BRIDGING THE GAP BETWEEN BASIC SCIENCE AND CLINICAL TRANSLATION:
UNDERSTANDING THE ASSOCIATIONS BETWEEN MONOCYTES/MACROPHAGES AND INFLAMMATION IN PATIENTS UNDERGOING CARDIAC SURGERY

by

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To my family and friends for their constant love and support that helped me get to where I am today.
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ABSTRACT

Cardiovascular disease (CVD) is the leading cause of death worldwide. Work from our lab group and others has shown that monocytes/macrophages are key mediators in the inflammatory response involved in CVD and the development of myocardial fibrosis. Inflammation is characterized as a double-edged sword. While its classical response is healing/repair, it may have detrimental effects on the inflamed tissue. In this thesis, we explored the significance of circulating monocytes and cardiac resident macrophages in patients undergoing cardiac surgery. We demonstrated a pro-inflammatory shift in monocyte phenotype in blood post-surgery as was defined by CD14++CD16+ expression, likely due to the use of cardiopulmonary bypass (CPB). We were also, able to correlate our CD16 data with neutrophil-to-lymphocyte ratio (NLR), a biomarker used for clinical outcomes assessment. Additionally, we were able to successfully isolate cardiac macrophages from human right atrial appendages that were significantly different from circulating monocyte phenotypes and correlate these findings with age and atrial fibrillation. This novel study has outlined the significance of studying monocytes/macrophages in the context of CVD and has helped set a path for further human macrophage research opportunities, in order to advance our understanding of their role and potential therapeutic implications.
**LIST OF ABBREVIATIONS USED**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Ang-II</td>
<td>Angiotensin-II</td>
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<tr>
<td>ANP</td>
<td>Atrial natriuretic peptide</td>
</tr>
<tr>
<td>CABG</td>
<td>Coronary artery bypass grafting</td>
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<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
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<tr>
<td>CBC</td>
<td>Complete blood count</td>
</tr>
<tr>
<td>CD11b</td>
<td>Cluster of differentiation molecule 11B</td>
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<tr>
<td>CD14</td>
<td>Cluster of differentiation 14</td>
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<td>CD16</td>
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<td>CD62L</td>
<td>L-selectin</td>
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<tr>
<td>CD68</td>
<td>Cluster of differentiation 68</td>
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<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CPB</td>
<td>Cardiopulmonary bypass</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
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<td>Cardiovascular disease</td>
</tr>
<tr>
<td>CX3CR1</td>
<td>CX3C chemokine receptor 1</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3′- diaminobenzidine</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EF</td>
<td>Ejection fraction</td>
</tr>
<tr>
<td>HF</td>
<td>Heart failure</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule 1</td>
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IHD  
Ischemic heart disease

IL-1  
Interleukin 1

IL-10  
Interleukin 10

IL-18  
Interleukin 18

IL-6  
Interleukin 6

LDL  
Low density lipoprotein

LOS  
Length of stay

LPS  
Lipopolysaccharide solution

LVAD  
Left ventricular assist device

Ly6C  
Lymphocyte antigen 6 complex, locus C1

MCP-1/CCL2  
Monocyte chemoattractant protein 1

MFI  
Mean fluorescence index

MI  
Myocardial infarction

MLR  
Monocyte-to-lymphocyte ratio

MMP  
Matrix metalloproteinase

MNR  
Monocyte-to-neutrophil ratio

NET  
Neutrophil extracellular trap

NK Cells  
Natural killer cells

NLR  
Neutrophil-to-lymphocyte ratio

NYHA  
New York health administration

PBMC  
Peripheral blood mononuclear cell

PBS  
Phosphate buffered saline

PCT  
Procalcitonin
PLR  Platelet-to-lymphocyte ratio
PMN  Polymorphonuclear leukocytes
POAF Post-operative atrial fibrillation
PS   Phosphatidylserine
REACH Restitution Enhancement in Arthritis and Chronic Heart Disease
ROS  Reactive oxygen species
SEM  Standard error mean
SR   Sinus rhythm
STS  Society of thoracic surgeons
TGF-β Transforming growth factor beta
TIMP Tissue inhibitor of metalloproteinase
TLR7/8 Toll-like receptor 7/8
TNFα Tumor necrosis factor alpha
VCAM-1 Vascular cell adhesion protein 1
WBC  White blood count
WT   Wild type
αSMA Alpha smooth muscle actin
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Life has a funny way of working out just when you start to believe it never will. I am grateful for the countless individuals who were by my side throughout this journey of accomplishing this degree. I did not think I had it in me.
CHAPTER 1 - INTRODUCTION

1.1 Cardiovascular Disease

Cardiovascular disease (CVD) is one of the leading causes of death worldwide. In fact, it accounts for an estimated 17 million deaths per year\(^1\). CVD is a general term used to describe a group of heart and blood vessel disorders including, arrhythmias, heart valve disease, coronary artery disease, and heart failure\(^2\). With that being said, my research aimed to better understand the inflammatory cellular components involved in heart failure in patients undergoing heart surgery.

1.1.1 Heart Failure

Heart failure (HF) is defined as the inability of the heart pump efficiently and supply peripheral tissues with their required level of blood and oxygen for their metabolic needs\(^3, 4\). It leads to a clinical syndrome typically characterized by symptoms that include dyspnea and ankle swelling and signs such as raised jugular venous pressure, tachycardia, peripheral edema, or cardiac death. However, HF develops secondary to left ventricular systolic or diastolic dysfunctions\(^5\). The most common causes of heart failure are a group of diseases grouped under the umbrella of coronary artery disease (CAD), also known as ischemic heart disease\(^6-8\). After an ischemic event occurs, the blockage of coronary vessels leads to decreased blood flow and oxygen supply to cardiac and peripheral tissues. In the event of insufficient restoration of this occlusion, myocardial infarction ensues, which if not resolved may lead to low-nutrient tissue injury and cardiac death. In the case of restored blood flow to the ischemic tissue, reperfusion injury occurs, which results in cardiac inflammation, adverse cardiac remodeling and eventually heart failure\(^9\).
Tissue remodeling is defined as the initial stage of healing, or towards it, where the myocardium undergoes a series of structural, cellular, neuro-humoral, and molecular adaptations coordinating in order to maintain physiological function \(^{10}\). In cases of normal healing, the inflammatory response shifts towards an anti-inflammatory or pro-fibrotic phenotype leading to resolution of inflammation and healing to recover cardiac function. However, in cases of chronic stress or improper repair, inflammation persists and leads to cardiac hypertrophy, increased sympathetic activity, formation of scar tissue, and inadequate cardiac blood pump, which eventually results in HF \(^3,^4,^11\). Therefore, HF in a general sense is an endpoint cardiovascular condition defined as the functional inability of the heart to effectively pump blood. Despite the numerous surgical and therapeutic advances in HF prevention and treatment, HF remains to be one of the leading disease burdens globally \(^{12,^13}\). It is estimated that 1 in 5 Canadians over the age of 40 years are said to develop HF at some point in their lifetime \(^4\).

1.1.2 Myocardial Fibrosis

In essence, the heart is constantly attempting to maintain a homeostatic environment in order to meet the blood demands of peripheral tissues, while maintaining its own structural integrity \(^{14,^15}\). Under stressed conditions like myocardial infarction, the myocardium is faced with an increased burden to try to maintain both function and organ structure, which may result in what is known as myocardial fibrosis \(^{16-18}\). Myocardial fibrosis is characterized by the excessive deposition of non-contractile ECM proteins (largely fibrillar collagen \(^{19}\)) in response to injury \(^{17}\). Despite, ECM proteins forming the backbone of tissue organization and structure, excessive accumulation (whether over-deposition, or lack of effective removal) can be detrimental to the contractility of the heart \(^{20}\). In normal physiology, ECM proteins are constantly
being replaced by a variety of enzymes like matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) to regulate deposition and degradation \(^{20,21}\). Therefore, myocardial fibrosis can be outlined as a result of two processes, (i) an imbalance in the deposition and degradation of ECM proteins and more importantly (ii) the inability of these proteins to contract \(^{22}\). In reality, cardiac fibrosis is seemingly a pathological feature that impairs cardiac function ultimately leading to heart failure, however, it could be thought of as an overcorrected repair/healing mechanism \(^{23}\). Moreover, cardiac fibrosis is initiated as a reparative response to stabilize the injured heart and aid in maintaining its function, while it is still pumping, by depositing a variety of ECM proteins to compensate for cardiomyocyte loss (apoptotic/necrotic) and structurally reinforcing the interstitial spaces between them \(^{24-26}\). However, excessive deposition of ECM proteins leads to abnormal thickening of the cardiac muscle tissue, known as cardiac fibrosis, which has been linked to myocardial stiffness, systolic and diastolic dysfunction \(^{26}\). It is important to note that this is a rather oversimplified description of the mechanisms and factors involved in cardiac fibrosis. Conversely, experimental evidence shows that the inhibition of fibrosis post MI has resulted in maladaptive structural changes that can lead to organ rupture in mice \(^{27}\). Therefore, cardiac fibrosis still remains to be a crucial poorly understood area of CVD.

### 1.2 Inflammation

So far, I have outlined the overarching disease pathology of HF and myocardial fibrosis, however, my research aimed to better understand the inflammatory portion of these cardiovascular states. Inflammation, in simple terms, is an orchestrated series of cellular and molecular events necessary to allow tissue healing as part of the normal response to injury. It can
be thought of as a vital destructive process meant to protect the body from harm. So how can something destructive be protective? Well, when an injury/infection occurs, a stimulus is elicited which involves a series of events including the infiltration of circulating leukocytes to the injured site, the activation of tissue leukocytes and the production of inflammatory mediators, such as cytokines and chemokines, to further recruit additional leukocytes, fight off harmful pathogens and heal the injured site. Traditionally, it has been difficult to differentiate between the normal beneficial inflammatory response needed for healing and the detrimental components of it that can be linked to disease progression. Therefore, it has led various investigators to further these findings primarily aimed to solve better understand inflammation in the context of heart disease.

In the context of ischemic heart disease, inflammation is a key player in the removal of dead cells and debris post injury. Yet, while serving their protective role, inflammatory responses almost invariably contribute to tissue damage. Moreover, inflammation is beneficial with that respect to prepare for the following wave of healing. However, in its chronic state, it is likely to be maladaptive, degrade normal tissue and promote scar tissue formation. Therefore, it is important to understand the different types of inflammation and inflammatory states.

1.2.1 Types of Inflammation in CVD

(a) Acute Inflammation

An acute inflammatory response could be thought to have double-edged sword properties. While it has an initial protective role in host defense, it may have detrimental effects on the myocardium due to its general/imprecise area of effect. Acute inflammation can be induced by high intensity sterile or non-sterile stimuli. Sterile inflammation is triggered by
physical, chemical, or metabolic noxiae, while non-sterile is most commonly triggered by invading pathogens such as viruses or bacteria \cite{33, 34}. In the event of sterile trauma such as ischemia, cardiomyocytes undergo necrosis initiating a strong inflammatory cascade by releasing a variety of pro-inflammatory cytokines like TNF-$\alpha$, IL-6, IL-1, and IL-18 that stimulate the endothelium in order to allow cross-membrane migration of leukocytes from circulation to the injured site \cite{34-36}. Furthermore, the injured site is rapidly (within minutes) infiltrated with an initial wave of polymorphonuclear cells (PMN) dominated by neutrophils, which remove dead/necrotic cells forming a pro-inflammatory environment by the release of reactive oxygen species (ROS) and extracellular traps (NETs) \cite{37, 38}. Since neutrophils have a short life span, they undergo apoptosis within 12-18 hours, leaving their inflammatory environment behind. Then comes the second wave of recruited cells consisting of mononuclear leukocytes, mostly monocytes, that again play double roles in both the progression and resolution of the inflammatory response. The goal of this section was to give you an overview of acute inflammation, I will be talking more about the complex monocyte role(s) in the coming sections of this introduction.

\textit{(b) Para-inflammation (low-grade chronic inflammation)}

The next type of inflammation is termed para- inflammation due to its unconventional activation and has characteristics between the basal/homeostatic condition and inflammatory states \cite{39}. Moreover, it is not triggered by any massive tissue injury or infection, but is generally activated by tissue stress (such as the presence of hyperglycemia, hypercholesterolemia, or an excessive amount of free radicals) resulting in an inflammatory response of lower intensity than acute inflammation \cite{30}. Innate tissue-resident macrophages are said to sense stressed cells and act
by mounting an immune response to restore tissue homeostasis with little to no circulating leukocyte recruitment. Unlike classical inflammatory responses, signals inducing para-inflammation still remain poorly understood. The physiological outcome of para-inflammation is aimed to helping cells adapt to noxious conditions while avoiding tissue malfunction, making it an important immunological mechanism. Moreover, in cases of persistent tissue malfunction, para-inflammation can develop into chronic inflammation preventing tissues from adapting gradually, becoming a disease pathology. Therefore, para-inflammation can be described as low-grade chronic inflammation. Which leads to the third and final type of inflammation, which is chronic inflammation.

**(c) Chronic Inflammation**

As described previously, chronic inflammation is a prolonged inflammatory state that coexists with tissue injury and reparative attempts. You can think of chronic inflammation as “an abandoned turned on lightbulb”, where nobody is making use out of its light and the longer it stays on the more likely the filament will break/melt. In the context of the heart, it is characterized by its continuous attempt at repairing an injury that does not exist and the longer it persists, the higher the risk of developing CVD. Atherosclerotic plaque formation is a common result of cardiac chronic inflammation, where persistent lipoprotein retention and inflammatory cellular recruitment amplifies the formation of layers of foam cells. This results in a positive feedback loop between inflammation and the pathological process it accompanies that disables the resolution of inflammation. Developed plaque may remain asymptomatic leaving the patient with sub-clinical condition, become obstructive inducing coronary heart disease (CHD), or elicit acute thrombosis which may lead to ischemic heart disease (IHD). Traditional risk
factors such as high cholesterol concentration, smoking, diabetes mellitus, and hypertension are triggers of chronic inflammation 45. Therefore, it is important to understand the immune components that play important roles in the regulation of inflammation that when dysregulated may lead to the degradation of normal tissue resulting in its replacement with scar tissue (fibrosis) 46-49.

1.3 Leukocytes & Acute Inflammation

Slowly after an ischemic event, endothelial cells become leaky and present a variety of adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1), CD62L, and β2-integrins that trigger leukocyte extravasation 50. Neutrophils are amongst the first responders that arrive to the infarcted myocardium within minutes of injury and accumulate in order to destroy pathogens and dead tissue while initiating the inflammatory response 28. They dominate the overall leukocyte population with a life span of 5-8 hours 51, 52. Neutrophils release a wide range of pro-inflammatory cytokines, such as tumor necrosis factor (TNF-α) and interleukin 1 beta (IL-1β) and chemokines, like monocyte chemotactic protein-1 (MCP-1/CCL2), that in turn amplify the inflammatory response by recruiting and activating T lymphocytes, monocytes, natural killer cells (NK) and dendritic cells (DC) 52. During this response, neutrophils accumulate at the injured site, which has been shown to be detrimental to the myocardium in MI disrupting endothelial homeostasis and inducing oxidative stress 53, however, they eventually undergo apoptosis and are removed by recruited monocytes. The second wave, as mentioned previously, consists of the recruitment of mononuclear cells to the site of inflammation via chemotactic gradients. Mononuclear cells include lymphocytes, but mainly circulating monocytes.
1.3.1 Monocytes/Macrophages

Monocytes account for 3-8% of peripheral blood leukocytes. Due to their phenotypic spectrum of expression, monocytes are able to play a variety of roles in the inflammatory/healing process. They range from pro-inflammatory, that promote the inflammatory mediators, to anti-inflammatory, that promote healing/pro-fibrotic factors. Also, circulating monocytes have the ability to differentiate into macrophages and dendritic cells (DCs) to further play different roles in tissue. 

Monocytes/macrophages can be divided into different sub-populations on the basis of their surface receptor expressions. In humans, monocyte/macrophage subsets can be characterized by their variable expression of lipopolysaccharide receptor CD14 and Fcγ-III receptor CD16. Traditionally divided into classical (CD14++CD16−, M1, Classical), intermediate (CD14++CD16+), and non-classical (CD14+CD16+, M2, non-classical) monocytes/macrophages. The M1/classical subset performs the functions classically associated with monocytes/macrophages such as strong phagocytic activity and promoting inflammation. The M2/non-classical subset got its name by fulfilling unconventional roles as a monocyte/macrophage. Furthermore, this subset carries out the anti-inflammatory and pro-fibrotic response to cardiac injury. In between these two spectrums comes a generally labeled phenotype called the intermediate population. This population is said to play important roles in cardiac inflammation, however, remains poorly understood/poorly studied. Labeling the different monocyte/macrophage subsets is an oversimplification of the infinite spectrum of activation they are characterized by.
1.3.2 Monocytes in Circulation

Initially, monocytes in circulation were considered leukocytes that sense the environment and replenish the pool of tissue macrophages and dendritic cells when needed \(^{58}\). It was not until recently, studies have discovered that monocytes are a heterogenic population that can be divided into specific subsets serving various functions \(^{59}\). The classical/M1, CD14++CD16-, subset is the most abundant phenotype found in circulation \(^{54,60,61}\). Classical monocytes have major functions in phagocytosis. They highly express CCR2, a chemokine receptor mediating monocyte chemotaxis during inflammation, and CD62L (L-selectin), a cell adhesion molecule, and low levels of CX3CR1, a chemokine receptor mediating resident monocyte accumulation \(^{60}\).

Compared to classical monocytes, the human CD14++CD16+ (intermediate) monocyte subset has a lower phagocytic capacity, expresses lower levels of CCR2, expresses higher levels of CX3CR1, and produces less reactive oxygen species (ROS) \(^{54}\). Several clinical studies have demonstrated the importance of the intermediate monocyte phenotype as a result of its increased levels in human inflammatory diseases, including rheumatoid arthritis, CAD, atherosclerosis, and Crohn’s disease \(^{62-64}\). Moreover, higher circulating CD16+ monocyte levels were shown to positively correlate with concentrations of atherogenic lipids \(^{65}\) and plaque vulnerability \(^{66}\), whereas they were shown to negatively correlate with cardiac function such as ejection fraction post-acute MI \(^{64}\).

One study suggested that elevated non-classical, CD16+ monocytes, correlated with both obesity and subclinical atherosclerosis in low risk individuals \(^{44,58}\). Moreover, investigators have suggested that the inflammation underlying both atherosclerosis and acute coronary syndromes is strongly related to the monocyte heterogeneous roles, where monocytes play important roles in
both initiation and destabilization of atherosclerotic plaques and contribute to infarct healing by residing in the myocardial tissue \(^{67-69}\).

1.3.3 Cytokine Dilemma in Monocyte Subset Characterization

To further demonstrate how complicated monocyte characterization can be, tremendous controversies exist in literature trying to understand their inflammatory role and individual activation states. One such controversy is the poor agreement on their respective cytokine production. Before the separation of monocyte subsets into classical, intermediate, and non-classical, studies were combining the intermediate and non-classical phenotypes into CD16\(^+\) monocytes. Investigators found that CD16\(^+\) monocytes were the main producers of TNF-\(\alpha\) after LPS stimulation \textit{in vitro} \(^{70,71}\). After the separation of the CD16\(^+\) super-population into intermediate and non-classical sub-populations, Cros et al. \(^{72}\) demonstrated that isolated non-classical monocytes were poor producers of several cytokines in response to LPS, which included TNF-\(\alpha\), IL-1\(\beta\), CCL2, IL-10, IL-8, IL-6, and CCL3 while responding strongly to TLR7/8 ligands and instead intermediate monocytes \textit{in vitro} produced the most TNF-\(\alpha\), IL-1\(\beta\) and IL-6. Rossol et al. \(^{73}\) further confirmed these findings by showing that the intermediate monocyte subset \textit{in vitro} produced the most TNF-\(\alpha\) and IL-1\(\beta\) after LPS treatment. However, in the same year, Wong et al. \(^{74}\) showed that isolated non-classical monocytes produced the highest levels of TNF-\(\alpha\) and IL-1\(\beta\) after LPS stimulation. In a more recent study, Boyette et al. found that the intermediate population produced similar levels of TNF-\(\alpha\) to the classical subset, while having a significantly lower IL-1\(\beta\) secretion post stimulation with Flagellin \(^{75}\).

Similarly, identification of the major IL-10 producers have been inconsistent in literature. Skrzelowska-Moncznik et al. \(^{76}\) identified isolated intermediate monocyte population as the main
producers of IL-10 after LPS stimulation. However, recent studies have shown that classical monocytes are perhaps the major producers of IL-10 \textsuperscript{72,74,77}. Moreover, another study published that their findings failed to reach significance between the different subsets in terms of IL-10 secretion \textsuperscript{75}. Therefore, monocytes are not as easy to understand as pro-inflammatory and anti-inflammatory, the role of each specific monocyte subset still remains controversial and will require further investigation. Which sets the stage for my next section of background leading up to my study, what is heart surgery with cardiopulmonary bypass and how does it relate to inflammation and disease pathology.

\textit{1.3.4 Cardiopulmonary Bypass Systemic Inflammation}

To switch gears and avoid any confusions of where I am heading with this section, I will be giving you a quick overview of cardiac surgery, which is a common feature of the patients I conducted my research on. In a nutshell, I was studying cellular markers of inflammation from blood and atrial samples from patients undergoing elective (non-emergent) heart surgery. The study population as a whole shares a common feature of utmost importance in surgery induced inflammation, known as cardiopulmonary bypass (CPB). CPB is a technique used to perform the majority of cardiac revascularization surgeries. Since the 1950s, cardiopulmonary bypass (CPB) has been extensively used in various cardiac surgery procedures \textsuperscript{78}. CPB is utilized to temporarily replace heart and lung function of circulating blood and oxygen to the body while surgery is being performed \textsuperscript{79}. However, as mentioned previously, it is known to induce the release of pro-inflammatory cytokines and the activation of various leukocytes resulting in a systemic inflammatory response and in some cases, raise the risk of post-operative complications \textsuperscript{78,80}. The pathophysiology of CPB-induced inflammation is multifactorial and has not yet been
described to follow a specific mechanism, but for simplicity’s sake, it can be divided into two broad phases, (1) early and (2) late.

The early phase is characterized by the contact of blood with non-endothelialized surfaces resulting in an interplay between humoral and cellular responses. These responses include the activation of (1) the complement cascade causing capillary permeability, vasodilation and the activation of neutrophils and platelets, (2) the activation of both intrinsic and extrinsic coagulation cascades, and fibrinolytic system, which in turn activate endothelial cells, platelets, neutrophils, monocytes and lymphocytes. As CPB duration increases, blood contact activation has been shown to decline as the CPB circuit becomes more biocompatible.

The second/late phase of inflammation is caused by the reperfusion of the ischemic myocardium (unclamping of the ascending aorta) and the release of endotoxins. These are responsible for endothelial injury, neutrophil activation, release of interleukins and the activation of complement cascades. Aside from different phases of inflammation, CPB is associated with increased levels of soluble adhesion molecules believed to be responsible for the dysfunction of multiple organ systems post-operatively. However, the mechanisms by which CPB alters leukocyte recruitment remain unknown. I purposely did not discuss these various cascades and crosstalk mechanisms in great as it is beyond the scope of my thesis, however, the take home message here is that cardiac surgery with CPB is a vigorous trigger of systemic inflammation and can raise the risk of post-operative complications. One of the main areas of my study was circulating leukocyte analysis from samples collected pre-operatively, 5 days after surgery, and at a later follow-up appointment at 90 days, which gave me the unique ability to study circulating monocyte subset shifts via their variable expression of CD14 and CD16.
Despite all the leaps in cardiac surgery advancements, atrial fibrillation (AF) still remains to be a common post-operative complication. Its incidence is between 15% and 50%, depending on patient profile and the type of surgery being done. AF is defined as an irregular cardiac rhythm (or arrhythmia) characterized by rapid atrial depolarization with chaotic fibrillary waves. Post-operative AF tends to occur 2-4 days after the procedure and can in some cases lead to short or long-term consequences. Additionally, it was linked to increased risk of congestive heart failure development and prolonged hospital stay. Therefore, various efforts have been made to identify proarrhythmic precursors in patients going into cardiac surgery, which increase the chance of POAF development. Needless to say, there has not been a single defining mechanism of how POAH is induced, however, several associations with biomarkers have been studied in retrospective studies. Systemic inflammation has been linked to POAF in a study showing that higher pre-operative WBC were associated with a higher risk of developing post-operative AF. Another study demonstrated that preoperative WBC count may independently predict both the incidence and duration of POAF after CABG surgery. Perioperative WBC response, however, has been shown not to associate with the development of POAF. Additionally, the incidence of POAF after CABG surgery was seen to increase with the amount of pre-existing fibrosis in right atrial appendages harvested from patients during surgery. Therefore, our study could potentially add further value to this finding by correlating atrial fibrosis with POAF as well as other variables measured.

1.3.5 Cardiac Macrophages

So far, the prevailing hypothesis is that inflammation if dysregulated, can exacerbate tissue injury and up-regulate the pro-fibrotic response replacing the normal architecture of the
myocardium with non-contractile tissue. Macrophages in particular have been shown to be capable of influencing this complex balance between inflammation and its’ resolution. The role of macrophages in the heart is not limited to promoting inflammation as they can exhibit various activation states including pro-inflammatory, pro-angiogenic, pro-fibrotic, and anti-inflammatory falling under a diverse phenotypic spectrum of expression. Moreover, macrophages represent the tissue-bound form of the monocyte lineage. Until recently, cardiac macrophages have been believed to be solely derived from circulation/monocyte recruitment and absent in the healthy myocardium. It was not until Pinto et al. conducted their study in 2012 that discovered an abundant population of resident cardiac macrophages existing in murine hearts at steady-state. This discovery forced investigators into re-assessing the importance of immune cells in the heart and especially macrophages. Therefore, several studies were conducted to try to understand the role of tissue macrophages and assess their dynamics in both steady and disease states. Since human cardiac macrophage isolations have not been performed prior to my research, animal models are our only source of isolation characterizations, therefore, for the majority of this section, I will be reviewing cardiac macrophage literature from murine-based disease models. As previously mentioned, macrophages and monocytes alike are not uniform cell types and lie on a wide spectrum of expression. The notion of classical vs. non-classical or M1 vs. M2 is only an oversimplification of the dynamic states of activation that we use to describe the various subsets. The various macrophage phenotypes have been shown to individually play important roles in cardiac repair in mice. Most of what we know about their role in-vivo comes from experiments involving the selective depletion of macrophage populations. The M1 subset performs the classical functions associated with macrophages such as strong phagocytic activity and promoting inflammation. Moreover, the loss of cardiomyocytes in
ischemic models requires M1 macrophages to aid in the phagocytosis of apoptotic cells and to promote the recruitment of other leukocytes such as neutrophils to phagocytize necrotic cells. In the absence of M1 macrophages, dead cells in the myocardium are not removed, which handicaps the start of the healing/remodeling phase. Additionally, M1 macrophages were found to produce a wide array of pro-inflammatory cytokines and chemokines, like TNF-α, IL-1β, IL-8 and CCL2. Therefore, one can deduce that M1 macrophage-induced inflammation is necessary for the sufficient removal of dead tissue leading to the initiation of the healing/resolution of inflammation phase. Furthermore, non-selective depletion models have reported that when monocytes and macrophages were depleted, markers of remodeling such as αSMA, Collagen, and TGF-β significantly decreased.

The M2 sub-population of macrophages, being at the other end of the spectrum, carries out anti-inflammatory and pro-fibrotic functions in cardiac disease. Several studies have shown M2 macrophages accumulation in the heart at later stages of healing (~3-7 days post Ang-II induced cardiac injury). And since the M2 phenotype has been associated with the healing phase, its specific depletion led to abnormal and insufficient cardiac remodeling. Furthermore, M2 macrophages were found to constitute to a large proportion of macrophages found in murine hearts at steady-state. This has led to a pool of confusion which raised questions to the origins of these cells, and the role they play in the healthy heart.

Unfortunately, not a lot of literature is out there regarding various macrophage subsets found in human hearts, both in steady and disease states. Also, the majority of the research aimed to characterize leukocytes and inflammation of the myocardium in humans has been limited to routine histology and immunohistochemistry, which are unable to effectively identify resident macrophages. Nevertheless, Azzawi et al. (1997) were one of the first groups to characterize
macrophages in normal human heart sections. With the aid of CD68 immunohistochemistry, they identified that macrophages were found to be more abundant in ventricles than in atria while showing a scattered distribution along interstitial spaces and close to blood vessels. Eight years later, Azzawi et al. (2005) expanded their study into exploring macrophage content in diseased heart tissue. Their CD68 stain findings identified significantly higher macrophage content in all four chambers of diseased hearts when compared to controls. Moreover, they discovered that unlike normal heart tissue, macrophage number in diseased hearts was higher in atrial sections than ventricles.

Macrophage recruitment into the human fibrillating atrium has been investigated in a small study analyzing the migration and adhesion of immune cells using immunohistochemistry in left atrial appendages collected from 16 patients undergoing surgery (5 with sinus rhythm (SR) and 11 with AF). Adhesion and migration molecules such as ICAM-1, VCAM-1 and CCL2 were more prominent in AF patients when compared to SR. Furthermore, CD68+ macrophages comprised the majority of cells seen in the atrial samples, implying that active recruitment of macrophages across the endocardium occurs in fibrillating atria compared to SR. The lack of immune cell isolation and characterization from human myocardial samples was our greatest motivation to pursue this study, as previous work from our lab and others combined have shown the importance of studying resident immune cell populations in the hearts of animals. Which leads me to the next section of this introduction highlighting the various studies done in our laboratory.
1.4 Previous Work Done in Our Lab

Our laboratory has conducted extensive research on leukocytes (mainly macrophages) and inflammation showing their key roles in cardiovascular disease and cardiac fibrosis. A variety of experiments were performed including: in vitro cell culture assays studying cellular differentiation under variable conditions, in vivo time point studies extensively using a well-established Angiotensin-II (Ang-II) infusion model of hypertension-induced myocardial fibrosis, and the use of knockout animals to further understand the phenotypic roles of various macrophage populations. The Ang-II model is a non-ischemic hypertension induced myocardial fibrosis model, where continuous Ang-II is infused into mice over a period of time. This model enabled our lab to better understand the relationship of hypertension and fibrosis in terms of monocytes/macrophages. Significant monocyte infiltration was shown to accumulate as early as 1 day post infusion, therefore, preceding the development of myocardial fibrosis, which was shown to develop by day 3 \(^{49, 118-121}\). Conversely, CCR2 KO animals had significantly lower monocyte infiltration into the myocardium than normal rodents, and were shown to be protected from Ang-II induced myocardial fibrosis, thus highlighting the importance of the CCR2 chemotactic axis in recruitment and fibrosis development \(^{103}\). Furthermore, CCR2 KO resident macrophages were shown to favor a more anti-inflammatory phenotype characterized by their lower Ly6C expression, reduced TNF-α and increased IL-10 compared to WT \(^{103}\). Together these findings suggest the importance of M2 resident macrophages as therapeutic targets in the protection against Ang-II induced myocardial fibrosis. Therefore, the novelty of isolating resident macrophages from human atrial samples is key for us to further understand and translate the apparent role of cardiac tissue macrophages in myocardial fibrosis.
1.5 Rationale & Objectives

1.5.1 Rationale

The roles of monocytes and macrophages in cardiovascular disease have been extensively studied in animal-based studies; however, limited research has been done in humans, especially those involving their characterizations using surface markers in the context of cardiac surgical revascularization \(^{115-117}\). There has been growing evidence supporting the use of surface markers present on monocytes namely CD14 and CD16 expression as indicators of disease, prognosis, and adverse outcomes \(^{60, 62, 71, 122-125}\). Therefore, studying blood and heart tissue samples from patients undergoing cardiac surgery could expand our understanding of leukocyte changes as well as offering an avenue for clinical based correlations.

Our lab group has extensively studied and characterized cardiac macrophages in ischemic/steady-state murine models in the past and demonstrated their mechanistic role in cardiac fibrosis and heart failure. However, cardiac macrophage role/characterization has not yet been explored in human samples. Therefore, our novel isolation of cardiac macrophages from human atrial tissues as well as identification of their phenotypic spectrum of expression could enable us to understand their role in the disease pathology.

As mentioned previously, inflammation plays an important role in cardiovascular disease, specifically chronic heart disease, therefore, cellular components, circulating monocytes and cardiac macrophages in particular, of the inflammatory response may provide avenues for therapeutic targets.
1.5.2 Objectives

The objectives of this study were to: (1) Identify and characterize circulating monocytes from patient undergoing heart surgery, assess the effect surgery has on circulating leukocytes, and correlate with clinical data (CBCs, patient information, post-operative outcomes), (2) Determine the feasibility of macrophage isolation from human atrial samples and separate patient groups by using age and atrial fibrillation as grouping criteria to further understand their role.
CHAPTER 2 - MATERIALS AND METHODS

2.1 Study Population

Patients were enrolled prospectively based on admission to the QEII Health Science Centre (Halifax) with ischemic heart disease requiring surgical revascularization. Informed consent was obtained for all participating patients and all patients received standard care in terms of treatment. Patient enrollment was determined based on participating surgeons and availability of the laboratory team to perform immediate processing of blood and tissue sampling. This also meant that the majority of patients had a history of prior acute coronary syndrome (ACS) and were recovering from their myocardial injury at the time of study. The scope of this research encompasses two research programs, (1) Restitution Enhancement in Arthritis and Chronic Heart disease (REACH) and (2) Atrial Natriuretic Peptide (ANP).

As part of the REACH project, blood and atrial tissue were obtained from post-MI patients undergoing surgery. The purpose of this study was to investigate the critical processes associated with successful disease resolution in post-MI inflammation. Blood samples obtained pre-operatively (D0), at prior to hospital discharge 5-7 days post-surgery (D5), and at a later follow up appointment >42 days post-operatively (D90), were used to conduct the blood portion of my study. It is important to learn these abbreviations, as they are used all throughout the thesis.

The purpose of the ANP study was to investigate the electrical signaling in patients with valvular heart disease in both the presence and absence of atrial fibrillation. Cardiac samples were pooled from both studies in order to maximize sample size. Figure M1 shows a Venn diagram of the patient population from both studies and how they were incorporated into each side of the research.
2.2 Study Procedures

*Intraoperative Management*

In all patients, blood samples were collected prior to skin incision and the atrial samples were taken at the time of cannulation. Blood tubes used contained sodium heparin in order to allow the collection of heparinized plasma. Atrial tissue samples were divided into various pieces for multiple immediate and future analyses: (1) cellular isolation, (2) Histology (paraffin)/immunohistochemistry, (3) RNA/protein. All samples not used for immediate use were stored in a -80°C freezer. Cardiac surgery was performed with CPB and anticoagulation was achieved using intravenous heparin given at a dose of 400 IU/kg with a target activated clotting time greater than 450 s. Antifibrinolytic agents were given to all patients and consisted mainly of

![Figure M1- Venn diagram outlining the source and number of patient samples used in each study](image)
tranexamic acid. Intermittent cold blood cardioplagia was delivered in an antegrade or retrograde manner based on surgeon preference. Protamine sulfate was given for reversal of heparin in all patients.

Patients also received routine baseline 12-lead electrocardiograms upon admission to the cardiovascular intensive care unit. Resumption of routine postoperative medications occurred as indicated and included anti-platelet agents within 24 hours, statins, and β-blockers. All patients were monitored in the cardiac intensive care unit for a minimum of 24 hours and an intermediate care unit for an additional 24 hours. Therefore, a minimum of 48 hours of continuous cardiac monitoring was used in all patients to detect atrial fibrillation. New atrial fibrillation was defined according to the Society of Thoracic Surgeons (STS) definition as any episode of atrial fibrillation occurring in-hospital after CABG surgery that required intervention (beta-blockers, calcium channel blockers, amiodarone, anticoagulation, or cardioversion).

Variable Selection

Preoperative clinical characteristics of interest included age, gender, New York Heart Association (NYHA) functional class, urgency of surgery (urgent if required within 24 h, in-hospital urgent if the patient required hospitalization until the time of surgery, and elective or outpatient), and diabetes. Intraoperative variables included pump time and clamp time. Blood samples were obtained pre-surgery, prior to hospital discharge (5-7 days post-surgery), and >42 days post-surgery. Standard blood analysis, including complete blood counts, neutrophil-to-lymphocyte ratio, platelet-to-lymphocyte ratio, peak troponin (collected within 24 h of surgery), and monocyte counts were obtained.
2.3 Atrial Tissue Processing

Atrial samples were all processed immediately for the purposes of cellular isolation for flow cytometry. In brief, tissue samples were finely minced into manageable pieces with razors followed by mechanical and enzymatic digestion using a cocktail of enzymes consisting of Collagenase II (1 mg/mL, Worthington Biochemical Corp., Lakewood, NJ, USA) and dispase (2 mg/mL) in serum free Hank’s Balanced Salt Solution (HBSS, 2mmol/L l-glutamine, 100 U/mL penicillin and 100mg/mL streptomycin), typically 5 mL of solution per sample. In order to activate the digestive enzymes, samples were put on a bench shaker-incubator at 37°C for 45 minutes at 250RPM. Further, samples were rinsed in complete HBSS (HBSS-C, 10% heat-inactivated fetal bovine serum) solution and pushed through a 70 μm filter and washed twice with HBSS-C for 10 min at 400 rcf at 4°C. Cell isolates were resuspended in FACS buffer (Dulbecco’s phosphate buffered saline, DPBS, 1% bovine serum albumin, 0.1% sodium azide, NaN₃). Then cell counts and viability were assessed via hemocytometer and trypan blue. Following the counts, cell isolates were purified over a Lymphoprep Ficoll-Paque gradient (GE Healthcare UK, Ltd., Buckinghamshire, UK) and centrifuged for 30 min at 400 rcf with no brake and minimal acceleration. Mononuclear cells at the interphase were collected, counted, and resuspended in FACS buffer and prepared for flow cytometry. An illustration can be found to display a visual representation in order to clarify the isolation procedure (Fig M2).
In brief, heparinized blood samples (100μL) were incubated with anti-human antibodies linked to different fluorescent labels, CD45-PerCp-Cy5.5 (BioLegend, San Diego, CA), CD11b-FITC (BioLegend, San Diego, CA), CD14-PE (eBioscience, San Diego, CA), and CD16-APC (eBioscience, San Diego, CA). Stained whole blood samples were then incubated with 1x FACS Lysing Solution (BD Biosciences, San Jose, CA) in order to lyse red blood cells. Samples were then fixed with 1% paraformaldehyde solution prepared in PBS with 0.1% sodium azide. Purified atrial mononuclear cell isolations were incubated with the same antibody panel, followed by 1% paraformaldehyde fixation to prepare them for flow cytometry analysis. Isotype
controls were used for negative gating to identify the level of non-specific binding. Data were acquired using the FACS Calibur (BD Biosciences, San Jose, CA) then analyzed using FlowJo (Flowjo LLC, Ashland OR). Monocyte/macrophage gating was performed by gating on cells with the use of forward and side scatter (FSC & SSC), which was then applied to a CD45 x SSC dot plot. The CD45+ events were gated and applied to a CD11b x SSC dot plot. The CD11b+ events were gated and applied on a CD16 x CD14 dot plot for characterization of distinct monocyte subsets and CD16 mean fluorescence index (MFI).

2.5 Histological analysis

Atrial heart sections were processed for histological analyses by fixing with 10% neutral buffered formalin (NBF) for 24 hours and then paraffin-embedded. Blocks were sectioned into 5μm using a microtome and placed on microscope slides for further staining.

Sirius Red/Fast Green Analysis

In order to assess fibrosis (% collagen deposition), a Sirius Red/Fast Green staining was prepared. Briefly, paraffin-embedded heart sections were stained with Sirius Red then counterstained with Fast Green. Slides were deparaffinized in a series of degrading alcohol gradient and fixed in Bouin’s solution (Sigma) for 1 hour. Then, slides were washed in running water until Bouin’s cleared and then placed in 0.1% Fast Green/ddH₂O for non-collagenous proteins. Slides were then washed in 0.1% acetic acid before staining with 0.1% Sirius red solution for 30 min and then dehydrated and mounted with xylene-based mounting medium. Collagen was semi-quantified using a pixel based method with the use of Adobe Photoshop CC 2017, originally described by Underwood et al. In brief, 500x500 pixel grids were blindly
overlaid on top of the compiled images of the heart sections by the observer. Further, red pixels were positively selected and summed for a total number of red pixels representing collagen. Non-background (green) pixels and red pixels were summed for the total number of heart pixels. Collagen content was measured by calculating the percentage of red pixels by total heart pixels. Consistency was ensured by using the same red color palette, green color palette, and by processing all tissues simultaneously.

**Hematoxylin-and-Eosin Staining (H&E)**

Paraffin-embedded heart sections were stained with hematoxylin and eosin (H&E) for analysis of basic myocardial histology and cellular composition. Slides were deparaffinized by subjecting to degrading alcohol gradient consisting of 2x xylene, 2x 100% ethanol, 2x 95% ethanol, and 70% ethanol before submerging into water. Slides were then differentiated in Harris’s hematoxylin solution, washed in running tap water to remove excess staining, differentiated in 1% acid alcohol, subjected to Scott’s solution for bluing, and then counterstained with 0.5% eosin with 0.5% CaCl$_2$ solution. Slides were dehydrated and mounted with xylene-based mounting medium. Images of atrial cross-sections were taken at 5x objective by light microscopy using Zeiss Axiovision 4.6 digital image analysis program (Carl Zeiss, Toronto, ON, Canada) on slides, visualized with AxioCam HRc camera (Carl Zeiss) and analyzed using Adobe Photoshop CC (2017) by compiling images into whole heart cross-sections.
2.6 Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded atrial appendage cross-sections for CD68 (Abcam, Cambridge, MA). Cross sections were deparaffinized and subjected to degrading alcohol gradient as described previously and then treated for antigen retrieval using a pressure cooker before staining. Sections were quenched for endogenous peroxidases using 3% hydrogen peroxide (H₂O₂), non-specific staining was blocked using 10% normal goat serum (NGS) and primary antibodies were incubated overnight at 4°C. Following the primary antibody incubation, a secondary biotin-specific antibody was incubated for 1 hour at room temperature. After incubation, the antibody complexes were then conjugated using an avidin-biotin complex, Vectastain ABC kit (Vector Laboratories Inc., Burlington, ON) and developed using 3,3’-diaminobenzidine (DAB) as the chromagen (Dako-Cytomation, Mississauga, ON). Images were captured in a similar manner to SR and H&E histology.

2.7 Luminex Assay & Analysis

Approximately 10 mg of human atrial tissue was cut into small pieces and suspended in 1X phosphate buffered saline (PBS) with cOmplete protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). A Qiagen Tissue Ruptor homogenizer was used to homogenize the tissue. Protein concentrations in the homogenate were determined via Bradford assay (BioRad, Hercules, CA, USA) and the homogenate was stored at -80°C. Supernatants collected from human atrial tissue homogenates from patients, were examined using the luminex assay. Fluorescence-coded magnetic micro-particles coated with antibodies specific for the desired cytokines and chemokines were purchased from R&D Systems (Minneapolis, MN, USA) and eBioscience (San Diego, CA, USA). The luminex assay was conducted according to
manufacturer’s instructions. The samples were read using a Bio-Rad Bio-Plex dual laser (BioRad, Hercules, CA, USA). Similarly, this procedure was performed on blood plasma samples (10mg) to assess Galectin-3 expression.

2.8 Statistical Analysis

This study was a double blind cross-sectional study, where patient characteristics were hidden, in order to avoid any examiner bias. Patient samples were referred to as codes. All data are represented as means ± standard error of mean (SEM). All data analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA) and significance was determined if p <0.05. Direct comparisons between two groups were evaluated using the student two-tailed t-test for changes in relative expressions compared to baseline. Categorical variables were reported as frequencies and percentages and were analyzed by chi-square. When two or more independent groups were compared, a one-way ANOVA test was used. For example, when circulating monocyte expression of CD16 MFI was compared between the different time points (D0, D5, and D90), one-way ANOVA test was used to assess the difference between the means of D5 and D90 to the baseline, D0.
CHAPTER 3 - RESULTS

3.1 Objective 1

3.1.1 Patient Characteristics

During the REACH study period, a total of 25 patients were screened and approached for consent with 17 patients consenting; yielding a 68% enrollment rate as our study population. The majority of patients were males (76.5%), with normal EF (70.6%), requiring surgery while in hospital after a recent MI (non-ST elevation myocardial infarction 82.4%), undergoing surgical coronary revascularization or CABG surgery (100%). The preoperative clinical information of the patients enrolled in the study is listed in Table 1. All patients received the same standard of care. A total of 13 patients’ samples were used for our study, of which, 11 had prior to hospital discharge (D5) samples collected and 7 came back for the later follow-up appointment (D90) blood sample.

3.1.2 Circulating monocytes CD16 expression is upregulated post-surgery

Cardiopulmonary bypass (CPB) has been associated with significant inflammation due in part to activation of circulating leukocytes \(^{78-81}\). Therefore, we became interested in studying the specific effect cardiac surgery with CPB has on monocyte subset distribution. As mentioned previously in the methods section, a standardized gating strategy was used to characterize the various monocyte subsets (Fig 1A). We found that pre-operatively, the most abundant monocytes were the M1/classical phenotype at 88.76 ± 1.12% of the overall monocyte population. By contrast, the intermediate subset represented 7.41 ± 0.98% and the M2/non-classical at 2.99 ± 0.51% of the overall population (Fig 1B). We further looked at changes that occur in the sub-population distribution post-surgery. Blood samples were drawn from the patients at prior to
hospital discharge after surgery (D5) and at a later follow-up appointment (D90). We observed visual differences in monocyte number and phenotype post-surgery (Fig 2A). Upon quantification, a significant expansion of the intermediate and non-classical population was observed at D5 at the expense of the classical phenotype (Fig 2B-D) suggesting a significant shift towards an M2 phenotype. Both intermediate and M2 monocytes share high CD16 expression and therefore, we took it one step further by quantifying CD16 as a mean fluorescence index (Fig 3A). We were able to see a similar trend to the individual populations at D5 where CD16 MFI significantly increases at D5 and seem to revert back to baseline levels at D90. However, we did not expect to see a significant CD14 MFI downregulation at D5 since both classical and intermediate monocytes share high CD14 expression (Fig 3B). At this point, we were only looking at the mean values of all the patients’ samples (n=13) and the change observed could have been as a result of a specific pre-surgery value of CD16. To verify this, we compared individual patient samples from the various time points (Fig 4) and found that CD16 MFI changes at D5 are consistent with our previous data, however, in several patients, CD16 MFI did not seem to revert back to baseline levels at D90. Upon closer analysis, the two samples that did not seem to revert back to baseline levels of CD16 MFI at D90 as seen in Fig 4, were different in terms of post-operative outcomes, where one experienced outcomes while the other patient did not.

CD16 also known as FcγRIIIA can be expressed on monocytes, macrophages, NK cells, and possibly neutrophils. While our data indicated that CD16 expression was upregulated in monocytes following surgery, we could not omit the potential in seeing changes in other leukocytes that express it. Therefore, we also investigated its expression levels on lymphocytes and neutrophils. Our findings suggest that CD16 expression was unchanged in both lymphocytes
(Fig 5A) and neutrophils (Fig 5B), therefore, indicating that the elevation in CD16 expression observed post-surgery was specific to circulating monocytes.

3.1.3 CD16 MFI positive correlation with plasma Galectin-3 levels & clinical NLR

Thus far, our results have only demonstrated the monocyte sub-population dynamic with relation to CD16 expression post-surgery, however, the meaning of this change still needed to be further understood, which prompted us to the next series of correlations. We examined the absolute change in CD16 MFI pre- and post-surgery correlated with two well-described variables known to influence inflammation after CPB: myocardial injury (troponin level post-surgery, cTnT nanograms/milliliter) and duration of CPB (pump time)\textsuperscript{127}. Blood samples for cTnT analysis were obtained for all patients within the rst 24h post-surgery. The peak cTnT levels (highest cTnT value in patients with multiple samples) were found to be 544.1 ± 125.2 ng/mL. Correlation analyses indicated that there was no significant relationship between peak troponin and change in CD16 MFI (Figure 6A) or pump time (minutes) and CD16 MFI values (Figure 6B).

Edelmann et al. examined the association of plasma levels of galectin-3 obtained from patients with stable HFpEF with patient characteristics and clinical outcomes\textsuperscript{128}. Galectin-3 is a protein belonging to the beta-galactoside-binding lectins that is secreted by activated macrophages and promotes myofibroblast proliferation\textsuperscript{129,130}. Therefore, it is a marker of myocardial fibrosis\textsuperscript{131}. Furthermore, Edelmann and colleagues have suggested that an increase in galectin-3 post-surgery was associated with an increased risk of death or hospitalization. Moreover, high galectin-3 levels at baseline were associated with worse clinical outcomes. Plasma protein luminex analysis was performed by Dr. Ian Haidl. Galectin-3 was amongst the
several proteins tested during the experiment. Compared to baseline, galectin-3 levels did not significantly increase at D5 samples (Fig. 7A). However, interestingly, baseline galectin-3 levels positively correlated with D0 monocyte CD16 MFI (Fig. 7B).

Neutrophil-to-lymphocyte ratio (NLR), as the name suggests, is the ratio between neutrophils and lymphocytes found in circulating blood. NLR has been used by some investigators as a measure for the burden of inflammation\textsuperscript{132-134}. Complete blood counts were obtained at the hospital from all patients prior to surgery and prior to hospital discharge of 5 days post-surgery (Figure 8A). To scratch the surface, we looked at differences between the two-time points and found that the overall leukocyte counts (WBC count) did not change significantly between day 0 and day 5 (Figure 8A). However, there was a significant 2.0-fold increase in NLR between baseline and 5 days post-surgery (Figure 8B). Similarly, there was a 1.7-fold increase in monocyte concentration in blood between baseline and 5 days after surgery (Figure 8C). Our next step was to compare routine clinical blood data with our flow cytometric analysis. When we initially plotted the relationship between pre-operative levels CD16 MFI and NLR, we were not able to find a correlation. However, by plotting the relationship between monocyte CD16 MFI and NLR from both D0 and D5 samples (Fig 9), we were able to determine a positive correlation between NLR and CD16 MFI suggesting that universally higher NLR levels were associated with increased numbers of non-classical monocytes in circulation.

3.1.4 Pre-op NLR levels correlated with the occurrence of adverse outcomes

Higher levels of NLR obtained from patients prior to surgery has been reported to be an independent predictor for the development of adverse events in patients undergoing cardiac surgery\textsuperscript{132, 133, 135}. Adverse cardiovascular outcome (AE) was defined as any one of the
following: prolonged length of hospitalization (LOS) greater than 9 days, readmission to hospital for any cardiac reason, persistent HF at follow-up NYHA class III or IV, and inability to wean diuretics for symptoms of congestion (dyspnea, peripheral edema). A total of 59% (10/17) of patients suffered AE with the majority (47%) of patients hospitalized for longer than 9 days and 12% of patients being readmitted after discharge for cardiac reasons. Additionally, our unadjusted findings suggest that patients who develop AE have a significantly higher blood NLR value pre- surgery than patients who did not develop AE (Fig 10A). However, even though we found a correlation between NLR and monocyte CD16 MFI, pre-operative monocyte CD16 MFI failed to reach significance between the two different patient groups (Fig 10B). Similarly, there was no significant difference between the two groups of patients in terms of D0 galectin-3 plasma levels (Fig 10C).

3.1.5 Other clinical blood values did not correlate with neither CD16 MFI nor AE

You might be asking yourself, why not look at the various other ratios that can be extrapolated from clinical CBCs. Platelet-to-lymphocyte ratio (PLR) has recently been gaining traction as a potential inflammatory marker of cardiovascular diseases. Several studies have suggested that PLR can be used as an independent predictor of in-hospital cardiovascular mortality in patients with ST-elevated acute myocardial infarction and a predictor of long-term cardiovascular outcomes in patients with ACS \(^{136-141}\). Nevertheless, our unadjusted findings revealed that pre-operative levels of PLR did not differ significantly between patients developing AE and ones that did not, therefore was not a predictor (Fig 11A). Moreover, there was no difference of PLR between D0 and D5 samples and no correlation was found with CD16 MFI.
Monocyte-to-lymphocyte ratio (MLR) has been proved by many to be a prognostic factor in patients with malignancies and tuberculosis \(^{142-145}\). To further these findings in the context of CVD, Ji et al. (2017) found MLR to be a novel independent risk factor of atherosclerosis and predictor of lesion severity \(^{146}\). Our data shows that there was a significant 2.28-fold increase in MLR at D5, however no significance was reached when the different patient groups were compared, and no correlation with monocyte CD16 MFI was observed (Fig 11B).

Monocyte-to-neutrophil ratio (MNR) in peripheral blood has been shown to be a risk factor in infections, where lower ratios corresponding to higher chances of infections \(^ {147}\). Our data shows that MNR did not significantly change between D0 and D5 samples, which corresponds to the absence of infection. Therefore, infection could be omitted as a possible contributor to the changes observed. However, this data would have to be compared with procalcitonin (PCT) and C-reactive protein (CRP), which are established markers of infections in order to verify.

### 3.2 Objective 2

#### 3.2.1 Patients Characteristics

Atrial tissue samples were obtained for analysis from a total of 18 patients. Samples were obtained from both the REACH and ANP studies in order to maximize study population and account for insufficient tissue sizes and lost samples in the optimization of isolation protocol. Patient characteristics are outlined in Table 2. The majority of patients were males (78%), with normal ejection fraction (72%), requiring surgery while in hospital (56%), and undergoing a spectrum of cardiac surgical interventions ranging from CABG, valve, combined procedures and LVAD for chronic heart failure. A third of the patients had pre-operative atrial fibrillation.
3.2.3 Successful isolation of cardiac macrophages from atrial samples

Prior to our experiments, macrophage composition in the human heart has been limited and not fully explored. Therefore, we created a novel isolation protocol highlighted in the methods section, in order to study these cells in patients undergoing heart surgery. Macrophages were isolated from atrial tissue and divided into sub-populations according to their variable expression of surface receptors. We visualized/quantified the macrophage composition using a unique flow cytometric gating strategy (Fig 12). The average number of CD45+ cells per gram of tissue was 3.35x10^6 cells (Fig 13A) with CD45 positivity composing of 18.9% of the PBMC isolation (Fig 13B). When we further subdivided the CD45+ (leukocyte) population we found that 26.21% were macrophages, 57.07% were lymphocytes, and 16.72% were other (Fig 13C).

Since our main area of focus was macrophages, our flow cytometry panel aided us in the characterization of the different phenotypes. Moreover, the macrophage population was found to be mostly CD14++CD16- (M1) at 80.78 ± 1.90% (Fig 14A). Both the CD14++CD16++ (intermediate) and the CD14+CD16++ (M2) sub-populations were found to equate to a small proportion of the overall macrophage population at 2.77 ± 0.49% and 2.71 ± 0.85% respectively. Like our blood flow cytometry analysis, we used CD16 MFI in order to quantify the non-classical macrophage population (Fig 14B).

To further prove that we were indeed isolating cardiac macrophages and not monocytes from contaminated blood in tissues, we performed our next series of immunohistochemical analyses. CD68 is a human transmembrane glycoprotein heavily expressed on tissue macrophages, immunohistochemical analysis was performed on the atrial cross sections (Fig 15A). The presence of CD68 positive binding enabled us to accumulate further evidence that our macrophage isolation was successful.
In order to add an additional verification test to solidify our findings, we compared patient pre-operative monocyte data with the atrial macrophage data (Fig 16). As expected, flow cytometry dot plots looked different and resembled differences in terms of the sub-populations between blood and atrium (Fig 16A-B). Additionally, upon quantification of our study population, blood samples had a significantly higher M1 and intermediate subset, while not reaching significance for the M2 phenotype (Fig 16C). CD16 MFI also appeared to be significantly higher in blood with a 2.75-fold versus atrium (Fig 16D). In order to make sure that the difference in CD16 MFI expression was not due to chance, we individually plotted each patient’s atrial sample CD16 MFI with their respective blood value and found that in 94.4% of patients, CD16 MFI was found to be higher in blood (Fig 16E). A final suggested measure to ensure that our results were not skewed by any contaminating blood was calculating available leukocyte population ratios in our samples. By calculating macrophage/monocyte-to-lymphocyte ratios extrapolated from our flow cytometry data, we found that blood had a significantly lower ratio than atria (Fig 16F). Therefore, our cardiac macrophage isolation was deemed successful.  

3.2.3 Cellular infiltration in the myocardium was not seen via H&E stain

In order to identify any cellular infiltration in the myocardium, we processed and stained the atrial samples with H&E. A representative section of the atrial tissue from one patient is seen (Fig 17A) demonstrating that by H&E staining, cellular infiltration could not be identified within the myocardium, only normal bundles of cardiac muscle fibers were seen.
3.2.4 Fibrosis & age correlation

Sirius Red/Fast Green staining was used to assess collagen deposition in the atrial sections (Fig 17B). As previously mentioned in the methods section, collagen deposition was semi-quantitatively assessed using atrial sections stained with SR/FG. A correlation between fibrosis and age was observed (Fig 17C, \( r^2=0.28; p=0.037 \)), which was consistent with previous studies.\(^\text{148, 149}\).

3.2.5 Macrophage CD45 MFI & CCL2 correlation with atrial fibrillation

In an attempt to draw more correlations between our lab data and patient outcomes/characteristics pre- and post-surgery, we used atrial fibrillation to group patients. We considered any event of atrial fibrillation as per the Society of Thoracic Surgeons’ (STS) definition, which includes both pre-operative AF and POAF to be the combined grouping variable.\(^\text{150}\). One-third of patients had pre-operative atrial fibrillation, but according to our grouping criteria, 50% of patients were put in the AF group. By looking at previously outlined variables, we were not able to demonstrate that age or atrial fibrosis were significantly different between the two groups (Figure 18A). We were able to demonstrate that length of stay (LOS) was significantly higher (\( p=0.05 \)) in the AF group. Moreover, macrophage CD16 MFI was not significantly different between the two groups. However, when we gated on macrophages and assessed their CD45 MFI expression, we noticed that it was almost twice as high in the AF group than the No AF group. This may suggest an important, but partially skewed relationship between macrophages and atrial fibrillation, as CD45 is meant to be a pan leukocyte marker and does not particularly express differently on varying macrophage subsets. Stephanie Legere performed the luminex analysis of the atrial tissue and studied a variety of cytokines/chemokines. However,
due to how sensitive trademarking/originality may be, we chose to only display CCL2 data to aid with my research. CCL2 expression in tissue, an important monocyte recruitment chemokine, was notably higher in the AF group.
3.3 Tables & Figures

Table 1- Patient Characteristics

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*EF- Ejection Fraction  
BMI- Body Mass Index  
CABG- Coronary Artery Bypass Grafting
Figure 1. Whole blood leukocytes isolated from patients’ blood samples were stained with CD11b, CD45, CD14, and CD16 and frequencies of monocyte subsets were measured by flow cytometry. 
(A) Representative images of flow cytometry gating strategy used to identify monocyte phenotypes from whole leukocyte blood samples. (B) Represents sub-population percentage findings from 13 patients in terms of M1, Intermediate and M2 at day 0 or just prior to surgery.
Figure 2. Monocyte subsets changes over time in circulating blood. (A) shows the flow cytometry dot plots of an individual patient at D0 (n=13), D5 (n=11), and D90 (n=7) post-surgical intervention. (B) Graph demonstrating the change in the M1 monocyte sub-population over time showing a decrease at D5 from baseline (p=0.0301). (C) Intermediate subpopulation showed a mean 1.47-fold increase at D5 from baseline (p=0.0025). (D) M2 subpopulation was observed to have a 1.69-fold increase at D5 from baseline (p=0.0257). D90 samples were not significantly different from either baseline or D5 samples.
Figure 3. Monocyte CD16 and CD14 mean fluorescence index (MFI) values in various time points. (A) Graph showing CD16 MFI differences between the various blood collection time points. There was an increase in CD16 MFI on D5 when compared to baseline (p=0.0051). (B) CD14 MFI was not significantly different between D0, D5 and D90. D0 (n=13), D5 (n=11), and D90 (n=7) samples.
Figure 4. Individual patient samples. CD16 MFI values for individual patients at D0, D5, and D90. It appears that all patients experience an increase in CD16 MFI 5-days post-surgery, however, not all patients returned to baseline levels of CD16 MFI at D90.
Figure 5. Lymphocytes & Neutrophils contribution to CD16 MFI. (A) Representative images of flow cytometry dot plots showing the lymphocyte population and its contribution to CD16 expression over time (ns). (B) Representative images of flow cytometry dot plots showing the neutrophil population and its contribution to CD16 expression over time (ns).
Figure 6. Relationship of peak troponin and pump time with CD16 MFI absolute change. Graphs representing correlations between absolute CD16 MFI change between D0 and D5 and (A) cardiac peak troponin T (cTnT) levels and (B) pump time. Both parameters were not significant with CD16 MFI (n=11).
Figure 7. Positive correlation between CD16 MFI & plasma concentration of Galectin-3.
(A) Graph showing no significance between Galectin-3 plasma concentrations at D0 and D5. (B) Plot demonstrating the relationship between pre-operative Galectin-3 plasma concentration and pre-operative monocyte CD16 MFI; netting a positively correlation.
Figure 8- Neutrophil-to-lymphocyte ratio & monocyte change 5 days post-operatively. (A) Table showing CBC data. (B) T-test was performed to compare NLR between D0 and D5 samples (p=0.0028). (C) Monocyte counts per μL in D0 and D5 samples (p=0.0074).
Figure 9. Relationship between CD16 MFI and NLR values. Graph showing a positive correlation between CD16 MFI and NLR in patients’ blood samples. Sample size included both D0 and D5 findings to show the universal relationship between the parameters tested (n=24).
Figure 10. Differences between patients who developed adverse events post-operatively and patients who did not develop in terms of pre-op blood findings. (A) Graph showing a higher mean NLR value in pre-op samples of patients who developed AE when compared to pre-op samples of patients who did not develop AE post-surgery (p=0.0065). (B) Graph representing CD16 MFI mean values at baseline in the AE and No AE patient groups (significance was not reached). (C) Graph representing mean plasma galectin-3 concentration at baseline in the AE and No AE patient groups (significance was not reached).
Figure 11 - Other leukocyte ratios that have been shown to be predictive of adverse outcomes in literature. (A) Changes in PLR post-surgery were insignificant and did not correlate with CD16 MFI and AE. (B) Changes in MLR post-surgery were significant, however, did not correlate with CD16 MFI and AE. (C) Changes in MNR post-surgery were insignificant and did not correlate with CD16 MFI and AE. **No AE, n=5; AE, n=8.
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Figure 12. Atrial PBMC isolation flow cytometry gating strategy. (A) Flow cytometry ungated dot plot of overall isolated cells. (B) CD45 x CD14 gating was applied to exclude non-leukocyte events, resulting in a cleaner population of lymphocytes and macrophages (C). Followed by a CD11b x SSC positive gate (D), then a FSC x SSC gate on macrophages (E), enabling us to visualize the different macrophage sub-types with the aid of CD16 x CD14 plot (F).
Figure S1. Atrium PBMC isolation specific binding verification via isotype controls. Levels of non-specific background signals caused by primary antibodies in the antibody panel is negligible when following the same gating strategy explained in Figure 12 on the isotype samples.
Figure 13. Leukocytes found in the atrium. (A) CD45+ cells per gram of tissue calculated from flow cytometry CD45+ data (n=18). (B) Pie chart highlighting the percentage of isolated cells that were CD45+. (C) Further characterization of the CD45+ population found in atrial samples.
Figure 14. Characterization of macrophage subsets from flow cytometry. (A) Flow cytometry dot plot representing gated macrophage populations (CD16 x CD14) as a mean value (n=18). (B) CD16 MFI values in atrial tissue isolates.
Figure 15. CD68 immunohistochemical cross-section. (A) Representative atrial appendage cross-section stained with CD68 highlighting macrophage presence with red circles.
Figure 16. **Atrium vs blood flow data.** (A,B) Flow cytometry dot plots from an individual patient’s atrium and blood respectively. (C) Bar graphs demonstrating the comparison between the different macrophage/monocyte phenotypes in atrium and blood. (D) CD16 MFI values in atrium and blood. (E) Individual patient data on CD16 MFI to further show that CD16 MFI shows a similar trend in all patients where CD16 MFI is lower in atrium than in blood. (F) Monocyte to lymphocyte ratio was significantly higher in atrial samples using flow cytometry data.
Figure 17. Fibrosis SR/FG stain analysis. (A) H&E stain of the atrial appendage cross-section (B) Cross section of the atrial appendage stained with SR/FG, where red indicated the presence of collagen and green stains muscular fibers. (C) Positive correlation demonstrated between patient age and percentage of fibrosis quantified from cross sections.
Figure 18. Grouping patients in terms of Atrial Fibrillation. (A) Table showing the differences between patients who did not have or develop AF and patients who did. (B) Patients who had or developed atrial fibrillation had significantly higher macrophage CD45 MFI. (C) Fibrosis was not different between the two different groups.
CHAPTER 4 - DISCUSSION

It is increasingly recognized that inflammation plays a crucial role and may be responsible for disease progression in conditions like cardiovascular disease\textsuperscript{29,151}. Similarly, inflammation has proven to be an important component of the normal host response to injury and necessary for healing. The seemingly conflicting role of inflammation highlights how clinically relevant efforts in this area remain\textsuperscript{151}. However, to date our ability to understand inflammation using simple biomarkers in the context of cardiovascular disease remains poorly defined. In this thesis, attention was turned towards two aspects of inflammation: (1) peripheral blood leukocyte analysis (systemic) and (2) atrial macrophage isolation from patients undergoing cardiovascular surgery (local).

4.1 Summary of Findings in the Context of Literature

4.1.1 Circulating Leukocytes

Our understanding of circulating monocytes and their phenotype has potentially important implications, as circulating monocytes are precursor cells to tissue macrophages\textsuperscript{152}. In the context of heart disease, increasing efforts have been made to better understand the balance between seemingly opposite pro-inflammatory M1 macrophages and non-classical, M2, macrophages\textsuperscript{97}. One should note that some groups have suggested that larger proportions of classical CD14++CD16- monocytes would be more predictive, but this was not the case in our study as the expression of CD14 did not change after surgery from baseline levels in our patient samples, indicating that classical (M1) monocytes were not predictive\textsuperscript{123}. The full significance of this observation remains to be determined; however, it could be that persistence of CD16 is indicative of a failure to resolve inflammation by M2 macrophages (providing negative feedback regulation) in the normal course of healing. To date the use of CD16 expression as a biomarker
to identify patients at risk of cardiovascular complications has yet to be widely applied and remains limited to case series \textsuperscript{58,124,125}. We argue that an important reason for flow cytometric analyses of blood have not been routinely clinically used is at least partly related to the fact that such assays are time consuming, labor intensive, and as such very expensive.

To the best of our knowledge, we are the first to describe changes in CD14 and CD16 expression pre-operatively, at prior to hospital discharge 5 days postoperatively, as well as at a later follow-up appointment at 90 days. Our findings suggest that circulating monocytes undergo significant phenotypic changes toward M2 phenotype early after surgery at the expense of M1 phenotype. Consistent with previous data, reperfusion injury post-CPB was a pro-inflammatory stimulus \textsuperscript{53,78-81,87}. This was seen in the shift described 5 days post-surgery, majorly in the form of the CD14++CD16+ intermediate sub-population, which are known to secrete increased amounts of TNF-\(\alpha\) and can exert pro-inflammatory functions \textsuperscript{153}. Therefore, this finding shows the extensive effect CPB has on inflammation as it was seen 5 days post-surgery and could offer an avenue to further understand the onset of inflammation and other leukocytes that may be playing a role in the shift towards intermediate monocytes. In cases of off-pump surgery, monocyte activation and their secreted inflammatory cytokines have been shown to be lower compared to on-pump CABG surgery, which has translated into reduced post-operative morbidities \textsuperscript{154,155}. Activation-dependent monocyte surface expression changes such as elevated CD11b levels was shown to be eliminated in off-pump variants of CABG surgery \textsuperscript{155}. However, a study has shown that although off-pump surgeries have a lower inflammatory burden, the impact on patients’ outcomes remain unaffected, showing that surgery alone is responsible for the overall outcomes \textsuperscript{156}. Thus, while our findings were observed in on-pump patients, it could likely not be solely due to the CPB alone. Furthermore, we demonstrated a positive correlation
between baseline CD16 MFI and baseline plasma levels of Galectin-3. Galectin-3, as previously mentioned, plays an important role in macrophage activation and macrophage-induced myofibroblast proliferation. Galectin-3 levels have been shown to be important in predicting heart failure prognosis. Higher Galectin-3 at baseline have been shown to be sicker patients, and higher Galectin-3 elevation over the course of 12 months post-surgery was associated with an increased risk of death or re-hospitalization in patients with HFpEF. Our data suggests that similar to CD16 MFI, plasma concentrations of Galectin-3 were not significantly different between patients who developed AE and ones that did not, but seemed to be following a similar trend (Fig 10C). Therefore, a larger study population would have probably enabled these values to reach significance. Recently, evidence has accumulated showing that the ratio between neutrophils and lymphocytes (NLR) predicts cardiovascular diseases and is slowly emerging as an independent, useful prognostic parameter for CVD. The predictive superiority of NLR over inflammatory cells alone, such as WBC counts, may be due to many reasons including the fact that it is less likely to be influenced by various physiological conditions. Furthermore, NLR is a ratio of two different yet complementary immune pathways, thus integrating the deleterious effects of neutrophils, which are responsible for active non-specific inflammation and lymphopenia, which in general, are markers of poor general health and physiological stress.

We demonstrated a significant increase in NLR after surgery without a significant change in overall leukocyte counts. Moreover, a significant increase was observed in the overall neutrophil and monocyte counts at the expense of lymphocyte counts. Our observation is not unique and has been previously described in the context of cardiac surgery. What is novel about our observations is that we also correlated our findings with monocyte subsets based on CD16 expression. We demonstrated that NLR is significantly correlated with CD16 expression on
circulating monocytes. While not fully understood, monocyte-CD16 expression has been suggested to be a marker of maturity, sharing many features with tissue macrophages that have been described as non-classical M2 macrophages in murine models. It may therefore suggest that this persistence of non-classical M2 cells follows a heightened (NLR) preoperative state of inflammation that is unresolved postoperatively and thus contributing to the development of AE. Furthermore, we demonstrated a significant correlation between CD16 expression and clinical NLR values. Determining NLR, in contrast to CD16 MFI, can be obtained simply from a complete blood count. Complete blood counts are readily available in all patients undergoing surgery and are therefore convenient. In our study, we report that NLR at the time of surgery was predictive of an increased likelihood of developing AE after surgery supporting its use as a potential biomarker to predict outcomes. One should note that in contrast to NLR, CD16 MFI was not predictive of AE despite showing a trend. Previous studies have shown that cardiac surgery patients that developed AE were six-fold more likely to be frail at the time of surgery. Clinical frailty at the time of cardiac surgery has been shown by our group to be a major risk factor for AE also.

It is possible that NLR findings in this study not only reflect patient’s inflammatory state at the time of surgery, but could also reflect an association between inflammation and frailty. An important observation from this study is the fact that prolonged hospitalization was the most common cause of AE (80%) and driven mainly by significant postoperative delirium (p = 0.044). Delirium is a complex clinical condition that manifests clinically as an acute confused state characterized by fluctuating mental status, inattention, and either disorganized thinking or altered level of consciousness. Relevant to this point are reports linking delirium with inflammation, but the mechanisms for this relationship require further study. It is also important to
acknowledge the effects patient medications, like statin, may have on leukocyte changes observed in our study population. Statins are known to reduce the risk of developing adverse outcomes and mortality in patients with CVD undergoing cardiac surgery \(^{167, 168}\). Along with its main purpose of lowering LDL cholesterol in circulation, it has been associated with reducing vascular inflammation and thrombus formation, while improving endothelial function. These pleiotropic effects of statin, in particular, may have contributed to the data presented in this thesis \(^{167, 168}\). Therefore, further investigation of the use of medication, may provide a more conclusive story in human CVD research.

A series of studies have described significant changes in cell surface expression of other markers of activation (HLA-DR, CD163, TM) of various subsets of immune cells like lymphocytes, B cells, and monocytes associated with CPB \(^{169-171}\). Our data did not show significant changes in lymphocytes in terms of CD16 expression, which makes our monocyte CD16 expression change exclusive only to monocytes, however, we were not able to fully conclude this, since we did not have the sufficient lymphocytes and B cell markers. It is noteworthy that the changes we observed early after cardiac surgery did resolve over time suggesting that some of these changes may not only be related to the surgery, but may also be important in healing and/or involved in regulating either the initial inflammatory response or its resolution.

4.1.2 Macrophage isolation from human atrial appendages

In the context of atrial tissue others have suggested that atrial tissue inflammation is associated with atrial remodeling and the substrate by which atrial fibrillation can develop \(^{172, 173}\). In the second half of the study, we were able to demonstrate feasibility in isolating and purifying
immune cell populations from human atrium from patients undergoing heart surgery. We were able to isolate $3.21 \times 10^6 \pm 0.66$ mononuclear cells per gram of tissue with 19% of them being CD45+ which is a common leukocyte marker. To the best of our knowledge we are the first to demonstrate this and show that human cardiac macrophages likely play a critical role in atrial tissue homeostasis that has yet to be fully described.

Until recently, limitations in experimental tools and a prevailing dogma that immune cells in heart tissue were mainly derived from the circulation led investigators to largely disregard resident immune cells. New evidence of a robust and heterogeneous resident cardiac macrophage population has forced investigators to reconsider their understanding of the complex role of immune cells and specifically macrophages in the heart $^{100, 101, 114}$. Macrophages appear particularly important given their ability to display a large array of activation states ranging from pro-inflammatory (M1) to immunomodulatory (M2) $^{23, 174}$. Initial work by Pinto et al. suggested that resident macrophages were largely an M2-like phenotype or alternatively activated $^{100}$. Most of the evidence describing resident macrophages has been obtained from mice model with to the best of our knowledge no human tissue evidence. We demonstrate that the majority of macrophages isolated from human atrium were pro-inflammatory CD14++/CD16- with only a small proportion of CD16+ macrophages (M2). Previous studies performed in our lab have shown that in mice myocardium, a large proportion of M2 phenotype macrophages were identified. Our approach to the isolation was rigorous, standardized and adapted from identical methodology previously described by our group supporting our findings $^{97, 103}$. The isolation protocol was refined several times in order to maximize cell yield and purity. In order to validate that we were successfully isolating macrophages rather than artifacts (or contamination), CD68 immunohistochemical staining was used and showed the availability of macrophages in the atrial
tissue cross-sections. Furthermore, as a second step of validation, we compared blood monocyte subset distribution to macrophages isolated from the atrial appendage, using flow cytometry, and found that the distribution was significantly different between them. Further, plotting individual patient data of atrium and baseline blood samples to show that they are indeed different in all samples and not due to chance.

Our findings show major differences in cardiac macrophage population dynamics between human and previously studied mice most notably that mice do not express CD16, but instead Ly6C is a marker used to identify the M2 phenotype. Furthermore, recent evidence indicates that embryonic resident macrophages are gradually being replaced with monocyte-derived macrophages from circulation, significantly reducing the M2 phenotype by adulthood. This might be the reason why we saw a small population of M2 macrophages in our samples, however, we do not have proof to support the origin of the macrophages.

Resident and infiltrating macrophages may have different, competing, or complementary roles in regulating myocardial homeostasis. With how our study was set-up, we were not able to determine the origin of the macrophage population that is being described. The majority of isolated macrophages were pro-inflammatory based on their CD14 and CD16 expression or M1 phenotype. We demonstrated that the isolated macrophages phenotype from atrial cells was different than circulating monocytes suggesting that our isolation strategy did not result only from blood contamination. Our isolation strategy was designed to characterize mononuclear cells (PBMC) in particular macrophages. This explains why we did not isolate neutrophils as these would have been eliminated from the isolation protocol. Neutrophils were not seen on routine histological sections and as such do not likely represent a large population. On the other hand, a large population of lymphocytes were identified based on CD45+, SSC and FSC distribution.
Since we were limited by the size of atrial tissue sampled, we did not have additional lymphocyte markers in our panel, and therefore, were not able to conclusively identify them. However, it is noteworthy that a significant population of lymphocytes appears to be CD16+ suggesting that a population of NK cells can possibly be isolated from the atrium.\textsuperscript{176}

Atrial fibrillation is a common clinical condition seen in patients undergoing heart surgery and often considered a marker of severity of heart disease.\textsuperscript{177} The exact etiology in the development of atrial fibrillation remains unclear. In our study, 9/18 patients had atrial fibrillation pre- or post-operatively, which is in keeping with what is usually described in patients undergoing heart surgery.\textsuperscript{178} We combined atrial fibrillation events, pre- and post-operatively, to have comparable patient group sizes. Our findings suggest that patients at risk of post-operative atrial fibrillation (POAF) had significantly higher numbers of CD45+ macrophages in the atrium as well as higher concentrations of CCL2, which is a chemokine involved in the recruitment of classical monocytes playing a key feature in the early inflammatory phase.\textsuperscript{106} This link between inflammation and atrial fibrillation has been suggested by other investigators based on association of several inflammatory cytokines like CRP, TNF-\(\alpha\), IL2, IL6, IL8.\textsuperscript{172, 173} Yet, whether inflammation is the cause of atrial fibrillation still remains unknown. However, a possible explanation could be that inflammation promotes atrial fibrillation and/or that atrial fibrillation can create an inflammatory environment. Other biomarkers, such as oxidative stress biomarkers, have been shown to be associated with POAF.\textsuperscript{179}

Atrial fibrillation has been shown by some to be associated not only with inflammation (macrophages), but also atrial fibrosis.\textsuperscript{117} Fibrosis results in conduction abnormalities, commonly leading to increased atrial fibrillation vulnerability. However, we could not
demonstrate an association between atrial fibrillation and fibrosis in our study. Furthermore, atrial structural remodeling or fibrosis is linked to disturbances in ECM turnover with TGF-β1 and AngII levels higher in patients at risk of POAF. In a mouse model, TGF-β1 overexpression resulted in significant susceptibility of atrial fibrosis. Moreover, strategies to limit TGF-β expression in a canine model, was able to reduce fibrosis and atrial fibrillation. Pathological features of atrial structural remodeling seen in atrial fibrillation: cardiomyocytes lose sarcomere starting in perinuclear area “myocytolysis”, interstitial fibrosis with some perivascular fibrosis, while inflammation less certain. In a sheep model, both Galectin-3 and TGF-β1 have been shown to be upregulated in atria of persistent atrial fibrillation animals. Inhibition of Galectin-3 using GM-CT-01, reduced atrial fibroblast migration and proliferation, myocyte hypertrophy, atrial fibrillation and fibrosis, therefore, forming an avenue for Galectin-3 inhibition therapies to be used to prevent the progression of atrial fibrillation. However, findings linking atrial fibrillation to fibrosis have not always been consistent. In a series of 46 patients undergoing surgery, right and left atrium were sampled and no difference in ECM content could be demonstrated between atrial fibrillation and normal sinus rhythm group. Similarly, in a goat model of atrial fibrillation the authors were able to demonstrate changes in myocytolysis, but not fibrosis. What we were able to demonstrate is that age was strongly associated with atrial fibrosis. Similarly, we were able to show that older patients showed an increased number of CD45+ macrophages in their right atrium samples suggesting a link with inflammation. Furthermore, consistent with previous literature, a positive correlation was seen between fibrosis and age in humans. Age related relationship to AF is likely multifactorial including neuro-hormonal activation (RAAS system) and inflammation, which supports our findings.
4.2 Limitations

A few challenges were met along the way of my research. Despite having identified a significant correlation between NLR and CD16 MFI in blood, I was not able to adjust for differences in characteristics and outcomes between patients, which makes the presented data completely unadjusted. Therefore, it is not possible to omit the possibility that patient characteristics such as gender, age, BMI, diabetes, etc. could have an effect on the findings. In order to tackle this in future research, the sample size would need to be estimated in terms of the grouping variable being tested. Therefore, reliable retrospective identification of patient population dynamics is crucial. In a multivariate study like this one, the most efficient way to estimate the study size would be to first choose the variables of interest (ex: sex, age group, BMI, complications etc.), identify the proportions for all the chosen ones and use the highest ratio to calculate an estimated sample size (for example, 1:9 females in an experiment looking at sex-based differences).

Moreover, our population of patients in the atrial study was compiled from two separate studies and thus, is a very heterogeneous sample size, ranging from elective patients undergoing CABG to LVAD. These patients required a variety of different cardiac surgeries, which might have accounted for the variability in the data produced (high deviations). Which explains why I focused my attention on common outcomes such as atrial fibrillation and characteristics such as age for possible grouping. For the blood portion of the study, only samples from the REACH project were used as the study design included 3 different times where blood was provided (pre-operatively, 5 and 90 days post-operatively). Our study also illustrates that while cell isolation is possible from human right atrial appendages, significant technical challenges were met. Sample
size was a very impactful limitation. Depending on the surgeon performing the surgery, tissue size was variable, compromising one or more experiments in order to meet minimum tissue size per test. Isolation protocol optimization (since isolation was never done in humans before) led to the data loss of first few patient samples until protocol became consistent and efficient. Also, there was potential cell loss during isolation, which meant that the findings might not be reflective of the whole leukocyte population in the atrium. Moreover, the first few patients’ tissue sample analysis was deemed unusable due to flow cytometry (FACS Fortessa) difficulties. This included the inexperience of the FACS Fortessa technician with dealing with compensation in tissue samples, therefore, the FACS Calibur was used, which limited our initially proposed 16 multi-leukocyte specific antibody panel into only 4 monocyte/macrophage specific antibodies. Additionally, we did not test the lymphoprep pellets, which consisted largely of cardiomyocytes, for the availability of macrophages to ensure that none were lost in the isolation protocol.

Although our novel isolation protocol enabled us to isolate macrophages, it might have had important impacts on the isolated cells and their expression of surface receptors. These impacts could include the loss of CD16 expression on macrophages as observed by our very small population size, the loss/diminished CD11b expression, which was crucial for our gating strategy, but unfortunately, we were not able to use other experiments to validate our protocol, such as the use of CD16 immunohistochemistry on atrial appendage cross-sections. Another very important limitation is our inability to compare atrial findings obtained from patients to normal controls. This would have enabled us to characterize the macrophage content at steady-state and compare it to the diseased samples. Therefore, we were not able to conclude that our atrial findings were exclusive to patients with cardiovascular disease undergoing surgery and not found in healthy atria.
4.3 Future Directions

The main objective of this thesis was (1) to characterize circulating leukocyte changes as a result of surgery and draw main correlations with well-known routinely collected markers of inflammation and predictors of adverse events and (2) to determine the feasibility of leukocyte (specifically macrophages) isolation from human right atrial appendages and to characterize their sub-population dynamics, protein expression and relevance to the overall disease pathogenesis and patient characteristics. With the current set of observations presented in this thesis, several directions can be taken from here in order to fill the gaps currently present in understanding the role of leukocytes in CVD.

It is of utmost importance to acknowledge that a portion of my study was designed to be an initial stepping stone in human CVD research (i.e. leukocyte isolation from atrial samples), therefore, some limitations were due to overexerting the data in order to get the most out of my experiments. This enabled me to form an array of possible future directions in studying heart failure in humans. Since we saw a shift in the monocyte distribution towards a pro-inflammatory intermediate in blood taken 5-days post-operatively, this could be attributed to a late inflammatory response. Thus, further experimentation exploring shorter blood collection time-points (peri-operatively, 1 day and 3 days) could offer an indication of the onset of CPB induced inflammation, define time-based leukocyte changes, and correlate differences with patient outcomes, while identifying the micro-stimulus that induces this change. Additionally, it could provide avenues for possible delivery or modulation therapies to alter specific monocyte phenotypes at different stages post-cardiac surgery to promote healing rather than expand the inflammatory response. One study suggested the use of liposomes consisting of
phosphatidylserine (PS) to mimic apoptotic cells in mice. It has been shown that monocytes/macrophages secrete IL-10 and TGF-β in response to their uptake of apoptotic cells, fundamentally shifting towards an M2/anti-inflammatory phenotype. Also, previous research done in our lab has shown proof of concept that the use of liposomes can be used in modulating macrophages in cardiac healing. Therefore, selective liposome treatments could offer a great opportunity for manipulating circulating monocyte phenotype to promote healing post-surgery.

As much as we think we covered in terms of leukocyte isolations from human atrial tissue, we do not necessarily answer all of the questions. Is it feasible? Yes. Should it be explored further? Definitely. We only scratched the surface by showing the varying macrophage populations found in the human right atrial appendage. But, are our isolated macrophages unique to diseased individuals only? We know from literature that at steady-state, resident cardiac macrophages exist in mice, therefore, normal healthy control tissue from donors should be compared to these patients. This will enable us to further characterize the macrophage populations available in the heart and understand their functions and role in disease. Moreover, comparing right atrial macrophage dynamics with the remaining 3 chambers could give us a better understanding of the overall macrophage presence in the myocardium.

Thus far throughout this thesis, the main focus was on a single cell type, the monocyte/macrophage, however, in order to fully understand disease pathology and the various pathways involved, one must look at other cell types involved. We were able to show, although not-conclusively, that a proportion of our cardiac PBMC isolation consisted of CD16+ NK cells. NK cells have been shown to become dysregulated in cardiac fibrosis and modulate inflammation. NK cell depletion in mice resulted in an increased severity of myocarditis and attenuated cardiac
Interestingly, several clinical studies have shown that NK cell presence in circulation significantly diminishes in patients with CAD and other ischemic heart diseases $^{194-196}$. Moreover, the reduction of circulating NK cell presence could possibly be due to the recruitment of NK cells into the myocardium as described by the presence of NK cells in our samples. Therefore, would be a great experiment to try to determine the relationship of NK cells, macrophages and cardiac fibrosis.

### 4.4 Concluding Remarks

Looking back 2 years from the date of this thesis, I would have never thought my research would end here. This statement could be taken in both a positive and a negative way. Therefore, like anything between these two covers, I must defend my stance. I started this journey thinking it was similar to a simple math equation of $X+1=2$, where I was solving for $X$. A human disease was at hand to study in a human-based model, a specific target population was available to look at, tons of previous promising research in this area was conducted in animal models, funding is available to conduct my research, and two years ahead of me to study it. It was all pretty much set-up for a perfect story. All I need to do is actually conduct the research and things will work out to equal to “$X=1$”. It did not take me long to realize that it was a rather skewed, oversimplified way of addressing cardiac research, let alone human multivariate cardiac research.

At the start of my degree I had a very specific objective, which was to experience research and broaden my knowledge of cardiovascular research. The education portion, however, was not the only thing I learned. I spent countless hours at the lab learning new skills and lab techniques, making mistakes, and most importantly, learning from these mistakes. Coming from a biology background, I was both fascinated and overwhelmed with the huge world of pathology.
and immunology that I was not exposed to prior. At the beginning of this journey, I was faced with countless struggles along the way including studying fundamental immunology in just under a couple of months. I remember when I would sit in lab meetings with Dr. Légaré and my fellow lab mates waiting for English subtitles to kick-in in order to decrypt what they were talking about, “CD-this and CD-that”. I would constantly ask Alec, my former lab mate, the same questions over and over again and learn his shortcuts. Until I was able to stand on feet. Then writing my first paper hit me like a truck. I noticed that the numerous lab reports we as undergraduate students had to write, had nothing to do with actual manuscript writing. But with the help of Dr. Légaré I was able to publish my first 1st author paper.

I truly enjoyed the program I was involved in for the past 2 years.

Scientific research, one of the main pillars of advancement.

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Thank you for making it to the end of my thesis.
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