deficient in IL-6 exhibited significantly fewer MΦ and less myocardial fibrosis following AngII infusion. In order to further investigate this finding, Ma et al. co-cultured M1 MΦ[xiv] with

fibroblasts to determine whether this was in part due to an interaction between the cell types. M1 MΦ were able to stimulate fibroblasts to produce IL-6, which set off a cascade of events associated with fibrosis, including:

(i) upregulating MΦ expression of MMP-1: a key enzyme involved in the degradation of collagens type I, II and III,

(ii) increased production of TGF-β and its signaling via the Smad3 pathway,

(iii) increased fibroblast expression of αSMA,

(iv) and increased fibroblast expression of Collagen type 1 (Col-1).

Finally, in the pièce de résistance, these effects were ablated in fibroblasts lacking IL-6 signaling (i.e. IL-$6^{-/-}$) or in which IL-6 signaling was blocked using anti-IL-6 antibodies. Thus, while beyond the scope of work presented in this thesis, IL-6 may be a key switch in the progress from M1-driven inflammation to cardiac remodeling. In summary, the likely role of M1 MΦ in early cardiac healing is to promote a sufficient inflammatory response (e.g. leukocyte recruitment and debris clearance), but with a theoretical aim of moving toward resolving inflammation and promoting remodeling, as dictated by environmental changes.

The M2 subset of MΦ picks up where the M1 subset let off, carrying the anti-inflammatory and pro-fibrotic response to cardiac injury. As such, the M2 subset performs functions not typically associated with MΦ. Importantly, not all M2 MΦ are created equally: in vitro and in vivo experiments have demonstrated that the environment shapes the M2 response[60,145,159–161,174,175,180,197,220–222]. In an oversimplification of an infinite spectrum of activation, the different M2 states have been termed M2a, M2b, M2c,

and M2d[125,145,175,180,223]. The conditions that promote their differentiation and their subsequent cytokine and receptor expression are described below:

(i) IL-4/IL-13 → **M2a** → Arginase1, FIZZ1, CD206, TGF-β, CD301, IL-10

(ii) Immune complexes + LPS or IL-1β → **M2b** → iNOS, IL-10, IL-6, TNF-α

(iii) IL-10 + TGF-β or glucocorticoids → **M2c** → CD206, CD163, Arginase1, IL-10, TGF-β

(iv) Adenosine + IL-6 → **M2d** → VEGF, IL-10, IL-12, TGF-β, TNF-α

Despite M2a, M2b, M2c, and M2d being an oversimplification of MΦ differentiation, researchers can still try to form links between the cardiac environment (e.g. IL-10, TGF-β, IL-4/13) and MΦ subset characterization (e.g. CD206, CD163, CD301). Unfortunately, there continues to be little information on which M2 subsets are involved in cardiac injury and repair. Even studies in infarct and ischemic models largely lack specific characterization for M2 markers and are limited to CD206 expression[72,100,102,197]. It is known that M2 subsets accumulate in the myocardium during the later stages of healing (~3-7d post infarct) and that their depletion is associated with abnormal and insufficient cardiac remodeling[59,100,154,224]. In addition, M2 cells as characterized by $CD206^+$ and $CD301^+$ – M2a-like phenotype – accumulate in the later stages (~3-7d) of AngII-mediate cardiac injury[72,100]. Consistent with this finding, IL-4 and IL-13 – cytokines upstream of M2a differentiation – are upregulated at the same time point, supporting the idea that AngII favours a later M2a-like phenotype[100]. There are serious limitations to these experiments though. For example, the researchers only reported the percentage of live cells expressing M2 markers – not the absolute number of M2 cells. Moreover, CD206 is also a marker of the M2c phenotype, for which the necessary

environment to induce their differentiation (i.e. TGF-β and IL-10) is likely present in the AngII model[72,100]. In further support of a role for an M2c-like phenotype, AngII infusion in IL-10$^{-/-}$ mice is associated with unresolved inflammation and increased MMP expression[225].

Thus, in the case of M2 MΦ and their spectrum of activation, much remains unknown, but we can begin to piece together how these subsets may resolve inflammation and begin the remodeling process. For example, in contrast to M1 MΦ, the M2 subsets are characterized by their increased production of IL-10 and decreased production of IL-12[xv175]. This would theoretically begin to limit a TH1 response in T-cells that feeds the M1 activation of MΦ. AngII models have also suggested that IL-10 decreases TNF-α and IL-6 signaling, which would ebb the heart's inflammatory response and the paracrine signaling between M1 MΦ and fibroblasts[225]. In the lead up to increased IL-10 expression, one can also imagine that the IL-6 signaling between M1 MΦ and fibroblasts that encourages the former to produce TGF-β could create the conditions necessary to begin changing M1 MΦ to M2 MΦ. In addition, the M2a and M2c subsets express high levels of Arginase1, which utilizes the substrate L-arginine[145,175,180]. Importantly, L-arginine is also the substrate for iNOS and its depletion can also regulate T-cell cell-cycle progression[226]. This provides another mechanism by which M2 subsets can regulate inflammation.

While regulating inflammation is an important step in beginning the remodeling process, in order for myocardial fibrosis to develop, a shift is required in the ratio of MMP-dependent ECM degradation to ECM production. There are 2 potential mechanisms by which M2 MΦ promote fibrosis: (i) interactions with fibroblasts and (ii)

the direct production of ECM proteins[145,148,160,161,178,179,227]. The production of TGF-β by M2 MΦ is thought to be the most important MΦ-dependent pathway in promoting fibrosis[145,148,160,161]. As mentioned previously, it is well-established that TGF-β promotes the differentiation and proliferation of fibroblasts, as well as enhancing their production of ECM proteins. Indeed, an increase in myocardial TGF-β is observed in tandem with the accumulation of M2 MΦ in the AngII-exposed myocardium[xvi100]. TGF-β also plays an important role through inhibiting MMP production and increasing TIMP production[62,135].

The ability for M2 MΦ to directly contribute to fibrosis through the production of ECM proteins is arguably better supported by literature, but introduces a world of confusion[39,178,228–230]. As mentioned above, some MΦ subsets express the enzyme Arginase-1, which competes with iNOS for the substrate L-arginine. Through a series of steps that are beyond the scope of this thesis, these Arginase-1-expressing M2 MΦ are able to convert L-arginine into collagen through an L-proline intermediate[231,232]. The confusion arose – and persists – as a result of MΦ expressing collagen and smooth muscle cell actin: markers thought to only be expressed by mesenchymal lineage cells[233–237]. Researchers even went so far as to call these a new cell type: fibrocytes[238]. Fibrocytes are/were cells that express markers of hematopoietic and mesenchymal lineages[47,71]. Perhaps they migrated to tissues in a way that resembled progenitor cells, but the evidence was lacking. Rather, there was more evidence to suggest that they migrated like monocytes. In addition, fibrocytes expressed many of the same markers as monocytes and MΦ, including CD68, CD14, and CD16[179].

As the depth of M2 activation developed, the lines between M2 MΦ and fibrocytes blurred. I came to this realization while sitting in a lecture at a transplantation conference in Quebec City. As you read on in this thesis, you will find that our laboratory – myself included – studied the elusive fibrocyte. During the talk at the conference, the presenter brought up numerous markers that we had used to characterize fibrocytes, but he was discussing M2 MΦ. It was a moment of research enlightenment. All of a sudden, there was not just new literature to read, but more literature as well. After all, MΦ were a much larger and older topic than fibrocytes.

Some researchers have held on to the concept of fibrocytes, suggesting they are a unique monocyte-derived lineage[178]. My faith waned; I found more coincidences between M2 MΦ and fibrocytes. For example, Serum Amyloid P (SAP) has been suggested to inhibit fibrocyte differentiation, but change 'fibrocyte' for 'M2 MΦ' and it appears to do the same for the latter[239–241]. This could be attributed to the fact that "both" cell types express the receptors for SAP, FcγRs[xvii]. The binding of SAP to MΦ, however, encourages the production of IL-10 to dampen inflammation in a process that is eerily similar to M2b activation[239,242]. In addition, PPARγ activation has been implicated in the IL-4/IL-13-dependent activation of M2 MΦ, as well as the differentiation of fibrocytes[243–245]. Perhaps most disconcerting was how research groups bastardized flow cytometry to demonstrate the presence of fibrocytes: antibodies against type-1 collagen that were never meant for flow cytometry often showed bizarre results such as all leukocytes expressing collagen[93]. I do not think it was done intentionally, but it was definitely systemic in groups trying to study fibrocytes. For example, we received one fibrocyte flow cytometry protocol that suggested using multiple tubes for the positive

label and isotype and using the "best one". I am not arguing against the existence of a monocyte or MΦ lineage that expresses collagen or αSMA. The fact that they express these markers supports that they may have a role in cardiac remodeling. Alas, fibrocytes are likely just another point along the M2 MΦ activation spectrum. It is still unknown whether ECM-producing M2 MΦ are key contributors to fibrosis. Moreover, the collagen observed within MΦ may be a consequence of other roles in fibrogenesis including phagocytizing ECM proteins[246].

While largely deductive or loosely supported by in vitro modeling, together, these observations suggest that M2 MΦ likely play an important role in creating an environment conducive to fibrosis. Moreover, the cumulative effect of these processes shift the scales from increased ECM degradation to increased ECM deposition, thus precipitating scar formation.

1.1.5.3 Revelation 2 – rCMϕ

Around the time I switched my research focus from fibrocytes to MΦ, I was in the process of submitting a manuscript. The manuscript, as you will read later on, described the effects of monocyte depletion on AngII-mediated myocardial fibrosis[72]. And then we got a question back from a reviewer: what about rCMΦ?

A few years ago and to the surprise of many, the heart was found to have a significant resident MΦ population that originated prenatally[157,247–249]. The embryonic cardiac MΦ tended to exhibit cardioprotective effects, including mediating tissue regeneration – a process once thought to be impossible[159]. Overtime, it was shown that this resident population of MΦ can also exist on a spectrum of activation phenotypes[247–

[249]. Fortunately for me writing this section and you reading it, the same rules that applied in Revelation 1 for MΦ activation also apply to rCMΦ and do not need repeating. As such, this section will focus on the point that is most relevant to this thesis: (i) the turnover of resident MΦ and how this may influence their phenotype and function. Importantly, since this is a relatively novel area of research (i.e. only discovered as of 2012), there is still much debate in the literature on the characterization of populations and their rates of proliferation and turnover[247–250]. However, researchers are becoming increasingly aware that the time scales used in experiments can drastically change the context of an observation (e.g. if the yolk-sac derived cardiac resident MΦ naturally declines from ~15% at birth to just 3.2% at 42wk post-natal, then observations about rCMΦ are explicitly time-dependent)[250].

As mentioned above, rCMΦ develop during the embryonic stages of development and primarily exhibit an M2 phenotype, as supported by surface markers and transcription profile[157,248–250]. At birth, rCMΦ are almost entirely characterized as F4/80^{+} CX3CR1^{+} MHC II^{-} CCR2^{-}[250]. During development, additional populations begin to appear in the myocardium, which are described below with their percent composition of the whole:

Table 1.2 - Summary of changes in mouse rCMΦ composition with age starting at neonatal

	Phenotype	% of Total RCMΦ					
		0wk	1wk	3wk	8wk	18wk	30wk
Pop. 1	F4/80+ CX3CR1+ MHC II-	95.7	91.6	65.7	29	21.8	23.9
Pop. 2	F4/80+ CX3CR1- MHC II-	2.89	5.48	4.02	18.3	28.5	28.2
Pop. 3	F4/80+ CX3CR1+ MHC II+	1.01	2.45	28.5	47	34.8	33.3
Pop. 4	F4/80+ CX3CR1- MHC II+	0.408	0.367	1.9	5.79	14.9	14.4
Adapted from Molawi et. al, 2014 [250]							

The current understanding is that the founder population (Pop. 1) is able to replenish the CX3CR1^{+} (Pop. 1 and 3) populations during steady state conditions[248,250]. In contrast, the CX3CR1^{-} populations are largely replenished from circulating CCR2^{+} Ly6C^{high} classical monocytes[248,250]. In addition, the expression of MHC II appears to serve as a marker of maturation for rCMΦ independent of their etiology[250]. This is further supported by the finding that Pop. 1 (i.e. founder) and Pop. 2 exhibit higher levels of proliferation than either Pops. 3 or 4[248]. Indeed, the cell turnover in steady state is sufficiently slow that it requires mice to be studied over the course of weeks to identify differences[248–250]. It may also be the case that Pops. 1 and 3 can be replenished from circulating CX3CR1^{+} Ly6C^{low} non-classical monocytes; however, this has yet to be proven. In support of such a theory, sophisticated studies using labeled yolk-sac-derived (E9) MΦ have demonstrated that proliferation of the founder population alone is insufficient to maintain the >20% of rCMΦ expressing its phenotype[250]. Moreover, the protective founder population is continually lost with age due to normal cell turnover, suggesting that its protective function is also lost with age[159,250]. Relevant to this thesis, clodronate liposomes are able to partially deplete rCMΦ populations[159,248]. All populations exhibited some proliferative capacity, which, together with an unknown contribution from circulation, was able to return populations to pre-depletion levels by 17d[248]. As such, one can also imagine that processes that accelerate MΦ turnover, such as tissue injury, could exacerbate the loss of this population. Indeed, during conditions of stress such as hypertension, all rCMΦ populations can be replenished from a combination of CCR2^{+} classical monocytes derived from circulation and local proliferation[248,249,251]. In

support of local proliferation, $CCR2^{-/-}$ mice still demonstrated expansion of Pop. 1[248]. In contrast, Pops. 3 and 4 showed the greatest contribution from $CCR2^{+}$ monocytes[248]. The exact contribution of recruitment versus proliferation remains unclear though. Together, these processes of MΦ turnover could lead to fundamental differences in the overall cardiac resident MΦ phenotype and in turn, their functions.

As for the specific functions of the rCMΦ populations, much remains unknown. Thus far, researchers have been able to show that, in general, $CCR2^{+}$ rCMΦ exhibit a shift toward pro-inflammatory functions, while $CCR2^{-}$ rCMΦ exhibit a shift toward anti-inflammatory functions[157,248–251]. In support, Lavine et al. demonstrated that the protective functions of embryonic cardiac resident MΦ are associated with a protective M2 phenotype characterized as lacking CCR2[252]. These $CCR2^{-}$ rCMΦ were able to encourage neonatal cardiomyocyte proliferation and angiogenesis. In contrast, $CCR2^{+}$ cells were associated with a pro-inflammatory phenotype including the upregulation of inflammasome genes[248]. Indeed, the $CCR2^{+}$ MΦ were required for the typical increase in IL-1β observed during AngII infusion. The $CCR2^{+}$ cardiac MΦ also promoted cardiomyocyte hypertrophy and fibrosis and were unable to promote cardiomyocyte proliferation or angiogenesis[159,249]. In another paper by the same group, Epelman et. al demonstrated that – not surprisingly – the MHC II^{+} rCMΦ exhibit a greater ability for antigen presentation[248]. Thus, while the literature on the roles of the individual cardiac resident MΦ populations is broad, the balance of these populations – for better or worse – is likely imperative to the myocardium's ability to heal.

1.2 Summary of Introduction

In this introduction, I have attempted to set the stage for the manuscripts in this thesis. I have described the personal and global burden of heart failure and the link to hypertension. In addition, I have tried to reduce these clinical syndromes and conditions to some of their underlying causes (e.g. myocardial fibrosis) and the cells responsible (e.g. fibroblasts and MΦ). Importantly, this introduction was written in hindsight – knowing what I know now, my hypotheses and, in turn, my manuscripts would be vastly different. As you continue reading through the manuscripts that comprise this thesis, I believe the information outlined in the introduction provides the context for my hypotheses, observations, and conclusions. Again, taking a step back and examining my work as a whole rather than individual pieces, the overarching theme I have tried to reinforce is as follows:

When I began my degree, myocardial fibrosis was largely believed to be a single-cell type process, driven by ECM-producing fibrocytes. Over time, as the concept of a fibrocyte dissolved, MΦ emerged as a key effector cell in both myocardial injury and fibrosis through their interactions with fibroblasts. As my degree comes to an end, MΦ have taken an eminent role in the heart and fibrocytes have faded into oblivion.

CHAPTER 2 HYPOTHESES, RATIONALES, AND OBJECTIVES

2.1 Study 1: Fibrocyte migration/progenitor cell mobilization in the AngII-infusion model of myocardial fibrosis

Rationale: There was growing evidence for the involvement of a cell type expressing mesenchymal and hematopoietic markers (i.e. fibrocyte) in models of fibrotic disease[238,253,254]. Specifically, we as well as others had demonstrated an early (~3d) abundance of fibrocytes in the myocardia of animals receiving exogenous AngII[47,254]. At the time, many researchers believed fibrocytes were either a type of progenitor cell or derived from a monocyte pre-cursor that emigrated from the bone marrow to populate organs and promote fibrosis[47,155,255,256]. Fibrocytes had been shown to express chemokine receptors CXCR4 and CCR2, which correspond to the chemokines CXCL12 and CCL2, respectively[93,256–258]. CXCL12 had been shown to be an important chemokine for the recruitment of other progenitor populations to the heart, while CCL2 was a well-established chemokine for classical monocytes[259,260]. Moreover, we had also observed early increases in CXCL12 and CCL2 myocardial transcript during AngII infusion[47].

It followed that if (i) fibrocytes were contributing to myocardial fibrosis and they migrated similar to (ii) progenitor cells or (iii) classical monocytes, then blocking or inhibiting the CXCL12 and/or CCL2 chemotactic pathways would prevent fibrocytes accumulating in the AngII-exposed heart and in turn, reduce myocardial fibrosis.

Objectives: Determine the ability to block the fibrocyte recruitment to the AngII-exposed myocardium using a CXCR4 antagonist and $CCR2^{-/-}$ mice. Determine how manipulating

these chemotactic pathways in the AngII-infusion model impacts leukocyte recruitment and myocardial fibrosis.

2.2 Study 2: Monocyte/MΦ depletion and an alternative chemotactic pathway in the AngII-infusion model of myocardial fibrosis

Rationale: Fibrocyte research had begun heading in a different direction – fibrocytes were now primarily considered to be monocyte-derived and thus, believed to behave more like monocytes[71,93]. Others had demonstrated that clodronate liposomes could deplete monocytes and some had even proposed the fibrocytes could be targeted with the same approach[200,261,262]. In turn, we hypothesized that the accumulation of fibrocytes or their monocyte-precursors could be prevented by depleting circulating monocytes.

In addition, monocytes/MΦ were no longer viewed as a single cell type, but rather a spectrum of phenotypes exhibiting pro-fibrotic functions[263]. There was reason to believe that fibrocytes may be (i) derived from non-classical monocytes and/or (ii) be a subtype of M2 MΦ. In turn, we focused on the non-classical monocytes chemotactic pathway CX3CL1-CX3CR1 as a potential means for fibrocyte migration[154].

Lastly, this manuscript was submitted at a time when Pinto et al. discovered a resident MΦ population in the heart[157]. As such, as an afterthought, we had to consider a potential role for rCMΦ in our observations, particularly with regard to observations in the CX3CR1$^{-/-}$ mice.

Objectives: Determine the effects of (i) monocyte depletion using clodronate liposomes and (ii) impaired non-classical monocyte recruitment using CX3CR1$^{-/-}$ mice on the accumulation of fibrocytes in the AngII-exposed myocardium. In turn, we also aimed to evaluate how these depletion strategies (e.g. liposomes and chemokine receptor knockouts) influence AngII-mediated myocardial fibrosis.

2.3 Study 3: Characterize baseline rCMΦ phenotypes between WT and CCR2-/- mice

Rationale: In our previous manuscript, we observed that CX3CR1$^{-/-}$ mice exhibit a potentially pro-inflammatory shift in the overall rCMΦ phenotype[72]. This pro-inflammatory shift was associated with worsened inflammation and fibrosis in the context of AngII infusion. Importantly, for monocytes, the complementary chemotactic pathway to CX3CL1-CX3CR1 is CCL2-CCR2.

In the first manuscript presented in this thesis, we demonstrated that CCR2$^{-/-}$ mice are partially protected from AngII-mediated myocardial inflammation and fibrosis[71]. In addition, there was growing evidence for the importance of the rCMΦ population in the ability to influence cardiac healing[159,248]. Specifically, the embryonic and yolk sac-derived rCMΦ conferred the ability to promote cardiac regeneration; however, this population is also replaced over time with monocyte-derived MΦ recruited via CCR2[159,250]. As such, the heart loses its ability to promote regeneration with age. Thus, our hypothesis was that (i) if CX3CR1 is complementary to CCR2 and (ii) CX3CR1$^{-/-}$

mice exhibit a pro-inflammatory rCMΦ phenotypic shift, then (iii) CCR2$^{-/-}$ mice may exhibit an anti-inflammatory rCMΦ phenotypic shift.

Objectives: Investigate and compare the phenotypes of rCMΦ in CCR2$^{-/-}$ mice relative to WT mice. Using literature as a guide, analyze the findings in the context of (i) genotype and (ii) age-dependent rCMΦ turnover.

CHAPTER 3 METHODS

3.1 Animals

Animal experiments were performed in accordance with the Canadian Council on Animal Care and approved by the Dalhousie *University Committee on Laboratory Animals*. Male C57BL/6 mice (8-10wk old; wildtype: WT) were purchased from Jackson Laboratory (Bar Harbour, ME, USA), male $CCR2^{-/-}$ mice (8-10wk old) on a C57BL/6 background were provided by Dr. Thomas Issekutz from a routinely genotyped colony, and male $CX_3CR1^{-/-}$ mice (8-10wk old) on a C57BL/6 background were acquired through an material transfer agreement with Taconic[264]. The absence of CCR2 and CX3CR1 were verified using conventional PCR, as previously described[71]. Mice were housed in the Carleton Animal Care Facility at Dalhousie University and provided food and water *ad libitum* for 1wk prior to beginning experimentation.

3.2 Saline/AngII/AMD3100 Infusion

Osmotic mini-pumps (Alzet, Palo Alto, CA, USA) were implanted as previously described[47,68,155]. Animals were randomly assigned treatments of vehicle (saline), AngII (2.1/2.8 mg/kg/d; Sigma Aldrich, Oakville, ON, Canada), AMD3100 (11.4mg/kg/d; Tocris Bioscience, Ellisville, MO, USA), or AngII + AMD3100. AMD3100 dosing was based on previous work by others that demonstrated reduced fibrocyte recruitment to the lungs[265]. Osmotic mini pumps were used to avoid reduced trough levels, since the plasma half-life of AMD3100 is approximately 3.6hrs[266]. The pumps remained in for 6hr, 12hr, 1d, 3d or 28d, during which, the animals were observed for morbidity. In time comparison groups (i.e. 3d vs. 28d), a lower dose of AngII (2.1 mg/kg/d) was infused in

order to dose-match previous studies and reduce risk of mortality[93]. Animals were weighed and blood pressure measurements were taken using the Coda2 non-invasive tail cuff system (Kent Scientific, Torrington, CT, USA) for minimum of five measurements per animal. At the time of harvest, hearts were extracted, flushed with saline, and weighed.

3.3 Liposomes

Liposomes were generated as previously described, with modification [224]. In brief, egg phosphotidylcholine and cholesterol (82.4:15.6% mol/L ratio, respectively) in chloroform were added to sterile pear-shaped flasks, mixed well, and placed under rotary evaporation until excess chloroform was removed to dryness and a uniform film developed. Lipid-soluble fluorescent DiO was added for liposome binding and uptake studies. The film was subsequently hydrated with either clodronate (300mg/mL; Sigma Aldrich) or saline and placed on a shaker for 2hrs. Following the shaker, liposomes were left to swell overnight at RT. Liposomes were then sonicated (4min at 55Hz) and washed of free clodronate by centrifuging 4-5 times at 24000 x *g* for 5min at 10°C. Prior to injection, liposomes were passed through a 3.0µm pore filter (Millipore). The final concentration of lipid in the liposomes was 33.07mM. Liposomes were injected in 100µL doses via tail vein every 24hrs beginning at -1d before AngII infusion in order to deplete monocytes before introducing myocardial injury.

3.4 Blood Pressure

Animals were weighed and blood pressure measurements were taken using the Coda2 non-invasive tail cuff system (Kent Scientific, Torrington, CT, USA) for minimum of five measurements per animal.

3.5 Echocardiography (ECHO)

Prior to ECHO, mice were anesthetized and the fur removed using Nair hair removal cream. Images and recordings were acquired on a G.E. Vivid 7 Ultrasound. Short-axis M-mode measurements for left ventricular posterior wall (LVPW) and intraventricular septal wall (IVSW) thickness were performed in Adobe Photoshop CS6.

3.6 Tissue Harvest

At the time of harvest, animals were weighed and hearts were extracted, flushed with saline, and weighed. Experimental hearts were harvested and weighed. Cardiac mass index was calculated by dividing the heart weight by the change in body weight, as previously described. Subsequent processing dependent on the fate of the tissue:

3.6.1 Heart

(i) *Paraffin histology and mRNA*: the base portion of the heart was processed for histology and the rest of the heart was vertically bisected and snap frozen for molecular analysis. Hearts were processed in 10% buffered formalin for 24hr and paraffin-embedded. Serial-sections (5μm) were stained for histological and immunohistological analysis. Due to overlapping experimental groups previously

published, some historical samples from AngII and saline infused animals were used for this study to compare to new experimental groups[47,68].

(ii) *Frozen histology*: anesthetised mice were perfused with saline followed by 4% paraformaldehyde (PFA). The hearts were extracted and placed in 4% PFA overnight. The hearts were transferred to 30% sucrose and 10μm sections were cut on a cryostat.

(iii)*Cell isolation*: in brief, hearts were mechanically and enzymatically digested in a collagenase solution (1 mg/mL collagenase II; Cedarlane Laboratories, Ltd., Burlington, ON, Canada) in Gibco DMEM (Life Technologies Corp., Burlington, ON, Canada) at 37°C, with agitation for 30 minutes. Cell isolates were washed in complete DMEM containing 10% heat inactivated fetal bovine serum, 2 mmol/L L-glutamine, 100 mg/mL streptomycin, and 100 U/mL penicillin.

a. *Fibrocyte enrichment*: isolated cells were plated in T25 flasks coated with 0.1% gelatin, incubated for 3 days at 37°C and 5% CO2, at which point all non-adherent cellular debris was removed and medium was replenished. These cells were subsequently used in proliferation assays, flow cytometry, and immunofluorescence.

b. Cell isolation for MΦ studies: following washes to remove collagenase, cell isolates were further purified over a Percoll gradient (30% and 70%). The cells at the interface were collected for subsequent characterization, as previously described[72,157].

3.6.2 Blood

For AMD3100 mobilization studies and in vitro fibrocyte characterization, mice receiving saline or AMD3100 were sacrificed 6hrs into infusion and approximately 1mL of blood was collected followed by ammonium chloride potassium (ACK) lysis. Isolated leukocytes were counted per volume of blood on a haemocytometer and some were characterized for CD133 expression, as described below in the Flow Cytometry methods. The remaining leukocytes from saline infused animals were resuspended in complete RPMI (10% heat-inactivated fetal bovine serum, 2mM L-glutamate, 100 µg/ml streptomycin, and 100 U/mL penicillin). Isolated leukocytes were plated at $1.5x10^6$ cells per mL on gelatin-coated in either 6-well plates (1mL/well) or in T25 flasks (5mL/flask) with complete RPMI for 3d. At 3d, non-adherent cells were removed by gently washing the wells or flasks twice with serum-free RPMI. Adherent cells were then further processed for characterization by immunofluorescence.

For flow cytometry characterization of 3d fibrocyte cultures, cells were lifted from culture using 0.25% trypsin (Life Technologies, Burlington, ON, Canada). Detachment was monitored under a microscope and stopped using complete RPMI. Cells were subsequently used for flow cytometry, as described below.

3.6.3 Bone Marrow

Bone marrow cell isolation: in brief, animals were anaesthetized then sacrificed and the femurs and tibias were isolated. Cells were flushed from the marrow using a 30G needle attached to a syringe containing DMEM (Dulbecco's Modified Eagle Media) complete (DMEM-C: DMEM, L-glutamine, Pen Strep, 10% FBS).

3.7 Blood Smears and Leukocyte quantification

At 3d, mice were sacrificed and blood was collected for smears. Smears were stained with Giemsa (GS500, Sigma Aldrich) for nuclear morphology and leukocyte populations were counted by a blind-observer. A total of 350 cells were characterized per slide.

3.8 Flow Cytometry

Isolated leukocytes and cultured fibrocytes were washed twice in FACS buffer (DPBS, 1% BSA, 0.1% NaN3) and counted using trypan blue exclusion. For studies labeling intracellular antigens, cells were permeabilized with Cytofix/Cytoperm (BD, Mississauga, ON, Canada) as per manufacturer's instruction. If cells were just labeled for surface antigens, then no permeabilization was performed. All cells were F_c blocked (Miltenyi Biotec, Auburn, CA, USA or Biologend, San Diego, CA, USA) as per manufacturer's instruction. The following antibodies were used in studies:

(i) αCD133 (FITC; Millipore, Billerica, MA, USA)

(ii) αCCR2 (PE or FITC; R&D Systems, Minneapolis, MN, USA)

(iii) αVimentin (PerCP; Santa Cruz Biotechnology, Santa Cruz, CA, USA; Vimentin was used as the mesenchymal marker due to availability of the antibody being conjugated and tested for flow cytometry.

(iv) αCXCR4 (APC; R&D).

(v) αCD11b-APC (eBioscience, San Diego, California, US)

(vi) αLy6C-PE (Biolegend).

(vii) αTNF-PE (Biolegend)

(viii) αIL-10-PE (Biolegend)

(ix) αF4/80-PerCP-Cy5.5 (Biolegend, San Diego, California, USA

(x) αCX3CR1-PerCP-Cy5.5 (Biolegend)

Following incubation, cells were twice washed with FACS buffer and fixed with 1% formalin/FACS buffer. Single label controls were used for compensation and isotype antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Data was acquired using a BD FacsCalibur and analyzed using WinList (Verity Software, Topsham, ME, USA) or FlowJo (FlowJo, LLC, Ashland, OR, USA). MΦ gating was performed by gating on cells using forward (FSC) and side scatter (SSC), which was then applied to a CD11b x F4/80 dot plot. The $F4/80^{+}$ events were gated and applied to a CD11b x Ly6C dot plot for the characterization Ly6C mean fluorescent index (MFI) of mature and immature MΦ populations in the myocardium. When F4/80 labeling was not performed, CD11b x SSC was used to distinguish polymorphonuclear cells (SSC^{high}) and monocyte-derived cells ($SSC^{low\text{-}mid}$).

3.9 Histological Analysis

3.9.1 Staining and Analyzing Paraffin Sections

Basic myocardial histology and cellular infiltrate were assessed using heart sections stained with hematoxylin and eosin (H&E). The area of the heart affected was calculated as previously described with modification[47]. In brief, fibrotic deposition was examined using heart sections stained with Sirius Red (SR) and the counter stain Fast

Green (FG). Collagen content was semi-quantified by photographing representative SR/FG whole heart-sections at 5x magnification. Using Adobe Photoshop CS5, red pixels were positively selected and summed for a total number of red (collagen) pixels. Subsequently, non-background pixels were summed for the total heart pixels. Collagen pixels were divided by the total heart pixels to provide a semi-quantitative measurement of the percent collagen content in the heart. All tissues were processed simultaneously for SR/FG and the same red colour palette was used to select red pixels.

Immunohistochemistry for KI-67 (DakoCytomation, Mississauga, ON, Canada) was performed on paraffin-embedded sections, which required antigen retrieval and blocking for endogenous peroxidase (3% H_2O_2), biotin (DAKO Biotin Blocking System, DakoCytomation), and non-specific secondary antibody binding (normal serum from the secondary antibody host). Sections were incubated with primary antibody overnight at 4°C, followed by a biotin-conjugated secondary antibody. Secondary antibody was conjugated to an avidin-biotin complex (Vecstatin ABC kit; Vector, Burlington, ON, Canada) and developed with 3,3' diaminobenzidine (DAB, DakoCytomation).

The proliferation index was calculated as the number of KI-67^+ cells over the total number of infiltrating cells in 5 fields of view at 40x in representative myocardial sections.

3.9.2 Staining Frozen Sections

Immunofluorescence and immunohistochemistry was performed on both frozen heart sections and fixed co-cultures for CD11b (AbD Serotec, Raleigh, NC, USA), CD45 (BD Biosciences, San Jose, CA, USA), collagen type-I (Rockland, Gilbertsville, PA),

F4/80 (Abcam, Cambridge, MA), α-smooth muscle cell actin (αSMA; Sigma Aldrich), CD206 (AbD Serotec), CX_3CR1 (Abcam), and CD107b (AbD Serotec)[xviiixix]. In brief, sections were permeabilized with 0.1% Triton-X-100/PBS, blocked with 5% BSA/PBS, and then incubated with primary antibodies overnight at 4°C. Sections were then incubated with AlexaFluor488, AlexaFluor555, AlexaFluor647, or biotin-conjugated secondary antibodies. Biotin-conjugated antibodies were linked to avidin and developed with DAB. In fluorescence, nuclei were stained with Hoechst. Slides were visualized using a Zeiss Axiovert 200M and photographed with a Hamamatsu Orca R2 Camera.

3.9.3 Staining in vitro cultured cells

Enriched fibrocyte cultures were used to evaluate fibrocyte phenotype and the expression of CXCR4 and CCR2. Following the removal of non-adherent cells at 3d, fibrocytes were fixed with 4% paraformaldehyde and subsequently washed with PBS before conducting immunofluorescence. In brief, non-specific binding of secondary antibodies was blocked with secondary host serum. Following blocking, fibrocytes were incubated with primary antibodies against CD133 (AbCam, Cambridge, MA), collagen-1 (Col-1; Rockland, Gilbertsville, PA), and either CXCR4 (AbCam) or CCR2 (AbCam). Fibrocytes were then incubated with Cy2-, Cy3-, or Cy5-conjugated secondary antibodies. Hoechst was used to label nuclei. Slides were visualized using a Zeiss Axioplan II and photographed with an AxioCam HRC Colour Camera.

3.10 Co-culture

The MΦ-fibroblast co-culture was conducted as previously described with modification[72,121,216]. Bone marrow-derived MΦ (BMDMΦ) were generated as previously described[267]. Following the isolation, washing and straining of bone marrow, cells were resuspended in DMEM complete with 15% L929 conditioned medium and plated in T75 flasks.

3.10.1 *Co-culture 1 – MΦ-fibroblast co-culture*

Media was replaced on day 5. On day 7, the BMDMϕ were lifted using 0.25% trypsin, washed with DMEM-C, counted, and re-plated in 12-well plates at a density of $2x10^5$/well. Additional MΦ were screened for CD11b and Ly6C expression using flow cytometry as described below. NIH/3T3 (ATCC, Manassas, VA) served as fibroblasts in mono- and co-cultures. NIH/3T3 were maintained in DMEM-C until lifted using 0.25% trypsin, washed with DMEM-C, counted, and re-plated at $2x10^5$/well. Mono- and co-cultures were allowed to sit down overnight in DMEM-C before being serum-starved for 24hrs. The next day, the media was replaced with DMEM-C and cells were incubated for 48hrs. At 48hrs, the supernatant was removed and the cells were washed with PBS then fixed in 4% PFA.

3.10.2 Co-culture 2 – differentiated MΦ-fibroblast co-culture

Media was replaced on day 3. On day 7, media was changed to (1) DMEM-C for control, (2) DMEM-C + 100ng/mL LPS + 10ng/mL IFN-γ for M1 differentiation, or (2)

DMEM-C + 10ng/mL IL-4 for M2 differentiation. On day 9, MΦ populations were lifted using 0.25% trypsin, washed with DMEM-C, counted, and re-plated in 12-well plates at a density of $2x10^5$/well. Samples of MΦ populations were screened for F4/80, CD11b, Ly6C, CD206, TNF-α, and IL-10 expression using flow cytometry as described below. NIH/3T3 (ATCC, Manassas, VA) served as fibroblasts in mono- and co-cultures. NIH/3T3 were maintained in DMEM-C until lifted using 0.25% trypsin, washed with DMEM-C, counted, and re-plated at $2x10^5$/well. Mono- and co-cultures were incubated for 72hrs, at which point, the supernatant was removed and the cells were washed with PBS then fixed in 4% PFA.

The cells were immunofluorescently labeled as described above and wells were read for fluorescence using a Tecan infinite M200 Pro (Tecan, Männedorf, Germany) plate reader. αSMA expression was standardized to Hoechst fluorescence intensity for co-cultures and fibroblast monocultures. The co-culture expression was then calculated relative to fibroblast monoculture expression. Immunofluorescence and flow cytometry were used to quantify cell-purity.

3.11 Proliferation Assay

ECM-producing cells isolated from 3d AngII hearts were used to assess proliferation in vitro, as previously described[47,68,155]. Following 3d in culture, cells were lifted from T25 flasks with 0.25% trypsin-EDTA and re-plated in black-walled, clear-bottomed 96-well plates at a concentration of 10^4 cell/well. All treatments were carried out in triplicates. Cells were allowed to adhere overnight in complete RPMI and then serum-starved (RPMI 1640 supplemented with 1% BSA, 2 mmol/L L-glutamine, 100

mg/mL streptomycin, and 100 U/mL penicillin). The media was replaced with mouse CCL2 (Biolegend, San Diego, CA, USA), mouse CCL12 (R&D Systems), complete RPMI, or serum-free RPMI, as described above. Complete RPMI served as the positive control. Cells were cultured at 37°C in 5% CO_2 for 48hrs, after which CellTiter-Blue (Promega, Madison, WI, USA) viability solution was added to each well, mixed, and incubated for 2hrs. After 2hrs, the plate was excited at 579nm and absorbance was acquired at 584nm on a Tecan infinite M200 Pro (Tecan, Männedorf, Germany) plate reader. Proliferation was measured as change in fluorescence relative to serum-free conditions.

3.12 Genotyping

Post-sacrifice, ears were isolated from CCR2$^{-/-}$, CX3CR1$^{-/-}$, and WT mice and snap-frozen in liquid nitrogen for later genotyping. Genotyping was performed by technicians Maria Vaci and Terry LeVatte using the Kapa Mouse Genotyping Kit (Kapa Biosystems, Wilmington, Massachusetts) according to the manufacturer's instructions.

(i) CCR2 (forward: CTTGGGTGGAGAGGCTATTC; reverse: AGGTGAGATGACAGGAGATC)

(ii) CX3CR1 (WT allele: forward: GGCCTGTTATTTGGGCGACAT; reverse: TGGGGTGACGCCACTAAGAT; KO allele: forward: GACCGCTTCCTCGTGCTTTA; reverse: TGGGGTGACGCCACTAAGAT)

3.13 qPCR

RNA isolation, cDNA generation, and quantitative polymerase chain reaction (qPCR) were performed as previously described[47,71,155]. Primers were designed against mRNA sequences of the following:

(i) CX_3CL1 (forward: GCAACCCCACCCCTTATCAA; reverse: CTTGCCAGCCCTCAGAATCA),

(ii) TGF-B (forward: CTCTTGAGTCCCTCGCATCC; reverse: GGTCTCCCAAGGAAAGGTAGG),

(iii) CTGF (forward: TAGAGCAGGTCTGTCTGCAAG; reverse: TCAACCTCAGACACTGGTTTC),

(iv) IL-1B (forward: TCCTCGGCCAAGACAGGTCGCT; reverse: CCCCCACACGTTGACAGCTAGGT);

(v) Collagen type 1, A1 subunit (Col1A1) (forward: CAACAGTCGCTTCACCTACAGC, reverse: GTGGAGGGAGTTTACACGAAGC;

(vi) TNFα (forward: TCTCATGCACCACCATCAAGGACT, reverse: ACCACTCTCCCTTTGCAGAACTCA

(vii) CCL12 (forward: GAGAGACACTGGTTCCTGACT; reverse: CGCATCTGGTCCAGCCAATA)

Expression was normalized to the 18S (forward: TCAACTTTCGATGGTAGTCGCCGT; reverse: TCCTTGGATGTGGTAGCCGTTTCT) ribosomal gene using the Pfaffl method.

3.14 Statistics

Results are expressed as mean ± SEM. Multiple group comparisons were performed by one-way analysis of variance (ANOVA) followed by Bonferroni post-test

for comparing means. One-sample t-tests were used for relative comparisons between experimental groups and standardized controls.

CHAPTER 4 EARLY FIBROCYTE MIGRATION TO THE ANGII-EXPOSED MYOCARDIUM IS NOT CXCL12 OR CCL2 DEPENDENT AS PREVIOUSLY THOUGHT

This work appears in part in the publication:

Falkenham A, Sopel M, Rosin N, Lee TD, Issekutz T, Légaré JF. Early fibroblast progenitor cell migration to the AngII-exposed myocardium is not CXCL12 or CCL2 dependent as previously thought. Am J Pathol. 2013 Aug;183(2):459-69. doi: 10.1016/j.ajpath.2013.04.011. Epub 2013 Jun 1.

Contribution:

AF – designed the study, carried out the experimentation collected and analyzed data, and prepared the manuscript

MS, NR – assisted with experimentation and data collection

TL, TI, JFL – assisted with study design and critical review of the manuscript

4.1 Introduction

The development of scar tissue in the heart, termed myocardial fibrosis, is a common pathological feature seen with many CVDs. Myocardial fibrosis is characterized by excess deposition of ECM proteins, which lack the contractile ability of cardiomyocytes and enhance stiffness. Thus, myocardial fibrosis is detrimental to both systolic heart function by affecting the strength of contraction and diastolic function by affecting myocardial relaxation, cumulatively contributing to the development of heart failure[50,268,269].

Recently, we and others have identified a population of circulating progenitor cells, termed fibrocytes, which are believed to be important contributors to myocardial fibrosis[47,68,270]. Fibrocytes are mononuclear cells that co-express hematopoietic (CD45, CD34, CD133) and mesenchymal (vimentin, αSMA, collagen-1) markers[179]. They have been shown to originate in the bone marrow, migrate to the myocardium, differentiate into myofibroblasts, and produce ECM proteins, directly contributing to myocardial fibrosis and eventually heart failure[47,69,93,271]. As such, fibrocytes have been suggested to be a novel therapeutic target for reducing the development of pathological fibrosis[69,93,256,265].

While targeting fibrocytes in models of fibrosis has been explored, there remains significant controversy as to the major chemokine used by these cells to migrate, specifically to the myocardium[69,93,227,270,272,273]. The evidence to date would suggest that both the CXCL12-CXCR4 and CCL2-CCR2 axes could be involved in the development of myocardial fibrosis, but the exact mechanism by which this occurs remains unclear[93,256,274–276]. Myocardial CXCL12 and CCL2 expression has been shown to be

upregulated in response to a variety of cardiac insults, supporting a role for both of these chemokines in regulating myocardial fibrosis[47,93]. CXCR4 antagonists have been used in models of pulmonary fibrosis and shown to inhibit fibrocyte recruitment and decrease fibrosis, suggesting that CXCL12 may be the key chemokine involved in fibrocyte recruitment, but this has yet to be shown in myocardial tissue[227,256]. Alternatively, myocardial fibrosis in $CCL2^{-/-}$ animals appears to be reduced with less cellular infiltration, suggesting that CCL2 is responsible for fibrocyte recruitment, but none of the work looked at early (within 3d) fibrocyte infiltration, which our laboratory has shown to be a key time point for fibrocyte migration in AngII-infused animals[47,69,270].

Taken together, there is evidence to suggest that CXCL12 and/or CCL2 play some role in fibrocyte recruitment, but this role has yet to be clarified with regards to early myocardial infiltration by fibrocytes. Our aim was to evaluate the potential blocking of fibrocyte migration using the CXCR4 antagonist AMD3100 in combination with $CCR2^{-/-}$ mice, thus examining the dual inhibition of these two chemotactic pathways in the early development of myocardial fibrosis.

4.2 Results

4.2.1 AngII Exposure Increases Myocardial Transcription of CXCL12 and CCL2

Previous work in our laboratory using a well-established model of hypertension and hypertrophy with AngII infusion has demonstrated that fibrocyte infiltration to the myocardium precedes the development of myocardial fibrosis[47,68,270]. Furthermore, using this model we have previously established that infiltration starts by 1d and peaks at 3d of AngII infusion, thus making it an important time-point to evaluate fibrocyte migration.

Fibrocyte infiltration has been suggested to be in response to CXCL12 and/or CCL2, particularly to the myocardium[47,69,93,227,256,265]. We used qPCR to detect changes in CXCL12 and CCL2 transcript in the myocardium after AngII infusion. By 1d of AngII infusion, CXCL12 mRNA expression was significantly upregulated to 4.60 ± 0.39 fold greater than control ($p<0.01$) (Fig. 4.1A). The increase in CXCL12 transcript corresponded to the increased fibrocyte infiltration seen at 1d, as previously described[47]. CCL2 transcript was also increased in the myocardium as early as 6hr of AngII infusion (7.83 ± 1.70 fold greater than control) (Fig. 4.1B). Increased CCL2 transcription was maintained until 12hr of AngII infusion (12.26 ± 2.54 fold greater than control; $p<0.05$), but did not persist beyond 1d. When compared to CXCL12, CCL2 expression appeared to peak earlier and was several fold higher.

Flow cytometry was used to confirm that fibrocytes express the receptors for CXCL12 (CXCR4) and CCL2 (CCR2). PBMC were cultured for 3d to obtain enriched fibrocytes for flow cytometry, as previously described[47]. Cultured cells were labeled for a fibrocyte phenotype (co-expression of CD133 and vimentin) and both CXCR4 and CCR2. Cells positive for the fibrocyte phenotype were gated on to evaluate the receptor expression. In Fig. 4.1C, one can see that $CD133^{+}$/$Vimentin^{+}$ fibrocytes also co-express CXCR4 (y-axis) and CCR2 (x-axis); representative flow cytometry from 3 replicates demonstrated that a large percentage (98.7%) of cultured fibrocytes co-express both receptors. Our findings suggest that there are not two separate subsets of fibrocytes: one that expresses CXCR4 and one that expresses CCR2, but instead concurrent expression of both CXCR4 and CCR2 by fibrocytes.

We confirmed chemokine receptor expression on enriched fibrocytes using immunofluorescence. Cultured cells were labelled for a progenitor marker (CD133) and a mesenchymal marker (Collagen-1) to confirm the fibrocyte phenotype (co-expression), as well as being labelled for either CXCR4 or CCR2. Nuclei were labelled blue (Hoechst). A representative photomicrograph is shown in Fig. 4.1D with collagen fluorescing green, CD133 fluorescing orange, and CXCR4 fluorescing pink. Similarly, a representative photomicrograph is shown in Fig. 4.1E with collagen fluorescing pink, CD133 fluorescing orange, and CCR2 fluorescing green. The co-localization of these markers in both micrographs appears red. Red co-localization can be seen in both photomicrographs, supporting our flow cytometry results and confirming that fibrocytes taken from the circulation and purified in vitro express CXCR4 and CCR2. We then aimed to evaluate the blockade of CXCL12 and CCL2 on fibrocyte migration in the AngII in vivo model.

4.2.2 CXCR4 Antagonism with AngII Exposure Increases Cellular Infiltration and fibrosis In the Myocardium

As we have shown previously, AngII infusion resulted in significantly increased myocardial cellular infiltrate (Fig. 4.2C), which was absent in saline controls (Fig. 4.2A). We used a previously described standard grid counting technique to quantify cellular infiltration[47,68,270]. Quantitatively, we observed that 29.99 ± 4.01 % of the myocardium was affected by cellular infiltrate in representative sections following AngII infusion (Fig. 4.2I). Unexpectedly, blocking the CXCL12-CXCR4 axis with AMD3100 led to an increase in cellular infiltration when compared to all other experimental groups (Fig 4.2E). Quantitatively, animals that received AngII+AMD3100 had 75.09 ± 2.90 % of the myocardium in representative sections being affected by cellular infiltrate (Fig. 4.2I) – a

significant 2.5-fold increase in percentage of grids affected in the myocardium relative to AngII alone ($p<0.01$). AMD3100 infusion alone was insufficient to recruit cells to the myocardium (Fig. 4.2G)[277].

We quantified collagen deposition (marker of fibrosis) in the myocardium using a standard Sirius Red stain[47,69,254,270]. Following AngII infusion, collagen content in the myocardium increased to 11.50 ± 1.12% of representative sections compared to control (2.11±1.25%) (Fig. 4.2B, D, J). Consistent with the increase in infiltrating cells seen with CXCR4 blockade, simultaneous infusion of AngII and AMD3100 significantly increased collagen content (28.05 ± 2.83%) in the myocardium compared to both control and AngII infusion (Fig. 4.2F,J) ($p<0.01$). AMD3100 infusion alone did not lead to significant changes in collagen content (Fig. 4.2H, J).

4.2.3 Infiltrating Cells Are Phenotypical of Fibrocytes

In AngII infusion models, fibrocytes are suspected of contributing to the deposition of ECM and as such, inhibiting their recruitment in pulmonary fibrosis models with CXCR4 antagonism or neutralization has been shown to reduce fibrosis[69,93,265,278]. Contrary to expectations, we now show that with CXCR4 blockade there is a several fold increase in myocardial cellular infiltration leading to worsening fibrosis. Frozen myocardial tissue from animals infused with AngII and AngII + AMD3100 were stained for the pan-leukocyte marker CD45 and the mesenchymal marker SMA. Co-localization of these two markers is a phenotype characteristic of fibrocytes[227,277]. A representative photomicrograph from an AngII + AMD3100 heart is shown in Fig. 4.3 with CD45 fluorescing green and SMA fluorescing red. The co-localization of these markers appears

yellow and is present on a large proportion of infiltrating cells (Fig. 4.3). These findings confirm that blockade of CXCR4 resulted in significant ($CD45^+/SMA^+$) fibrocyte migration to the myocardium. Serving as a positive control, fibrocytes were also observed in the myocardium of AngII-infused animals, as previously described (data not shown)[47,270].

4.2.4 CXCR4 Antagonism Mobilizes Progenitor Cells, but Does Not Increase Blood Pressure or Proliferation

Antagonizing CXCR4 is known to mobilize large numbers of hematopoietic stem cells into circulation which likely includes fibroblast progenitor cells or fibrocytes[279]. We confirmed this effect in our model by quantifying circulating blood leukocytes. Following AMD3100 infusion, the concentration of leukocytes (absolute number per known volume of blood) in the circulation rose to 2.23 fold greater than saline control (Fig. 4.4A) ($p<0.05$). Flow cytometry confirmed that the increase in circulating leukocytes was associated with a corresponding increase in cells expressing the progenitor marker CD133 (Fig. 4.4B,C). This data provides evidence that AMD3100 increases the concentration of circulating progenitor cells. It also supports the idea that increased circulating progenitor cells may be responsible for the increase in infiltrating cells in the myocardium. Our data does, however, suggest that the mechanism of migration to the myocardium may not be dependent on CXCR4-CXCL12 axis.

Previous work has shown that infiltrating cells seen after AngII exposure can significantly proliferate, potentially explaining the significant difference in infiltration seen in animals also receiving AMD3100. Proliferation of infiltrated cells was therefore assessed by KI-67 immunohistochemical labelling, which was used to calculate a

proliferation index. The proliferation index was based on the number of KI-67^{+} cells over the total number of infiltrating cells. In the AngII group, 56.9 ± 4.4% of infiltrating cells (Fig. 4.5B) were found to be proliferative, as indicated by KI-67 positivity compared to 54.6 ± 2.1% in the AngII + AMD3100 group (Fig. 4.5C, D; p=ns). The high proliferative index in both groups supports evidence that the majority of infiltrating cells are fibroblast progenitor cells rather than terminally differentiated leukocytes (Fig. 4.5D), but our results suggest that the increase in infiltrating cells observed with AngII+AMD3100 infusion is not due to enhanced proliferation. Saline control animals showed minimal proliferation in the myocardium (Fig. 4.5A).

AMD3100 also did not have a significant effect on blood pressure with or without co-infusion of AngII (Fig. 4.6A). Consistent with our blood pressure findings, hypertrophy was also not significantly different between AngII and AngII + AMD3100 animals (Fig. 4.6B). Thus, the increase in infiltrating cells in the myocardium is likely the consequence of increased cell mobilization from the bone marrow by CXCR4 antagonism, not changes in proliferation or changes in blood pressure between experimental groups.

4.2.5 Fibrocyte Migration and Fibrosis in the Absence of CCR2

Others have previously shown that CCL2-CCR2 axis blockade using CCL2$^{-/-}$ and CCR2$^{-/-}$ animals resulted in less myocardial fibrosis[69,274,280,281]. This led researchers to believe that fibrocyte migration was CCL2-CCR2 dependent; however, these studies were limited to time points 14d or longer, which contrasts to work in our laboratory that suggests important fibrocyte migration occurs in the first 3d[47]. As such, we examined

whether early cellular infiltration and fibrosis observed with AngII ± CXCR4 antagonism would be reduced in CCR2$^{-/-}$ mice. Following 3d of AngII or AngII + AMD3100 infusion, representative sections from CCR2$^{-/-}$ mouse hearts were stained for H&E and SR/FG. Despite the absence of CCR2, significant cellular infiltrate was still observed in AngII and AngII + AMD3100 myocardium (Fig. 4.7C, E, respectively). Through grid counts of the H&E stained sections, we found that there was no significant reduction in cellular infiltration at the 3d time point (Fig. 4.7G). Similarly, there was significant myocardial fibrosis present in AngII and AngII + AMD3100 infused hearts in CCR2$^{-/-}$ animals (Fig. 4.7D, F, H). Thus, at this early time point, CCR2$^{-/-}$ mice failed to show a reduction in either cellular infiltrate or fibrosis compared to WT. Infiltration was absent in saline controls (Fig. 4.7A).

Given that CCR2$^{-/-}$ mice relative to WT reportedly show reductions in myocardial infiltrate and fibrosis following AngII infusion, we investigated potential differences in the experimentation that may account for why our results differed[93]. We first lowered our AngII dose to 2.1mg/kg/d and extended our length of infusion up to 28d as done by others. We observed significant reductions in both cellular infiltrate (Fig 4.8A, C) and fibrosis (Fig 4.8B, D) in CCR2$^{-/-}$ mice relative to WT at 28d, but this was not the case at 3d, supporting our previous findings in which early infiltration was not affected by the absence of CCR2. Quantitatively, CCR2$^{-/-}$ animals that received AngII had a 48.7% reduction (Fig. 4.8E) in the percentage of grids affected in the myocardium relative to WT ($p<0.01$). Consistent with myocardial infiltration findings, there was a significant reduction in fibrosis (Fig. 4.8F; 51.0%) in CCR2$^{-/-}$ animals receiving AngII compared to WT controls, but only at 28d ($p<0.01$). In both CCR2$^{-/-}$ and WT mice, the collagen

appeared to have undergone maturation, increasing in density and colour, as seen by Sirius Red staining (Fig. 4.8B, D).

4.2.6 CCR2 drives fibroproliferation in the AngII-exposed myocardium

Previous *in vitro* studies have demonstrated that isolated circulating fibrocytes proliferate in response to an alternative ligand for CCR2, CCL12[282]. As such, we investigated CCL12 transcript levels in the AngII-exposed myocardium and whether there were any changes in proliferation at the time of peak infiltration (3d).

AngII infusion led to a significant increase in myocardial CCL12 transcript by 3d relative to saline controls (Fig. 4.9A) (*$p<0.05$; n=4). At the same time point, we evaluated proliferation of infiltrating cells in the myocardium of $CCR2^{-/-}$ and WT mice again using the proliferation marker KI-67. We found that 43.20 ± 1.59 % of infiltrating cells in the myocardium of WT mice were proliferating, as indicated by KI-67 positivity (Fig. 4.9B, C). Infiltrating cells in the myocardium of $CCR2^{-/-}$ mice, however, exhibited significantly lower proliferation with only 31.42 ± 3.18 % of infiltrating cells expressing KI-67 – a 27.3% relative reduction in proliferation compared to WT.

We then verified that the observed in vivo reductions in infiltrate were due to changes in CCR2-dependent proliferation. We evaluated the proliferation of isolated ECM-producing cells from 3d AngII WT and $CCR2^{-/-}$ hearts in response to CCL2 and CCL12. After 2d in culture in the presence of CCL2, CCL12, or complete RPMI, we compared proliferation relative to serum-free conditions. Neither WT, nor $CCR2^{-/-}$ cells exhibited proliferation in response to CCL2 stimulation, as has previously been suggested by Ekert et al. (Fig. 4.9C)[282]. Proliferation was, however, significantly increased in WT

cells in response to 50ng/ml, 100ng/ml, and 200ng/mL of CCL12 (Fig. 4.9d, $p<0.05$, n=4). $CCR2^{-/-}$ cells, however, only displayed significantly increased proliferation at the highest tested CCL12 concentration, 200ng/mL.

It follows that the reductions in infiltration we observe in $CCR2^{-/-}$ relative to WT mice at 28d of AngII infusion are due to early (3d) changes in infiltrating cell proliferation. These changes appear to be at least in part due to the inability of CCL12 to interact with its primary receptor, CCR2.

4.3 Figures

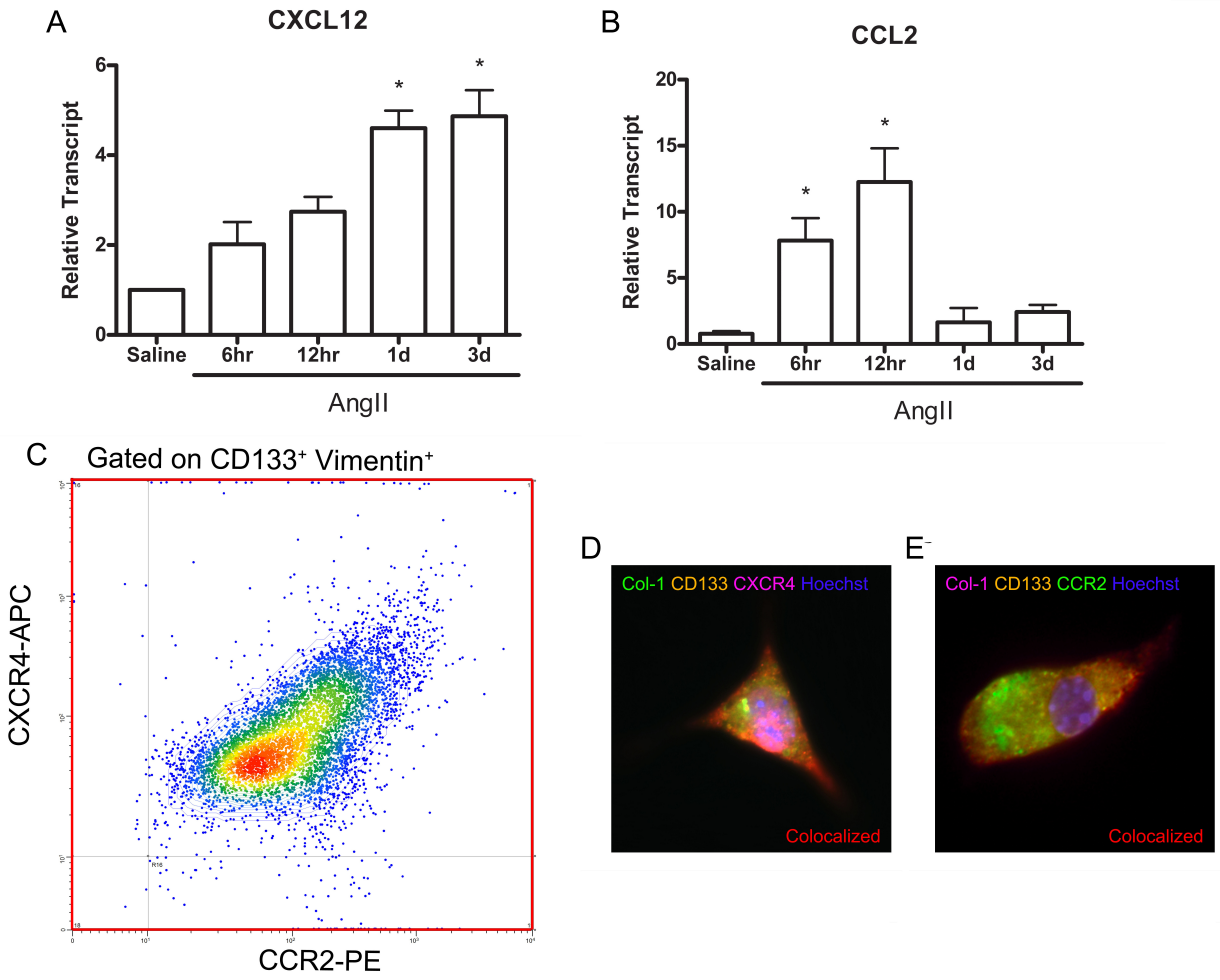


Figure 4.1 - AngII increases myocardial CXCL12 and CCL2 mRNA. Using qPCR, we measured myocardial expression of CXCL12 and CCL2 transcript levels relative to 18s ribosomal gene expression. ***(A)*** *Relative mRNA transcript levels for CXCL12 were significantly increased in AngII-infused animals at 1 and 3 days (**p<0.01).* ***(B)*** *Transcript levels for CCL2 were also significantly increased in AngII-infused animals by 6hrs and remained significantly elevated at 12hrs. (**p<0.01).* ***(C)*** *Representative flow cytometry confirmed that cultured fibrocytes ($CD133^{+}$ Vim^{+}) predominantly co-express the receptors CXCR4 and CCR2. As further support, representative immunofluorescence is shown for the expression of* ***(D)*** *CXCR4 and* ***(E)*** *CCR2 on fibrocytes cultured from circulating leukocytes. The co-localization of the fibrocyte markers and either receptor overlay as red. Images were captured at 100x. Data is expressed as the mean ± SEM.*

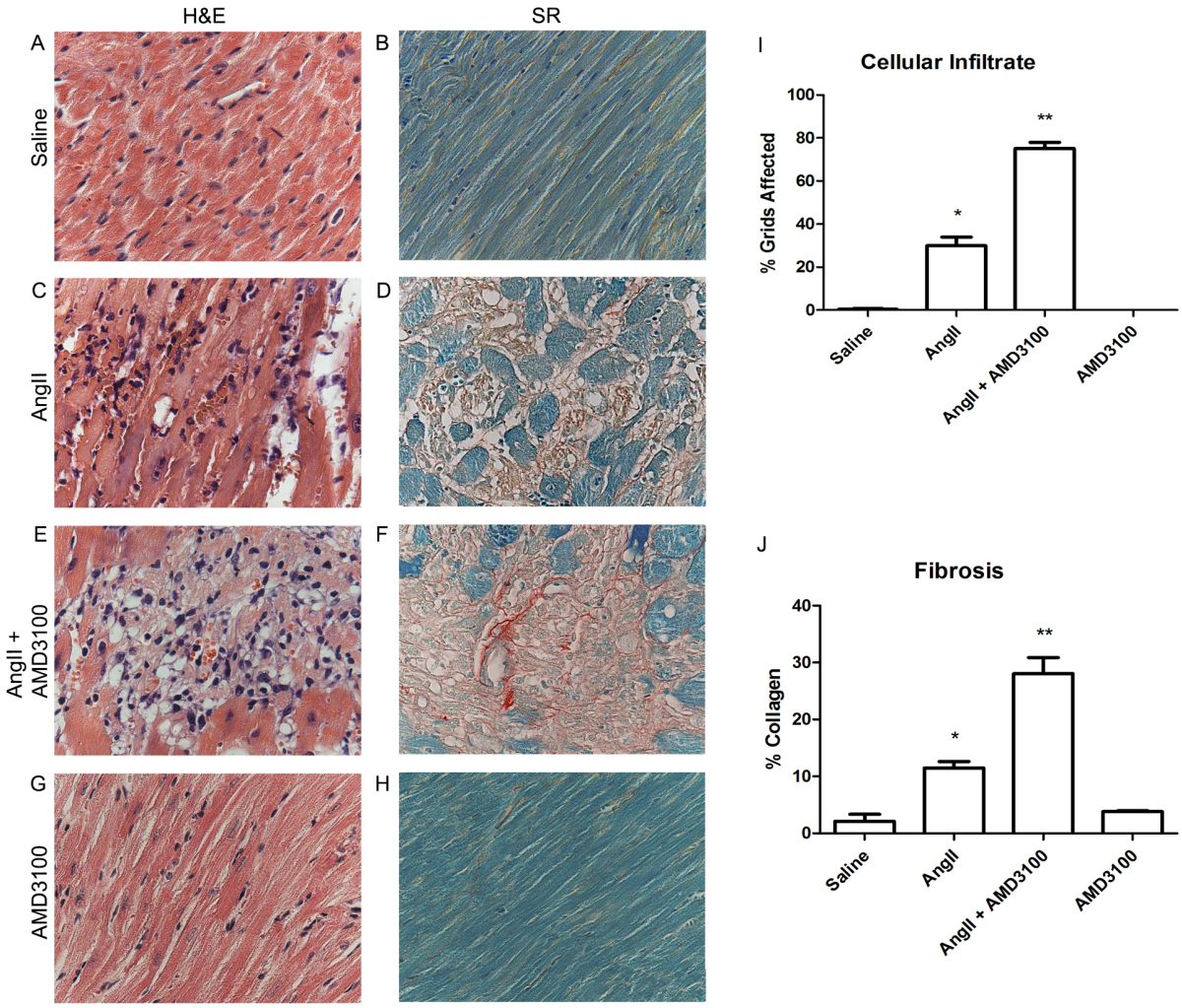


*Figure 4.2 - CXCR4 antagonism increases cellular infiltrate and fibrosis. Representative sections of 3d hearts stained with H&E (**A, C, E, G**) and SR (**B, D, F, H**): saline control (**A, B**), AngII (**C, D**), AngII + AMD3100 (**E, F**), and AMD3100 (**G, H**). AngII-infusion led to significant cellular infiltrate in the heart (*p<0.01) relative to saline controls. Infiltrate was significantly exacerbated by CXCR4 antagonism relative to AngII-infusion alone (**p<0.01). AngII-infusion also led to increased myocardial collagen content relative to saline controls (*p<0.01). The significant increase in cellular infiltrate observed with CXCR4 antagonism (**I**) was associated with significantly increased collagen content relative to AngII-infusion alone (**J**), as semi-quantified by digital image analysis (**p<0.01; n=5). Images were captured at 40x. Data is expressed as the mean ± SEM.*

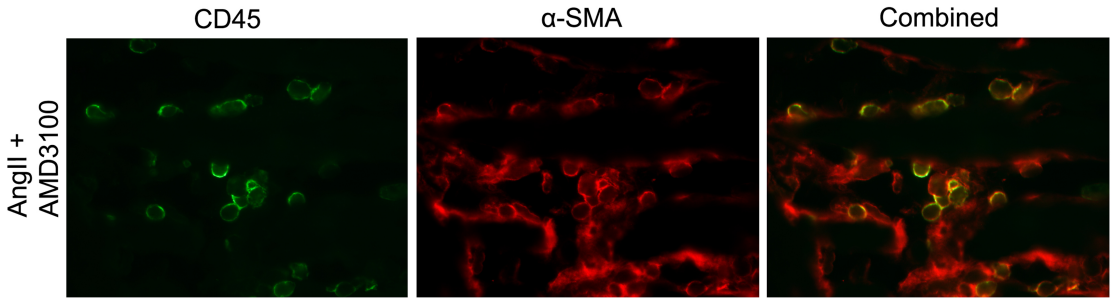


Figure 4.3 - Infiltrating cells are predominantly fibrocytes. Myocardial sections were labelled for the leukocyte marker CD45 (green) and the mesenchymal marker α-SMA (red). The overlay of these markers showed co-localization (yellow) of infiltrating cells in the myocardium of AngII + AMD3100 infused animals. Fibrocytes were found in the myocardium of AngII infused animals as well (data not shown). Images were captured at 63x.

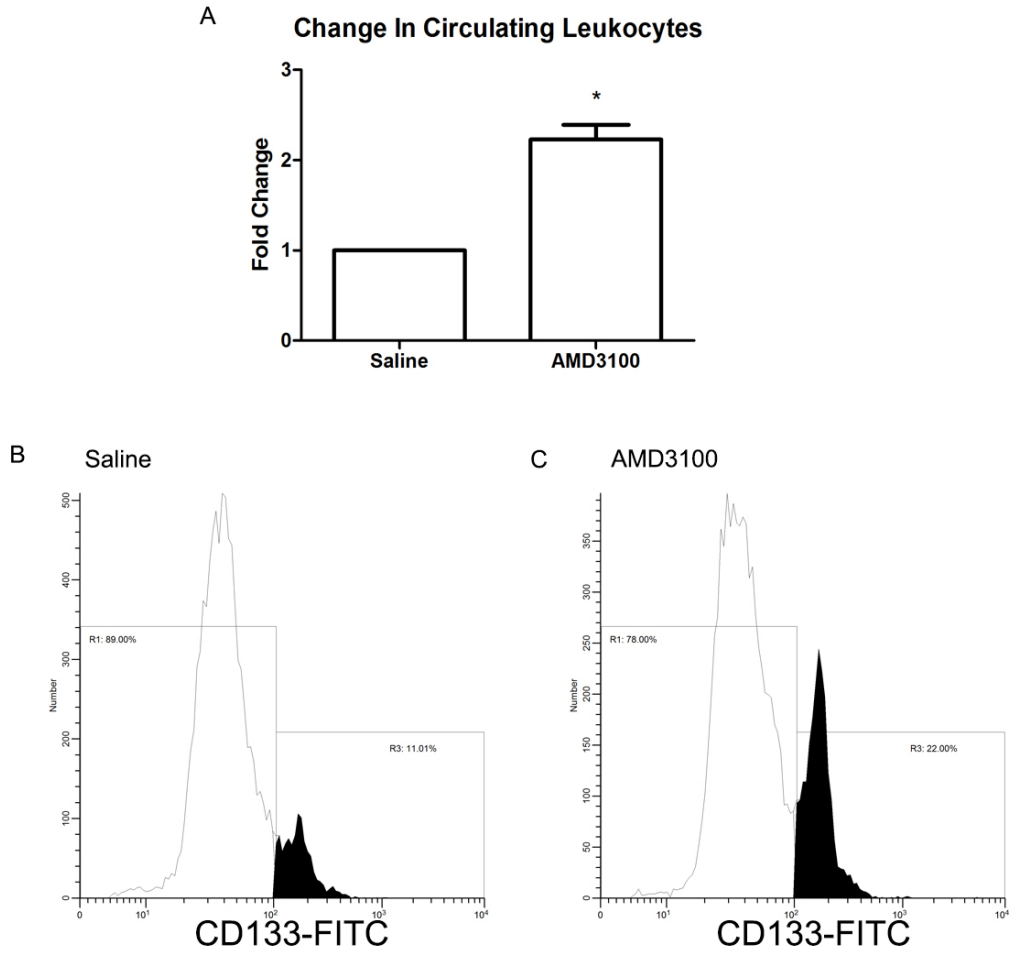


*Figure 4.4 - CXCR4 antagonism increases circulating CD133+ leukocytes. **A** Following 6hrs of AMD3100 infusion, the absolute number of circulating leukocytes per known volume of blood were significantly elevated compared to saline infusion. The same circulating leukocytes were labelled for the progenitor marker CD133 (n=4). (**B**) Representative flow cytometry demonstrated that $CD133^+$ cells were present in the circulation of saline infused animals; however, **(C)** AMD3100 infusion led to a significant increase in circulating $CD133^+$ leukocytes relative to saline infusion (*p<0.05). Data is expressed as the mean ± SEM.*

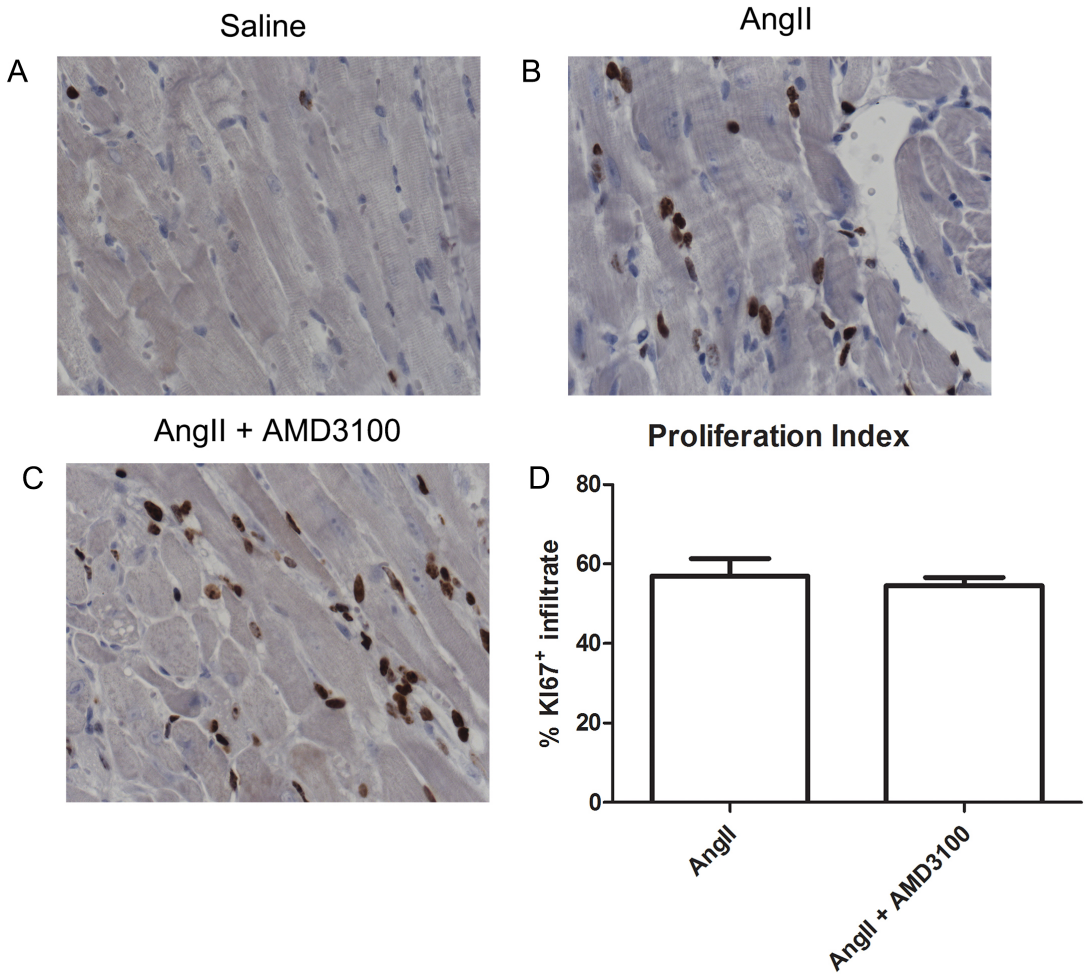


*Figure 4.5 - Increased cellular infiltrate is not due to proliferation. KI67 labelling was used to assess proliferation in 5 fields of view at 40x in representative sections of myocardium for each experimental group – (**A**) saline, (**B**) AngII, and (**C**) AngII + AMD3100. (n=3). AngII and AngII + AMD3100 infused animals both showed large proliferating populations, which were absent in saline controls. (**D**) The myocardial proliferation between the AngII and AngII + AMD3100 infused animals did not differ significantly. Data is expressed as the mean ± SEM.*

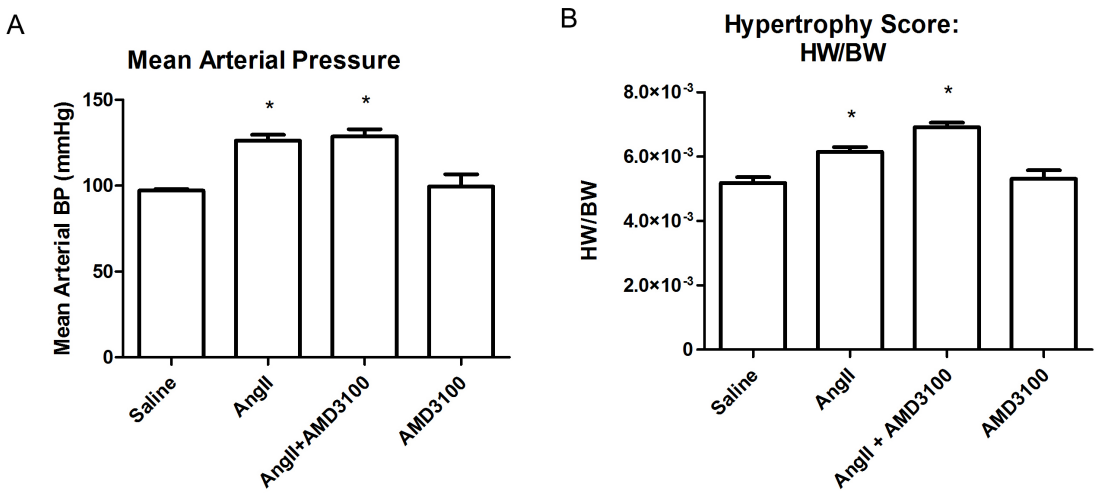


*Figure 4.6 - Increased infiltrate and fibrosis is not blood pressure or hypertrophy dependent. (**A**) Tail cuff measurements at the 3d endpoint demonstrated that AngII infusion significantly increases mean arterial pressure; however, AMD3100 did not significantly affect blood pressure (n=4). (**B**) Hypertrophy scores for AngII and AngII + AMD3100 were also significantly elevated relative to saline controls (*p<0.01), but did not significantly differ between the experimental groups (n=5). Data is expressed as the mean ± SEM.*

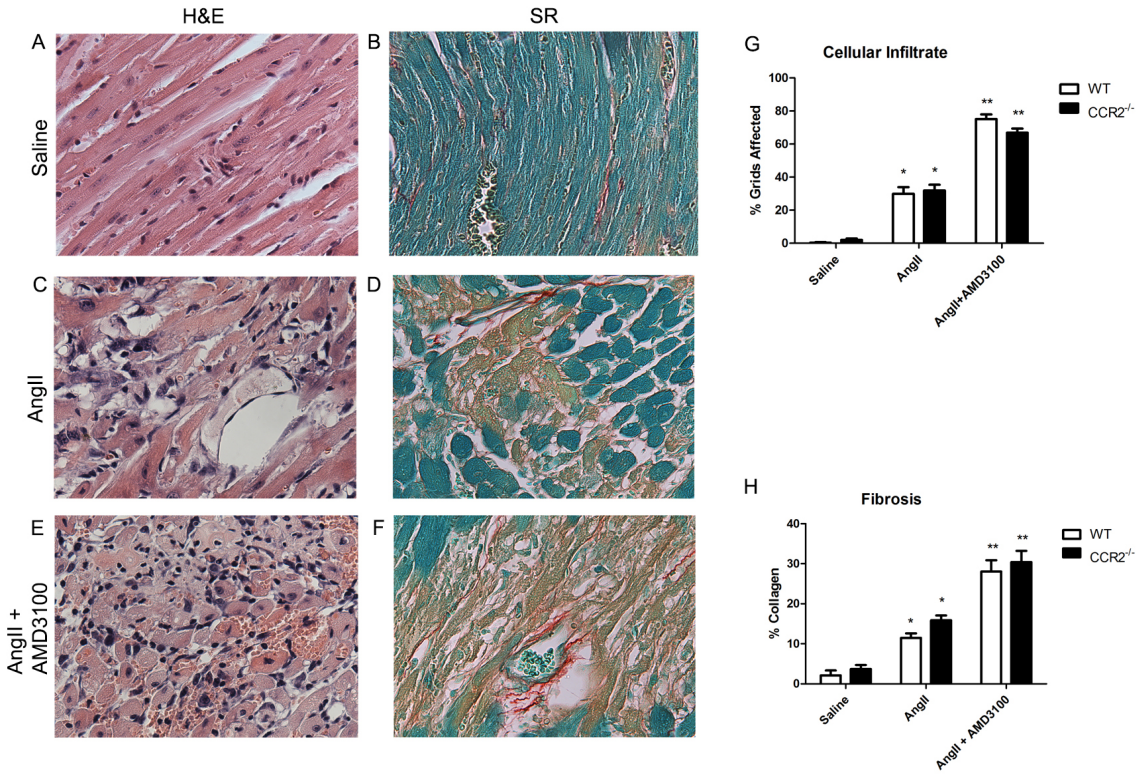


*Figure 4.7 - CCR2-/- mice do not exhibit reduced infiltrate and fibrosis in the heart following 3d of high-dose AngII infusion. Representative sections of 3d hearts stained with H&E (**A, C, E**) and SR (**B, D, F**) **are shown**: saline control **(A, B)**, AngII (2.1mg/kg/d) (**C, D**) and AngII + AMD3100 (**E, F**). Myocardial infiltrate and fibrosis were compared between CCR2$^{-/-}$ and WT mice. AngII led to significant cellular infiltrate and fibrosis compared to saline controls in both CCR2$^{-/-}$ and WT mice (*p<0.01, n=3), which was again exacerbated by CXCR4 antagonism relative to AngII-infusion alone (**p<0.01, n=3). Despite the absence of CCR2, infiltrate (**G**) and fibrosis (**H**) were not significantly reduced at the 3d time point relative to WT experimental groups. Images were captured at 40x. Data is expressed as the mean ± SEM.*

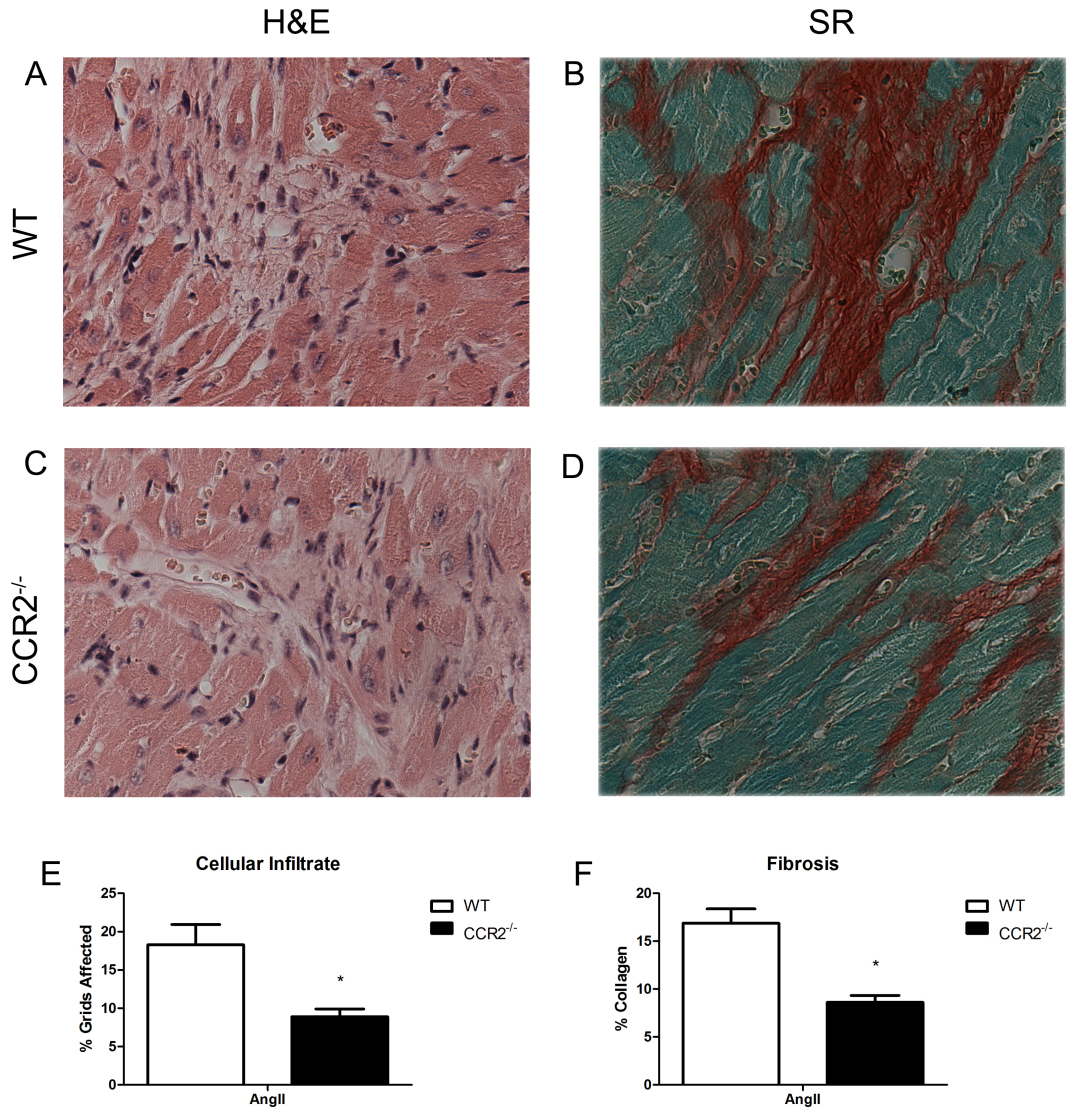


*Figure 4.8 – CCR2-dependent reductions in infiltrate and fibrosis are time-dependent. Representative sections of hearts stained with H&E (**A, B**) and SR (**C, D**) following 28d of AngII infusion: (**A, C**) WT and (**B, D**) CCR2$^{-/-}$. Both WT and CCR2$^{-/-}$ mice demonstrated reductions in infiltrate (**E**) and fibrosis (**F**) relative to their dose-matched (2.1mg/kg/d) 3d counterparts; however, CCR2$^{-/-}$ showed a greater reduction in both features relative the time-matched WT counterpart. (**p<0.01, n=4-5). Images were captured at 40x. Data is expressed as the mean ± SEM.*

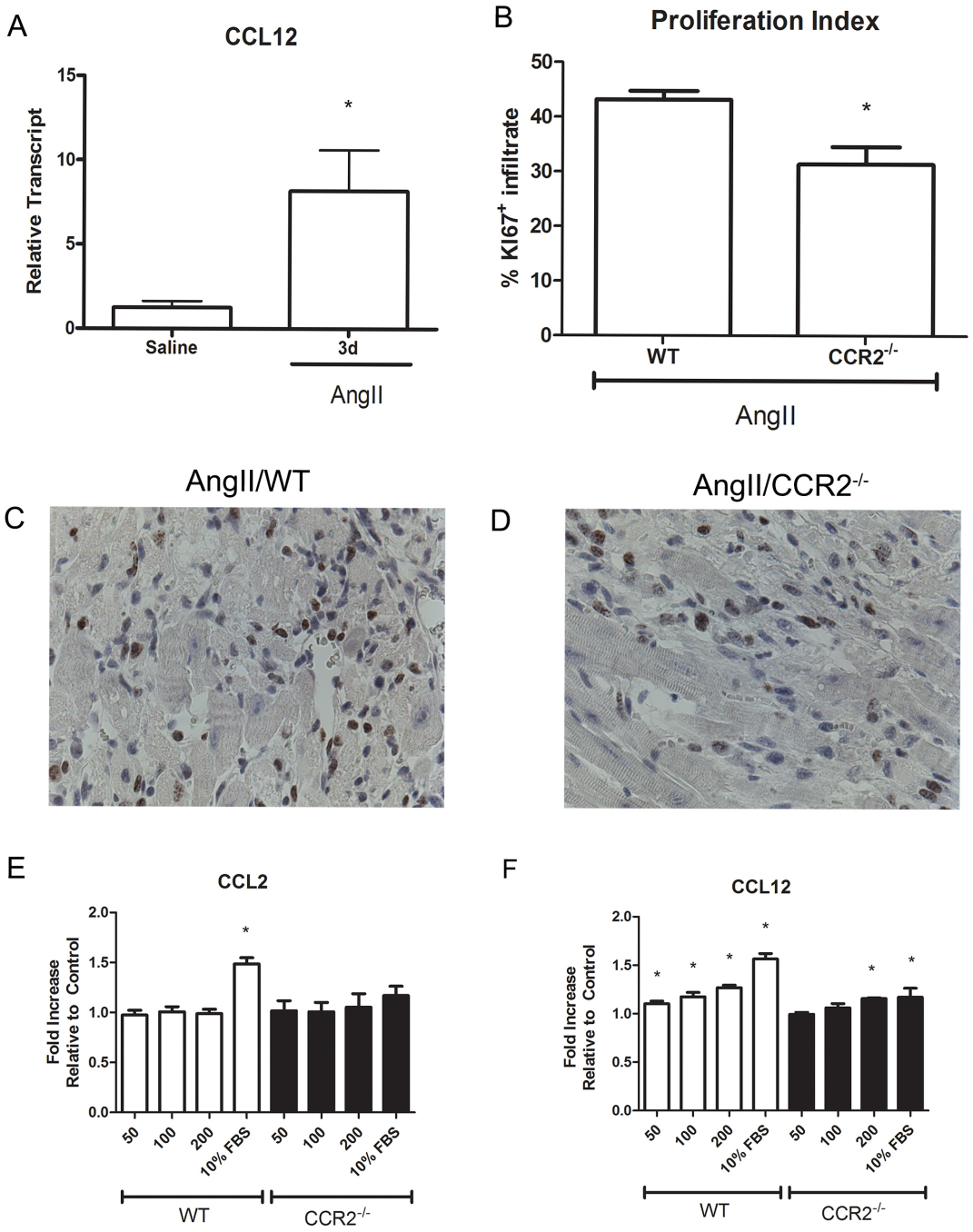
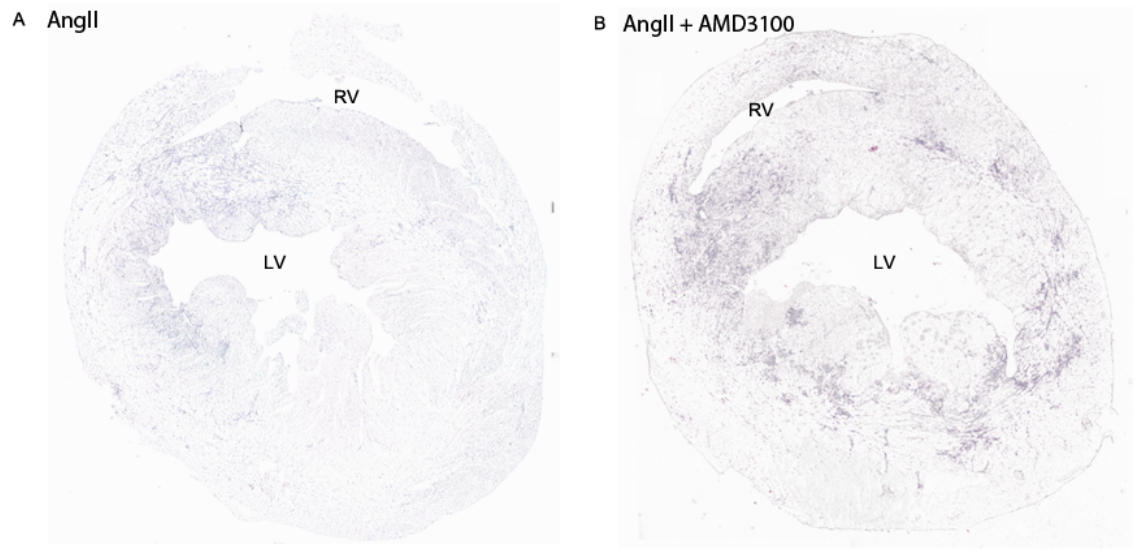


Figure 4.9 – ECM-producing cells proliferate in response to CCL12, which is upregulated in the AngII-exposed myocardium. ***(A)*** *CCL12 – a CCR2 ligand known to increase fibrocyte proliferation – exhibited significantly increased myocardial transcript levels following 3d of AngII infusion relative to saline controls (*p<0.05, n=4). KI67 labelling was used to assess proliferation in 5 fields of view at 40x in representative sections of myocardium from* ***(C)*** *CCR2$^{-/-}$ and* ***(D)*** *WT mice (n=3). Both* ***(C)*** *WT and* ***(D)*** *CCR2$^{-/-}$ mice exhibit proliferation of infiltrating cells following 3d of AngII infusion.* ***(B)*** *CCR2$^{-/-}$ mice, however, exhibit reduced proliferation relative to WT mice (*p<0.05).*

Saline controls showed minimal proliferation. In vivo observations were confirmed using an in vitro proliferation assay on ECM-producing cells exposed to CCL2 or CCL12. ***(E)*** *CCL2 failed to induce proliferation in ECM-producing cells isolated from the myocardium of AngII infused WT and CCR2$^{-/-}$ mice (n=4).* ***(F)*** *ECM-producing cells did, however, show proliferation in response to CCL12 stimulation in both WT and CCR2$^{-/-}$ cells (n=4, *p<0.05). These significant increases were limited to the highest dose (200ng/mL) in CCR2$^{-/-}$ cells. WT cells also proliferated in response to our positive control, 10% FBS (n=4, *p<0.05), while CCR2$^{-/-}$ cells failed to exhibit increases in proliferation in response to FBS. All concentrations are expressed in ng/mL.*



*Supplementary Figure 4.1 - Comparing myocardial infiltrate between AngII and AngII + AMD3100 by haematoxylin staining. Representative myocardial sections from **(A)** AngII and **(B)** AngII + AMD3100 stained with haematoxylin. These sections better demonstrate the dramatic increase in cellular infiltrate and infiltrate density following co-infusion with AngII and AMD3100 relative to AngII infusion alone.*

4.4 Discussion

Fibrocytes are important mediators of fibrosis in a variety of pathologies and have been proposed as a therapeutic target for alleviating the burden of scar tissue formation in organs. In the development of potential therapies targeting fibrocytes, researchers have identified chemokines that may be responsible for recruiting fibrocytes to these tissues. Some researchers have reported benefits in treating pro-fibrotic conditions by manipulating the chemotactic axes of either CXCL12 or CCL2[69,93,227,256,265,273]. However, most of the published work has been in models other than myocardial fibrosis and limited to later time points, in which fibrocyte migration may be less clearly established[93,273]. The main objective of the present study was to examine fibrocyte migration at an earlier time point in which fibrocyte migration is well-described and manipulate both axes concurrently[47,68].

CXCL12 is a potent chemotactic factor for progenitor cells and is upregulated in many CVD[47,283–285] . We observed an increase in CXCL12 transcription in the heart following exogenous AngII infusion – a hypertensive model of myocardial fibrosis[47]. Peak CXCL12 expression was at 1d, which coincided with the early (1d-3d) recruitment of bone marrow-derived fibrocytes and the development of fibrosis[47]. It followed that CXCL12 may be recruiting fibrocytes in AngII-mediated myocardial fibrosis. In support of this idea, others have suggested that using a receptor antagonist for CXCR4 in pulmonary fibrosis leads to reduced fibrocyte recruitment and consequently reduced fibrosis[227,256,265]. Under the same premise, we hypothesized that CXCR4 antagonism with AMD3100 would inhibit the infiltration of fibrocytes into the myocardium, ultimately decreasing myocardial fibrosis.

In conjunction with AngII, we administered a dose of AMD3100 that was previously shown to be effective in blocking CXCR4 and reducing fibrocyte recruitment to the lungs. To our surprise, CXCR4 antagonism with AMD3100 did not reduce fibrocyte migration. Instead, we observed a significant increase in cellular infiltration and fibrosis at 3d of infusion. We confirmed that infiltrating cells were $CD45^{+}/\alpha SMA^{+}$ fibrocytes, similar to what was seen in AngII animals, and also showed that the degree of proliferation was not different between groups, suggesting that the observed difference was due to increased fibrocyte migration. AMD3100 is a known to mobilize progenitor cells, due to its ability to interfere with attractive force of the CXCL12-CXCR4 axis in the bone marrow[279]. We did find that AMD3100 administration significantly increased the number of $CD133^{+}$ circulating progenitor cells. As such, administering AMD3100 increased the concentration of progenitor cells in circulation, which we believe may be responsible for the increased cellular infiltration observed following AngII and AMD3100 infusion by simply allowing a larger pool of circulating cells capable of migration to the myocardium. Consistent with what others have shown, infusion with AMD3100 alone did not result in infiltration or fibrosis[277].

In contrast to pulmonary fibrosis models, which demonstrate benefits from concurrent CXCR4 inhibition, our results indicate that the recruitment of fibrocytes or role of CXCL12 in tissue fibrosis may be dependent on the model and organ affected[227,256,265]. While tissue fibrosis appears to involve similar mechanisms in the liver and heart, lung and skin models may differ in the key chemokines and cytokines involved, as supported by our findings[227,265,276,286]. Our results cumulatively suggest that CXCR4 antagonism in our model of AngII-mediated myocardial fibrosis exacerbates

fibrocyte recruitment and fibrosis, likely secondary to the significant mobilization of progenitor cells into the circulation, which were then available to migrate to the myocardium in a CXCL12-independent manner.

Alternatively, CCL2 has also been suggested as a chemokine involved in fibrocyte migration, including studies in heart tissue[69,93,273]. Using knockouts for its receptor ($CCR2^{-/-}$), investigators have been able to show reduced fibrosis in the myocardium. This reduction in fibrosis appeared to be associated with reduced fibrocytes observed in the myocardium leading investigators to conclude that fibrocyte migration was CCL2-CCR2 dependent. It is, however, important to note that these investigators looked at fibrocyte migration later (14-28d). This is in contrast to work in our laboratory that has characterized extensive fibrocyte migration within 3d of AngII[47,270] . In the present study we therefore initially evaluated the ability for fibrocytes to migrate to the myocardium following 3d of AngII infusion in $CCR2^{-/-}$ mice. Once again, to our surprise, myocardial cellular infiltration and fibrosis did not differ between wildtype and $CCR2^{-/-}$ mice, suggesting that early fibrocyte migration can also be CCR2-independent. Similarly, $CCR2^{-/-}$ mice failed to demonstrate reductions in cellular infiltrate and fibrosis when AngII was administered concurrently with AMD3100. On the surface, our findings contrast what others have shown using slightly different doses of AngII and longer duration of exposure to assess fibrosis (14-28d)[69,93]. We sought to replicate experiments of Xu. et al in which the AngII dosage was adjusted (2.8mg/kg/d vs. 2.1mg/kg/d) as well as the length of infusion (3d vs. 28d)[93]. Once again, using this approach we found that at 3d, $CCR2^{-/-}$ mice did not have a reduction in fibrocyte migration or fibrosis. We found, however, that there was a significant difference in both cellular infiltrate and fibrosis in

the myocardium following 28d of AngII infusion in CCR2$^{-/-}$ mice relative to wildtype, as reported by other investigators[93]. Taken together, these findings suggest that the recruitment of fibrocytes early (3d) in the development of myocardial fibrosis is not dependent on CCR2, but that later migration of fibrocytes may still be CCR2-dependent.

Alternatively, our results suggest that the beneficial changes observed at 28d of AngII infusion in CCR2$^{-/-}$ mice may be unrelated to the chemotactic effects of CCL2-CCR2 and are at least in part due to modulated proliferation of ECM-producing cells. In support of this explanation (non-chemotaxis), Moore et al. demonstrated that CCR2$^{-/-}$ mice showed reduced pulmonary fibrosis at 21d, but this observation was independent of decreased cellular infiltrate or changes in infiltrate composition at the earlier time point of 7d[280]. In agreement with our observations, Ekert *et al*. examined the proliferative capacity of fibrocytes in response to CCL2 and CCL12[282]. They found that while human fibrocytes proliferate in response to CCL2, mouse fibrocytes proliferate to CCL12 exposure. CCL12 is found to be upregulated in pulmonary fibrosis models as well[280,287]. In our AngII model, we observed increased myocardial CCL12 transcript following 3d of infusion in WT mice. We also observed reduced proliferation of infiltrating cells in CCR2$^{-/-}$ mice relative to WT. We then demonstrated that ECM-producing cells isolated from the myocardium following 3d of AngII exhibit proliferation in response to CCL12, but that these effects were limited in CCR2$^{-/-}$ cells. Since CCL12 was able to promote proliferation at higher concentration in CCR2-/- cells, it suggests that CCL12 may have effects independent of CCR2, potentially through an unidentified, low-affinity receptor. CCR2$^{-/-}$ cells also failed to significantly respond to our positive control, 10% FBS. This may be due to factors in the FBS that interact with CCR2, but this would require further

investigation. Finally, there are still proliferating cells in the CCR2$^{-/-}$ myocardium following AngII infusion, confirming that CCR2 ligands are not the only factors influencing fibrocyte proliferation.

Sakai *et al.* have also demonstrated the importance of CCR2 in fibrogenesis by examining the production of pro-fibrotic factors by MΦ in response to CCL2 stimulation[275]. Changes in pro-inflammatory and pro-fibrotic cytokines have been noted in several models of fibrosis that implicate the CCL2-CCR2 axis[228,274,276,280]. As such, in AngII-mediated myocardial fibrosis, fibrocytes recruited within the first 3d of infusion may respond to CCL2 by regulating pro-fibrotic factors, thus promoting ECM deposition[275]. As fibrosis develops over the course of 3d to 28d in CCR2$^{-/-}$ mice receiving AngII, reduced fibrocyte proliferation with or without reduced production of fibrotic factors may be occurring as a consequence of abolished CCR2 signaling; however, the latter would need to be evaluated.

Based on our findings, we believe that CCR2 and its ligands – specifically CCL12 – remain valuable therapeutic targets capable of regulating myocardial fibrosis. However, our findings do not support a prominent role for CCL2 in early fibrocyte migration or proliferation in the first 3d of AngII infusion. We provide evidence that the mechanism by which the absence of CCR2 confers benefit is modulated fibrocyte proliferation due to the loss of CCL12-CCR2 interaction. Similarly, while CXCL12 plays a role in the development of myocardial fibrosis – be it positive or negative – it does not appear to be in the early recruitment of fibrocytes. While CXCL12 may have a role in the mobilization of progenitor cells, including fibrocytes, inhibition of its receptor, CXCR4, appears capable of worsening myocardial fibrosis – something that may have clinical

implications, as AMD3100 is used in some patients for autologous bone marrow transplantation[288]. Reducing fibrocyte recruitment in fibrotic conditions could be an important therapeutic strategy; however, further work is necessary to establish which chemokine(s) may be responsible for the early recruitment of fibrocytes to the myocardium.

CHAPTER 5 NON-CLASSICAL RESIDENT MACROPHAGES ARE IMPORTANT DETERMINANTS IN THE DEVELOPMENT OF MYOCARDIAL FIBROSIS

This work appears in part in the publication:

Falkenham A, de Antueno R, Rosin N, Betsch D, Lee TD, Duncan R, Légaré JF. Non-classical resident macrophages are important determinants in the development of myocardial fibrosis. Am J Pathol. 2015 Apr;185(4):927-42. doi: 10.1016/j.ajpath.2014.11.027.

Contribution:

AF – designed the study, carried out the experimentation collected and analyzed data, and prepared the manuscript

RdA – aided in study design, carried out the experimentation

DB – assisted with experimentation and data collection

TL, RD, JFL – assisted with study design and critical review of the manuscript

5.1 Introduction

Myocardial fibrosis is a common feature in CVD and is suspected to be the final pathway in the development of heart failure[289]. The characteristic feature of myocardial fibrosis is the abnormal accumulation of ECM proteins. As ECM proteins accumulate, the heart stiffens and myocardial dysfunction develops that can ultimately culminate in heart failure. In the US alone, almost 6 million people are afflicted with heart failure, which contributes to an estimated over 300,000 deaths per year, emphasizing the importance of understanding the mechanisms underlying the development of myocardial fibrosis[290].

Fibroblasts have been described as the effector cell responsible for ECM deposition; however, there is increasing evidence that MΦ may be key orchestrators of fibrosis through their interactions with fibroblasts[121,291–295]. An evolving understanding of MΦ plasticity has added complexity to the relationship between MΦ and fibrosis. We now know that MΦ are a heterogeneous population capable of pro-inflammatory (classical), regulatory/anti-inflammatory (non-classical), and intermediate phenotypes. In humans, these populations represent a wide spectrum of potential differentiation and activation state and are characterized by their expression of CD14, CD16, CCR2, and CX3CR1, among other markers. On one end of the spectrum, the classical or M1 population, is $CD14^{high}$ $CD16^{-}$ $CCR2^{high}$ and $CX3CR1^{low}$ [184,296]. Conversely, on the other end of the spectrum, the non-classical or M2 population, is $CD14^{low}$ $CD16^{low\text{-}high}$ $CCR2^{low}$ and $CX3CR1^{high}$ [184,296]. This chemokine receptor expression is mirrored in mice; however mice do not express CD16, the M1 to M2 transition is characterized by the loss of Ly6C expression[184,296]. Thus, the consistency of the M1/M2 paradigm between mice and

humans supports the use of mouse models to characterize the roles of monocyte and MΦ subsets in disease.

Although M1 MΦ-mediated inflammation can worsen tissue injury and exacerbate fibrotic responses, M2 MΦ can also produce anti-inflammatory and pro-fibrotic factors such as interleukin (IL)-10, TGF-β, CTGF, and platelet-derived growth factor (PDGF) to directly promote fibroblast proliferation and ECM production[293,297,298]. These MΦ activation states do not exist exclusively from one another. Rather, the contribution of MΦ subsets to fibrosis is part of a dynamic response determined by the demands of healing tissues and characterized by differential monocyte recruitment and the ability to shift between functional phenotypes.

MΦ phenotype change during fibrotic responses has been suggested in a number of fibrosis models including liver, lung, and kidney; however, the evidence in the myocardium is only suggestive and limited to ischemic injury[291,298–300]. Work in our laboratory using a hypertension model of myocardial fibrosis has shown that myocardial fibrosis is preceded by a mass influx of circulating mononuclear cells characteristic of MΦ, expressing markers such as ED1, CD11b, and F4/80 [47,270]. Infiltrating monocytes can dramatically expand the MΦ population in the heart.

Recent findings have also demonstrated that an important resident MΦ population exists in the myocardium, thus providing an additional source of MΦ-fibroblast interactions but their role in myocardial fibrosis is unknown[157]. These new knowledge could mean that monocytes and MΦ may represent important therapeutic targets for myocardial fibrosis rather than targeting the final effector cell, the fibroblast. However, elucidating the contributions of the different MΦ populations and specific subsets in

myocardial fibrosis needs to be better understood and is a necessary step toward MΦ-dependent therapies. Liposomal monocyte depletion and chemokine receptor knockouts are valuable tools for characterizing the contribution of MΦ subsets[174,301,302].

Here, we use the well-established AngII model to study myocardial fibrosis, which is dependent on sustained hypertension and RAAS activation, as is observed clinically in patients[47,68,71,155,270]. The AngII model is characterized by significant MΦ infiltrate, a pro-fibrotic milieu, and significant ECM deposition without the loss of healthy tissue as seen in ischemic injury[47,270,303]. Furthermore, our work with CCR2$^{-/-}$ mice in this model suggests that the non-classical CX3CR1 pathway may be important to non-ischemic myocardial healing[71]. This model offers the unique opportunity to study myocardial fibrosis without conflicting ischemia that is seen post MI and focus the depletion to the fibrotic stage of the response.

We have characterized early MΦ accumulation and the effects of monocyte depletion on the development of AngII-mediated myocardial fibrosis. Specifically, we examined the effects of liposome monocyte depletion on cellular infiltrate, the pro-fibrotic environment, and the development of myocardial fibrosis. In addition, we are the first group to characterize myocardial healing in the absence of CX3CR1 signalling – the outcomes of which could have important therapeutic implications.

5.2 Results

5.2.1 MΦ Are Present in the Myocardium Following 3d AngII Infusion

Previous work in our laboratory using a well-established AngII infusion model of hypertension and hypertrophy has demonstrated that bone marrow-derived leukocyte

infiltration precedes the development of myocardial fibrosis [47,68,71,155,270]. Furthermore, we have established that infiltration starts by 1d and peaks at 3d of AngII infusion, thus making it an important time-point to characterize infiltrating cells.

Consistent with previous findings, the majority of infiltrating cells in the AngII-exposed myocardium were mononuclear (Fig. 5.1A). We then further characterized the infiltrating cells using flow cytometry and immunohistochemistry. In saline controls, 2.58% ± 0.01 of non-myocytes isolated from the heart expressed the $CD11b^{+}$ SSC^{low} MΦ phenotype, likely representing rCMΦ, as recently described (Fig. 5.1B, D) [157,251,304]. Following AngII infusion, the percentage of cells expressing the MΦ phenotype significantly increased to 19.33% ± 1.53 – a 7.49-fold increase over saline controls (Fig. 5.1C,D; $P < 0.0001$). Consistent with non-classical MΦ, cells expressing the chemokine receptor $CX3CR1^{+}$ were observed in the areas of MΦ accumulation (Fig. 5.1E). These MΦ were further characterized by flow cytometry for Ly6C expression.

In both the saline- (Fig. 5.1F) and AngII-exposed (Fig. 5.1G) myocardia, the $CD11b^{+}$ SSC^{low} population displayed a shift toward $Ly6C^{low}$ expression, indicative of a non-classical phenotype. When quantified in the AngII infused group, the $Ly6C^{low}$ population represented 54.56% ± 1.93, whereas the $Ly6C^{mid}$ and $Ly6C^{high}$ comprised 27.46% ± 0.74 and 18.05% ± 1.58, respectively (Fig. 5.1H; $P < 0.001$). Favoring the $Ly6C^{low}$ $CX3CR1^{+}$ MΦ phenotype is consistent with previous work in which $CCR2^{-/-}$ mice did not demonstrate a reduction in early cellular infiltrate or fibrosis in the myocardium early after AngII exposure[71]. Furthermore, our previous work with GFP bone marrow chimeras indicates that these areas of cellular infiltrate are predominantly

derived from circulating monocytes[47]. It followed that these MΦ could be depleted using IV liposomes.

5.2.2 MΦ can be targeted with liposomes in circulation

Circulating monocyte targeting was evaluated by administering fluorescent, DiO^+ liposomes to mice during AngII infusion. Liposome administration was initiated one day prior to implanting an osmotic mini-pump containing AngII to saturate circulating monocytes with liposomes. Animals were sacrificed after 3d of AngII infusion and frozen sections of heart were characterized by immunofluorescence. In a representative section of myocardium, a population of $CD11b^+$ cells (Fig. 5.2A) were observed in areas of collagen deposition (Fig. 5.2A). In addition, punctate green fluorescence was observed in these same areas, consistent with DiO^+ liposomes (Fig. 5.2A). Cell nuclei were labeled with Hoechst (Fig. 5.2A). Immunofluorescence staining confirmed the co-localization of liposomes in CD11b expressing cells (Fig. 5.2B). Liposomes were not observed in non-MΦ, supporting intravenous phagocytosis by monocytes as the mechanism for targeting these cells and supporting our previous work, suggesting that a significant number of myocardial infiltrating cells are derived from the circulation. The appearance of liposome-laden MΦ in the myocardium following AngII infusion supports the premise that these MΦ can be targeted in circulation by IV liposome administration.

5.2.3 Monocyte depletion prevents MΦ accumulation in the AngII-exposed myocardium

To confirm that monocytes could be depleted with cytotoxic clodronate liposomes, mice were intravenously administered liposomes containing either PBS or

clodronate beginning 1d prior to AngII infusion and each subsequent day for a total of four treatments. Non-liposome animals infused with AngII served as controls. Following 4 liposome treatments, blood samples were characterized for monocyte populations using flow cytometry and taken for smears and stained for cell morphology using Giemsa. Flow cytometry supported a reduction of approximately 50% in F4/80^{+} CD11b^{+} cells in animals receiving clodronate liposomes (Fig. 5.3C) relative to PBS liposomes (Fig. 5.3A), which was further confirmed using blood smears. The ablation of monocytes with clodronate liposomes was consistent with the Ly6C expression profile of new monocytes (Ly6C^{high}) entering circulation from the bone marrow (Fig. 5.3D) relative to those differentiating toward non-classical (Ly6C^{low}) monocytes in animals receiving PBS liposomes (Fig. 5.3B). A blind observer assessed cells on blood smears for morphology and counted leukocyte subsets to a total of 350 cells per slide. Non-monocyte populations were unaffected by the administration of either PBS or clodronate liposomes (Fig. 5.3F). Control and PBS liposome animals exhibited 4.26% ± 0.20 and 3.53% ± 0.36 monocytes of the total leukocyte population, respectively (Fig. 5.4E-F). The clodronate liposome group, however, demonstrated a significant reduction in monocytes, exhibiting 0.51% ± 0.08 of leukocytes (Fig. 5.3E-F; $P < 0.001$). This represented an 88.0% and 85.5% reduction in circulating monocytes relative to control and PBS liposome animals, respectively. Thus, consistent with previous reports, intravenous clodronate liposomes can effectively deplete circulating monocytes[224].

5.2.4 Clodronate liposomes reduce monocyte infiltration in the AngII-exposed myocardium

Animals were infused with AngII and intravenously injected with, either PBS liposomes, clodronate liposomes, or clodronate solution and sacrificed at 3d. Myocardia from these animals were assessed for the percent of myocardium affected by infiltrating cells using a standardized grid as previously described[68,71,155,270,294]. AngII infusion led to obvious areas of cellular infiltrate in the myocardium as indicated by an abundance of perivascular and interstitial mononuclear cells – the same pattern of mononuclear cells was minimal or absent in saline controls as previously described[47]. Myocardia from animals that received AngII and either PBS liposomes or clodronate solution demonstrated similar patterns of mononuclear infiltrate relative to AngII alone (Fig. 5.3G-H and 5.1A). Quantitatively, AngII-exposed hearts exhibited 10.00% ± 1.34 of heart grids affected by infiltrating cells – a significant increase over saline controls (0.37% ± 0.37; Fig. 5.3J; $P < 0.001$). Consistent with AngII infusion alone, animals that received AngII and either PBS liposomes or clodronate solution had 14.66% ± 1.23 and 13.53% ± 1.50 of the heart affected, respectively (Fig. 5.3J). In contrast, to all the above controls animals that received AngII and clodronate liposomes showed little infiltration of cells in the myocardium (Fig. 5.3I). Myocardia from animals that received AngII and clodronate liposomes exhibited just 3.28% ± 0.26 in area of myocardium affected by infiltrating cells – a significant 77.6% reduction in infiltrating cells relative to AngII infused animals approaching that of control animals (Fig. 5.3J; $P < 0.001$). Immunofluorescence for CD11b (Fig. 5.3L) and F4/80 (Fig. 5.3N) in myocardia from animals receiving AngII and liposomes displayed little to no positivity confirming our findings.

Consistent with AngII infused animals, those receiving PBS liposomes and AngII still showed populations of $CD11b^+$ (Fig. 5.3K) and $F4/80^+$ (Fig. 5.3M), indicative of MΦ. AngII led to significantly increased cardiac transcript levels of TGF-β (4.706 ± 0.3498) relative to animals receiving saline (1.774 ± 0.9107) (Fig. 5.3O; $P < 0.05$). Surprisingly, clodronate liposomes in association with AngII further increased cardiac transcript levels of TGF-β (10.96 ± 2.693) relative to animals receiving AngII alone (Fig. 5.3O; $P < 0.05$). In contrast, col1α1 (13.62 ± 3.612) and CX3CL1 (1.335 ± 0.5201) transcript levels were significantly reduced relative to animals receiving AngII alone (97.16 ± 40.31 and 13.84 ± 2.649, respectively) (Fig. 5.3P-Q; $P < 0.05$), potentially supporting modulated non-classical MΦ involvement.

5.2.5 *Reduction in infiltrating monocytes is associated with reduced myocardial fibrosis*

Following the analysis of infiltrating cells, representative myocardia from individual groups were stained for collagen using the Sirius red and fast green stain as previously described[71,155]. Stained myocardia were assessed for the percentage of collagen in whole myocardial sections. Large areas of collagen deposition were observed in animals receiving AngII, as indicated by Sirius red staining and significantly higher than saline control as previously described[47,71,155]. Similar patterns of excess collagen deposition were observed in animals receiving AngII and either PBS liposomes or clodronate solution (Fig. 5.4A and B, respectively). In contrast, animals receiving AngII and clodronate liposomes had a significant reduction in collagen deposition when compared to AngII animals – rather collagen was largely localized to vasculature, as in saline controls. Quantitatively, collagen comprised 11.50% ± 1.12 of representative

myocardial sections from animals receiving AngII alone (Fig. 5.4D). Consistent with infiltrating cell data, this represented a significant increase over basal collagen levels observed in saline controls (2.11% ± 1.25; Fig. 5.4D; P <0.001). Myocardia from animals that received AngII and either PBS liposomes or clodronate in solution demonstrated similar changes– 14.47% ± 1.76 and 11.11% ± 0.69, respectively– which were not significantly different from AngII alone (Fig. 5.4D). In contrast, animals that received AngII and clodronate liposomes (Fig. 5.4C) had a significant reduction in (3.28% ± 0.26) myocardial collagen content; 71.5% reduction in collagen content relative to myocardia from animals receiving AngII alone corresponding to the significant reductions in infiltrating cells (Fig. 5.4D; P <0.001). Animals receiving AngII (and PBS liposomes) displayed clusters of αSMA^+ fibroblasts (Fig. 5.4E) – an observation consistent with our previous findings in animals that received AngII alone [47]. In support of the reduction in collagen content, animals infused with AngII receiving clodronate liposomes did not have clusters of αSMA^+ fibroblasts, suggesting reduced fibroblast differentiation to activated myofibroblast phenotype and supporting reduced fibrosis (Fig. 5.4F). Rather, αSMA^+ cells were primarily limited to vasculature as previously observed in historical controls and likely representative of smooth muscle cells that also can express αSMA (data not shown). Collagen α1α myocardial transcript levels were significantly elevated following AngII infusion (97.16 ± 40.31) relative to saline controls (1.238 ± 0.2920) (Fig. 5.3P; P <0.05). However, following the administration of clodronate liposomes, myocardial transcript levels were reduced to levels not-significantly different from baseline (13.62 ± 3.612). Myocardial transcript levels of TGF-B were significantly elevated following clodronate liposomes (10.95 ± 2.693) relative to AngII alone (4.706 ± 0.3498) (Fig. 5.3O;

P <0.05) and higher than in saline controls. Thus, the reduction in MΦ observed with clodronate liposome treatment is associated with significant reductions in myocardial collagen, potentially due to reduced fibroblast proliferation and/or activation into myofibroblasts.

5.2.6 MΦ Promote Fibroblast αSMA Expression In Vitro

Given the reduction in collagen and αSMA^+ cells in the myocardia of animals depleted of monocyte-derived MΦ, we evaluated whether MΦ could directly promote fibroblast activation. To characterize MΦ and fibroblast interactions, we set up a system of monocultures and juxtacrine co-cultures.

Following 2d of culture, MΦ (Fig. 5.5A), fibroblasts (Fig. 5.5B), and co-cultures (Fig. 5.5C) were photographed under light microscopy and harvested for immunofluorescence. Bone marrow-derived MΦ were characterized for purity using immunofluorescence for F4/80 (Fig. 5.5D). The 3T3 fibroblasts were characterized for purity and activation by the expression of collagen and αSMA, respectively (Fig. 5.5E). The purity of MΦ and fibroblasts was 97.3% ± 0.77 and 100.0% ± 0.0, respectively (Fig. 5.5G; P <0.001). The $CD11b^+$ cultured MΦ (Fig. 5.5H) were also found to exclusively express the $Ly6C^{low}$ non-classical phenotype (Fig. 5.5I). We demonstrated increased αSMA^+ fibroblast differentiation in the co-culture compared to the monoculture, as seen in the representative fields of view (Fig. 5.5E-F). The expression of collagen and αSMA was quantified relative to cell number by standardizing to the fluorescence intensity of nuclei per well of cultured cells. A significant difference in collagen and αSMA expression was observed in co-cultures relative to fibroblast monocultures (Fig. 5.5J-K; P

<0.05). It follows that MΦ, specifically of the non-classical (M2) phenotype, are sufficient to promote fibroblast activation and collagen production by promoting myofibroblasts differentiation.

Together, the majority Ly6C^{low} MΦ phenotype observed in the myocardium following AngII infusion, the reduction in CX3CL1 myocardial transcript following monocyte depletion, and the co-culture data suggest that non-classical MΦ may have an important role in the development of AngII-mediated myocardial fibrosis. To further evaluate the role of non-classical monocyte recruitment in this process, we administered AngII to CX3CR1$^{-/-}$ mice and characterized the effects on cellular infiltrate and myocardial fibrosis.

5.2.7 CX3CR1-dependent non-classical monocyte recruitment is protective in AngII-mediated myocardial fibrosis

The absence of CX3CR1 on the surface of circulating monocytes did not result in any reduction in cellular infiltration as predicted when compared to the cellular infiltrate in AngII-exposed WT myocardium (Fig. 5.6D). In contrast, myocardial changes appeared to be exacerbated in the AngII-infused CX3CR1$^{-/-}$ mice (Fig. 5.6E). In accordance with the CX3CR1-dependent non-classical monocyte recruitment, there was a marked reduction in myocardial CD206^{+} (a marker of M2 non-classical MΦ) cells in the AngII-infused CX3CR1$^{-/-}$ mice when compared to AngII WT counterparts assessed by immunocytochemistry (Fig. 5.6F). Cellular infiltrate was significantly increased in AngII-infused CX3CR1$^{-/-}$ (17.37% ± 2.13 of heart grids affected) mice relative to WT controls (10.00% ± 1.34 of heart grids affect; Fig. 5.6G). Furthermore, the increase in myocardial infiltrate in CX3CR1$^{-/-}$ mice was associated with a significant 2.54-fold

increase in collagen deposition (29.26 ± 2.58) relative to WT mice (11.50% ± 1.12; Fig. 5.6H). Saline controls from both CX3CR1$^{-/-}$ and WT mice were unremarkable (Fig. 5.6H). Based on the apparent reduction in myocardial CD206 expression in CX3CR1$^{-/-}$ relative to WT mice, we suspected that there was a shift toward a classical or intermediate phenotype and that in fact non-classical phenotype may provide a yet to be described protective role.

5.2.8 Total MΦ in CX3CR1$^{-/-}$ AngII-exposed hearts display higher Ly6C expression

Following AngII infusion, both WT and CX3CR1$^{-/-}$ total cardiac MΦ populations (resident+infiltrating) shifted toward lower Ly6C expression relative to saline controls (Fig. 5.7B and D to A and C, respectively). When quantified, it was found that the reduction in cardiac Ly6C expression between saline and AngII-infused mice was significantly reduced ($P < 0.05$; Fig. 5.7E). In contrast total cardiac MΦ in AngII/CX3CR1$^{-/-}$ mice continued to demonstrate a significant shift toward higher Ly6C expression relative to AngII/WT mice, thus favoring a pro-inflammatory phenotype ($P < 0.001$; Fig. 5.7E).

5.2.9 Infiltrating MΦ in CX3CR1$^{-/-}$ AngII-exposed hearts display higher Ly6C expression

Despite the presence of multiple dense, Ly6C^{high}, early MΦ populations in the AngII-infused WT hearts, the early MΦ populations in CX3CR1$^{-/-}$ demonstrated a significant increase in MFI of Ly6C ($P < 0.01$; Fig. 5.7F). This 1.4-fold increase relative to AngII-infused WT mice suggests a shift toward the pro-inflammatory, classical MΦ

phenotype, which could further exacerbate an overall pro-inflammatory environment set by the resident population.

5.2.10 *CX3CR1$^{-/-}$ AngII-exposed animals have a pro-inflammatory myocardial environment*

In support of the hypothesis that phenotypic pro-inflammatory MΦ favor an inflammatory cardiac environment, myocardial transcript levels of the pro-inflammatory cytokine IL-1B were significantly elevated in CX3CR1$^{-/-}$ relative to WT AngII-infused mice ($P < 0.05$; Fig. 5.7G). In addition, AngII-infused CX3CR1$^{-/-}$ displayed a significant reduction in myocardial transcript levels of the anti-inflammatory cytokine TGFβ ($P < 0.001$; Fig. 5.7I), while maintaining myocardial transcript levels of the pro-fibrotic connective tissue growth factor (CTGF; Fig. 5.7J).

5.2.11 *Resident MΦ in CX3CR1$^{-/-}$ control hearts display higher Ly6C expression*

To compare MΦ phenotypes in the hearts of control WT and control CX3CR1$^{-/-}$ mice, we isolated non-myocytes following 3d of saline infusion. Cells were labeled for F4/80, CD11b, and Ly6C as described above. In both saline infused WT (Fig. 5.7A) and CX3CR1$^{-/-}$ (Fig. 5.7B) control hearts, there were readily identifiable populations of F4/80^{+}CD11b^{low} cells observed in the left half of the dot plots, indicative of rCMΦ. These rCMΦ displayed a spectrum of Ly6C expression (y-axis); however, the rCMΦ in the CX3CR1$^{-/-}$ appeared to favor a Ly6C^{mid} phenotype, more consistent with the classical or intermediate phenotype. Quantification of Ly6C expression by MFI confirmed that even at baseline levels, Ly6C expression in CX3CR1$^{-/-}$ cardiac resident MΦ significantly

exceeded baseline levels in WT controls (P <0.01; Fig. 5.7F). Thus, if increased Ly6C expression indicates a pro-inflammatory MΦ, phenotype, then CX3CR1$^{-/-}$ mice may have a pre-disposition to cardiac inflammation or conversely, reduced protection to inflammation which could explain their higher susceptibility to AngII.

5.2.12 Differences in cellular infiltrate and collagen deposition independent of physiological parameters

AngII infusion led to a significant increase in MAPB relative to saline controls – 130.6 ± 5.32 mmHg compared to controls 101.3 ± 4.22 (Table 5.1; P <0.001). WT Animals receiving PBS liposomes, clodronate liposomes, or clodronate solution as well as CX3CR1$^{-/-}$ mice responded to AngII with similar increases in MABP – 123.3 ± 5.21 mmHG, 129.8 ± 2.88 mmHG, 124.4 ± 6.01 mmHG, 125.7 ± 4.57, respectively (Table 5.1). MABP did not significantly differ between experimental groups receiving AngII with or without liposomes or clodronate solution. Thus, the overall observations in this study appear to be blood pressure independent. Similar to blood pressure, animals receiving AngII exhibited significant weight loss (-2.30g ± 0.21) and myocardial hypertrophy (6.15mg/g ± 0.15) relative to saline controls (+0.23g ± 0.04 and versus 5.19mg/g ± 0.18 (Table 5.1). Weight loss and hypertrophy were comparable between experimental groups receiving AngII independent of liposomes or clodronate solution. AngII infused CX3CR1$^{-/-}$ mice also exhibited significantly increased hypertrophy (7.48mg/g ± 0.52) relative to AngII infused WT mice (P <0.01; Table 5.1). Finally, transthoracic echocardiography suggested that both AngII/WT and AngII/CX3CR1$^{-/-}$ mice exhibited significantly increased LVPW and IVSW thickness during systole and

diastole relative to their respective controls supporting the findings of hypertrophy ($P < 0.05$; Table 5.2).

5.3 Tables

*Table 5.1 - Physiological and morphological parameters. Hypertrophy as measured by heart weight:body weight (HW/BW). Change in body weight (ΔBW). Tail cuff blood pressure measurements as mean arterial blood pressure (MABP). Relative to saline/WT: **p<0.01, ***p<0.001; relative to AngII/WT †‡p<0.001. Data is expressed as the mean ± SEM.*

Physiological and morphological parameters.					
Strain	1° Treatment	2° Treatment	HW/BW (mg/g)	ΔBW (g)	MABP (mmHg)
WT	Saline	No Liposomes	5.19 ± 0.18	0.23 ± 0.04	101.3 ± 4.22
	AngII	No Liposomes	6.15 ± 0.15 ***	-2.30 ± 0.21 ***	130.6 ± 5.32 **
		Clodronate Liposomes	6.23 ± 0.16 ***	-2.79 ± 0.22 ***	129.8 ± 2.88 **
		PBS Liposomes	6.38 ± 0.17 ***	-2.37 ± 0.21 ***	123.3 ± 5.21 **
		Free Clodronate	6.33 ± 0.26 ***	-2.85 ± 0.48 ***	124.4 ± 6.08 **
CX3CR1 $^{-/-}$	Saline	No Liposomes	4.93 ± 0.28	0.29 ± 0.07	103.5 ± 2.41
	AngII	No Liposomes	7.48 ± 0.52 †‡	-3.64 ± 0.37 †‡	125.7 ± 4.57 **

*Table 5.2 - ECHO measurements. ECHO measurements for left ventricular posterior wall thickness in systole (LVPWs) and diastole (LVPWd) and intraventricular septal wall thickness in systole (IVSWs) and diastole (IVSWd). Relative to saline/WT: *p<0.05; relative to AngII/WT †p<0.05. Data is expressed as the mean ± SEM.*

ECHO measurements						
Strain	1° Treatment	2° Treatment	LVPWs (mm)	LWPWd (mm)	IVSWs (mm)	IVSWd (mm)
WT	Saline	No Liposomes	1.23 ± 0.10	0.92 ± 0.08	1.36 ± 0.17	1.08 ± 0.14
	AngII	No Liposomes	1.80 ± 0.22 *	1.20 ± 0.13 *	1.73 ± 0.14 *	1.51 ± 0.14 *
CX3CR1-/-	Saline	No Liposomes	1.20 ± 0.08	0.85 ± 0.09	0.98 ± 0.02	0.81 ± 0.03
	AngII	No Liposomes	1.65 ± 0.08 *	1.24 ± 0.06 *	1.29 ± 0.10 †	1.09 ± 0.09 †

5.4 Figures

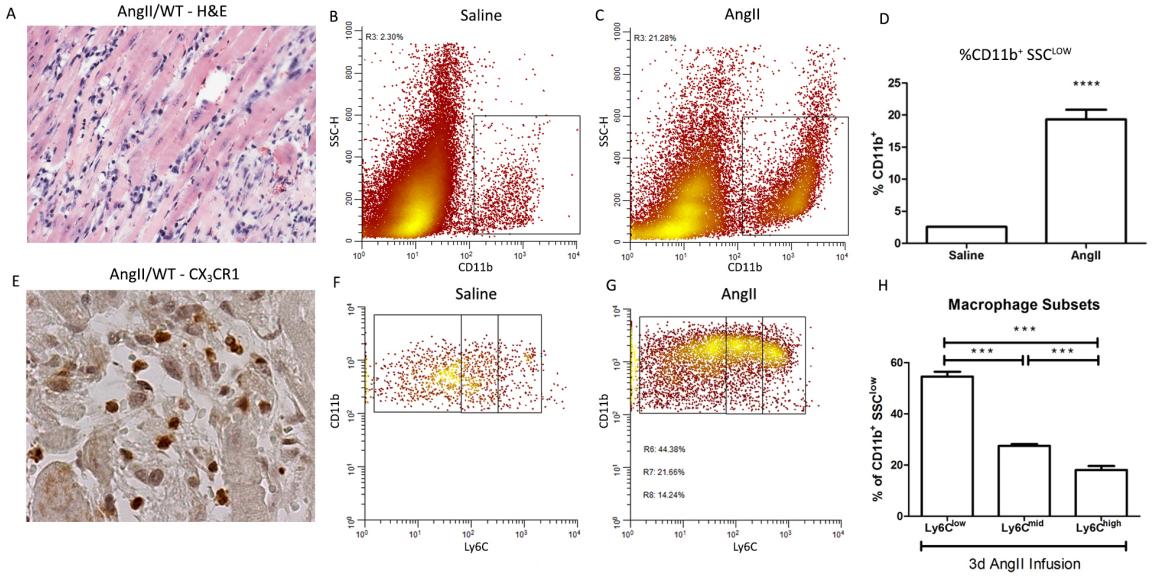


*Figure 5.1 - AngII leads to a macrophage influx in the heart. Representative image of myocardium stained with H&E from an animal receiving (**A**) AngII. Flow cytometry of overall cardiac macrophage (resident+infiltrating) populations in **(B)** saline control and **(C)** AngII-exposed hearts. **(D)** Quantification of $CD11b^{+}SSC^{low}$ macrophage populations of saline and AngII-exposed myocardia. **(E)** Representative immunohistochemistry for CX_3CR1 in myocardium from an AngII/WT animal receiving AngII (63x). Representative flow cytometry on $CD11b^{+}SSC^{low}$ populations from WT animals receiving **(F)** saline and **(G)** AngII animals characterized for CD11b (y-axis) and Ly6C (x-axis). **(H)** Semi-quantitative distribution of Ly6C expression on $CD11b^{+}SSC^{low}$ populations. (***p<0.001, ****p<0.0001, n=4-8). Data is expressed as the mean ± SEM.*

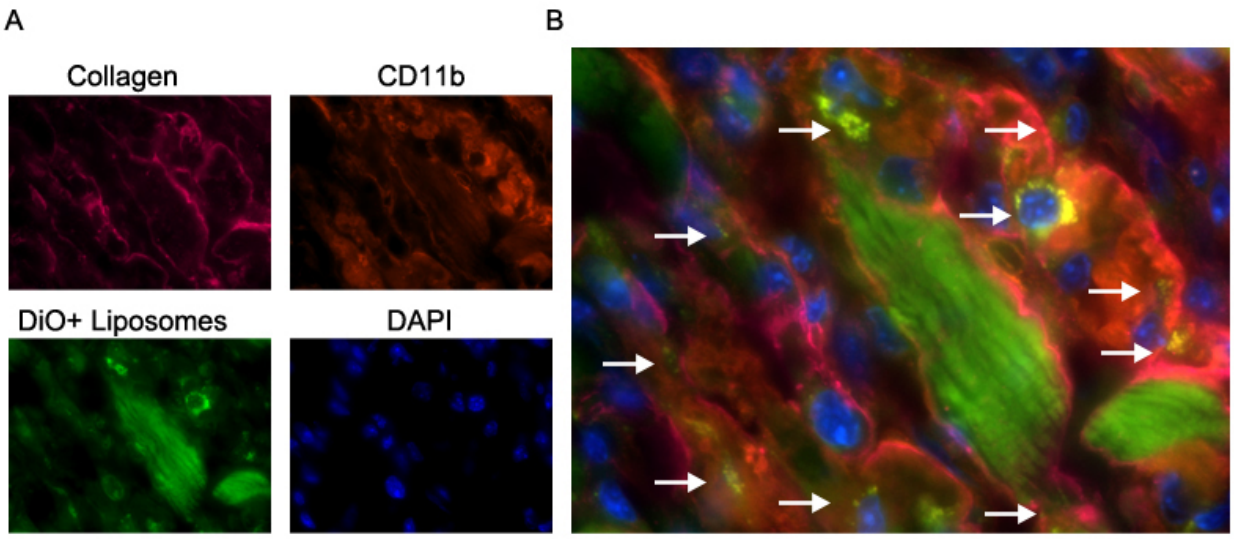


*Figure 5.2 - Cardiac MΦ in the AngII exposed myocardium can be targeted intravenously by liposomes. Fluorescent (DiO^+) liposome uptake by MΦ in areas of collagen deposition from representative myocardial sections. **(A)** Single labels for (I) collagen type-I, (II) CD11b, (III) DiO liposomes, and (IV) nuclei (DAPI). **(B)** Overlay of individual channels demonstrating punctate yellow fluorescence, characteristic of co-localization with DiO^+ liposomes. Arrows indicate examples of co-localization. Images were captured at 100x.*

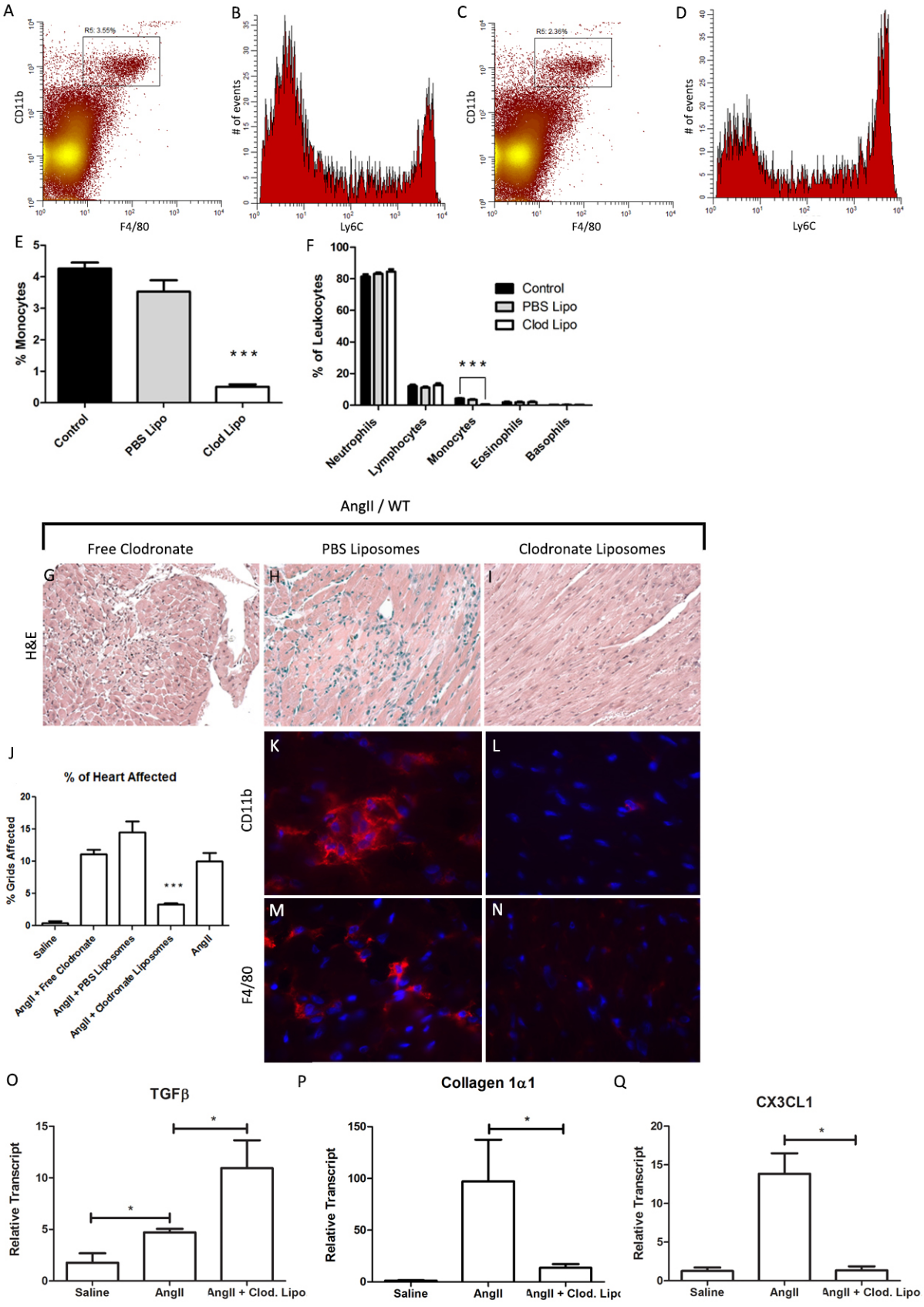

A
B
C
D
R5: 3.55%
R5: 2.36%
CD11b
F4/80
# of events
Ly6C
E
% Monocytes
***
Control
PBS Lipo
Clod Lipo
F
% of Leukocytes
Control
PBS Lipo
Clod Lipo
***
Neutrophils
Lymphocytes
Monocytes
Eosinophils
Basophils
AngII / WT
Free Clodronate
PBS Liposomes
Clodronate Liposomes
G
H
I
H&E
J
% of Heart Affected
% Grids Affected
***
Saline
AngII + Free Clodronate
AngII + PBS Liposomes
AngII + Clodronate Liposomes
AngII
K
L
M
N
CD11b
F4/80
O
TGFβ
P
Collagen 1α1
Q
CX3CL1
Relative Transcript
Saline
AngII
AngII + Clod. Lipo

Figure 5.3 - Monocyte depletion significantly decreases the macrophage influx following AngII infusion. ***(A)*** *Representative gating of a double-positive population in an F4/80 x CD11b plot from an animal receiving PBS liposomes and* ***(B)*** *its Ly6C expression.* ***(C)*** *Representative gating of the same double-positive population from an animal receiving clodronate liposomes and* ***(D)*** *its Ly6C expression.* ***(E)*** *Percentages of neutrophils, lymphocytes, monocytes, eosinophils, and basophils based on blood film nuclei counts.* ***(F)*** *Magnified percentages of monocyte percentages from blood film nuclei counts. (***p<0.001, n=5). Representative images from myocardial H&E sections from animals receiving AngII and either* ***(G)*** *clodronate solution,* ***(H)*** *PBS liposomes, or* ***(I)*** *clodronate liposomes (20x).* ***(J)*** *Quantification of area of heart affected by mononuclear infiltrate (***p<0.001, n=3-14). Immunofluorescence confirming that animals receiving PBS liposomes had myocardial* ***(K)*** *$CD11b^+$ populations that were absent in animals receiving* ***(L)*** *clodronate liposomes. $F4/80^+$ cells were also observed in animals receiving* ***(M)*** *PBS liposomes, but were not apparent in animals receiving* ***(N)*** *clodronate liposomes (40x). Myocardial transcript levels of* ***(O)*** *TGF-B,* ***(P)*** *collagen 1α1, and* ***(Q)*** *CX_3CL1 following AngII infusion with and without clodronate liposome (*p<0.05, n=4-6). Data is expressed as the mean ± SEM.*

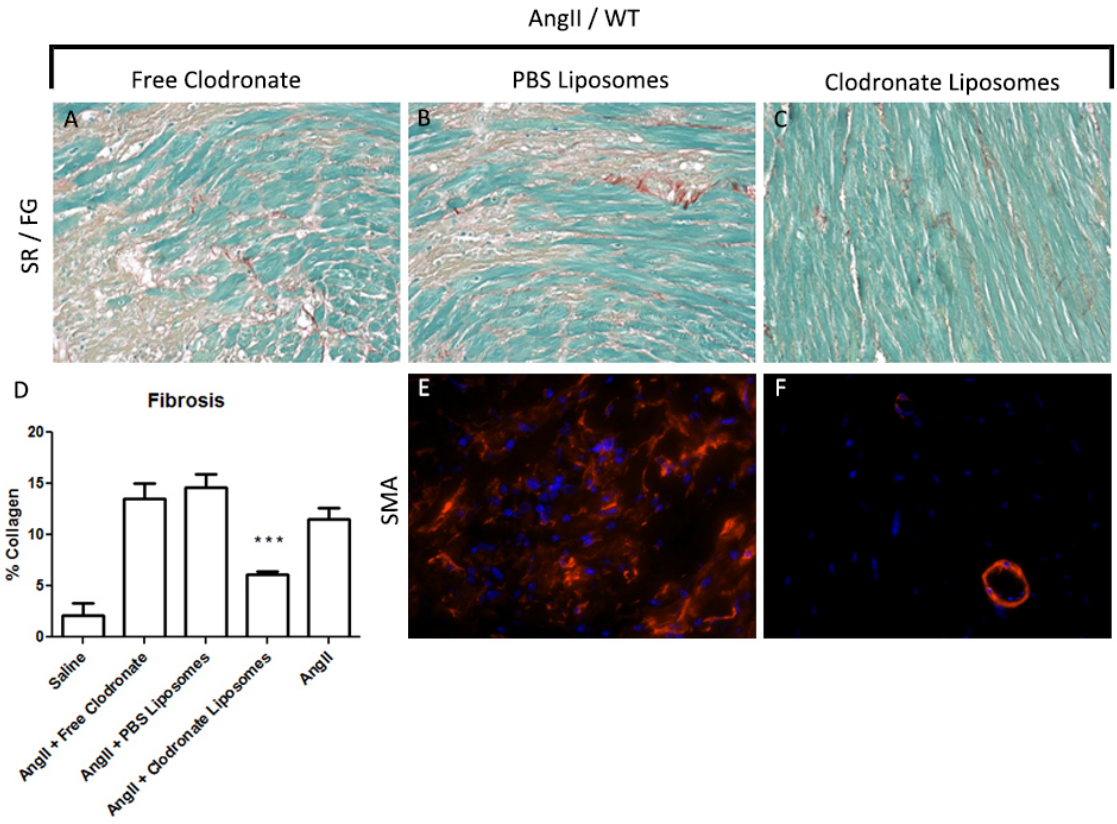


Figure 5.4 - Myocardial fibrosis is significantly reduced in the absence of the macrophage influx. Representative images from myocardial sections stained with SR from animals receiving AngII and either ***(A)*** *clodronate solution,* ***(B)*** *PBS liposomes, or* ***(C)*** *clodronate liposomes (20x). (collagen: red; cardiomyocytes: green).* ***(D)*** *Quantification of SR/FG collagen content (***p<0.001, n=3-14). Representative immunofluorescence showing large clusters of α-SMA^{+} myofibroblasts in animals receiving* ***(E)*** *PBS liposomes relative to those receiving* ***(F)*** *clodronate liposomes. (40x). Data is expressed as the mean ± SEM.*

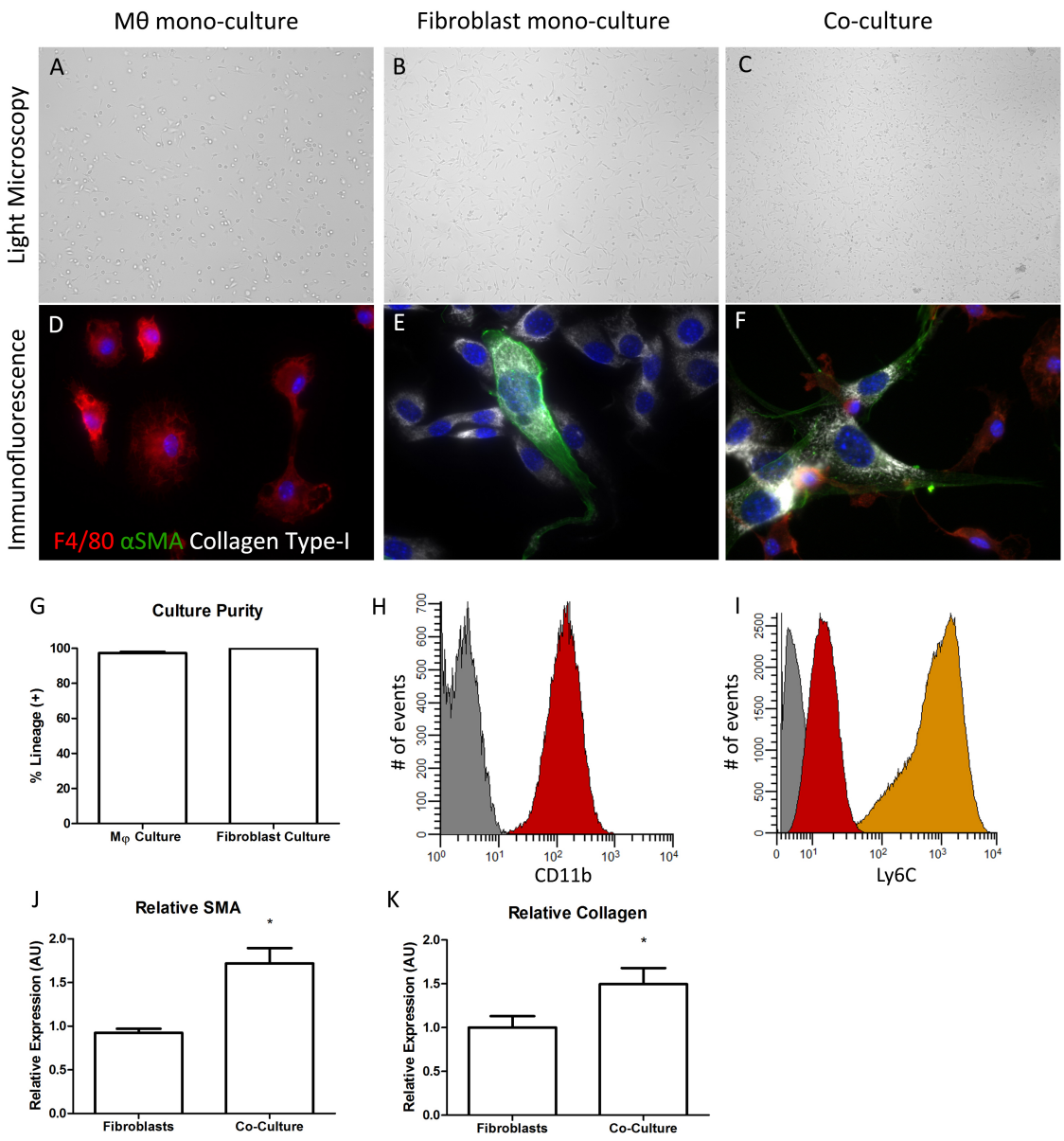


*Figure 5.5 - Non-classical MΦ promote fibroblast activation and collagen production in vitro. BMDMθ and 3T3 fibroblasts were cultured in monoculture and co-culture at equal ratio. Macrophage and fibroblast monocultures and co-culture were characterized using light microscopy (**A, B, C,** respectively) (10x) and immunofluorescence for F4/80 (red), collagen type-I (white), and α-SMA+ (green) (**D, E, F,** respectively) (63x). **(G)** Mono-culture purity was quantified from immunofluorescence (n=6). (D) BMMθ were further characterized for **(H)** CD11b and **(I)** Ly6C using flow cytometry. Quantification of co-culture expression demonstrated that non-classical MΦ promote both fibroblast activation as indicated by **(J)** α-SMA+ and **(K)** collagen production relative to monocultures (*p<0.05, n=4-6). Data is expressed as the mean ± SEM.*

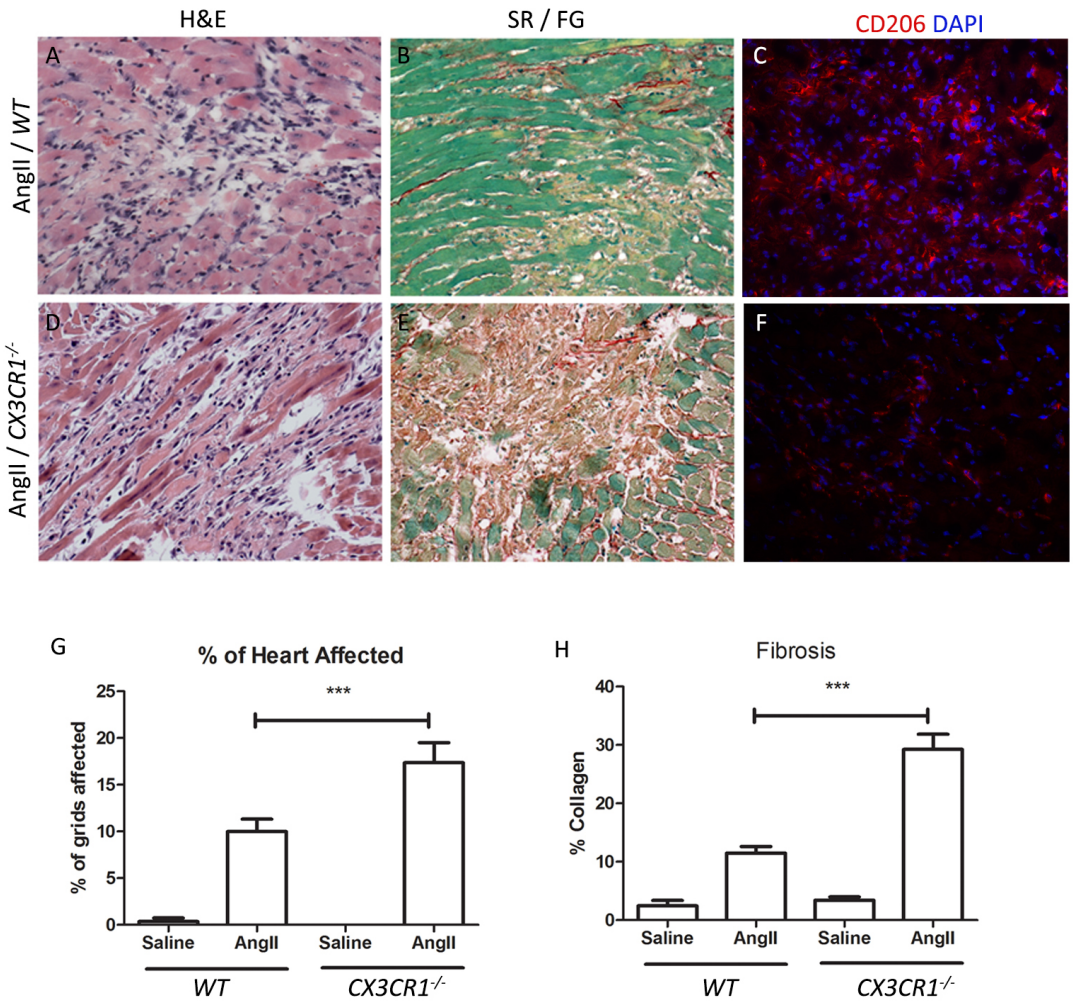


Figure 5.6 - Non-classical macrophage inhibition exacerbates myocardial infiltration and fibrosis following AngII infusion. Representative heart sections from AngII infused WT ***(A, B, C)*** *and $CX_3CR1^{-/-}$ mice* ***(D, E, F)*** *animals stained with H&E, SR/FG, and CD206, respectively (20x). Semi-quantification of* ***(G)*** *infiltrate and* ***(H)*** *fibrosis. (***$p<0.001$). Saline $CX_3CR1^{-/-}$ and WT mice were unremarkable. Data is expressed as the mean ± SEM.*

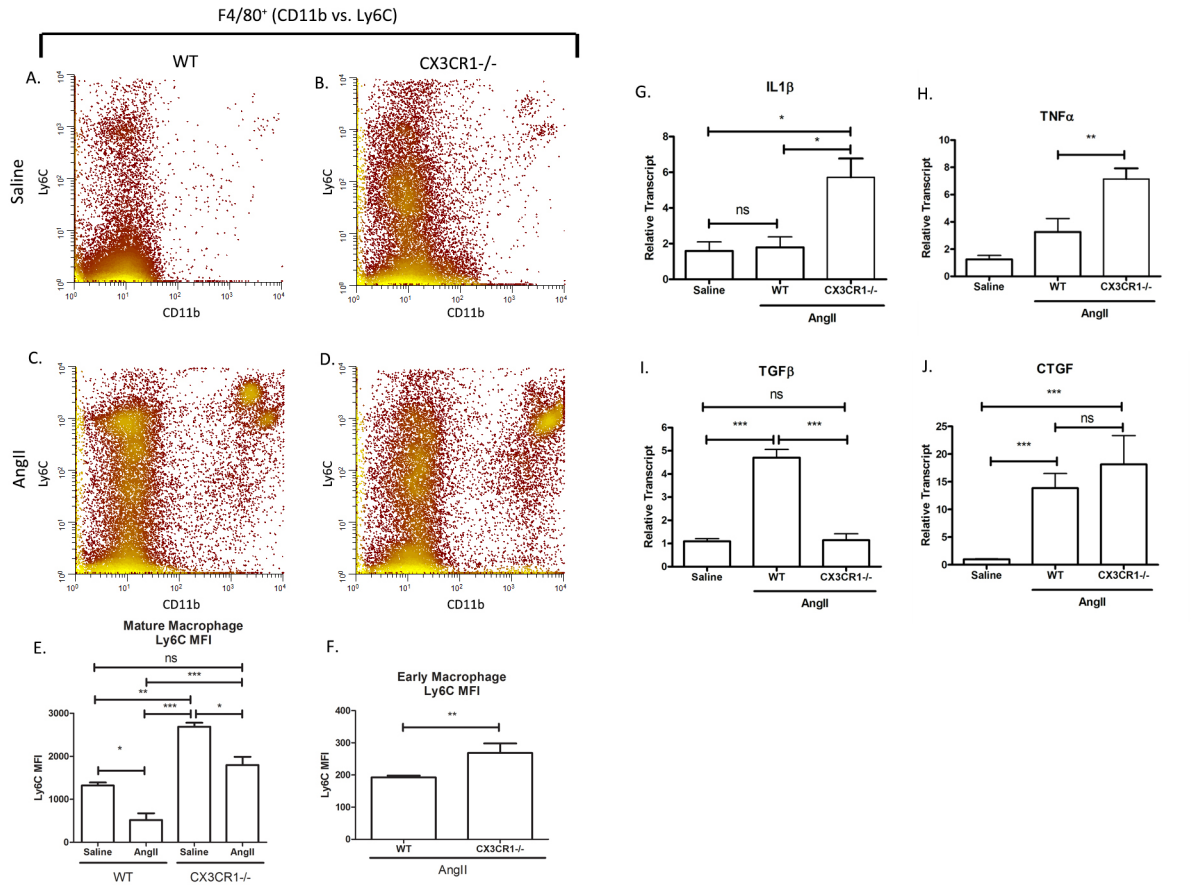
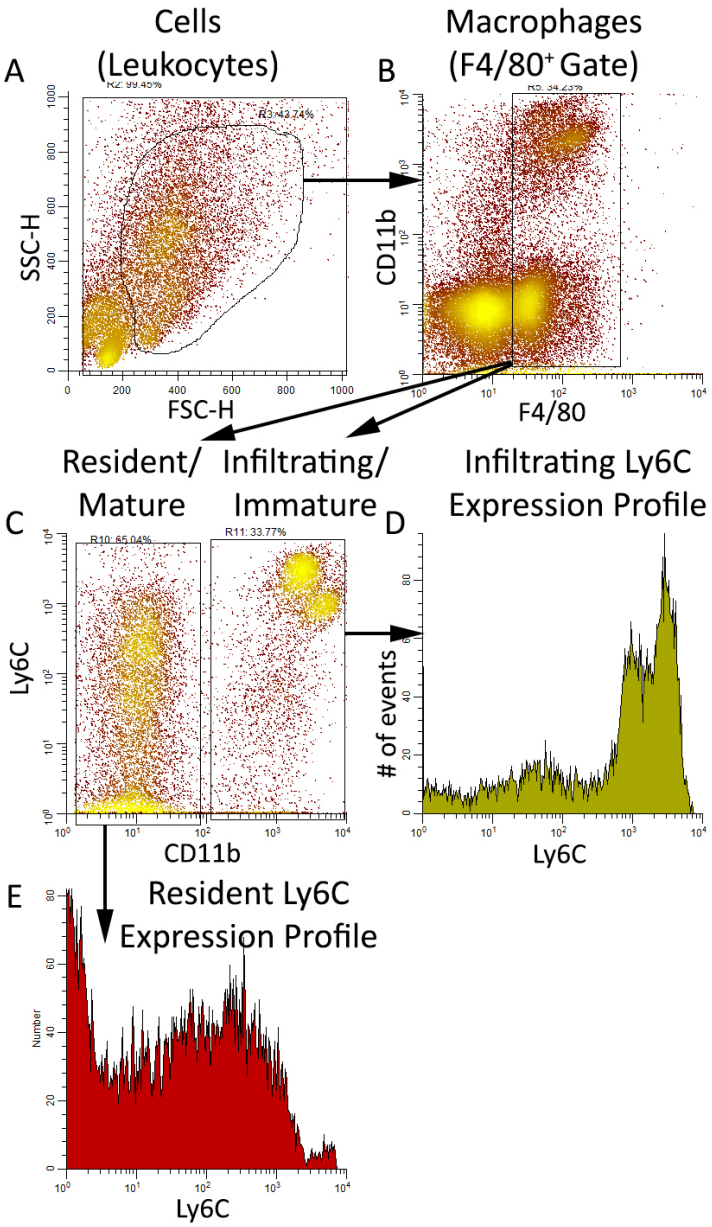


*Figure 5.7 - Resident and infiltrating MΦ in CX3CR1-/- myocardia favour a pro-inflammatory phenotype and environment. Representative flow cytometry for F4/80, CD11b, and Ly6C in saline-infused **(A)** WT and **(B)** $CX_3CR1^{-/-}$ myocardia and AngII-exposed **(C)** WT and **(D)** $CX_3CR1^{-/-}$ myocardia. Resident or mature MΦ are on the left side of dot plots and infiltrating or early MΦ are on the right side of dot plots. MFI of Ly6C in **(E)** resident and **(F)** infiltrating cardiac MΦ from both $CX_3CR1^{-/-}$ and WT mice. Infiltrating MΦ could only be characterized in animals receiving AngII. (***p<0.001). Myocardial transcript levels of **(G)** IL-1B, **(H)** TNF-α, **(I)** TGF-B, and **(J)** CTGF (*p<0.05; **p<0.01; ***p<0.001). Data is expressed as the mean ± SEM.*



Supplementary Figure 5.1 - Representative Gating Strategy For Characterizing MΦ in the Heart. ***(A)*** *Flow cytometry dot plot for forward scatter (FSC) and side scatter (SSC) to exclude debris.* ***(B)*** *FSC x SSC gate applied to F4/80 x CD11b dot plot and the gate used to include MΦ (F4/80^{+}) and concurrently exclude neutrophils (F4/80^{-}).* ***(C)*** *F4/80^{+} gate applied to CD11b x Ly6C dot plot, in which MΦ can be differentiated by their expression of CD11b: resident or mature MΦ are CD11b^{low} and infiltrating or immature MΦ are CD11b^{high}. The Ly6C expression of these macrophage populations can be visualized in the dot plot above or by applying the gates in C to Ly6C histograms* ***(D, E)***.

5.5 Discussion

MΦ are experiencing a renaissance in research, largely in part to the recent discoveries of their phenotypic and functional heterogeneity[263,305,306]. MΦ function is now known to span from pro-inflammatory to anti-inflammatory to pro-angiogenic to pro-fibrotic, which together serve an essential role in tissue healing, particularly in the context of the myocardium[1,74,154,186,263,306–308]. It is suggested that the role of MΦ in ischemic injury is characterized by a biphasic response, in which early classical MΦ drive the inflammatory phase and non-classical MΦ resolve the inflammation and promote remodelling[1,154]. The role of MΦ in non-ischemic models such hypertension secondary to AngII infusion is less clear. In the well-established AngII-dependent model of hypertension, we and others have demonstrated an absence of tissue necrosis and associated polymorphonuclear cell infiltrates, suggesting that this model may not require the mass influx of classical MΦ[47,68,71,270,294]. We have also characterized that early myocardial infiltration in AngII-exposed hearts is largely unaffected in CCR2$^{-/-}$ mice, suggesting redundancy in chemokines or implicating CX3CR1-dependent non-classical MΦ recruitment[71]. Despite this body of evidence it remained to be demonstrated whether MΦ were mediating the cellular changes observed in the myocardium and the ECM remodelling following AngII infusion. Here, we characterized the role of infiltrating MΦ via liposomal clodronate depletion and then characterized the contribution of CX3CR1-dependent non-classical recruitment using a CX3CR1$^{-/-}$ mice[195,297,309,310].

As previously shown, a dramatic influx of mononuclear cells, precedes the development of myocardial fibrosis during AngII infusion [47,270121]. Here, we characterized the infiltrating cells as predominantly MΦ with further characterization

based on Ly6C expression. We were able to demonstrate an overall trend toward Ly6C^{low} expression and CD206 positivity, consistent with non-classical MΦ as the predominant phenotype of the accumulating cells within the myocardium[1,74,154]. A similar mass influx of non-classical MΦ has been observed in ischemic myocardial injury, as well as non-ischemic models of lung, liver, and kidney injury, thus supporting our findings[291,298–300,311]. Non-classical MΦ are generally associated with the production of pro-fibrotic factors and the fibrotic phase of healing[154,312]. Given the presence of non-classical MΦ in the AngII-exposed myocardium and their pro-fibrotic role, it followed that the non-classical MΦ would be the key candidate cells capable to mediate and promote the observed myocardial fibrosis.

In our initial approach, we used IV clodronate liposomes to successfully deplete the majority of circulating monocytes, as has been previously described[165,224]. Monocyte depletion resulted in a significant reduction of MΦ in the heart following AngII infusion, which also resulted in significant reduction in myocardial fibrosis. Furthermore, the reduction in infiltration and fibrosis was independent of blood pressure. Other compensatory mechanisms observed with AngII infusion such as hypertrophy were not affected by MΦ depletion or the reduction in myocardial fibrosis. As such, other structural compensatory mechanisms may be sufficient to overcome the increased stress on the myocardium without the need for ECM fibrotic stabilization. This is in stark contrast to ischemic myocardial injury that requires early classical MΦ to remove necrotic tissue to progress to neovascularization and normal remodelling. In the absence of this classical MΦ response, the myocardium becomes structurally unstable and is associated with increased mortality[59,154,313,314]. Non-classical MΦ infiltrate then ensues to

sufficiently replace lost tissue with ECM deposition (replacement fibrosis) – a hallmark of myocardial fibrosis[154,313]. As shown in our model, there is a robust infiltration of Ly6C^{high} MΦ following AngII infusion, but the overwhelming phenotype in the heart is Ly6C^{low}, which corresponded to a non-classical resident population. We have also identified the presence of a non-classical resident MΦ population in the heart of control animals prior to AngII exposure supporting recent findings by Pinto et al regarding a significant resident MΦ population being present in the myocardium[157]. Furthermore, this resident population expresses CX3CR1 and exhibits non-classical, anti-inflammatory characteristics, but its exact contribution to the infiltrating population is unclear and beyond the scope of the present study[157]. We suspect that liposomal depletion prevented the accumulation of infiltrating MΦ, but may have left the resident population intact. Our findings suggest that the infiltrating population, expressing a Ly6C^{high} phenotype, may be promoting inflammation, which is then counter-balanced by the resident Ly6C^{low} population.

Surprisingly, we observed a significant increase in cardiac transcript of TGF-β following clodronate liposome treatment. We acknowledge that TGF-β is commonly associated with fibrotic conditions; however, the pleiotropic growth factor has also been shown to have potent anti-inflammatory effects in the myocardium[315] Specifically, TGF-β has deactivating properties that can prevent MΦ-mediated pro-inflammatory injury[316]. Moreover, there have been no studies examining the specific contribution of rCMΦ to TGF-β signalling. While we did not investigate the mechanism by which TGF-β may reduce myocardial fibrosis in our model, future work could take into consideration MΦ deactivation, particularly in the context of downstream signalling. The pleotropic effects

of TGF-β may be underlying our observations from the MΦ-fibroblast co-culture. There is evidence to suggest that IL-6 mediates fibroblast activation and collagen production via TGF-β pathways[121]. Although the combination of IL-6 and TGF-β signalling may promote a fibrotic response, a reduction of inflammatory IL-6 signalling, as may be the case following the depletion of infiltrating monocytes, may elicit an alternative, anti-inflammatory response. Ideally, one would examine the interactions between different MΦ activation states with fibroblasts; however, this is a shortcoming of BMDMΦ, which inevitably acquire a $Ly6C^{low}$ phenotype in the absence of exogenous signalling and suggests that our findings need to be interpreted with these caveats. Thus, in the absence of the infiltrating MΦ, the resident population may confer some protection for the myocardium from hypertensive or inflammatory changes normally seen after AngII exposure.

Despite our important findings that monocyte depletion leads to obvious benefits with regard to myocardial cellular infiltration and remodelling, it would be of little therapeutic value due to the systemic immunosuppressive effects. However, eventual refinement in techniques using liposomes may provide novel yet to be described opportunities to target specific monocyte subsets or influence their phenotype [147]. As such, liposomes could be used to modulate monocyte phenotype and function toward the anti-inflammatory, non-classical phenotypic to reduce remodelling in the myocardium while maintaining immunocompetence and myocardial stability. Harel-Adar et al provided one such method by which MΦ phagocytosis of phosphatidylserine presenting liposomes promoted the $CD206^{+}$, anti-inflammatory MΦ phenotype[147]. Shifting the MΦ phenotype toward the $Ly6C^{low}$ $CD206^{+}$, as observed in AngII/WT myocardia, was

sufficient to down-regulate pro-inflammatory cytokines such as TNF-α and increase anti-inflammatory mediators such as TGF-B. This approach to shifting MΦ phenotype has been shown to protect the myocardium following ischemic injury but has yet to be explored in non-ischemic models of myocardial fibrosis.

Given the above findings, we aimed to specifically inhibit non-classical MΦ recruitment during AngII infusion by using a chemokine receptor knockout for CX3CR1 – the predominant chemokine receptor expressed by non-classical circulating monocytes. Recent evidence in skin, liver, and kidney suggests that CX3CR1$^{-/-}$ mice may display reduced fibrosis and thus, CX3CR1-dependent MΦ recruitment could represent a therapeutic opportunity for fibrosis and remodelling[189,190,309,310]. Contrasting reports in a liver model suggested that impaired non-classical MΦ recruitment exacerbated inflammation and consequently remodelling[297]. Consistent with the liver model, we have demonstrated that by inhibiting the recruitment of non-classical MΦ, there is a shift toward a pro-inflammatory MΦ phenotype and environment in the heart, which resulted in worse myocardial cellular changes and remodelling. These findings could also be interpreted to suggest that non-classical MΦ play an important regulatory role and are protective in maintaining ECM homeostasis. Furthermore, recent studies on rCMΦ using GFP under the CX3CR1 promoter support that our findings are specific to the MΦ, as fibroblasts, endothelial cells, and cardiomyocytes were all negative[157].

One needs to be cautious of oversimplification when trying to understand the differing roles of MΦ subsets. Although the infiltrating MΦ populations are obviously important in cardiac injury and remodelling, one needs to take into consideration resident MΦ, which we have been able to identify in this study, but their exact role remains

largely unknown. Our findings are consistent with the resident MΦ population, described by Pinto et al, based on their expression of CX3CR1, and an anti-inflammatory phenotype[157]. We have demonstrated for the first time that the resident MΦ population can shift phenotype in the absence of CX3CR1 signalling – something yet to be reported and perhaps important to our observations in which $CX3CR1^{-/-}$ animals had a shift in resident MΦ towards $Ly6C^{high}$ potentially making them more susceptible to AngII exposure. Although recent findings indicate that cardiac MΦ are largely replenished from self-renewal, one cannot rule out that their initial recruitment is CX3CR1-dependent[317251]. One also cannot rule out that CX3CR1 may be involved in MΦ survival, as has been suggested by Landsman et al[318]. Our observations may be dependent on a rCMΦ population that would be unaffected by IV liposomal clodronate, but modulated in the $CX3CR1^{-/-}$ mice. As the rCMΦ population can be affected phenotypically and perhaps functionally by either modulated turnover or survival, they may still represent a valuable therapeutic target.

In summary, monocyte-derived MΦ are the primary infiltrating cell in the early development of AngII-dependent myocardial fibrosis. For the first time, we have demonstrated that depleting circulating monocytes prevents the accumulation of myocardial MΦ in non-ischemic injury. In the absence of the MΦ influx, there is a marked reduction in cells expressing an activated fibroblast phenotype and a reduction in collagen deposition. Notably, we have demonstrated that the resident MΦ population in the heart can shift its phenotype and may be an important modulator of myocardial healing. Together, infiltrating and resident MΦ may be potential therapeutic targets in the development of non-ischemic myocardial fibrosis. Future studies should examine

whether modulating the phenotype of cardiac MΦ, rather than depleting all circulating monocytes, can lead to clinically important reductions in myocardial fibrosis, while maintaining immune-competence.

CHAPTER 6 IMPLICATIONS FOR THE ROLE OF MACROPHAGES IN A MODEL OF MYOCARDIAL FIBROSIS: CCR2$^{-/-}$ MICE EXHIBIT AN M2 PHENOTYPIC SHIFT IN RESIDENT CARDIAC MACROPHAGES

This work appears in part in the publication:

Falkenham A, Myers T, Wong C, Légaré JF. Implications for the Role of Macrophages in a Model of Myocardial Fibrosis: CCR2$^{-/-}$ Mice Exhibit An M2 Phenotypic Shift In Resident Cardiac Macrophages. J Cardiovasc. Pathol. 2016. Accepted for publication.

Contribution:

AF – designed the study, carried out the experimentation collected and analyzed data, and prepared the manuscript

TM, CW – assisted with experimentation and data collection

JFL – assisted with study design and critical review of the manuscript

6.1 Introduction

MΦ are important mediators in tissue development, homeostasis, and disease progression[174]. As resident MΦ localize to different tissues during embryonic development, they acquire tissue/organ-specific functions[176,247,319,320]. These tissue/organ-specific MΦ are now known to exhibit distinct functions and phenotypes in tissues including the central nervous system, liver, spleen, lungs, peritoneum, skin, and myocardium. This is particularly important in tissues like myocardium, in which the ability to regenerate tissue after injury is assumed to be minimal[321]. Moreover, circulation can contribute to tissue MΦ populations in function-specific manors, such that monocyte-derived MΦ can promote, resolve, or regulate inflammation[165154].

Until recently, cardiac MΦ were thought to be exclusively derived from circulating monocytes during injurious stimuli[157]. This was supported by extensive research in different cardiac models, including AngII infusion and myocardial infarction, in which MΦ influx the heart from circulating monocytes[72,100,102,186]. Using a GFP-linked CX3CR1, Pinto et al. demonstrated a $CX3CR1^{+}$ MΦ population in the adult murine myocardium that persisted at baseline and was distinct from bone marrow derived monocytes [157]. This observation has generated a resurgence in cardiac MΦ research that has focused on their phenotype, function, turnover, and etiology[72,102,248,252]. As our understanding of MΦ evolves, researchers have been increasingly able to separate the MΦ into a broad phenotypic spectra[167]. Notably, MΦ could be differentiated by their expression of the chemokine receptors CCR2 and CX3CR1[1,154]. The *classical* MΦ phenotype expressed high levels of CCR2 and low CX3CR1. Conversely, the *non-classical* MΦ phenotype was either CCR2 low or negative and expressed high levels of

CX3CR1. The non-classical population was associated with a mature phenotype, typically observed in both late-infiltrating phases and resident populations[154,157].

rCMΦ have since been separated into at least 4 distinct populations that can be distinguished based on their expression of markers including CX3CR1, MHC class II, Ly6C, CD11b, CD64, CD11c, and CD206[72,157,248,250]. Molawi et al. have classified the populations into (1) $CX3CR1^{-}MHC\ II^{-}$, (2) $CX3CR1^{-}MHC\ II^{+}$, (3) $CX3CR1^{+}MHC\ II^{-}$, and (4) $CX3CR1^{+}MHC\ II^{+}$, which we have also characterized by CX3CR1, CCR2, CD11b, and Ly6C. RCMΦ originate during embryonic development as $CX3CR1^{+}MHC^{-}$ cells. Notably, these embryonic-derived rCMΦ can confer protection against and favor resolution of injury by promoting cardiac regeneration and preventing remodeling[249,250]. While still under debate, rCMΦ appear to be replenished primarily from circulating $CCR2^{+}$ monocytes that express CX3CR1 and MHC class II[250]. Despite extensive work to characterize rCMΦ phenotypes, the functions of these different populations remain poorly characterized, particularly in models of disease.

It is known that animals lacking CCR2 exhibit impaired ability to turnover rCMΦ from circulating monocytes[250]. Moreover, work in our laboratory using $CCR2^{-/-}$ mice suggested that these animals were partially protected from AngII-mediated inflammation and fibrosis[71,93]. When we first described the benefits of CCR2-/- deficiency, we did not appreciate or explore the role of this genetic defect on rCMΦ. Notably, early MΦ infiltrate did not significantly differ from WT mice and the underlying mechanism(s) for the benefits in $CCR2^{-/-}$ mice remained unclear. Given the close relationship between MΦ activation and cardiac healing, comparing baseline rCMΦ populations between $CCR2^{-/-}$ and WT mice warrants investigation.

In order to elucidate the role of CCR2 on rCMΦ phenotype, we compared myocardial tissue and rCMΦ from $CCR2^{-/-}$ mice relative to WT controls. When we discovered that $CCR2^{-/-}$ displayed a significant shift in rCMΦ Ly6C expression relative to WT, we investigated the functional significance of such a shift in terms of MΦ phenotype and how this would influence fibroblast activation and thus, fibrotic remodeling.

6.2 Results

6.2.1 No baseline structural differences in myocardium between WT and $CCR2^{-/-}$ animals

We first investigated whether gross histological differences existed between $CCR2^{-/-}$ and WT mice at baseline. In order to evaluate baseline histology, 8-10wk (age-matched) $CCR2^{-/-}$ and WT mice were sacrificed and their hearts were characterized using H&E and SR/FG. Consistent with previous work from our laboratory, WT hearts did not contain observable areas of mononuclear cell accumulation (Fig. 6.1A). Similarly, $CCR2^{-/-}$ hearts were also void of mononuclear cell accumulation (Fig. 6.1B). Predictably, WT and $CCR2^{-/-}$ hearts did not contain the excess interstitial and perivascular collagen that is normally associated with disease models infusion (Fig. 6.1C and D, respectively).

Quantification of cellular infiltration using representative histology sections confirmed that there were no significant differences in mononuclear cell accumulation between $CCR2^{-/-}$ and WT animals (Fig. 6.2A; 0.3% ± 0.3 relative to 0.4% ± 0.4) or collagen deposition (Fig. 6.2C; 3.7% ± 1.0 relative to 2.1% ± 1.3) between $CCR2^{-/-}$ and WT hearts. As demonstrated by others rCMΦ are very difficult to identify by routine histology and are best characterized by cell isolation and purification. Using a standardized and well-established technique we proceeded to isolate and purify

mononuclear cell population from the heart of WT and CCR2$^{-/-}$ animals. This quantification of mononuclear cells isolated from WT and CCR2$^{-/-}$ hearts was performed prior to flow cytometric characterization. Consistent with visual analysis, the number of purified mononuclear cells isolated between WT and CCR2$^{-/-}$ mice did not significantly differ (Fig. 6.2B) – [(3.3 ± 0.6 vs. 2.8 ± 0.6) x 10^5, respectively]. Structural comparison of the hearts by heart weight:body weight ratio further confirmed that WT and CCR2$^{-/-}$ hearts do not significantly differ in hypertrophy score (Fig. 6.3D).

Together, these results suggest that there are no observable gross cellular or histological differences between WT and CCR2$^{-/-}$ hearts at baseline. As such, we next explored whether inherent differences were present in the baseline cardiac MΦ populations between WT and CCR2$^{-/-}$ mice. Specifically, we were interested in identifying if differences in rCMΦ existed between WT and CCR2$^{-/-}$ animals.

6.2.2 CCR2$^{-/-}$ rCMΦ exhibit and M2 shift in phenotype

In order to better examine potential cardiac differences at the cellular level, rCMΦ were isolated from the CCR2$^{-/-}$ and WT myocardia and processed for flow cytometry. In the cell isolate, rCMΦ were characterized for their expression of MΦ markers (i.e. CD11b, CCR2, and CX3CR1) and activation markers (i.e. Ly6C, TNF-α, and IL-10). Total rCMΦ were selected based on their expression of CX3CR1 as previously described, which did not significantly differ between WT and CCR2$^{-/-}$ (Fig. 6.3A and C, respectively). Within the CX3CR1 gated populations, however, there is a prominent CD11b^{high} Ly6C^{high} population in the WT myocardium (Fig. 6.3B) that is absent in the CCR2$^{-/-}$ myocardium (Fig. 6.3D). Moreover, when gated on, the CD11b^{high} Ly6C^{high}

population also expresses CCR2 (Fig. 6.3E; representative histogram of n=4). Thus, WT and CCR2$^{-/-}$ myocardia significantly differ in their baseline populations of MΦ, as characterized by their expression of CX3CR1, CD11b, and Ly6C.

Since Pinto's definitive characterization of a rCMΦ population in the heart, a growing body of literature supports that rCMΦ are not a uniform population, but rather phenotypically and functionally distinct populations. rCMΦ populations are, however, linked by their expression of CX3CR1[248], which provided an initial gate for excluding non-rCMΦ. We then assessed the frequency of CX3CR1+ populations based on their varied expression of CD11b and Ly6, which are markers of maturity and may reflect functional divergence. Largely, the MΦ populations did not significantly differ between WT and CCR2$^{-/-}$ mice (Table 6.1). Consistent with the appearance of the CD11b x Ly6C dotplot, the only group that exhibited a significant change was the CD11b^{+} Ly6C^{++} gate (Fig. 6.3F). CD11b^{++} Ly6C^{++} cells represented 2.2 ± 0.3% and 0.8 ± 0.2% of myocardial CX3CR1^{+} cells in WT and CCR2$^{-/-}$ mice, respectively – a 2.75-fold increase in WT over CCR2$^{-/-}$. Thus, there are distinct differences between WT and CCR2$^{-/-}$ rCMΦ populations at baseline.

While Ly6C is an indicator of MΦ phenotype, it does not necessarily correlate to function. As such, we next analyzed the expression of 2 functional markers of opposing MΦ polarities: inflammatory TNF-α and anti-inflammatory IL-10. The classical MΦ phenotype, which expresses higher levels of Ly6C, is typically associated with a pro-inflammatory functional state. In contrast, the non-classical MΦ phenotype expresses lower levels of Ly6C and is instead associated with an anti-inflammatory, pro-fibrotic, and/or pro-angiogenic functional state(s).

Intracellular flow cytometry was used to characterize the expression of the cytokines. rCMΦ populations were again defined as CX3CR1+ cells isolated from the myocardium. We then separated the populations based on their expression of CD11b into $CD11b^{low}$ and $CD11b^{high}$ gates. The representative contour plots for WT (Fig. 6.4A) and $CCR2^{-/-}$ (Fig. 6.4B), reveal a leftward shift toward lower TNF-α expression. Reflective of baseline differences, $CD11b^{low}$ rCMΦ – often considered the predominant resident population – did significantly differ in its expression of TNF-α (Fig. 6.4C; *$p<0.05$). WT $CD11b^{low}$ rCMΦ displayed higher TNF-α relative to the $CD11b^{low}$ population in $CCR2^{-/-}$, as measured by MFI. Interestingly, the $CD11b^{high}$ population did not differ in TNF-α expression between WT and $CCR2^{-/-}$, despite the WT $CD11b^{high}$ group containing the $Ly6C^{high}$ population described above (Fig. 6.5D). Similarly, an inverse trend – increased IL-10 expression – is observable in the representative contour plots for WT (Fig. 6.5A) and $CCR2^{-/-}$ (Fig. 6.5B) rCMΦ. In contrast to TNF- α expression, $CCR2^{-/-}$ rCMΦ in both (Fig. 6.5C) $CD11b^{low}$ and (Fig. 6.5D) $CD11b^{high}$ populations trended toward increased IL-10 expression, which reached significance in the former population (*$p<0.05$).

While significant findings were limited to the $CD11b^{low}$ population for both TNF-α and IL-10, the data suggests that $CCR2^{-/-}$ rCMΦ favor a lesser pro-inflammatory and stronger anti-inflammatory phenotype than their WT counterpart. This novel observation now offers some insight into potential mechanisms by which rCMΦ can influence the myocardial inflammatory response and in turn fibrosis.

6.2.3 Classical MΦ increase fibroblast activation in vitro

In previous work, we demonstrated a difference in the AngII-mediated myocardial fibrotic responses between CCR2$^{-/-}$ and WT mice, with the former exhibiting reduced fibrosis. In addition, we had also shown that early mononuclear infiltration does not significantly differ in the AngII response, suggesting potential baseline differences could account for the differences in fibrosis. In this manuscript, we have supported that theory by showing significant differences in CCR2$^{-/-}$ and WT rCMΦ populations at baseline; CCR2$^{-/-}$ and WT rCMΦ exhibit an inverse trend in their expression of TNF-α and IL-10 that may favour a more protective phenotype in CCR2$^{-/-}$ mice. To support our findings, we used an in vitro co-culture system designed to evaluate fibroblast activation in the presence of different MΦ phenotypes. The purpose of this experiment was to focus on how shifts in MΦ phenotypes (identified in vivo) influence fibroblast function and phenotype, rather than looking at direct connections with CCR2 signaling. As such, we opted to use differentiated WT MΦ in the fibroblast co-culture.

Bone marrow-derived MΦ were grown in culture to represent 3 different phenotypes as previously described: (1) non-induced MΦ, (2) classical MΦ using IFN-γ and LPS, and (3) non-classical MΦ using IL-4 – and all groups were then co-cultured with 3T3 fibroblasts for 24hrs[322]. Induced MΦ populations were characterized for their expression of CD11b, CD206, Ly6C, F4/80, IL-10 and TNF-α (Fig. 6.1S). Generally, classically induced MΦ exhibited dichotomous CD11b expression, increased Ly6C and TNF-α expression and reduced CD206 and IL-10 expression. In contrast, non-classically induced MΦ exhibited uniform CD11b expression, reduced Ly6C and TNF-α expression and increased CD206 and IL-10 expression. F4/80 expression remained consistent across groups.

Mono- and co-cultured fibroblasts were labeled for Col-1 as indicators of fibroblast phenotype and (Fig. 6.6A-C) αSMA or (Fig. 6.6D-F) KI-67 to demonstrate their activation and proliferation, respectively. Representative micrographs demonstrate the co-localization (yellow) of Col-1 (red) and αSMA (green) and the co-localization (pink) of Hoechst (blue) and KI-67 (red) on Col-1^{+} (green) cells. While Col-1 expression did not significantly differ between any groups (Fig. 6.6G), fibroblasts co-cultured with M1 MΦ exhibited significantly greaterαSMA expression, as measured by fluorescence emission (Fig. 6.6H, *$p<0.05$).

In contrast to the αSMA findings, M2 MΦ were the only subset to significantly increase fibroblast proliferation relative to fibroblast monocultures (Fig. 6.6I; ***$p<0.001$). In monocultures, 17.9 ± 1.9% of fibroblasts, characterized by co-localization of Col-1 and KI-67, were undergoing proliferation. MΦ+ Fibroblasts and M1 + Fibroblasts exhibited similar levels of proliferation at 28.95 ± 2.9% and 29.2 ± 1.9% of KI-67^{+} fibroblasts, respectively. In contrast, M2+Fibroblasts exhibited 40.2 ± 4.4% of fibroblasts undergoing proliferation – a 2.25-fold increase over the fibroblast monocultures. Thus, while M1 MΦ are able to promote fibroblast activation, M2 MΦ are able to promote fibroblast proliferation.

6.2.4 Results Summary

The findings of this manuscript are summarized below in Table 2. In brief, we observed an increase in Ly6C expression in WT relative to CCR2$^{-/-}$ mice. This increase is likely attributable to the presence of a CD11b^{++} Ly6C^{++} population that was absent in CCR2$^{-/-}$. In addition, the increased Ly6C expression in WT relative to CCR2$^{-/-}$ mice was

associated with an increase in rCMΦ TNF-α. Overall, this is representative of a shift in rCMΦ toward the M1 phenotype relative to $CCR2^{-/-}$ and the M1 phenotype was shown to favour fibroblast activation in vitro.

Conversely, $CCR2^{-/-}$ mice displayed a reduction in rCMΦ Ly6C expression relative to WT mice. This decrease was associated with increased IL-10 and a shift toward the M2 phenotype. Lastly, the M2 phenotype was shown to promote fibroblast proliferation in vitro.

6.3 Tables

*Table 6.1 - Quantification of rCMΦ phenotypes. Populations were separated based on their expression of CD11b – an indicator of phenotypic immaturity. Of the CD11b high rCMΦ, CCR2$^{-/-}$ exhibited a significantly lesser CD11b high Ly6C high population relative to their WT counterpart (n=4-6; ***p<0.001).*

	CD11b+			CD11b++		
	Ly6C-	Ly6C+	Ly6C++	Ly6C-	Ly6C+	Ly6C++
WT	22.9 ± 1.4	14.3 ± 1.2	4.0 ± 0.7	17.2 ± 2.6	10.2 ± 1.0	2.2 ± 0.3***
CCR2-/-	23.0 ± 0.8	14.0 ± 1.3	5.2 ± 1.0	13.3 ± 1.4	10.2 ± 1.1	0.8 ± 0.2

Table 6.2 - Summary of findings. This table represents a summary of our findings in this manuscript.

	Ly6C	TNF-a	IL-10	rCMΦ shift	How rCMΦ shift affects fibroblasts
WT	⬆	⬆	⬇	M1	Activation
CCR2-/-	⬇	⬇	⬆	M2	Proliferation

6.4 Figures

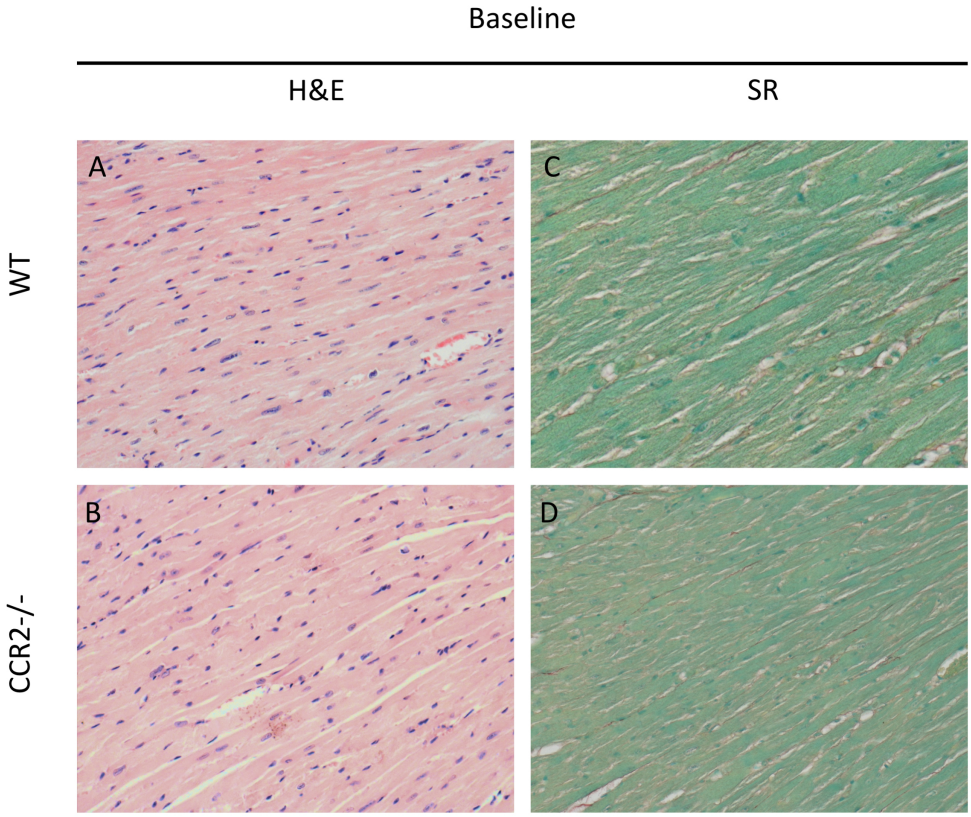


Figure 6.1 – Baseline myocardia were assessed for gross histology. Representative H&E stained baseline myocardial sections from ***(A)*** *WT and* ***(B)*** *CCR2-/- mice. Representative SR/FG stained baseline myocardial sections from* ***(C)*** *WT and* ***(D)*** *CCR2-/- mice. (n=6-8)*

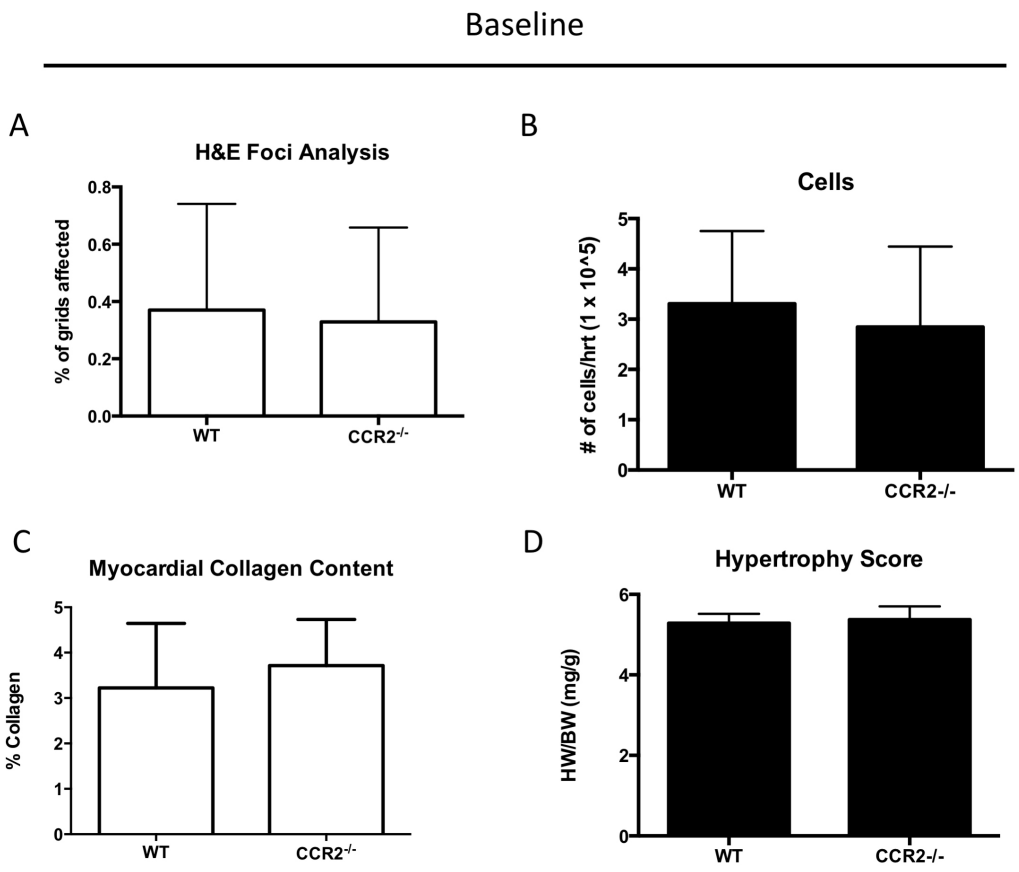


Figure 6.2 - Baseline myocardia were characterized for gross differences. ***(A)*** *Myocardial MΦ accumulation was assessed using H&E sections from each group.* ***(B)*** *In support of the histology findings**,** hearts were mechanically and enzymatically digested and the isolated leukocytes were counted and compared between groups.* ***(C)*** *Collagen content, as quantified in SR/FG stained myocardial sections, did not significantly differ between WT and CCR2-/- mice.* ***(D)*** *Myocardia were weighed at the time of harvest to generate a hypertrophy score, which did not significantly differ between WT and CCR2-/- myocardia (n=6-8).*

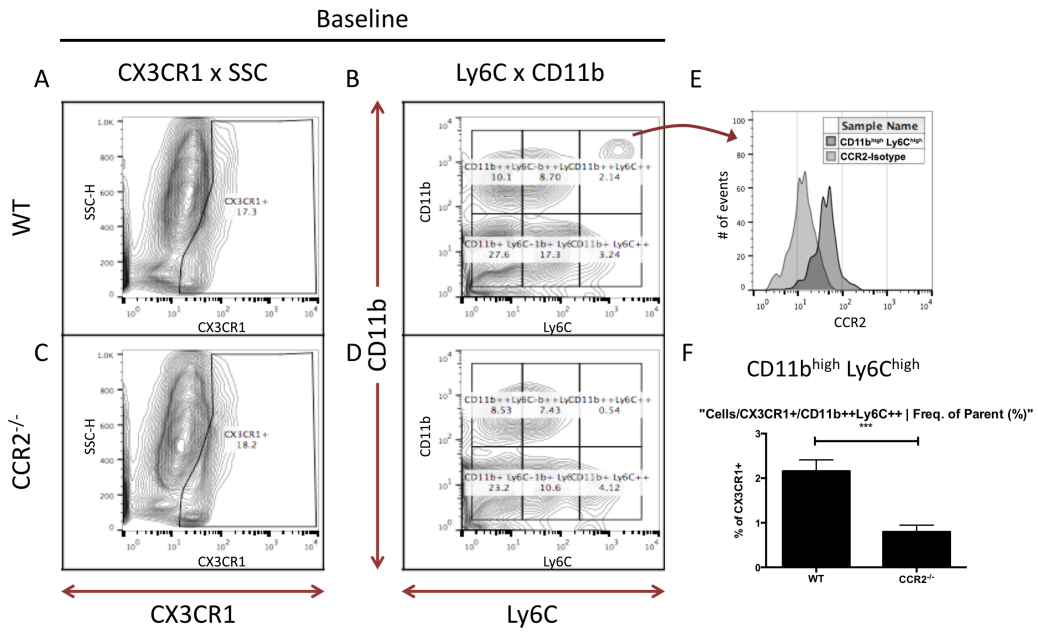


Figure 6.3 - Flow cytometric characterization of isolated myocardial leukocytes labeled with CX3CR1, Ly6C and CD11b. ***(A)*** *CX3CR1+ cells, consistent with a MΦ phenotype, were selectively gated on in order to encompass the entirety of the myocardium's MΦ population.* ***(B)*** *Further characterization by Ly6C and CD11b identified a population of CD11b++ Ly6C++ cells that were absent in CCR2-/- myocardia.* ***(C)*** *A CX3CR1+ population in CCR2-/- myocardia indicated the preservation of an rCMΦ population.* ***(D)*** *Further characterization, we were able to observe all but one rCMΦ population (i.e. CD11b++ Ly6C++) in CCR2-/-. This population was also CCR2+ and the only population that significantly differed in percentage between CCR2$^{-/-}$ and WT mice. (n=4-6).*

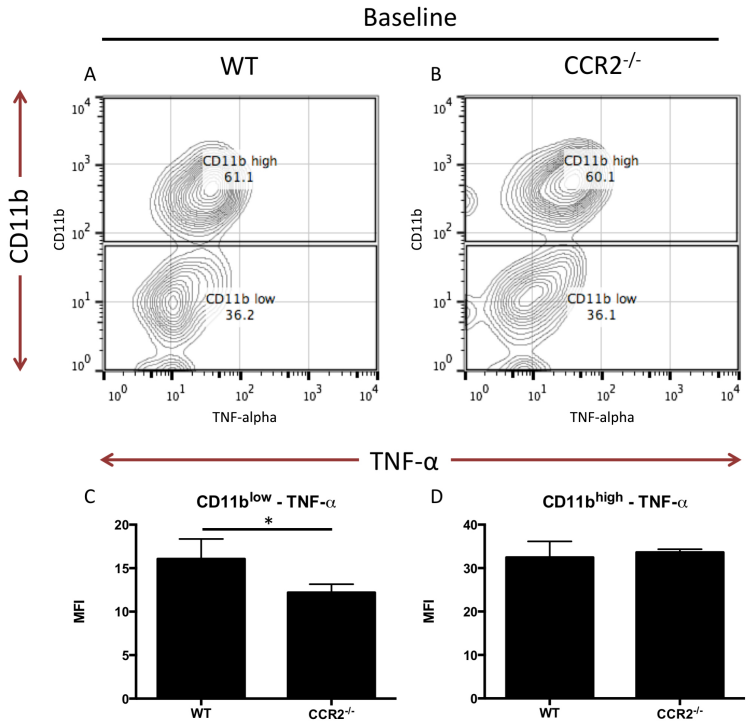


*Figure 6.4 - Individual rCMΦ populations characterized for their expression of TNF-a. Representative flow cytometry for TNF-a expression in CX3CR1+ cells in **(A)** WT and **(B)** CCR2-/- myocardia. Gates were applied to CD11b low and high populations for quantitative analysis. TNF-a MFI in CD11b **(C)** low and **(D)** high rCMΦ. (n=4-6; *p<0.05).*

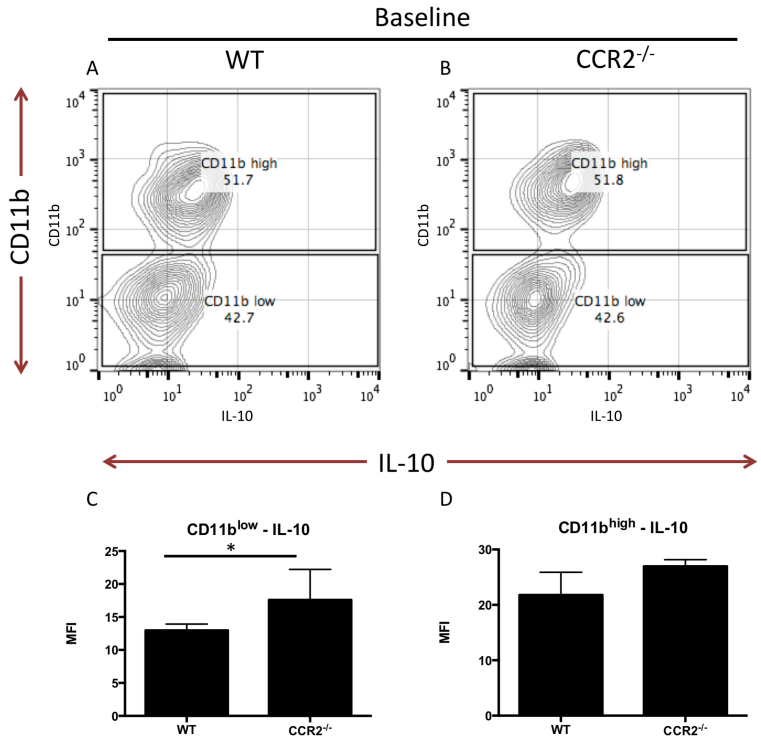


*Figure 6.5 - Individual rCMΦ populations characterized for their expression of IL-10. Representative flow cytometry for IL-10 expression in CX3CR1+ cells in **(A)** WT and **(B)** CCR2-/- myocardia. Gates were applied to CD11b low and high populations for quantitative analysis. IL-10 MFI in CD11b **(C)** low and **(D)** high rCMΦ. (n=4-6; *p<0.05).*

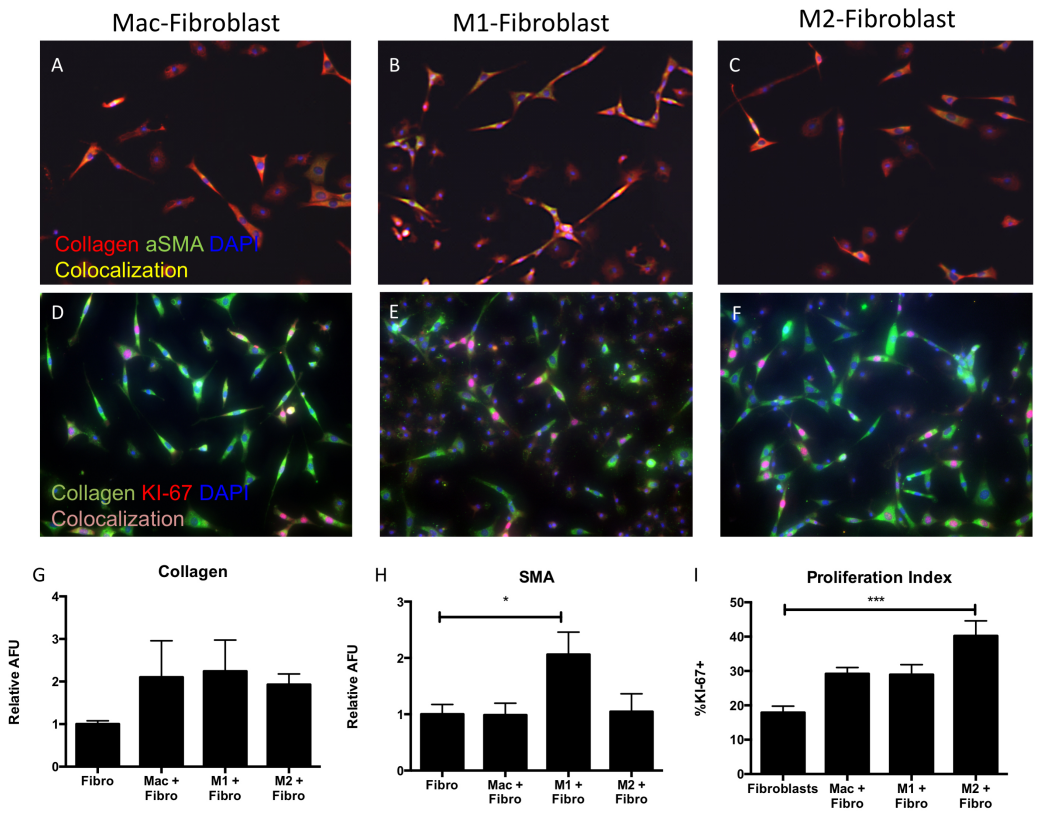


Figure 6.6 - M1 MΦ encourage myofibroblast differentiation, while M2 MΦ favour fibroblast proliferation. Representative fields of view stained for Collagen type I, αSMA, and DAPI are shown for the different MΦ co-cultures: ***(A)*** *undifferentiated MΦ + fibroblasts,* ***(B)*** *M1-differentiated MΦ + fibroblasts, and* ***(C)*** *M2-differentiated MΦ + fibroblasts. Similarly, Representative fields of view stained for Collagen type I, KI-67, and DAPI are shown for the co-cultures:* ***(D)*** *undifferentiated MΦ + fibroblasts,* ***(E)*** *M1-differentiated MΦ + fibroblasts, and* ***(F)*** *M2-differentiated MΦ + fibroblasts. Quantification of the* ***(G)*** *collagen type-I,* ***(H)*** *αSMA, and* ***(I)*** *KI-67 are shown below the representative immunofluorescence. Fibroblast monocultures served as controls (images not shown; n=4-6; *p<0.05, ***p<0.001).*

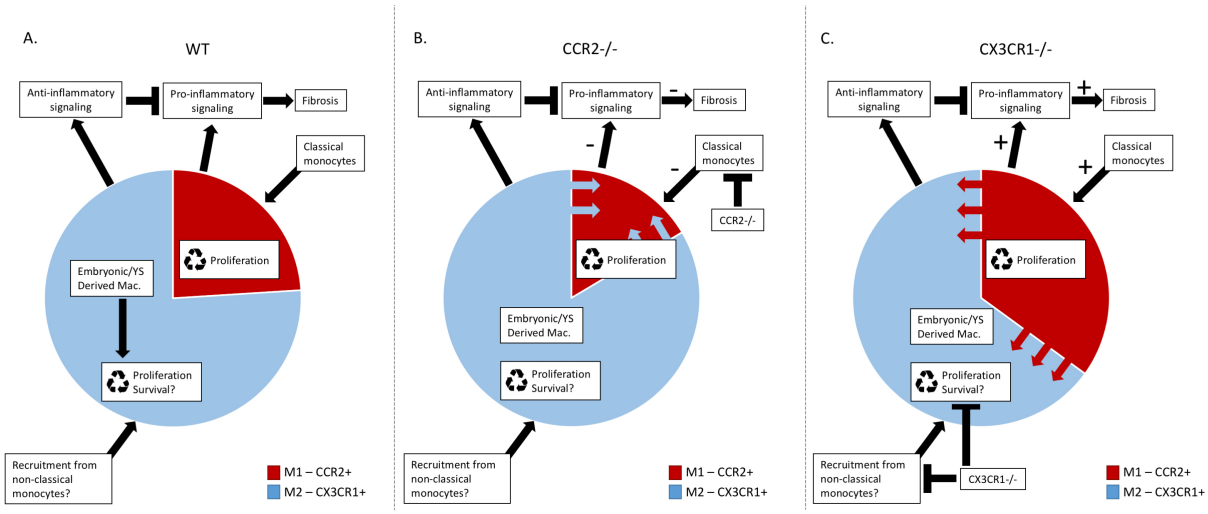
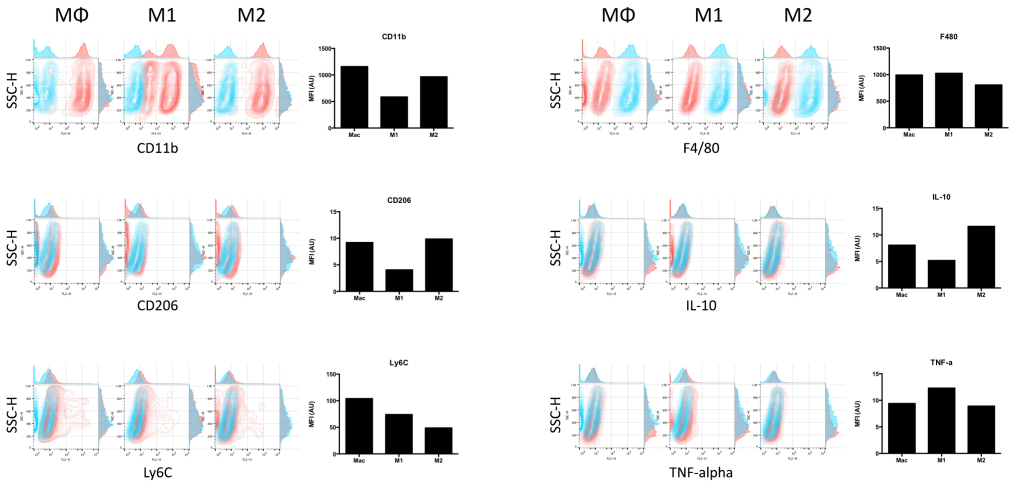


Figure 6.7 - A schematic of how rCMΦ turnover and contribution of M1 vs. M2 could be influenced in (A) WT, (B) CCR2-/-, and (C) CX3CR1-/- mice. (A) In WT mice, it is known that classical monocytes recruitment and local proliferation contribute to the CCR2^{+} portion of rCMΦ. There is evidence that CX3CR1^{+} cells undergo proliferation to replenish the CX3CR1^{+} portion of rCMΦ. It is also possible that this population is partially replenished from non-classical monocytes, but this has yet to be shown. (B) In contrast, we hypothesize that CCR2$^{-/-}$ exhibit impaired recruitment of classical monocytes, thus providing a mechanism by which the CCR2^{+} M1 population would contract relative to CX3CR1^{+} M2 rCMΦ. (C) As a complement to the CCR2 data, we have previously shown that CX3CR1$^{-/-}$ mice exhibit an increased CCR2^{+} rCMΦ population. As such, we hypothesize that the normally CX3CR1^{+} M2 population contracts in CX3CR1$^{-/-}$, thus increasing the proportion of M1 to M2 rCMΦ relative to rCMΦ in WT and CCR2$^{-/-}$ mice. (Symbols: - indicates inhibition of a pathway and + indicates enhancement of a pathway)



Supplementary Figure 6.1 - Representative flow cytometry for Macrophage cultures post-differentiation. Shown are SSC x 'marker of interest' in the different culture conditions and the calculated MFI for said markers.

6.5 Discussion

A growing body of literature has expanded our understanding of MΦ population composition, diversity, and activation[72,174,176,247,249,250,319]. We now know and must consider how the complex activation, recruitment, and etiology of MΦ in the heart influences tissue development, homeostasis, and disease[72,154,248,250]. Recently, our laboratory demonstrated that rCMΦ do not necessarily maintain a constant phenotype and that their phenotype can be associated with differences in the expression of CX3CR1[72]. Moreover, we also previously demonstrated that $CCR2^{-/-}$ mice are in part protected from AngII-mediated cardiac fibrosis in a process that is not dependent on early infiltrating MΦ[71]. In the current manuscript, we built upon that observation by investigating rCMΦ phenotypes in the context of $CCR2^{-/-}$ mice.

There is no debate that MΦ can influence fibroblast function; however, the association is likely only partially responsible for the observed differences in cardiac fibrosis between $CCR2^{-/-}$and WT mice[216,323]. In the current manuscript, we assessed the baseline non-myocyte cell count, the myocardial collagen content, and the hypertrophy to determine whether there were gross myocardial differences prior to initiating AngII infusion. The lack of gross differences at baseline suggests that any inherent differences in rCMΦ between $CCR2^{-/-}$ and WT mice do not significantly affect normal cardiac function and development. Important in the context of this manuscript is whether potential underlying cellular differences between $CCR2^{-/-}$ and WT mice, not obvious at baseline, could affect cardiac healing.

Anti-inflammatory/pro-healing $CX3CR1^{+}$ rCMΦ are initially derived from embryonic and fetal MΦ populations, but are progressively replaced with age from

circulating monocytes in a process largely dependent on CCL2-CCR2[154,248,252]. In addition, baseline $CCR2^{+}$ rCMΦ exhibit upregulated genes involved with inflammasome activation and become important contributors of pro-inflammatory IL-1β in the AngII model of myocardial fibrosis. As such, the contribution of the $CCR2^{+}$ rCMΦ could change how the heart responds to stressors[11]. In support of this process, we identified a $CCR2^{+}CD11b^{high}Ly6C^{high}$ MΦ population in WT hearts that was absent in $CCR2^{-/-}$ hearts. This is particularly interesting in the context of recent observations in $CX3CR1^{-/-}$ mice: at baseline, $CX3CR1^{-/-}$ mice show a greater contribution of $CCR2^{+}CD11b^{high}Ly6C^{high}$ to their rCMΦ relative to WT controls. Moreover, $CX3CR1^{-/-}$ mice exhibited exacerbated inflammation and fibrosis during AngII infusion. The process by which this occurs is summarized in a diagram representing the theoretical contribution of M1 and M2 MΦ to the total rCMΦ population (Fig. 6.7; WT values adapted from Molawi et al.)[16]. Together, this supports the premise that at the onset of stressors, $CCR2^{-/-}$ mice may be partially protected from inflammation while $CX3CR1^{-/-}$ mice may be predisposed to inflammation. In turn, the corresponding chemokines, CCL2 and CX3CL1, likely have competing roles in preserving and replacing the anti-inflammatory rCMΦ population, respectively.

In order to provide a mechanistic link between MΦ phenotype/activation and myocardial fibrosis, we studied a co-culture system in which differently activated bone marrow-derived MΦ were incubated with fibroblasts in vitro. While we acknowledge that isolated rCMΦ would have provided a better representation of the in vivo interactions with fibroblasts, rCMΦ cannot realistically be isolated in sufficient quantities to perform such an experiment. As such, bone marrow-derived MΦ were chosen as an appropriate analog. Consistent with previous findings, MΦ were able to stimulate fibroblast

activation into myofibroblasts[72,323]. A growing body of research suggests that inflammation can promote fibroblast activation through processes that involve TNF-α, fibroblast activation protein, and IL-6 signaling[216,324]. Moreover, when MΦ are co-cultured with fibroblasts, their interactions promote feedback loops that favour a pro-fibrotic response. Notably, MΦ production of TNF-α directly promotes fibroblast proliferation, as well as other pro-inflammatory cytokine production. In addition, pro-fibrotic fibroblasts also produce TNF-α. Thus, even in the co-culture system these interactions are not one directional, but rather a cohesive response to promote fibrosis during inflammation. This may serve a protective role for replacing tissue lost during early inflammatory processes. At the same time, this reparative process can become corrupted in chronic inflammation, leading to normal tissue being degraded and replaced with scar tissue[325]. In our co-culture, we observed pro-inflammatory M1 MΦ increasing fibroblast activation, as demonstrated by increased αSMA expression. Interestingly, M2 MΦ – not M1 MΦ – promoted fibroblast proliferation, suggesting that the biphasic MΦ response observed in cardiac healing is much more complex than a single cell type. Moreover, it suggests that the healing/reparative response traditionally associated with the M2 phenotype may (i) begin with the M1 phenotype and (ii) have fibroblasts bridging the gap in the biphasic response. Thus, inflammation may serve as a precursor to fibrosis through fibroblast activation, which is temporally followed by fibroblast proliferation during the reparative phase.

Together, our findings support the importance of rCMΦ in fibroblast function and in turn, cardiac healing. Specifically, we have provided additional support for how changes in rCMΦ phenotype and/or activation can influence cardiac healing through the

susceptibility to inflammation and the subsequent fibrotic response. Future studies could examine how systemic changes in inflammation, such as that which occurs with obesity or metabolic syndrome, alter rCMΦ phenotype and/or activation and in turn, also susceptibility to cardiac injury. We have also provided a novel mechanism by which $CCR2^{-/-}$ mice may be protected from stressors such as AngII-mediated myocardial fibrosis. While previous data still supports the role of reduced proliferation in the protecting $CCR2^{-/-}$ mice from myocardial fibrosis, our novel findings support the link between MΦ and fibroblasts in this process. Notably, MΦ phenotype may be linked to a biphasic fibroblast response (i.e. activation followed by proliferation). Lastly, in concert with our previous findings, we have raised the question of whether CCR2 and CX3CR1 may have competing roles in the replacement, preservation, and/or survival of rCMΦ. This manuscript paves the way for future studies to explore the seemingly opposing roles of the chemokine receptors CCR2 and CX3CR1 in rCMΦ homeostasis and how this balance impacts the development of myocardial fibrosis.

CHAPTER 7 DISCUSSION

In this thesis, I have presented my work on characterizing the phenotype and function of MΦ in the heart during both steady-state and AngII-dependent hypertension[71,72]. The overarching aim was to understand and potentially characterize therapeutic strategies against aberrant fibrotic processes that can lead to cardiac dysfunction and culminate in heart failure. In many respects, what I set out to accomplish at the beginning of my degree is vastly different from that which I actually accomplished. When I began this degree, my specific aims related to the migration of a contested cell type – the fibrocyte – and a progenitor cell chemokine – CXCL12. And as I arrive at the end of my degree, I find myself studying another 'novel' cell type – rCMΦ – and characterizing their phenotype at baseline in various knockout animals. In this discussion, I will elaborate on (i) my cumulative contributions in the context of literature, (ii) the future direction of the field, (iii) the clinical context of my work, and (iv) some closing comments.

7.1 My contributions in the context of literature

In retracing the last 5 or so years of my life, my work began with a detour into the realm of fibrocytes, which was spurred on by confusion in the literature. At times, my attitude toward my first manuscript may come off as self-deprecating, but that is reflective of my initial hypothesis being wildly incorrect: blocking CXCR4 would prevent the recruitment of fibrocytes to the myocardium and in turn, reduce myocardial fibrosis in the AngII model. That being said, if we designed hypotheses based on known outcomes, then research would go in circles and we would make no progress.

To refresh your memory, fibrocytes are cells that express markers of mesenchymal and hematopoietic origin[238,326,327]. My focus was attempting to block fibrocyte migration to the myocardium in the AngII model by targeting a chemotactic pathway – CXCL12-CXCR4. Importantly, CXCL12 had been implicated in the recruitment of progenitor cells to the myocardium[259]. These observations were largely related to endothelial progenitor cells (EPC), which are involved in revascularization post-infarct. In contrast to the beneficial effects of EPC, fibrocytes were thought to have a negative effect on cardiac healing by promoting fibrotic deposition and the ensuing cardiac dysfunction[47,68,155,254]. This theory hinged on an important assumption: fibrocytes were directly and aberrantly producing the ECM proteins that contributed to myocardial fibrosis. In addition, fibrocytes were also observed during pro-fibrotic phases of healing including those in lung, liver, kidney, skin, and heart models[255–257,271,328]. The assumption also revolved around the premise that these cells were "fibroblast-like". There is evidence that a population of monocyte-derived cells can express typical fibroblast markers including collagen, vimentin, and even αSMA[179,236,327,329–331]. Researchers have even gone so far as to say their physical shape looks like a fibroblast and therefore, these cells are undergoing a differentiation process toward becoming a fibroblast[29,238,332–334]. Thus, one can understand how researchers could arrive at the conclusion that fibrocytes were important to the fibrotic process – an assumption that prefaced my first and second manuscript for this thesis.

As I mentioned in my introduction, the realization that fibrocytes were likely just a MΦ subset happened by chance. If I had not been attending that conference in Quebec City, who knows what I would be studying right now. Even from the beginning of my

degree though, there appeared to be holes in the fibrocyte façade that made me question the cell type I was studying. As one re-examines the assumptions and observations in the paragraph above, the fibrocyte story falls apart. Yes, fibrocytes were present during pro-fibrotic phases of healing, but they were also present during the early inflammatory stages as well[335–337]. For example, in the AngII model, cells that one could classify as fibrocytes (e.g. $CD14^+$, $CD11b^+$ $CD45^+$ $vimentin^+$) appear between 1-3d post-infusion[47,68,72,93,100]. During this phase of healing, there is an abundance of pro-inflammatory factors in the environment such as IFN-γ, TNF-α, IL-1β, and IL-6[47,68,72,93,100,101,215]. Not dissimilar to MΦ, fibrocytes have even been shown to produce pro-inflammatory factors including IL-6, IL-8, CCL2, CCL3, and CCL4[336,338,339]. As such, the idea that these cells are a phenomenon of the pro-fibrotic stage of healing does not stand up to the evidence. Phenotypically, the expression of collagen, vimentin, and αSMA by fibrocytes may not be enough to assume that these cells are differentiating toward a fibroblast and have an active role in fibrotic deposition. Importantly, that assumption is premised on the idea that these are "fibroblast" markers and not just markers of a particular phase of healing, for example. Again, I cannot argue against the idea of a monocyte-derived cell expressing ECM proteins because even I demonstrated $CD45^+\alpha SMA^+$ cells in the myocardium following AngII infusion[72]. I can, however, call into question the ability for researchers to demonstrate monocyte-derived cells expressing collagen using flow cytometry[93,227]. For example, antibodies against collagen type I are typically unconjugated and polyclonal and thus, more likely to bind non-specifically than conjugated monoclonal antibodies. This is particularly important when labeling cells that abundantly express Fc receptors, which largely mediate non-specific antibody binding.

Interestingly, many of the antibodies used to label leukocytes for collagen were not even manufacturer-tested for flow cytometry. The cells characterized as fibrocytes using flow cytometry against Col-1 also went through an in vitro culture period, during which they likely changed their phenotype[93,238,327]. Moreover, Col-1 is a secreted protein, suggesting that flow cytometry may not be the most appropriate method for its detection. The only antibody of which I am aware that labels fibrocytes for a relevant ECM marker and is manufacturer-tested for flow cytometry is against pro-collagen type I (Miltenyi Biotech). I welcome anyone to check the product page to determine whether they feel this is a suitable antibody for flow cytometry. Thus, the flow cytometric characterization of fibrocytes using antibodies against Col-1 is likely insufficient to support their existence.

Functionally, fibrocytes (i) produce significantly less collagen than fibroblasts and (ii) would comprise significantly fewer cells in the myocardium even during injury, suggesting that fibroblasts are still the primary effector cell in myocardial fibrosis. And then there's the shape – ugh. For a researcher in this day and age to use shape as an important characteristic cell feature is arguably absurd, particularly when that cell type is cultured in artificial conditions (e.g. plastic flasks with varying factors and growth media). When discussing fibrocytes, I have been known to use the analogy that if someone dressed up in a police officer costume, I can understand why one would believe that person is a policer officer, but it still does not necessarily make it true. It is a supportive piece of evidence, but a weak one at that. Thus, the case for fibrocytes as a unique cell type involved in the fibrotic process is somewhat lacking. In contrast, a stronger case can be made that the cells we set out to study were, in fact, monocytes and monocyte-derived MΦ.

Monocyte-derived MΦ are present in all stages of cardiac healing and serve and important role in shaping the myocardial environment[1,72,102,154,157,248–250]. The biphasic healing response, characterized by early classical then later non-classical monocyte recruitment, appears to be conserved in both ischemic and non-ischemic myocardial injury[100,154]. While the specific functions for these cells remain somewhat unclear, their general roles can be dichotomized into an early pro-inflammatory response and a later anti-inflammatory/pro-fibrotic response[1]. This would help explain (i) the early appearance of monocyte-derived cells in the myocardium following AngII infusion and (ii) the later gradual increase in ECM expression by monocyte-derived cells[47,72,100,155,254]. Importantly, if fibrocytes were to exist as a unique cell type, then their expression of hematopoietic (e.g. CD45, CD11b, CD14, CD68) and mesenchymal markers (e.g. collagen, vimentin, and αSMA) does not serve as a specific identifier. Indeed, even a paper that specifically set out to differentiate fibrocytes from monocyte-derived MΦ supports that collagen expression – the hallmark of a fibrocyte – cannot separate the 2 cell types[179]. Growing evidence suggests that under certain conditions, MΦ – predominantly M2 – can also express the same ECM markers, which may highlight their role in the healing phase of myocardial injury[235,236,340–342]. In addition, M2 subsets have been shown to internalize collagen for degradation, suggesting that collagen within MΦ can also be acquired from their environment[246].

Importantly, the classical monocytes that form the first wave of leukocyte migration to the heart have been described as immature and as such, are believed to have a greater ability to differentiate along the MΦ spectrum[165]. Moreover, it is possible that the $CD133^{+}$ cells previously reported to be in the AngII-exposed myocardium could be

an even less mature cell of the myeloid lineage with greater pluripotency[47,343]. Unfortunately, the CD133 antibody was discontinued without a viable replacement. Nevertheless, pluripotency could have important implications in animals receiving both AngII and AMD3100. If there were an increased concentration of immature myeloid cells released into circulation and subsequently recruited to the myocardium, then it is reasonable to believe that the early, pro-inflammatory myocardial environment would encourage M1 activation. In turn, this increased M1 activation could exacerbate the typical pro-inflammatory, AngII myocardial injury, which would require a sufficient but detrimentally greater fibrotic response. This could serve as the mechanism by which AMD3100, in addition to AngII infusion, appears to increase the presence of $CD45^+$ αSMA^+ cells in association with increased myocardial fibrosis. It is important to note that AMD3100 blocking the CXCL12-CXCR4 interaction in the bone marrow does not specifically increase the specific release of monocyte-precursors, but rather, all leukocyte precursors[344]. Thus, the response we observe with AMD3100 is likely an over-response by all leukocytes (e.g. T-cells, NK cells) involved in AngII-mediated myocardial injury and repair, but further characterization of the infiltrating cell populations would be required.

Lastly, given the established importance of CCL2-CCR2 in classical monocyte recruitment, there is still the lingering question as to why $CCR2^{-/-}$ mice would not be protected from early mononuclear cell accumulation in the myocardium[191]. I do not have a definitive answer, but it could relate to the fact that (i) classical monocytes can utilize alternative chemotactic pathways that may be upregulated in the AngII model (e.g. CCL3, CCL5) and/or (ii) the lack of vascular integrity following AngII-mediated injury

(i.e. non-specific chemotaxis)[117,131,345]. While not necessarily conclusive, there is evidence for the upregulation of myocardial CCL3 and CCL5 in the AngII model and classical monocytes express their receptors, CCR1 and CCR5[163,346–348]. In support of the former mechanism, we have observed significant hemorrhaging in some AngII-exposed hearts, particularly those also exposed to AMD3100. Furthermore, AngII has been shown to increase vascular permeability and is known to cause significant vascular injury[117,126,132]. Thus, outside CCL2-CCR2 signaling, there are alternative mechanisms by which classical monocytes could enter the AngII-exposed myocardium.

Building on the observations from the AMD3100, fibrocyte chemotaxis manuscript, I move onto the liposome and CX3CR1$^{-/-}$ studies: we were still investigating fibrocytes when I began these projects, but as the lines between monocyte and fibrocyte blurred, I investigated alternative approaches for preventing the accumulation of the monocyte lineage (i.e. MΦ). It was at this point that I discovered liposomes in the literature.

Liposomes were not a new technique, but it was an ever-refining method for targeting phagocytic cells[301,302,349]. To date, one group had successfully employed liposomes to label and deplete 'fibrocytes' in a model of hypoxia-induced vascular remodeling[261]. In turn, they had demonstrated a reduction in 'fibrocytes' in the vessel wall and linked this observation to a reduction in vascular remodeling. Similarly, we were able to demonstrate a significant reduction in mononuclear cell accumulation following AngII infusion in tandem with clodronate liposome injections. Importantly, we believed we were still targeting a single cell type and thus, took for granted any potential biphasic response that would involve 2 waves of monocyte migration. As such, the serial

liposome injections I provided throughout AngII infusion had prevented both waves of migration, but this oversight still led to an an important conclusion: monocyte depletion in a non-ischemic/infarct model was beneficial to cardiac remodeling. Dissimilar to infarct models, in which monocyte depletion was determined to be detrimental and even fatal, animals receiving clodronate liposomes and AngII did not exhibit any adverse responses or increased mortality[59,72]. This observation hints at the environmental differences and, in turn, demands between ischemic and non-ischemic myocardia.

As previously mentioned, the hypertension models do not typically exhibit the same extent of cardiomyocyte loss as those involving infarcts or ischemic[154,294]. As such, it could be argued that the fibrosis observed in hypertension models, including exogenous AngII infusion, is intended to reinforce the structural integrity of a more uniformly stressed organ. In contrast, infarcts and ischemia can lead to a significant breakdown in the structural integrity of a particular area of the heart, which, if it were not to repair sufficiently, is at risk for rupture. If an animal were to be continually infused with AngII for a longer duration without the MΦ-mediated pro-fibrotic response, the heart may also lose its structural integrity; however, to date, no such experiment has been performed. Findings in our AngII infused CX3CR1$^{-/-}$ mice indicate this could be the case. While these animals exhibited significantly greater fibrosis, the scar appeared unusually immature (e.g. looser fibres, lighter in colour), as indicated by SR/FG staining. Moreover, the hearts in these animals exhibited dilatation by just 3d of AngII infusion, supporting a loss of integrity. Unfortunately, one cannot separate this observation from observed increases in inflammatory markers, which may have precipitated the heart toward failure.

Our findings also provide clues as to how changes in MΦ accumulation in the myocardium can subsequently influence the myocardial environment. Some of the more interesting but perhaps understated findings of my liposome manuscript are the effects that clodronate liposomes have on the levels of TGF-β and CX3CL1 myocardial transcript. It may seem counterintuitive that TGF-β transcript is upregulated and both Col-1 transcript and collagen deposition are downregulated; however, while often considered pro-fibrotic, TGF-β is a pleiotropic growth factor with potent anti-inflammatory effects as well. I hypothesize that the increase in TGF-β could potentially be attributed to a few mechanisms:

(i) AngII is still exerting pro-inflammatory effects on the vasculature and inducing hypertension. In the absence of MΦ, the heart's inability to adapt through fibrotic deposition may exacerbate vascular stress and TGF-β may be serving a role in counteracting the vascular inflammation. Essentially, without MΦ as part of the normal cardiac repair mechanism, the heart assumes a new 'homeostasis', and/or

(ii) clodronate liposomes induce apoptosis and the phagocytosis of apoptotic cells by other MΦ is believed to induce a MΦ activation/deactivation that includes the production of TGF-β[147]. While many MΦ are depleted by clodronate liposomes, a population of rCMΦ appears to be resistant to depletion, and thus, may be responsible for clearing other apoptotic MΦ[248].

The reduction in CX3CL1 myocardial transcript also points to a fundamental change in the myocardial environment following monocyte/MΦ depletion. If CX3CL1 is

responsible for the second wave of monocyte (i.e. non-classical) recruitment, then its downregulation suggests 2 other potential mechanisms at play:

(i) the early wave of classical monocytes that likely become M1 activated MΦ are responsible for the production of CX3CL1 in the heart. Thus, in their absence, as would be the case with clodronate liposomes, less CX3CL1 will be produced and this would also prevent the second wave of monocyte recruitment, and/or

(ii) if the first wave of monocyte influx into the heart induces inflammation that causes injury, then the second wave of monocytes may be required to shift the heart into an anti-inflammatory/pro-fibrotic state to repair said injury. With a reduction in inflammatory injury, whichever cells are normally responsible for CX3CL1 production may no longer be receiving the appropriate signaling to trigger the second wave of monocyte recruitment. Thus, CX3CL1 production would be redundant.

Regardless of the underlying reasons for the decreased CX3CL1 transcript in AngII-infused, monocyte-depleted mice, our results still suggest a very important role for the chemokine in the resolution of inflammation and cardiac repair. In AngII-infused mice that lacked its receptor, CX3CR1, we demonstrated an increase in myocardial mononuclear infiltrate and fibrosis. This was in contradiction to our hypothesis that non-classical monocyte recruitment and their preferential differentiation into M2 MΦ were primarily responsible for AngII-mediated myocardial fibrosis. Yes, M2 MΦ – or a portion thereof – are the phenotype and activation state that can express ECM markers and correspond to the fibrotic stage of healing[235–237,341,342]. In addition, evidence from

infarct models supports M2 MΦ as predominantly being derived from infiltrating $Ly6C^{low}$ monocytes and as such, eliminating their primary chemotactic pathway (i.e. CX3CL1-CX3CR1) would impair their recruitment. In support of such a theory, AngII-infused $CX3CR1^{-/-}$ mice did appear to have fewer myocardial $CD206^{+}$ cells than their WT counterpart[xx]. Our hypothesis; however, was an oversimplification of (i) the mechanisms responsible for AngII-mediated myocardial fibrosis and (ii) the complex activation of MΦ (e.g. multiple M2 subsets) and its effect on healing.

I return to the idea that AngII-mediated myocardial fibrosis has been muddled by the idea of a single cell type (e.g. fibrocytes) driving the fibrotic process. It was loosely based on the idea that fibrocytes become fibroblasts, which produce ECM proteins that contribute to fibrosis[47,350,351]. Ergo, more fibrocytes equates to more fibrosis. Now, replace 'fibrocytes' with 'M2 MΦ' and it could be argued that more M2 MΦ should equate to more fibrosis. Arguably, the clodronate liposome, monocyte-depletion studies could reinforce this idea, if one were to still assume this a single cell type was predominantly responsible. After all, significantly reducing the accumulation of mononuclear cells in the myocardium dramatically diminished fibrotic deposition. In many respects, the $CX3CR1^{-/-}$ experiments were the proverbial nail in the coffin for the AngII model being an aberrant fibrotic process. It was not the case that mononuclear cells were entering the myocardium only to become ECM-expressing, fibrosis-depositing machines. Even groups that have touted fibrocytes for the better part of a decade report under 4% of all isolated non-myocyte myocardial cells expressing the fibrocyte (or M2, ECM-expressing phenotype)[100]. Rather, in the context of literature and our ever-growing understanding of MΦ in cardiac disease, the AngII model looked more and more like a

non-ischemic version of cardiac injury that still followed a biphasic role for MΦ subsets. Indeed, by examining the phenotype of early (i.e. 1-3d) versus late (i.e. 3-7d) myocardial MΦ and the corresponding myocardial environments, one can begin to understand the temporal progression of the AngII-mediated response. For example, Duerrschmid et al. demonstrated the accumulation of M1 activated MΦ (i.e. TNF-α-producing $CD68^+$ cells) by 3d followed by an expansion of 2 subtypes (i.e. $CD301^+$ and $CD206^+$) of M2 activated MΦ at 7d in the AngII model[100]. Moreover, the phase dominated by M1 activated MΦ was also associated with upregulation of the pro-inflammatory factors IFN-γ, TNF-α, IL-1β, IL-6, IL-12, and CCL2[47,71,100,125]. In contrast, the M2 dominated phase was associated with upregulation of the anti-inflammatory and pro-fibrotic factors IL-4, IL-13, and TGF-β[100]. As such, preventing the accumulation of myocardial M2 MΦ, as we achieved with $CX3CR1^{-/-}$ mice, in the same AngII model employed by Duerrschmid et al., appeared to impair the normal resolution of M1-driven inflammation[72]. This was supported by the concurrent increases and decreases in pro-inflammatory and anti-inflammatory cytokines, respectively. In simple terms, an early inflammatory response likely necessitates a later fibrotic response. As such, in myocardial healing, villainizing the M2 phenotype – ECM marker expression or not – can be thought of as analogous to blaming firefighters for water damage after a house fire.

But what if the heart had live-in arsonists and firefighters and they were in constant competition? This was the nature of the question we were asked by reviewers when submitting the liposome-$CX3CR1^{-/-}$ manuscript: have you considered your findings in the context of rCMΦ? No, we had not because we did not know such a cell existed, but it did fill a missing piece to the cardiac healing puzzle. Within 1 manuscript, I came to

the realization that (i) fibrocytes are likely a MΦ subset and we had been studying MΦ all along, (ii) MΦ subsets are imperative to myocardial healing – they both promote and resolve it – and (iii) the heart, like many other organs, has its own resident MΦ population. Additionally, we discovered that CX3CR1$^{-/-}$ mice exhibit an increase in Ly6C expression in their rCMΦ population, which is consistent with the more immature, pro-inflammatory M1 phenotype. This suggested that CX3CR1$^{-/-}$ mice have variations in their MΦ activation potential that could ultimately affect the ability for the heart to heal (i.e. a pre-existing balance between resident M1 and M2 activation states). And if it was the case that rCMΦ were dynamic, then what other perturbations in signalling – or variables – could influence their phenotype?

I continue to be fascinated by the dynamic rCMΦ concept: one heart could be more primed for injury than another. Conversely, a heart could also be more primed for protection than another. I imagined all the different sources of inflammation and regulation of inflammation that could influence rCMΦ phenotypes: obesity, infection, previous cardiac injury, autoimmunity, age, and hypertension. Obesity and the systemic inflammation with which it is associated also stuck out at me as an interesting research avenue for rCMΦ, but that could have been another graduate degree in itself. Rather, we chose to investigate the yin, CCR2, to the yang, CX3CR1[xxi].

Since rCMΦ demonstrated a more pro-inflammatory inflammatory phenotype in CX3CR1$^{-/-}$ mice than WT, it followed that rCMΦ in CCR2$^{-/-}$ mice may exhibit a more anti-inflammatory phenotype. This hypothesis was premised on these chemokine receptors representing the 2 poles of monocyte differentiation: classical/CCR2 and non-classical/CX3CR1[154]. At the time, I did not realize it, but I was also circling the

hypothesis that these 2 chemokine receptors could influence the turnover of rCMΦ from circulation. I also took for granted how profound an effect differences in rCMΦ could have on cardiac healing.

While I was personally comparing rCMΦ phenotypes between CX3CR1$^{-/-}$ and CCR2$^{-/-}$ mice, other researchers provided temporal and functional context to our observations. In collaboration with Dr. Légaré's previous PhD student, Dr. Nicole Rosin, we had actually begun to find connections between age and rCMΦ phenotypes. We demonstrated that older mice exhibit a pro-inflammatory shift in their rCMΦ, but this was still early on and I failed to truly appreciate the connection between age and rCMΦ phenotype[138]. I had not considered that this could be on an ever-changing spectrum that begins as an embryo, as demonstrated by other researchers. For example, Epelman. et al. and Molawi et al. both showed that rCMΦ originate from embryonic and yolk-sac-derived populations that are gradually replaced by circulating CCR2^{+} monocytes[248,250]. Importantly, these CCR2^{+} circulation-derived MΦ exhibit a more pro-inflammatory M1 activation state compared to the embryonic and yolk-sac derived populations. This added important context to the aging study by Dr. Rosin and our baseline observations in CCR2$^{-/-}$ mice at 8wk: if CCR2^{+} M1 populations replace M2 embryonic and yolk-sac derived populations, then this provides an underlying mechanism to the observation that CCR2$^{-/-}$ mice exhibit an overall anti-inflammatory shift in rCMΦ. In other words, CCR2$^{-/-}$ mice may have slower turnover of their initial resident MΦ populations, thus helping better preserve an overall M2 phenotype. Aurora et al. remarkably demonstrated the functional significance of this CCR2-dependent turnover: embryonic and yolk-sac derived rCMΦ can aid in the repair and regeneration of the heart following ischemic injury in

neonates[159]. Thus, as $CCR2^{+}$ MΦ replace embryonic and yolk sac-derived MΦ, the heart may also lose its ability to regenerate following injury. As such, the time point/age at which we study CVD becomes increasingly relevant to the observed outcomes.

Our lab has predominantly studied the AngII model in 8wk old mice. In the case of comparing $CCR2^{-/-}$, $CX3CR1^{-/-}$, and WT mice in CVD models at 8wks, we as well as others had long been under the assumption that we were specifically studying the migration of cells strictly during the disease process (e.g. AngII infusion or infarct). Yes, $CCR2^{-/-}$ mice also exhibit a reduction in the $Ly6C^{high}$ circulating monocyte population – an effect likely attributed to the importance of CCR2 in the emigration of monocytes from bone marrow[352]. However, when one is trying to block the recruitment of $CCR2^{+}$ cells by using a knockout, it matters less that there are fewer of the population with which to begin – only that fewer of these cells migrate to the heart, so that in their absence, we can understand their function as MΦ. The fact that the overall rCMΦ population is dynamically dependent on CCR2, age, and potentially CX3CR1, has the potential to require us as researchers to rethink all previous observations in CVD. It suggests that the time point at which we choose to study CVD is a snapshot on a continuum from (i) a baseline ability to promote cardiac regeneration to (ii) a state that may predispose one to worsened injury and ultimately fibrosis (i.e. the extent of a cardiac injury increases with age).

Unfortunately, while there is growing evidence for the role of CCR2 in the turnover of embryonic and yolk sac-derived MΦ, the role for CX3CR1 in the resident population is less clear[248,250]. The embryonic and yolk sac-derived rCMΦ are characterized by their expression of CX3CR1, which is absent or significantly reduced in

the $CCR2^{+}$ monocyte-derived MΦ. Moreover, as previously mentioned, these 2 chemokine receptors serve complementary roles in the recruitment of classical and non-classical monocytes to tissue. As such, it may be the case that CX3CR1 has an important role in the initial homing or preservation of the embryonic and yolk sac-derived populations. Importantly, CX3CR1 has been shown to promote MΦ survival in certain models of disease, but whether these observations can be extrapolated to the heart is unknown[194,196,353]. In turn, the mechanisms underlying our observations in the $CX3CR1^{-/-}$ mice remain hypothetical. However, in the context of our complementary study using $CCR2^{-/-}$ mice, our results still support a shift toward an M2 activation state as being protective for the heart.

There are still some whom find fault with M2 MΦ in the AngII model though. In reviewing the literature for this thesis and trying to piece together a cohesive image of the AngII model, a single paper stands out as a stumbling block: Interleukin-12p35 Deletion Promotes CD4 T-Cell–Dependent MΦ Differentiation and Enhances AngII–Induced Cardiac Fibrosis[125]. IL-12 is considered a pro-inflammatory cytokine produced by M1 MΦ often in response to IFN-γ signaling. In this paper, Yulin et al. demonstrated that knocking out the IL-12 subunit p35 is detrimental in the setting of AngII-mediated myocardial fibrosis. In other words, less of a pro-inflammatory cytokine exacerbates fibrosis. When the evidence seems stacked in favour of inflammation being the pre-cursor to AngII-mediated fibrosis, how then can more inflammation be a good thing? Moreover, in another study by the same group, inhibiting TNF-α and IL-1β in the AngII model led to a downstream reduction in IL-12, which was associated with a reduction in

myocardial fibrosis[215]. So which is it: is IL-12 good or bad? And for that matter, does this suggest M2 MΦ are detrimental in the AngII model?

These sorts of studies can threaten to overthrow prevailing theories (e.g. inflammation leading to fibrosis), just as the idea of fibrocytes threatened to detour our understanding of cardiac healing (e.g. one cell type on a mission to deposit ECM proteins). In turn, anomalies can force – or encourage – researchers to think outside the box. With respect to IL12p35$^{-/-}$ mice, it may be more likely the case that we are missing a vital piece of the puzzle. Importantly, the IL-12p35$^{-/-}$ studies made all their observations at 7d of AngII infusion – a time point at which inflammation has been shown to have somewhat subsided[125]. As such, the missing piece of the puzzle in the IL-12p35$^{-/-}$ story may lie between t=0d and t=7d. These studies can be like a magic trick in which you know the magician starts with a hat and a rabbit and then the rabbit seems to disappear: in order to understand this observation, you need to know what occurs in between those 2 events. Does removing IL-12p35 somehow exacerbate early inflammation, causing a secondary bump in M2 MΦ activation and fibrosis? Interestingly, the researchers failed to take into account that an IL-12p35$^{-/-}$ mouse would also knockout the lesser known IL-35, which promotes regulatory T-cell activation[xxii354,355]. Relevant to this theory, the adoptive transfer of regulatory T-cells reduced myocardial fibrosis in the AngII model[92]. Moreover, IL-35 has been shown to be protective in the context of some cardiac pathologies[356,357]. While this is a relatively novel area of research that requires further investigation, it permits the theory that inflammation precedes and even necessitates the ensuing myocardial fibrosis to remain intact.

Thus, I come to the end of discussing my findings in the context of the literature. The principal conclusions to which I arrive include:

(i) A progenitor cell population (e.g. fibrocytes) is not responsible for AngII-mediated myocardial fibrosis,

(ii) Indeed, the AngII model is not necessarily contingent on a single cell type driving the fibrotic response, despite the fact that depleting circulating monocytes with liposomes still prevents myocardial fibrosis,

(iii) Rather, the process we are studying represents an extension of observations in infarct models. Specifically, the AngII model (i.e. non-ischemic injury) also features a biphasic response in which M1 MΦ predominate during the pro-inflammatory stages of healing and are subsequently replaced or added to by M2 MΦ during inflammation resolution and fibrosis,

(iv) The AngII model is complicated by the fact that $CCR2^{-/-}$ does not prevent the accumulation of early M1 activated MΦ in the myocardium, but theories including increased vascular permeability, additional chemotactic pathways, and even the mass mobilization of classical monocytes from the spleen may contribute to the accumulation of the M1 phenotype,

(v) Cardiac pathology models are further complicated by the presence of rCMΦ, which likely has an important influence over the ability for the heart to heal versus scar during an injury. Moreover, the fact that resident MΦ turnover with age and shift toward a pro-inflammatory phenotype has important implications in how we study cardiac pathologies. As such, age

must always be considered as a variable for our observations in cardiac pathologies,

(vi) CCR2 and CX3CR1 may serve complementary roles in replacing and preserving rCMΦ, respectively.

(vii) Lastly, there are many questions left unanswered as to which other systemic or local changes have the ability to change the rCMΦ population phenotypes through turnover or environmental differences. For example, pathologies that increase MΦ turnover may predispose the heart to worse outcomes – an effect that may be additive (i.e. each subsequent cardiac event may reduce the ability for the heart to heal).

This last point is particularly important for future researchers and leads into the next section of my discussion: future directions.

7.2 Future Directions

There are many directions in which I could take this section, but I will try to focus it on a few key areas addressed in this thesis. Namely, the future of fibrocyte research, rCMΦ, and the AngII model.

There are still researchers plodding along in fibrocyte research, arguing that this elusive – a word used in their circles – is an important source of "fibroblasts" and responsible for the deposition of fibrosis in virtually all organs and tissues[178,358]. Indeed, researchers have demonstrated the presence of ECM marker-expressing, monocyte-derived cells in models of lung, liver, kidney, heart, and skin fibrosis[255–257,271,328]. Just as I have re-examined my results in the newly-discovered context of M2 MΦ subsets, I would

encourage other researchers to do the same. There are possibly conditions in which we have wrongly attributed pro-fibrotic functions to these cells, similar to our findings in the AngII model.

Many studies in Th2 lung pathologies have also linked the cytokines IL-4 and IL-13 with pro-inflammatory fibrocyte functions[262,359,360]. As previously mentioned, IL-4 and IL-13 are key cytokines in M2 activation and indeed, certain M2 subsets have been implicated in Th2-drive pulmonary inflammation[168,361]. In turn, the functions attributed to fibrocytes in these pathologies may be more reconcilable with M2 phenotypes. As researchers attempt to design studies and therapies around these potentially false attributions, they risk misdirecting attention and resources to more viable avenues (e.g. MΦ activation). Thus, it is likely the case that these misunderstood MΦ serve important functions in both pro-inflammatory and pro-fibrotic models that remain uncharacterized.

While researching literature for this thesis, I asked myself again: what is a fibrocyte? What do we know about fibrocytes? Some common themes arose:

(i) they can express some classical MΦ markers[179],

(ii) there are discrepancies in characterizing which circulating populations give rise to them[71,72,93],

(iii) they originate in the bone marrow and traverse circulation[155],

(iv) in tissue, they can decrease the expression of classical MΦ markers in favor of increased mesenchymal markers[47,71],

(v) they are present in the fibrotic/reparative phases of injury[47,71,93,100,254], and

(vi) they seem to migrate to many of the same chemokines as monocyte populations including classical monocyte chemokines such as CCL2 and CCL5[93,362].

It then struck me that if MΦ can shift phenotypes (i.e. M1 to M2) in response to changes in tissue environments (e.g. inflammation to fibrosis), then fibrocytes as a phenotype may indicate such a transition. In other words, if fibrocytes look like a transition between MΦ phenotypes, then perhaps they are a transitional phenotype. This could explain why fibrocytes seem to accumulate in the interphase between inflammation and fibrosis; however, more investigation is required to tease out the subtleties of MΦ differentiation, particularly in in vivo settings[47,327,363].

One approach for addressing whether fibrocytes are a transitional cell type would be to determine whether classical monocytes can differentiate into ECM-expressing M2 MΦ in the appropriate environment. In addition, one could investigate whether classical versus non-classical monocytes have differing abilities to undergo certain forms of MΦ activation (e.g. M1-like versus M2-like). There are still large discrepancies in the literature regarding which monocytes are beneficial versus detrimental in the context of different pathologies[162,364,365]. Classical monocytes, which are primarily recruited via CCL2-CCR2, are key feature in the early inflammatory phase of many pathologies[165,366,367]. As I have indicated in the discussion, this is not necessarily the only chemotactic pathway involved in the recruitment of classical monocytes[187,345,368]. Importantly, researchers have begun to consider classical monocytes as a naïve cell type with more differentiating potential than non-classical monocytes[163,165]. Classical monocytes are released into circulation only to learn from their environment and adapt to

the conditions to which they are recruited[163,165]. In addition, they provide a quick, accessible, vast source of pathological MΦ[102,163,165,171,247,366,367,369]. As such, in pro-inflammatory environments, classical monocytes have the ability to dramatically increase tissue M1 MΦ in order to ramp up the inflammatory response[154,165]. Alas, if one could shift the phenotype of classical monocytes in circulation before they reach inflammatory tissues, then there may be an opportunity to therapeutically resolve rather than promote inflammation.

Some have suggested this can be achieved with a type of liposome comprising phosphatidylserine (PS)[147]. PS is typically found on the cytosolic side of the cell membrane and is flipped toward the extracellular side when a cell undergoes apoptosis[370]. The PS can be recognized by monocytes and MΦ in order to aid in the phagocytosis of PS-expressing apoptotic cells. Moreover, it has been shown that the uptake of apoptotic cells by MΦ induces an anti-inflammatory M2 activation, characterized by the production of IL-10 and TGF-β[371,372]. Consequently, some have suggested liposomes containing PS can mimic apoptotic cells and in turn, therapeutically modulate monocyte and MΦ phenotypes toward M2 activation[147]. In preliminary experiments, we had attempted to modulate rCMΦ by administering liposomes containing PS. Our early results suggested that it was worth further investigation, but significantly more work was required to verify that these liposomes can (i) reach the rCMΦ and (ii) induce a therapeutically useful M2 phenotype. Importantly, it may not be as simple as presented by Harel-Adar, in which liposomes containing PS were suggested to improve recovery post-infarct[147]. Indeed, they report that liposomes containing PS were associated with a reduction in CD68 and an increase in CD206 – markers of M1 and M2, respectively. However, many of the

reported benefits, including increased scar thickness in the case of infarcts, appear to be attributal to liposomes, not PS specifically. Thus, while the theory makes sense in principle, there is currently insufficient evidence to support its validity.

Regardless of whether we can therapeutically manipulate rCMΦ, based on our work in CCR2$^{-/-}$ and CX3CR1$^{-/-}$ mice, it appears that changes in signalling can modulate their phenotype. As mentioned previously, there are many conditions associated with systemic inflammation that have the potential to affect rCMΦ phenotype and in turn, the ability for the heart to respond to injurious stimuli[168,171,373,374]. Recently, researchers have delved into age as a variable of influence in rCMΦ, but the underlying theme in age is the increased turnover of MΦ, which is likely featured in other conditions as well[248–250]. There were 2 environmental conditions that stood out as both particularly interesting and clinically relevant: (i) obesity and (ii) having had a previous cardiac event.

Current estimates suggest 1 in 4 adults and 1 in 10 children in Canada are clinically obese, which is characterized as a BMI of ≥30 kg/m^2[375]. Relevant to this thesis, obesity is generally associated with low-grade systemic inflammation and significantly increased risk of CVD[4,376–379]. In brief, obesity overwhelms the normal mechanisms and capabilities for energy storage in adipose tissue and leads to the apoptosis and necrosis of the adipocytes[380,381]. In turn, diseased adipose tissue produces pro-inflammatory adipokines (e.g. IL-6, CCL2, and TNF-α) that can also promote the recruitment and activation of pro-inflammatory leukocytes[174,380]. Indeed, obese individuals exhibit both increased adipose and serum levels of TNF-α and IL-6, suggesting that the pro-inflammatory effects of obesity can extend beyond the local adipose environment. This vicious pro-inflammatory cycle features a prominent role for M1 activated MΦ that is

believed to contribute to the systemic inflammation associated with obesity[380]. In contrast, lean adipose tissue produces anti-inflammatory adipokines (e.g. adiponectin) that favour M2 activation and the production of IL-10[174,380]. In essence, adipose becomes a potent paracrine and endocrine tissue, capable of modulating signalling throughout the body[382,383].

Perhaps capable of exerting a stronger effect on the myocardium, epicardial fat is also expanded in obesity and, similar to obese visceral adipose tissue, promotes inflammation involving MΦ phenotype modulation[384,385]. In support of proximal paracrine signalling, epicardial fat in obese individuals has been shown to modulate MΦ phenotypes toward M1 activation in coronary artery disease[385]. It follows that visceral and pericardial fat may also modulate rCMΦ populations via endocrine and paracrine mechanisms, respectively. In turn, changes in adipose composition may influence the ability for rCMΦ to promote versus prevent myocardial injury. Some researchers have begun to theorize such a connection between rCMΦ and obesity, but currently, the basic science is lagging behind speculation[386,387]. As an optimist, I see a potentially incredible research opportunity to understand the influence of obesity through rCMΦ on CVD.

Pre-existing injury is another interesting area of investigation in the context of rCMΦ. It is well-established that MΦ accumulate in the heart in many forms of CVD (e.g. hypertension/AngII model, ischemia, infarct, myocarditis)[102,197]. It is, however, less clear how long these MΦ persist and what contribution they make to the rCMΦ populations. Recent evidence indicates that Ly6C^{low} rCMΦ can be completely lost by 1d post-infarct[xxiii251]. Moreover, the rCMΦ appeared to be entirely replenished with Ly6^{high} monocytes infiltrating from circulation. It follows that the change in composition of

rCMΦ may influence the ability for the heart to heal following another injurious stimulus. While challenging mice with more than 1 infarct would likely lead to high mortality, alternatives such as hypertension models have also been shown to influence rCMΦ composition with reduced mortality[47,72,146,155]. One possible experimental approach could be a hypertensive model (e.g. AngII infusion) with a period of recovery followed by a second insult (e.g. another duration of hypertension or an infarct). As such, one would be able to assess (i) how cardiac insults affect the rCMΦ population over time and (ii) how changes in the population could impact the ability for the heart to heal during future injuries and stressors. Thirdly, age could be incorporated as a factor by characterizing the immune response (e.g. changes in rCMΦ populations, inflammatory markers, fibrosis markers) to injury at age intervals. This could potentially resolve or uncover the issue of age as a variable for influencing cardiac healing.

Thus far, the future directions and much of this thesis have focused on a single cell type (i.e. MΦ or MΦ by any other name). Importantly, one cannot expect to completely understand cardiac healing by focusing on a single cell type (e.g. MΦ or T-cells). This leads into the last future research area which I would prioritize: arguably, no paper to date is a masterpiece on the healing response to non-ischemic injury such as AngII exposure or hypertension. As with most research, there are important and valuable pieces of information that are seemingly scattered like an unfinished puzzle. No one has connected the dots from T-cells to MΦ to fibroblasts and all the links in between. The summary of findings in key papers in the AngII model are summarized in the table below (Table 7.1):

Table 7.1 – Summary of temporal findings from the AngII infusion model in mice. ↑ indicates upregulation or increase in expression relative to control levels. – indicates no known difference between AngII infusion and control at said time point. A dark cell in the excel indicates an unknown.

	<1d	1d	3d	7d	14d	28d	42d
Physiological/Structural							
ANP				↑			
β-MHC				↑			
Blood Pressure	↑	↑	↑	↑	↑		↑
Cardiomyocyte Size				↑			
EF				-			
Hypertrophy		↑	↑	↑	↑		↑
Fibrosis							
Fibronectin				↑	↑		
αSMA				↑	↑	↑	-
Collagen Deposition		-	↑	↑	↑	↑	↑
Collagen I				↑	↑	↑	-
Collagen III				↑	↑		-
CTGF	↑	↑	↑	↑	↑		
TGF-β		↑	↑	↑	↑		↑
Anti-Inflammatory							
IL-4		-		↑			
IL-10				↑			
IL-13		-		↑			
Pro-Inflammatory							
IFN-γ		↑	↑	↑			
IL-12			↑	↑			
IL-1β			↑	↑			
IL-6		↑		↑			
iNOS				↑			
Osteopontin		↑		↑			
TNF		↑	↑	↑	↑		-
Chemokines							
CCL2			↑	↑	↑		
CX3CL1			↑				
CXCL12		↑	↑	↑			
Cells							
CCR2+				↑			
CD3+		↑			↑		

CD4+				↑			
CD8+				↑			
CD34+				↑	↑		
CD45+		↑		↑	↑		-
CD45+ CD86+			↑	-			
CD45+ CD206+			-	↑			
CD45+ CD301+			-	↑			
Cellular Infiltrate		↑	↑	↑		↑	
F4/80			↑	↑	↑		
Mac-2				↑	↑		

68–72,92,93,100,101,155,215,225,294,388,389

The table (7.1) is not intended to be exhaustive, nor perfectly representative of the AngII model. While all studies used to generate this table used the AngII infusion model in mice, they may have differed in (i) the dose of AngII and the methods used for characterizing/quantifying (ii) cells and (iii) fibrosis. As such, there are obvious limitations in comparing such studies. Importantly, the table does highlight our knowledge regarding the AngII model and continuous, non-ischemic myocardial injury and repair. In addition, it also demonstrates that gaps in our understanding. For example, many of the studies I reviewed focused on 7d of AngII infusion, but little information is available before and after that time point. As such, the more we narrow our focus to time points or cell types, the more assumptions we are forced to make and the less we are able to describe the context for our observations.

Even in my efforts as a PhD student, I focused on resolving discrepancies in the literature on one cell type. It would be naïve to think that AngII-mediated myocardial fibrosis can be attributed to MΦ. In preparing this thesis, I came across papers that made sweeping claims about certain cell types and pathways in the pathogenesis of CVD[100,101,121,125,215]. Indeed, we simplify our messages to suggest that blocking one cell

(e.g. MΦ T-cells) or pathway (e.g. TNF-α, IFN-γ) is sufficient to abrogate a disease process. I think this is a reflection of the fact that we, as researchers, are supposed to "sell" our research and emphasize its importance. In my time as a graduate student, I began to form an appreciation for the complexity of cells and signaling that mediate cardiac injury and repair. As I read papers on AngII infusion and hypertensive models, I found myself trying to compile pieces of information into a cohesive image of AngII and hypertensive injury – a research version of "What comes first: the chicken or the egg?".

There are some whom suggest it is T-cells in the vascular wall that promote the inflammatory injury that results in perivascular fibrosis in the AngII model, but that leads into the question of how the T-cells got there in the first place[390]. It may be the case that the initial injury to the vasculature promotes T-cell recruitment to the vessels[101,126]. T-cells obviously have an important role in cardiac healing, particularly through interactions with MΦ that regulate different phases of healing[124,125,391,392]. Some hypothesize that T-cells produce the IFN-γ and TNF-α that promotes M1 MΦ activation in the myocardium; however, others hypothesize that M1 MΦ produce the IL-12 that promotes T-cells to produce IFN-γ and TNF-α[125,393]. Perhaps it is rCMΦ mediate the early events that dictate how the myocardium heals during an injury. After all, they are present at the onset of a cardiac injury.

I still do not know the answer as to which cell(s) fire the starting pistol in the race from injury to inflammation to fibrosis. In reality, we are attempting to model a complex series of processes that are likely interdependent on one another. I have, however, realized that a lot of the knowledge, tools, models, and techniques required for forming a cohesive, temporal image of AngII and hypertensive injury are available – it would just

need to be a research priority. Unfortunately, my understanding is that it is difficult to acquire funding for and to sell a more complete version of a research story when many of the individual pieces have already been seemingly completed. Moreover, in times of lean funding, we must remain focused on the topic of the next section: clinical significance.

7.3 Clinical Significance

In my Future Directions, I largely focused on basic science approaches because they can serve as a "sandbox" for ideas. In contrast, the clinic can provide the inspiration and ideas for researchers to explore in a laboratory. In the process of taking an idea from "bedside to bench", basic scientists are somewhat forced to take a reductionist approach in order to model complex human disease. When the real world contains so many more variables than models such as AngII infusion, it can be difficult to work in the opposite direction – "from bench to bedside".

While researching for this thesis, I formed a great appreciation for the complexity of disease. I will reiterate a fact from my introduction because I think it is very important and telling of what we cannot model in a laboratory: 2/3 of Americans are either pre-hypertensive (above 120/80mmHG, but below 140/89mmHg) or clinically hypertensive (>140/89mmHg)[12]. It was a sad moment when you as a researcher look at what you have achieved in the lab versus the true reality of a disease. Through further investigation, I realized that social determinants of health – many of which are outside our control – are the primary driving forces for hypertension and heart failure[6,7,17,394]. These social determinants include upstream factors such as education, occupation, socioeconomic status, and gene-environment interactions[395]. As such, if my objective as a graduate

student was to cure hypertension, I probably should have spent less time in a lab with hypertensive mice and more time petitioning government for social change. Nevertheless, this realization forced me to take a deeper look at the clinical significance of my work. Indeed, my objective as a graduate student was to contribute to the understanding and characterization of a disease process with the long-term aim of improving patient care. In a roundabout way, studying a disease process that is more prevalent in lower socioeconomic populations is still positively contributing to clinical outcomes.

As a basic scientist, there are different ways in which one can make a clinical connection to their work including (i) modeling human disease and (ii) studying pathways conserved between humans and another species to explore the ethically inaccessible. As a researcher, connecting bench to bedside and vice versa, will likely always have its limitations. The complexities of human disease, including life-long exposure to variables (e.g. comorbidities), prevent small animal models from being perfectly analogous. As researchers, we do, however, use multiple approaches to best replicate tissue environments (e.g. in vivo, in vitro, ex vivo).

I cannot confidently argue that the AngII infusion model is representative of a particular human disease, but an easier case can be made for its strength as a model. Fortunately, as a hormone that spans primitive nematodes all the way to humans, any study on AngII signalling can potentially be linked back to the clinic[396]. In mice, for example, the AngII pathways are identical to those in human, making it suitable for modelling AngII signalling[396]. As such, exogenous AngII infusion in mice could be argued to generally model the increases in AngII signaling observed in certain pathologies. Indeed, AngII serum concentration and, in turn, its signalling are often

upregulated in CVD such as hypertension and heart failure[397–403]. Many researchers have attempted to take the association between model and clinic a step further by comparing the plasma AngII concentrations in rodent infusion models to those in patients[47]. Unfortunately, there is not enough data to support the association. Based on literature, (i) AngII plasma concentrations range anywhere from 8.5pg/mL to 15000pg/mL and (ii) determining AngII concentrations in plasma is fraught with experimental artifacts[397,402–407]. Indeed, some companies selling AngII detection kits (i.e. Cayman Chemicals) list the normal (e.g. non-pathological) average AngII concentration plasma as between 10-30pg/mL; however, they provide no literature to support such a claim. Thus, despite the push from granting agencies and publishers to form a direct link between bench and bedside, there continue to be limitations to small animal models. Rather, the AngII infusion model is likely just a surrogate for studying disease processes such as myocardial inflammation, its resolution, and the consequential fibrosis to repair damaged tissue.

Importantly, our work in small animal models has helped describe disease processes that are ethically inaccessible in humans (e.g. studying MΦ in ventricles and having the freedom to explore different time points). In tandem with work by others, we have redefined myocardial fibrosis as a response to early inflammatory injury instead of an independent process guided by a single cell type (i.e. fibrocyte)[47,71,72,93,100,254]. In theory, had I continued along the fibrocyte path, then I may not have made any meaningful contributions to the potential clinical future of fibrosis. With the help of cardiac models including AngII infusion, we now know that MΦ accumulate in biphasic stages that reflect the phase of myocardial healing[72,100]. In non-ischemic injury, our work

specifically helped demonstrate that MΦ serve a starring role in the early inflammatory injury and the subsequent fibrotic response[72,100]. It would have been implausible – or unethical – to characterize this process in human tissue and as such, we would have remained in the dark without suitable animal models. As such, we have been able to use animal models to identify MΦ as a potential therapeutic target in the different phases of cardiac healing[72,154,186,313]. And through our work with liposomes, we have also demonstrated 'proof of concept' that liposomes can modulate MΦ in the context of cardiac injury and healing[72,154]. Thus, targeting MΦ with liposomes could be a therapeutic strategy for modulating MΦ phenotype and function in the context of CVD.

In addition, the discovery that the heart contains rCMΦ was largely made possible through mouse models, which served as justification for human studies[157,248,250]. Specifically, in our lab, we are currently characterizing the presence and phenotype of rCMΦ in human atrial samples (REACH Project, 2016, unpublished). The work in the AngII model has also potentially provided some important information regarding the immune response in sterile, non-ischemic cardiovascular injury. Through our work with monocyte depletion in the AngII model, we can now begin to argue that the MΦ accumulation in the non-ischemic heart may be an overreaction[72]. In contrast, work in infarct models has demonstrated that MΦ accumulation is necessary for sufficient healing[59,154]. Thus, the comparisons between different mouse models allows researchers and clinicians to infer the roles of various cell types as we design novel therapies.

Lastly and somewhat selfishly, the clinical relevance that I experienced during my degree was imagining how my heart was responding to stress and hypertension. I envisioned how cells of different phenotypes and functions were interacting in order to

compensate for increased blood pressure. As an extension of that thought process, I imagined the same processes happening in all patients with hypertension – my newfound knowledge became a window into the heart.

7.4 Limitations

In this thesis, I presented work using the murine AngII model of myocardial injury and fibrosis. As mentioned earlier in the thesis, this is one of the available hypertension models – the others include aldosterone infusion, aortic banding, nephrectomy, renal artery clamping, high-salt diet in susceptible rodents, denervation of sinoaortic baroreceptors, and psychogenic stimulation (e.g. air jet, emotional stimuli, psychosocial stress, electrical stimuli, immobilization)[408]. Each model has its benefits and limitations in modeling human disease.

The AngII model is a minimally invasive model, which has the benefit of technically ease and reduced procedural mortality[408]. Unfortunately, the AngII model also does not necessarily reflect a normal human disease state; to my knowledge, no clinical condition results in a sudden spike in plasma AngII concentration[409]. Indeed, murine AngII infusion can induce sustained hypertension at levels comparable to moderate human hypertension; however, AngII infusion also ignores or diminishes the effects of other pathways involved in human hypertension such as sympathetic nervous system activation[31,68,91,110,409–411]. In addition, as mentioned earlier in the thesis, AngII has also been shown to have strong pro-inflammatory effects beyond its vasoactive and blood volume properties[120,142]. As such, in the AngII model, there may be a disproportionate primary inflammatory response in addition to the hypertension-mediated inflammation.

In addition, our findings were also limited by some of the experimental techniques used in the studies included in this thesis. For example, we quantified mRNA transcript to characterize the myocardial environment. The technique – qPCR – has several advantages such as high throughput, sensitivity to detect changes, repeatability, and requiring less starting material than other techniques[412]. Unfortunately, it also has several disadvantages including not necessarily being reflective of protein expression and being prone to error[412]. As such, a further analysis of temporal changes in protein expression could clarify the true state of the myocardial environment at given times during AngII infusion.

Our second methodological limitation was the use of intracellular flow cytometry in an attempt to provide functional characterization of macrophage populations isolated from the heart. While we were able to characterize a relative difference in cytokine expression between WT and CCR2$^{-/-}$ mice, the protocol to digest heart tissue in order to isolate macrophages involves harsh conditions that may promote changes in MΦ phenotypes. As such, using this technique, one cannot compare macrophage cytokine expression from other tissues sources, such as blood, to that in heart. In the context of this thesis, we cannot reliably compare the MFI for either TNF-α or IL-10 expression in cultured cells to those isolated from myocardium.

The use of clodronate liposomes as an approach for systemic monocyte and MΦ depletion is another limitation of the work presented in this manuscript. As mentioned earlier in this thesis, there are different methods for depleting monocytes and MΦ including inducible approaches (i.e. diphtheria toxin receptor under monocyte/MΦ-specific promoters)[72,154,200–203]. None of the current methods are perfect – ideally, one

would be able to temporally deplete monocytes and MΦ without the large-scale apoptosis that likely has effects that shape and affect immune processes[147,370]. Conventional liposomes (e.g. containing phosphatidylcholine and cholesterol) delivered intravenously (i) primarily target monocytes and MΦ via vasculature and (ii) are short-lived in circulation[413]. Indeed, in our experiments, we had to deliver liposomes every 24hr in order to continually deplete circulating monocytes and we still did not achieve absolute depletion. Moreover, resident MΦ are not necessarily accessible to IV liposomes. For example, in preliminary work, we were only able to target ~10% of rCMΦ with IV liposomes (data not shown). In turn, we cannot confidently state that our observations with clodronate liposomes are entirely due to monocyte and MΦ depletion.

Lastly, we relied on histological methods such as SR/FG staining in order to quantify myocardial fibrosis. While our results were largely supported by qPCR data on COL1A1 transcript levels. Importantly, the actual quantity of collagen in a given section may differ from its visual representation due to differences in scar maturation. As a scar matures, the collagen forms tighter fibrils, which would visually present as less fibrosis by area[29,40,59]. In contrast, earlier collagen deposition comprises looser fibres that would visually present as more fibrosis by area. Moreover, in a state of increased inflammation (e.g. AngII/CX3CR1$^{-/-}$ or AngII + AMD3100), it may be the case that scar maturation is delayed, resulting in perpetuation of loose collagen fibres that give the perception of increased fibrosis by area.

7.5 Closing Comments

In this thesis, I have attempted – and hopefully successfully – to describe my contributions to research over the past 5 years. I also set out to write a different style of thesis: I have included all the science, but also anecdotes and personal perspectives. I wanted to create something accessible and all-encompassing, which has helped me consider the different perspectives in disease, research, science, academia, and beyond. As I conclude my degree and thesis, the most important question for me is: what am I taking away from this degree?

As previously mentioned, the scientific knowledge that I have acquired during my degree has been only part of my education. I started this degree with the intent of exploring research and I discovered a lot more along the way. I learned an important lesson about knowledge and my attitudes toward research. Knowledge without the skills is like a library without anyone to read the material. Conversely, skills without knowledge is like a hammer with no one to swing it. I now look at information as pieces of a puzzle and the research process as actually putting the puzzle together. When I began this degree, I was more focused on the puzzle pieces and not the puzzle as a whole. For example, when Dr. Légaré presented me with the issue of myocardial fibrosis in the AngII model, I thought my task was to get rid of fibrosis and then my mission would be accomplished (think George W. Bush on the aircraft carrier prematurely declaring an "end" to the Iraq war). In hindsight, this was a terrible way to look at research because my contribution was both (i) an oversimplification and (ii) a small piece of the enormous puzzle that is heart failure. Through the help of my supervisor – both through his own expertise and by enabling me to experience the patient perspective – I saw diseases from

the cells up to the individual and finally beyond to societies. It was the whole spectrum of pathology. In addition, my own experiences with Dengue and hypertension helped break the fourth wall between research and disease – I am now able to imagine what is transpiring, despite the inability to directly visualize it.

In August, I will be beginning another path – a more clinical path – in medical school. For now, I may be finished with research in the form in which I have been practicing it for the last 5 years – as Dr. Légaré knows all too well, I never enjoyed writing papers. I always loved the thinking though. During my degree, I spent many hours daydreaming about diseases and the way they work.

***

Thank you for taking the time to read my thesis.

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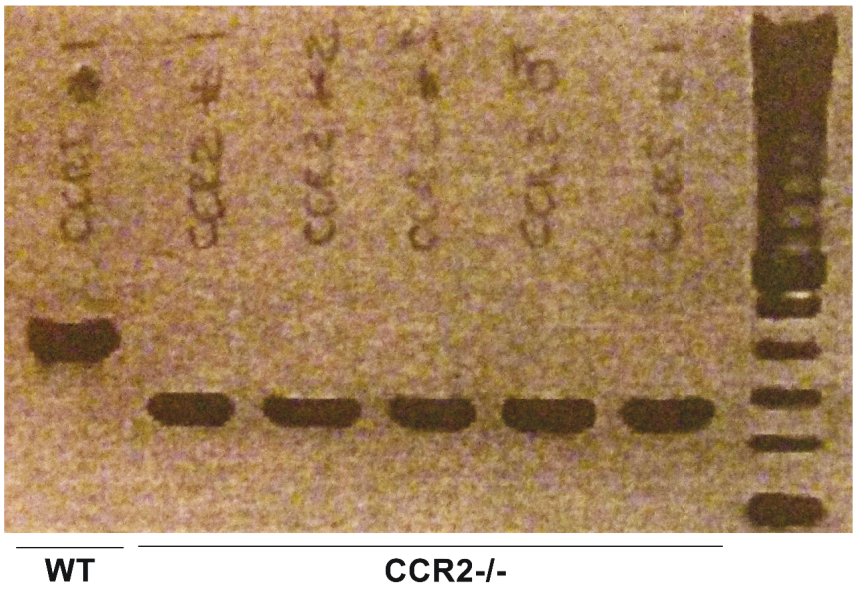
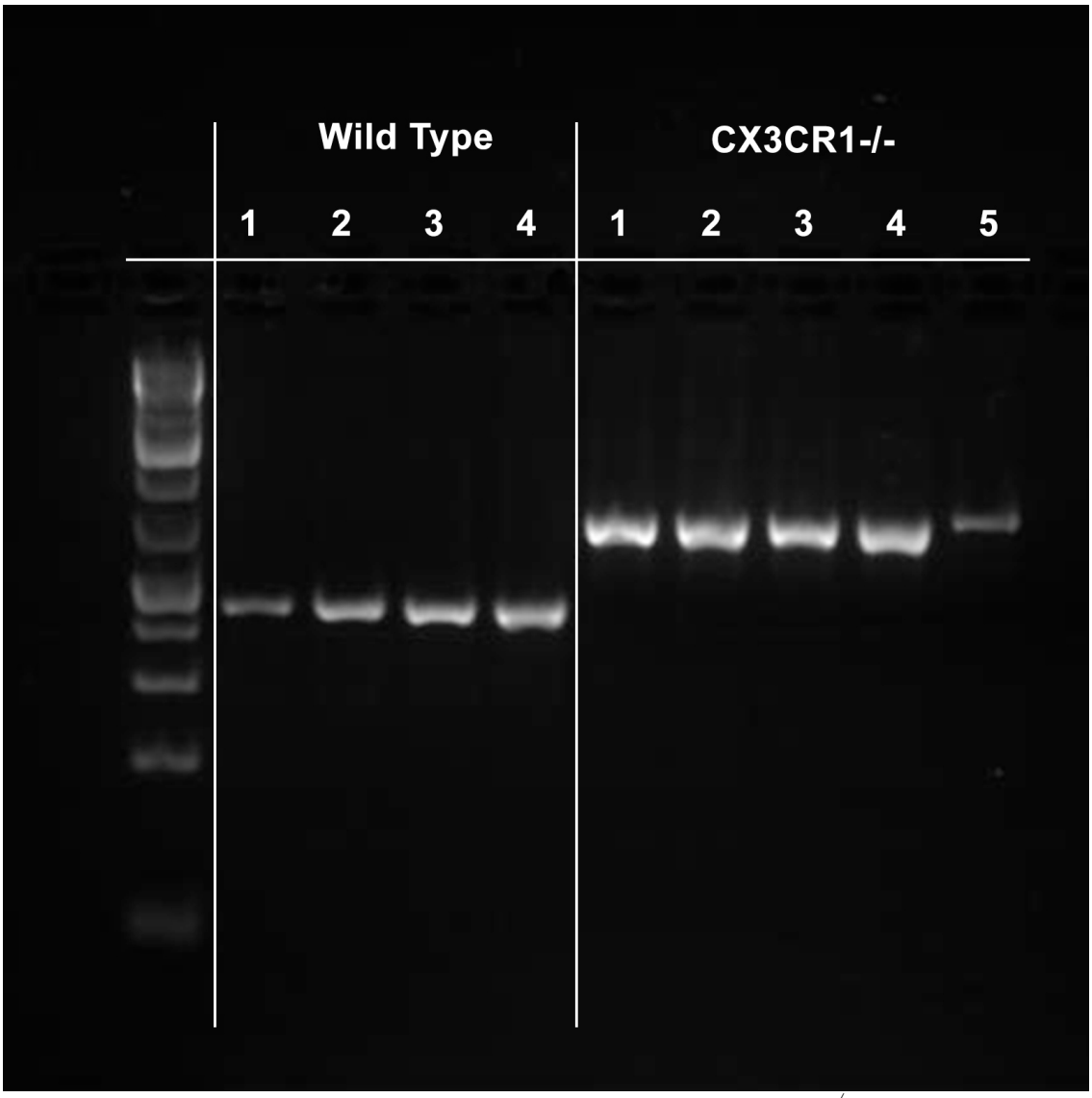
APPENDIX A - GENOTYPING



Appendix A - Figure 1 – Results from genotyping WT and CCR2$^{-/-}$ mice for CCR2. WT mice exhibited the full length gene (424 BP), while CCR2$^{-/-}$ exhibited a disrupted gene (280 BP), which was consistent with ongoing genome analysis in the breeding colony.



Appendix A - Figure 2 – Results from genotyping WT and CX3CR1$^{-/-}$ mice for CX3CR1. Consistent with previous reporting, WT mice exhibited the full length gene (10 kBP), while CX3CR1$^{-/-}$ exhibited a gene disrupted through the replacement of a fragment of the start codon with a neomycin resistance gene (12 kBP).

APPENDIX B – COPYRIGHT PERMISSIONS

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Date: May 26, 2016 at 12:21 PM
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Cc: Sarah Keith sarah.keith@library.tmc.edu, Winkler, Jason (ELS-PHI) (j.winkler@elsevier.com) j.winkler@elsevier.com

BL

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Best,
Jason

APPENDIX C – ENDNOTES

[i] Atria also contain baroreceptors for communicating sudden decreases in blood pressure to the hypothalamus. In addition, the atria are also home to the sinoatrial node – the origin of the heart's electrical signals.

[ii] Also known as heart failure with preserved ejection fraction.

[iii] Mechanical unloading of failing hearts is a relatively novel therapeutic approach that may permit the heart to repair under reduced stress conditions. Ventricular assist devices aid the heart in circulating blood. In turn, the heart does not have to contract with the same force, thus preventing the heart from having to work and repair simultaneously.

[iv] This is an area of active debate. While cardiomyocytes do not readily proliferate like some other cell types, there may be subsets of progenitor cells capable of replacing some lost cardiomyocytes. Importantly though, there does not seem to be a sufficient number of such cells to repair entire areas lost to infarct in normal individuals.

[v] AngII metabolite Ang-(1-7), which is generated via the enzyme ACE-II, can also bind the MAS1 oncogene receptor. In brief, AT4R is primarily expressed on vascular endothelial cells and is believed to play a role in the pro-coagulation effects of AngII by inducing increased expression of pro-coagulant factors and is not the focus of this thesis.

[vi] The role of CXCL12, also known as SDF-1, is a bit more ambiguous: this pleiotropic chemokine is often associated with the recruitment of progenitor cell populations from the bone marrow. In the case of CVD, upregulation of CXCL12 is believed to be involved in the recruitment of a particular progenitor cell population termed endothelial progenitor cells. In addition, CXCL12 is associated with cardiomyocyte survival in models of infarct and ischemia. The only non-ischemic cardiac models that potentially implicate CXCL12 in progenitor cell migration involve the contested cell type termed fibrocytes, but the evidence is unclear.

[vii] Evidence indicates that $CD4^+$ T-cells are responsible for the oxidative stress involved in vascular dysfunction – the details of which are beyond the scope of this thesis

[viii] NO promotes vasodilation

[ix] Fibrocytes are a contested cell type that I will be discussing further in the section on monocyte and MΦ phenotypes.

[x] CD11b is also expressed strongly on neutrophils and thus, this method of monocyte/MΦ depletion is relatively non-specific

[xi] This demand does not necessarily imply that the strength of the inflammatory response is warranted.

[xii] The specifics of DAMP release and signaling are beyond the scope of this thesis, but were important in highlighting the pro-inflammatory response in cardiac healing.

[xiii] Lymphocytes exposed to AngII produce IFN-γ and AT1R blockade significantly reduces lymphocyte IFN-γ production. These effects are believed to be mediated through $CD4^+$ T-helper cells, which have also been shown to have a role in AngII-mediated hypertension. AngII-exposed $Rag1^{-/-}$ mice exhibit reduced hypertension in a process dependent on T-cell infiltration into the vasculature. The production of IL-12p35 by MΦ may be upstream of these $CD4^+$ T-helper cell effects.

[xiv] While not ideal, M1 activation was induced through the collection of MΦ following peritoneal thioglycollate injection.

[xv] T-regulatory cells have also been shown to have a protective role in AngII and hypertensive models, which may be mediated by their production of IL-10

[xvi] To the best of my knowledge, no study has specifically shown M2 MΦ producing TGF-β in the myocardium though.

[xvii] Neutrophils also express FcγR and they're adhesion to the endothelium is inhibited by SAP

[xviii] In the AMD3100 study, we had to switch to CD45 as the hematopoietic marker because the CD133 antibody was suddenly discontinued with reference to non-specificity and no viable replacement could be purchased.

[xix] Immunohistochemistry was only used for CX3CR1 labeling.

[xx] Further characterization, including proper quantification, was impeded by the discontinuation of $CX3CR1^{-/-}$ mice by Taconic.

[xxi] The use of yang (positive) is intended to reflect the fact that CX3CR1 appeared more beneficial to retaining an M2 rCMΦ phenotype. In contrast, the use of yin (negative) reflects the fact that CCR2 could be detrimental to retaining an M2 rCMΦ population.

[xxii] IL-12 and IL-35 share a common p35 subunit.

[xxiii] Again, one cannot separate this observation from age. The animals in these experiments were 10-12wk old female mice.