Multifactorial Immune Cell-Based Development of Heart Failure

by

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A thesis submitted in conformity with the requirements for the degree Master of Science
Department of Laboratory Medicine and Pathobiology
University of Toronto

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2017

Abstract

The development of heart failure is a complex and multifactorial process. Risk factors such as prior viral cardiac infection and hypertension identify individuals at risk of developing heart failure, and the current literature has conflicting evidence about the role of individual cardiac immune cell subsets in these pathologies. Some of the contradictory results stem from the fact that immune cells within the myocardium have been characterized only superficially. In addition, the interaction between different risk factors, while clinically important, has been ignored at the basic science level. In this thesis, we first specifically define an important, yet uninvestigated cardiac immune subset – the dendritic cell (DC). We show the myocardium contains two specific conventional DC lineages (CD103$^+$ and CD11b$^+$), that possess unique, tissue-specific and lineage-specific mechanisms that regulate their life cycle and tissue residency. We identified cardiac CD103$^+$ DCs as critical in inducing antigen-specific CD8$^+$ T cell responses following subclinical viral myocarditis, and preventing the development of heart failure. In addition, chronic hypertension also predisposes mice to develop heart failure following subclinical viral myocarditis, highlighting that only by combining two risk factors, does heart failure develop.
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Abbreviations

ACE
Angiotensin converting enzyme

AIM2
absent-in-melanoma 2

Ang-II
angiotensin-II

AT1
Angiotensin type 1

BATF3
basic leucine zipper transcription factor ATF-like 3

BM
bone marrow

BrdU
bromodeoxyuridine

CCR2
C-C chemokine receptor 2

CCR7
C-C chemokine receptor type 7

cDC
Conventional/classical dendritic cell

CDP
common DC progenitor

CTL
cytotoxic T lymphocyte

DAMPS
damage-associated molecular patterns

DC
Dendritic cell

DN
Double negative

DT
diphtheria toxin
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<tr>
<td>DTR</td>
<td>diphtheria toxin receptor</td>
</tr>
<tr>
<td>EMCV-D</td>
<td>encephalomyocarditis variant D virus</td>
</tr>
<tr>
<td>FLT3</td>
<td>Fms Related Tyrosine Kinase 3</td>
</tr>
<tr>
<td>Flt3L</td>
<td>Fms related tyrosine kinase 3 ligand</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HF</td>
<td>heart failure</td>
</tr>
<tr>
<td>HFpEF</td>
<td>heart failure with preserved ejection fraction</td>
</tr>
<tr>
<td>HHV6</td>
<td>Human herpes virus 6</td>
</tr>
<tr>
<td>HTN</td>
<td>Hypertension</td>
</tr>
<tr>
<td>i.p</td>
<td>intraperitoneally</td>
</tr>
<tr>
<td>i.v</td>
<td>intravenously</td>
</tr>
<tr>
<td>ICAM1</td>
<td>intracellular adhesion molecule 1</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
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<tr>
<td>IFN-β</td>
<td>Interferon β</td>
</tr>
<tr>
<td>IL-4</td>
<td>interleukin 4</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol 1,4,5 – triphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
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<tr>
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<td>-----------</td>
</tr>
<tr>
<td>IRF4</td>
<td>Interferon regulatory factor 4</td>
</tr>
<tr>
<td>IRF8</td>
<td>Interferon regulatory factor 8</td>
</tr>
<tr>
<td>Klf4</td>
<td>Kruppel-like factor 4</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricular</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>MDA-5</td>
<td>Melanoma differentiation-association protein 5</td>
</tr>
<tr>
<td>MDP</td>
<td>Macrophage DC progenitor</td>
</tr>
<tr>
<td>MF</td>
<td>Macrophage</td>
</tr>
<tr>
<td>MHC-II</td>
<td>Major histocompatibility complex class II</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>NFkB</td>
<td>Activation of nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NICM</td>
<td>Non-ischemic cardiomyopathy</td>
</tr>
<tr>
<td>NK cells</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>NLRs</td>
<td>NOD-like receptors</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NR4A1</td>
<td>Nuclear receptor subfamily 4 group A member 1</td>
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</table>
PAMPS pathogen-associated molecular patterns
pDC plasmacytoid DCs
PRR pattern recognition receptor
PSGL-1 P-selectin glycoprotein ligand 1
RAS renin-angiotensin system
RIG-I retinoic acid-inducible gene I
RLRs retinoic-acid inducible gene I like receptors
S1RP1 sphingosine 1 phosphate receptor
TCR T-cell receptor
TGF-β1 transforming growth factor-β 1
TLR Toll-like receptor
TNF-α tumor necrosis factor alpha
VCAM-1 Vascular cell adhesion molecule 1
VEGF vascular endothelial growth factor
XCL1 X-C Motif Chemokine Ligand 1
XCR1 X-C Motif Chemokine Receptor 1
ZBTB46 Zinc finger and BTB domain containing 46
\textbf{\(\gamma\delta \text{ T cells}\)}  \quad \text{gamma delta T cells}
Introduction

1. Cardiovascular Disease

Cardiovascular disease remains the leading cause of mortality and morbidity worldwide [1]. Cardiovascular disease can be broadly divided into two categories, ischemic cardiomyopathy and non-ischemic cardiomyopathy (NICM) [2]. Worldwide, cardiovascular disease accounts for 32% of all deaths with the majority of mortalities attributed to some form of ischemic cardiomyopathy due to atherosclerotic heart disease and myocardial infarction (MI) [1]. The widespread frequency of ischemic cardiomyopathy has warranted it the major focus of scientific research on cardiovascular disease, with numerous animal models to study the initiation, progression, and long-standing effects of ischemic cardiomyopathy. NICM refers to the broad range of diseases affecting the myocardium which are not the result of reduced or absence of blood flow, and represents a clinically-relevant, yet understudied area of cardiovascular disease. There are many potential causes of NICM, such as haemodynamic pathology, infection, immunologic abnormalities, toxic injury, or genetic factors [2].

1.1 Viral Myocarditis

Myocarditis is defined as inflammation of the heart muscle that may be identified by clinical, histopathologic and imaging criteria, and is an important cause of heart failure (HF), [3]. Dilated cardiomyopathy is the most common form of cardiomyopathy following viral infection, and is characterized by increased myocardial mass and volume. The walls of the myocardium become thin, the contractile function of the heart becomes impaired, and chambers become dilated, leading to the clinical diagnosis of HF [4].
Myocarditis is often a secondary complication to infections, toxic substances, or autoimmune processes [5]. Most often, myocarditis manifests as a result of viral infections and is a disease with variable clinical presentation and progression [4, 5]. During the acute phase, a viral pathogen induces an immune response, which can range from transient, self-resolving injury to fulminant myocarditis and death. Acute subclinical viral myocarditis is defined as inflammation of the myocardium heralded by a non-specific flu-like illness while acute fulminant myocarditis is characterized by distinct onset of severe congestive HF or cardiogenic shock [6].

The true prevalence and impact of viral myocarditis has been highlighted in recent studies. For example, in a cohort of 498 patients with myocarditis or dilated cardiomyopathy, endomyocardial biopsy revealed that 64.7% of patients had viral genomes for the parvovirus B19 compared to 7.7% of control patients. Furthermore, approximately 40% of individuals less than 35 years old with unexpected death have evidence of myocarditis on histology [7-11]. The gold standard for diagnosis of viral myocarditis has been the Dallas criteria based on histopathology from an endomyocardial biopsy [12]. It has now been recognized that the Dallas criteria is not sensitive for myocarditis because it does not take into consideration the presence of viral genomes in the myocardium [13]. In a recent multicenter analysis of 624 patients in the United States with histologically proven myocarditis, 38% of them had presence of various viral genomes in their myocardium while only 1.4% of the 215 control patients had detectable viral genomes [14]. Even in the absence of classic histological myocarditis, viral genomes have been shown to be present in patients with idiopathic dilated cardiomyopathy [7, 14]. Although the presence of viral genomes has been shown in patients with dilated cardiomyopathy and acute viral myocarditis, no implication on cardiac outcome can be made. Some studies have shown the association between viral persistence in the heart and progressive cardiac dysfunction [9], while other studies have shown that the presence of viral genomes is not a predictor of heart transplantation or cardiac death
in patients with clinically suspected myocarditis [15]. In the latter study, the authors concluded that presence of infiltrating inflammatory T cells and/or macrophages (MFs) with enhanced expression of HLA class II molecules are reliable predictors of clinical outcome in patients with no viral genomes but positive for the Dallas criteria [15]. Certain aspects of the manifestation and progression of viral myocarditis remain unclear. Patients with fulminant viral myocarditis who require intense haemodynamic support can rapidly recover within a few days and have better long-term prognosis than patients with subclinical viral myocarditis, who have a worse prognosis due to development of dilated cardiomyopathy [16, 17].

Coxsackievirus B is the best-known primary infectious agent associated with viral myocarditis. However, infection with parvovirus and adenoviruses in the past decade has become more prevalent [7]. Other pathogens include the Eppstein-Barr virus, human herpes virus 6 (HHV6), parvovirus B19, and cytomegalovirus, which are common upper respiratory tract viruses. Although almost 90% of people will be infected with one or more of these viruses in their lifetime, it is unclear why only a selected few develop clinical cardiac symptoms [5].

Specific treatment of viral myocarditis is limited and is largely restricted to standard HF therapies that are focused on blockade of neurohormonal pathways (angiotensin converting enzyme (ACE) inhibitors, angiotensin-II (Ang-II) receptor blockers and β-adrenergic blockade). Since patients generally present weeks or months after viral infection, antiviral therapy for acute myocarditis has limited applicability [3]. Treatment of viral myocarditis to improve left ventricular (LV) function may be dependent on the particular pathogen and has presented mixed results. In a study performed by Kühl et al., 22 patients with persistent LV dysfunction were treated with the anti-viral cytokine Interferon-β (IFN-β) for 23 weeks. Clearance of viral genomes was observed in all 22 patients and LV ejection fraction was significantly increased [18]. Furthermore, a large
body of experimental evidence suggests that acute and some chronic myocardial injury in myocarditis is due to an immune response involving T lymphocytes and autoreactive antibodies [3]. However, clinical trials have shown mixed results when patients with acute myocarditis were treated with immunosuppressive drugs. For example, the US Myocarditis Treatment Trial, in which 111 patients with histologically confirmed myocarditis were randomly assigned to placebo or to prednisone (a synthetic corticosteroid) and either azathioprine (immunosuppressant – purine synthesis inhibitor) or cyclosporine (decreases production of inflammatory cytokines produced by T lymphocytes), has demonstrated no benefit in their transplant-free survival or change in LV ejection fraction [16]. Recent evidence has shown that symptom duration has an impact on the efficacy of the treatment. Generally, patients with symptom duration of less than 6 months do not benefit from active treatment, while in patients with dilated cardiomyopathy in which symptoms last for more than 6 months, treatment generally shows positive results. For example, in a trial of immunosuppression in patients with myocarditis and symptoms for more than 6 months, LV ejection fraction improved after treatment with azathioprine and prednisone [19].

These studies implicate viral myocarditis as a clinically prevalent condition. Considering that the majority of patients remain asymptomatic, develop HF symptoms later on in life, and with limited therapeutic options, it is necessary to study in-detail the pathogenesis of viral myocarditis to gain a better understanding of how to improve patient outcomes.

1.2 Animal Models of Viral Myocarditis

Currently, several animal models are available that resemble human viral myocarditis. Encephalomyocarditis virus (EMCV) is a common pathogen that is used to study myocarditis in
mice and pigs [20, 21]. EMCV is a small non-enveloped single-strand RNA virus belonging to the Picornaviridae family, the same family as Coxsackievirus B3, a known human pathogen. EMCV-D genome consists of a positive single-stranded RNA of approximately 7.8 kb that allows direct translation of the RNA into a polyprotein. Vascular cell adhesion molecule 1 (VCAM-1) is a sialoglycoprotein that has been identified as a receptor for EMCV on murine vascular endothelial cells. The main receptor for EMCV remains unknown [22]. EMCV primarily infects the central nervous system, myocardium and pancreas, inducing encephalitis, myocarditis, and type 1 diabetes, which largely depend on the variant of EMCV and host susceptibility. EMCV is classified into two main variants based on its organ tropism in mice; one is neurotropic (designated E) and produces a rapidly fatal infection, while the other is cardiotropic (designated M) and usually causes non-fatal illness with a few signs of central nervous system involvement [23]. Generally, the M variant of EMCV also has two variants termed the D and B variant. The D variant of EMCV is diabetogenic, while the B variant is non-diabetogenic in BALB/c mice. C57BL/6 mice are not susceptible to EMCV-induced diabetes. The D and B variant of EMCV have both been shown to induce myocarditis in BALB/c and C57BL/6 mice [24]. The D variant of EMCV has also been shown to infect the central nervous system of DBA/2 mice. EMCV has been described as a potential zoonotic agent although studies are limited [22].

EMCV-induced myocarditis is characterized by myocardial necrosis and cellular infiltration. The inflammatory response induced upon infection with EMCV has been shown to play a critical role in its pathogenesis, in which activation of immune cells may have a deleterious role in EMCV infection. Several studies have shown that EMCV virulence might be partly due to CD4+ and CD8+ T cells as well as MFs and mast cells [25-27]. For example, when BALB/c mice were infected with the M variant of EMCV, a paralytic syndrome was observed in 7-10 days. Pathologically, the white matter of brain and spinal cord showed well-demarcated areas of
perivascular cuffing, demyelination, and, during recovery, remyelination by oligodendrocytes, which are all suggestive of post-infectious encephalomyelitis. When CD4$^+$ or CD8$^+$ T cells were depleted with monoclonal antibodies, development of the paralytic syndrome was decreased by 79%, suggesting pathological activation of adaptive immunity [25]. Another study showed that treatment of mice with the tyrosine kinase inhibitor tryphostin AG126 prior to EMCV infection blocked the production of nitric oxide and prevented MF-mediated beta-cell destruction and lowered incidence of diabetes [28]. In addition, mast cells have also been shown to play a critical role in EMCV-induced myocarditis [29-31]. Infection of mast cell-deficient mice with EMCV mice showed attenuated myocarditis, diminished cellular infiltration and improved survival rate [29-33]. Activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) during EMCV infection has also been shown to have deleterious consequences for mice. Inhibition of NFκB upon EMCV infection in mice lowered the mortality of the animals, attenuated necrosis and cellular infiltration, and decreased the production of interleukins (ILs), IL-1β, IL-6, tumor necrosis factor alpha (TNF-α) and nitric oxide in the myocardium [30, 32]. Together, these studies implicate that a strong inflammatory response contributes to EMCV virulence and that immune cells activated immune cells can play a pathological role with regards to promoting tissue injury following EMCV infection.

Other studies demonstrate that immune cells and RNA-sensing receptors, melanoma differentiation-association protein 5 (MDA-5) and retinoic acid-inducible gene I (RIG-I), play a protective role against EMCV infection. For example, in a study done by Philip et al., the authors generated cardiac-specific MDA5 transgenic mice, which had overexpression of MDA5 in cardiac tissue. These mice showed increased baseline cardiac expression of antiviral cytokines and increased cellular infiltration but no alterations in cardiac function and structure. Mice
overexpressing MDA5 were less susceptible to EMCV infection, and had a significantly lower cardiac viral load compared with littermate control mice. Cardiac myocyte apoptosis was also attenuated in these mice. Furthermore, overexpressing MDA5 mice were protected against EMCV-induced myocardial dysfunction. *Mda5<sup>−/−</sup>* mice develop severe cardiac pathology and higher mortality was observed following EMCV-D infection [34, 35]. In another study using CD11c-DTR mice, which enables depletion of all CD11c-expressing cells after diphtheria toxin (DT) administration, higher mortality and viral loads were observed in EMCV-D infected mice after DT administration. A major limitation of this study is that the CD11c-DTR model depletes MFs, natural killer (NK) cells and other lymphocytes in addition to dendritic cells (DCs), therefore it is difficult to attribute protective/pathological roles to specific immune cells in the setting of viral myocarditis.

These results are important examples of the demonstration that the host immune response might be a delicate balance between the beneficial antiviral effects of immune cells and cytokines and the pathological consequence of excessive inflammation in tissue. These studies also implicate an important role for immune cells in the pathogenesis of viral myocarditis. To date, we lack an in-depth understanding of the specific immune cells involved, and their functional role in viral myocarditis. Furthermore, the functional role of cardiac DCs, which likely play a very important role in mediating the innate and adaptive immune responses, has received very little scientific attention in the setting of viral myocarditis.

1.3 Hypertension

High blood pressure affects nearly 30% of the world’s population [36]. It is well established that HTN is strongly linked to other cardiovascular diseases including stroke, MI, and HF.
According to WHO data, 62% of all strokes and 49% of coronary heart disease are attributable to high blood pressure [36]. Clinically, HTN is divided into two main branches: essential/primary HTN and secondary HTN. Primary HTN is defined as high blood pressure, in which secondary causes such as renovascular disease, renal failure, pheochromocytoma, aldosteronism, or other mendelian forms (monogenic) are not present. Primary HTN accounts for 95% of all hypertensive cases. The etiological factors of primary HTN are manifold and are often present simultaneously in individuals. Some factors include obesity, insulin resistance, high alcohol intake, high salt intake, aging, sedentary lifestyle, stress, low potassium intake, low calcium intake, and other genetic factors [37].

1.4 Renin-Angiotensin System

The renin-angiotensin system (RAS) is a major regulator of blood pressure homeostasis. Cleavage of angiotensin-I by angiotensin converting enzyme (ACE) yields angiotensin-II (Ang-II). The main effects of circulating Ang-II are increase in vasoconstriction, release of aldosterone, sodium and fluid retention, increase in inflammation, oxidative stress, and fibrosis. These effects are primarily mediated through the binding of the angiotensin type 1 (AT1) receptor. The AT1A receptor, the closest murine homologue to the human AT1 receptor, is expressed in the kidney, heart, brain, adrenal gland, vascular smooth muscle, liver, and several other tissues/immune cells. Upon binding to the AT1 receptor, Ang-II induces inositol 1,4,5-triphosphate (IP3), calcium, and adenylate cyclase to mediate its effects. The binding of Ang-II to the AT1 receptor raises blood pressure through vasoconstriction, sodium retention, and aldosterone generation [38, 39]. Genetic ablation studies have shown that the hypertensive and cardiac hypertrophic effects of Ang-II are primarily mediated through its actions on receptors in the kidney. However new studies have
shown that the AT1 receptor is ubiquitously expressed on cells of the innate and adaptive immune system [38, 40-45]. This area of research has not been extensively studied. Recent studies have shown both protective and deleterious effects on the direct impact on Ang-II on immune cells [44-46].

ACE inhibitors and AT1 receptor blockers are some of the most potent therapeutic options for treatment of HTN [38, 39, 46]. A powerful technique for simulating HTN is via the administration of Ang-II. Administration of Ang-II to induce HTN is clinically relevant, because in addition to providing insights into the mechanism by which Ang-II mediates hypertensive effects, it mirrors a clinically relevant problem, as studies have shown that almost 45% of patients being treated with ACE inhibitors have elevated levels of circulating Ang-II, a phenomenon called Ang-II escape [47, 48].

1.5 Cardiac Remodelling and Transition to Heart Failure

Cardiac remodelling consists of molecular, cellular, and interstitial changes to the heart during times of stress that result in morphological and functional changes to the heart. The transition from physiological to pathological cardiac remodelling is determined by intensity, duration and the specific change in cardiac function. The dichotomy between the two terms is further complicated, because what is believed to be physiological remodelling (occurring in healthy individuals such as during pregnancy and exercise, and ultimately does not lead to cardiac damage) versus pathological remodelling (occurring in patients who experience MI, HTN, or viral myocarditis) at the cellular level consist of changes that are often a form of compensatory mechanism to the specific cardiac stressor. Exacerbated compensation through intensity of the
stress and/or duration is what augments the beneficial compensatory response to LV dysfunction and ultimately HF [49].

Cardiac hypertrophy is a form of remodelling which occurs when the heart experiences increased workload. There are two main forms of cardiac hypertrophy: eccentric and concentric. Eccentric hypertrophy is characterized by dilation of the LV with thinning of the myocardial wall. In eccentric hypertrophy sarcomere units are added in series, with myocytes decreasing in width and increasing in length. Eccentric hypertrophy usually manifests during volume overload. Clinically, eccentric hypertrophy is often seen in patients with mitral or aortic valve regurgitation [49].

Concentric hypertrophy is characterized by thickening of the myocardium with reduction of the internal radius of the ventricle and little change in size of the heart. Concentric hypertrophy is characterized by sarcomere units added in parallel, with increase in myocyte width. Clinically, concentric hypertrophy is observed in pressure overload situations, such as in patients with HTN. Importantly, chronic hypertrophy can transition to eccentric hypertrophy, LV dysfunction and HF if pressure overload isn’t treated [49].

HTN is the leading cause of heart failure (HF) with preserved ejection fraction (HFP EF), which can lead to NICM, and is also the leading cause of NICM itself [50, 51]. There are currently no approved treatment options for HFP EF, even though these patients account for ~50% of all HF hospital admissions [50, 51]. Interestingly, not all patients with poorly controlled HTN develop HFP EF or NICM. Traditional risk factors account for only 50% of the risk for HF development, indicating that additional mechanisms promote susceptibility [50]. In large, well-characterized cohort studies, multivariable models adjusted for known risk factors, including systolic blood pressure, explained only 50% of the variability of LV mass as assessed by echocardiography and
60% to 68% by cardiac magnetic resonance imaging. These data suggest the presence of other unmeasured risk factors [51][52-54].

2.0 Cardiomyocyte sensing of PAMPs and DAMPs

The host response to injury is initiated via the release and/or recognition of pathogen-associated molecular pattern molecules (PAMPS), which are derived from microorganisms, and recognized by pattern recognition receptor (PRR)-bearing cells of the innate immune system as well many epithelial cells. In contrast, damage-associated molecular pattern molecules (DAMPS) are cell derived and are associated with trauma, ischemia and tissue damage with or without the presence of a pathogen [55].

 Resident cardiac immune cells are triggered by the detection of PAMPS and DAMPS by a fixed number of germline encoded PRRs. Examples of PAMPS include lipopolysaccharides of Gram-negative microorganisms, teichoic acid of Gram-positive microorganisms, the zymosans of yeast, the glycolipids of mycobacterium, and double-stranded RNA of viruses. DAMPs are localized within the nucleus and cytoplasm (HMGB1), cytoplasm alone (S100 proteins), exosomes [heat shock proteins (HSP)], the extracellular matrix (hyaluronic acid), and in plasma components such as complement (C3a, C4a and C5a). Examples of non-protein DAMPs include ATP, uric acid, heparin sulfate, RNA, and DNA. DAMPs can also be mimicked by release of intracellular mitochondria, consisting of formyl peptides and mitochondrial DNA [55]. Endogenous proteins and DNA from necrotic cells in the myocardium are also recognized by intracellular and extracellular PRRs [56].
Cardiac PRRs can be divided into two major classes based on their subcellular localization. Toll-like receptors (TLRs) and C-type lectin receptors are found on plasma membranes and endosomes, while retinoic-acid inducible gene I (RIG-I)-like receptors (RLRs), NOD-like receptors (NLRs) and absent-in-melanoma 2 (AIM2) receptors are found in intracellular compartments [57, 58]. Activation of PRRs through recognition of PAMPS and DAMPs trigger signalling cascades that activate NFκB, activator protein 1 (AP-1), and interferon regulatory factor (IRF) transcription factors. These cascades subsequently induce production of pro-inflammatory cytokines and IFNs which assist in recruitment of other immune cells and in controlling pathogen replication [59]. Other inflammatory responses in the heart are through another subset of PRRs that mediate the assembly of cytosolic protein complexes called inflammasomes [60]. Canonical inflammasomes convert pro-caspase 1 into the active caspase 1 protease, which is responsible for the production of IL-1β and IL-18 [60].

2.1 Immune cells involved in modulating cardiac tissue injury and repair.

Following cardiac injury, immune cells residing in the myocardium along with non-immune cells produce pro-inflammatory cytokines and chemokines that are responsible for recruiting inflammatory leukocytes into the area of injury [61]. This initial phase is characterized primarily by influx of neutrophils and bone marrow-derived monocytes. These cells assist in clearing necrotic cells and matrix debris, and release cytokines and growth factors that aid in the formation of granulation tissue, composed of connective tissue and new blood vessels. Entry of activated of T cells from circulation is the next step of the immune response to viral infection. Activated T cells play a critical role in viral clearance and induction of apoptosis in virus-infected
cells. The final step is the activation of endothelial cells and fibroblasts, which play a key role in angiogenesis and fibrosis formation [62].

2.11 Role of neutrophils in cardiac injury

During the very early stages of cardiac injury, mast cells and soluble complement proteins are important initiators of inflammation. Resident mast cells release pre-formed pro-inflammatory cytokines such as TNF and histamine, which act on neighbouring endothelial cells, resident MFs and infiltrating neutrophils [63]. Complement proteins exposed to the injured myocardium and endothelium trigger further mast cell degranulation, release of histamine, and vasogenic oedema [64]. Cleaved complement proteins such as C5a both attract neutrophils and induce their transendothelial migration into injured tissue via the CD11b-CD18 complex [65].

Neutrophils are one of the first responders to cardiac injury [66]. Neutrophils have been shown to play a critical role in preventing uncontrolled expansion of pathogens. For example, patients deficient in neutrophils or neutrophil function suffer from disseminated bacterial infection [67]. In the setting of sterile cardiac injury, however, the role of neutrophils is almost entirely pathological [56]. Neutrophil recruitment into injured myocardium is mediated via two mechanisms. The first method is through peripheral activation prior to infiltration. Mitochondria in all cell types, including cardiomyocytes produce DAMPs that closely resemble bacterial proteins. These DAMPs, such as formylated peptides and mitochondrial DNA are released and are sensed by formyl peptide receptor 1 and TLR9. Binding of these mitochondrial DAMPs to the receptors promotes neutrophil activation and their recruitment to inflamed tissue [68]. The second method of neutrophil recruitment is dependent on cardiac endothelial cells. Pro-inflammatory
mediators, such as TNF-α, IL-1β, and histamine, released by mast cells and the myocardium activate endothelium and induce upregulation of adhesion molecules that enable neutrophil transmigration between endothelial cells to the site of tissue injury [63, 69]. IL-6 has been shown to be an important cytokine in neutrophil recruitment. IL-6 released by the myocardium and recruited myeloid cells (neutrophils and MFs) induces the expression of intracellular adhesion molecule 1 (ICAM1) on cardiomyocytes, which promotes neutrophil binding and cytotoxic activity [70, 71]. Neutrophils play a central role in the inflammatory response by releasing oxidants and proteases that damage or kill tissues, and release inflammatory products that amplify the recruitment and activation of greater numbers of neutrophils into the effected myocardium, thereby extending the severity of tissue damage. Neutrophils are intimately involved in the pathogenesis of MI, vascular endothelial dysfunction, damage to the genetic apparatus, apoptosis and other manifestations of lethal injury in the acute phase following reperfusion [72].

2.12 Role of monocytes and macrophages in cardiac injury

At steady state, the majority of cardiac immune cells are MFs [73]. The traditional dogma that all MFs arise from blood monocytes has been disputed due to findings that brain microglia and liver Kupffer cells originate from yolk-sac precursors which are maintained throughout the lifetime of the animal without blood monocyte input [74-77]. Our recent findings have revealed that cardiac MFs demonstrate significant ontological heterogeneity, with the majority of MFs originating from yolk-sac precursors, which are also maintained throughout the lifetime of the organism independent of blood-monocyte input [73, 74]. There are two main subsets of circulating monocytes in mice, Ly6c<sup>hi</sup> and Ly6c<sup>low</sup> monocytes. Ly6c<sup>low</sup> monocytes adhere to and move along the endothelium, clearing damaged cells and triggering inflammatory responses without entering
tissue [78, 79]. Ly6c^+ monocytes progenitors give rise to Ly6c^{hi} monocytes and through a nuclear receptor subfamily 4 group A member 1 (NR4A1)-dependent transcriptional program, Ly6c^{hi} monocytes differentiate into Ly6c^{low} monocytes [76, 80, 81]. During cardiac stress, Ly6c^{hi} monocytes are recruited from blood and enter tissue. Recruitment of monocytes from blood is a process dependent on endothelial cells, B cells, and the myocardium. Some monocytes have been shown to originate from splenic reservoirs and also seem to have a protective role with regards to cardiac healing after injury [82-84]. Recruited monocytes secrete pro-inflammatory cytokines and chemokines, drive inflammatory responses, and ingest apoptotic cells, including neutrophils and necrotic cardiomyocytes [56]. Production of IL-23 by monocytes has been shown to be important in controlling the inflammatory response by inducing expression of IL-17A by γδ T cells. IL-17A drives neutrophil production in the bone marrow and causes cardiomyocyte death [85, 86].

In-depth understanding of the ontological origin and function of cardiac MFs has only recently been described by us and others [73, 74, 87]. Using genetic fate mapping, parabiosis and adoptive transfer techniques, resident cardiac MFs have been more accurately defined. Our previous work has identified three main cardiac MF populations based on their ontological origin and their expression of major histocompatibility complex class II (MHC-II) and C-C chemokine receptor 2 (CCR2). At steady state, the adult mouse contains MHC-II^{hi} and MHC-II^{low} MFs, which are CCR2^- . These two populations comprise the majority of cardiac MFs at steady state and are primarily of embryonic origin. After birth, there is some dilution of embryonically derived MFs by recruited monocyte-derived MFs in the heart [73, 88]. CCR2^+ MFs are the third subset of cardiac MFs and these cells are primarily comprised of hematopoietically derived, circulating blood monocytes. Embryonically derived cardiac MFs have been shown to self-renew in situ at steady state and during cardiac stress, while hematopoietically derived MFs require monocyte input prior to proliferative expansion in the tissue. CCR2^+ MFs are enriched in pro-inflammatory
genes such as those involved in the NLPR3 inflammasome which is required to deliver IL-1B to the heart during cardiac stress [73]. Excessive inflammation in cardiac tissue leads to adverse cardiac remodelling and hypertrophy and it is very likely that CCR2+ MFs play a key role in these processes. The robust pro-inflammatory signature of CCR2+ MFs is likely beneficial against invading pathogens, but in the setting of sterile inflammation likely results in unwanted pathology [89, 90].

Numerous studies have demonstrated that cardiac MFs play a major role in tissue repair, regeneration, and inflammation after injury. In mice lacking monocytes and MFs, scar formation and angiogenesis are reduced while increased mortality due to myocardial rupture has been observed after ischemic cardiac injury [91, 92]. In apolipoprotein-E-deficient mice, excessive MF expansion leads to excessive inflammation and impaired cardiac function, which ultimately resulted in impaired infarct healing post myocardial infarction [93]. In studies where monocytes are absent, such as in Ccr2−/− mice, mice had attenuated LV remodelling post MI at the expense of a prolonged inflammatory phase and delayed replacement of granulation tissue [94]. Lastly, the neonatal heart shows a remarkable capacity to regenerate in response to multiple forms of injury but this regenerative potential is lost in adult mice [87, 95, 96]. One possible explanation to these findings is attributed to the function of embryonically-derived cardiac MFs. MFs derived from embryonic precursors are more efficient at engulfing apoptotic cardiomyocytes, promoting endothelial cell activation and cardiomyocyte growth and generating minimal inflammation after stimulation through TLR and inflammasome pathways [87]. One fundamental difference between neonatal and adult heart is that neonates favour the expansion of embryonically derived MFs following injury as opposed to the recruitment of monocytes [56]. These studies indicate that cardiac MFs affect myocardial recovery after cardiac damage. The extent to which each cardiac
MF subset plays role in tissue regeneration and inflammation are ongoing investigations of our laboratory.

2.13 Transition from acute inflammation to fibrosis

The transition from acute inflammation to fibrosis is mediated by several subsets of immune cells and the cytokines they release. The first step in transitioning from inflammation to tissue healing is through resolution of neutrophil recruitment. This is largely mediated by apoptosis by cardiac MFs. Apoptosis of necrotic cardiomyocytes or neutrophils by MFs leads to changes in MF cytokine expression. MFs decrease expression of pro-inflammatory cytokines such as IL-1β and TNF-α, and increase anti-inflammatory and pro-fibrotic cytokines such as IL-10 and transforming growth factor (TGF-β1) [97, 98]. The second mechanism of transitioning to tissue healing involves γδ T cells and their production of IL-17A. MFs also regulate this process by inducing expression of IL-17A from γδ T cells by producing IL-23. IL-17A has been shown to induce neutrophil production and release from bone marrow and is directly toxic to cardiomyocytes [85, 99]. Resolution of inflammation through apoptosis of neutrophils and other necrotic material has been demonstrated through decreased IL-23 production from MFs and subsequent decreased IL-17A levels [85, 99-101].

Lastly, activation of cardiac fibroblasts and subsequent extracellular matrix deposition is an important component of cardiac injury and repair. Activation of cardiac fibroblasts is regulated by monocytes, neutrophils, MFs, and activated CD4+ and CD8+ T cells [102-104]. Deposition of extracellular matrix by cardiac fibroblasts has been shown to critically affect cardiac function by altering myocardial stiffness [102, 105-108].
2.14 Animal Models to study functional role of cardiac DCs

These studies have shown that immune cells in the heart play a major role in modulating injury and recovery following cardiac damage. To elucidate the multifactorial manifestation of HF, we took a bottom-up approach. Although the cardiac response to HTN has been extensively studied by us and other groups, immune cells involved in viral myocarditis manifestation has received relatively little scientific attention. DCs are one of the major initiators of the adaptive immune system; their role as antigen presenting cells (APCs) and subsequent activation of T cells is critical in mediating host protection against invading pathogens such as bacteria and viruses. At steady state, cardiac DCs contain three major populations; two subsets of classical/conventional dendritic cells (cDCs), which are integrin αE, CD103+ DCs, and integrin αM, CD11b+ DCs, and plasmacytoid DCs (pDCs) [109-111]. During an inflamed setting, blood monocytes also have the capacity to enter tissue and contribute to the CD11b+ DC subset [112, 113]. Functional analyses of cardiac cDCs in the setting of myocarditis is limited and often confounded partly because of our inability thus far to accurately differentiate them from other cardiac immune cells, such as MFs, because they share similar cell-surface markers such as CD11c and MHC-II [73]. In one study using CD11c\textsuperscript{DTR} mice, depletion of DCs lead to increased mortality and higher viral titers following EMCV infection [34]. One major limitation of the CD11c\textsuperscript{DTR} system is the depletion of MFs, NK cells and other lymphocytes in addition to DCs [34, 114-116]. Extrapolating these results to elucidate DC function is confounding and difficult. Zinc finger and BTB domain containing 46 (ZBTB46) is a transcription factor expressed on endothelial cells, erythroid progenitors, and cDC1 and cDC2 DCs [117]. Zbtb46\textsuperscript{DTR/DTR} chimera mouse models have become a novel tool to target specific depletion of cDCs without affecting monocytes, MFs and NK cells– a limitation of the cDC11c-DTR mouse model [110]. In these mice, diphtheria toxin (DT) receptor (DTR) is inserted
into the 3’UTR of the ZBTB46 locus as a mean to specifically deplete cDCs. Mice bearing this knock-in express DTR in cDCs but not other immune cell populations, and DT injection into Zbtb46DTR/DTR bone marrow chimeras results in effective cDC depletion [116]. Bone marrow chimeras are made in order to spare other ZBTB46-expressing cells, such as endothelial cells.

We now have the necessary mouse lines and in-depth understanding of other cardiac immune cells to elucidate the functional role of specific cardiac DC subsets in the setting of viral myocarditis – a clinically-relevant scenario where DC should play a central role.

2.2 Dendritic Cells

DCs are central players of the immune system that operate at the interface of innate and adaptive immunity. DCs are potent and versatile APCs, and their ability to migrate is crucial in the initiation of protective pro-inflammatory as well as tolerogenic immune responses. As myeloid immune cell sentinels, DCs are specialized in the sensing of pathogen challenges, and trigger diverse sets of immune responses to rid the host of invading pathogens.

DCs can be characterized as classical/conventional (cDCs) and plasmacytoid DCs (pDCs). pDCs mainly circulate in blood and lymphoid organs and enter lymph nodes through circulation. pDCs express endosomal nucleic acid-sensing TLR7 and TLR9 and upon recognition of foreign nucleic acids, acquire antigen-presentation capabilities and produce large amounts of type I IFNs [117]. This rapid production of type 1 IFNs during viral infections promotes an antiviral state by inducing cellular expression of IFN-stimulated genes and apoptosis of infected cells [118]. Other consequences of TLR activation in pDCs is the release of TNF-α and IL-12. These powerful immunostimulatory functions of pDCs contribute to the recruitment and/or activation of nearly all
immune cell types, establishing pDCs as a key link between innate and adaptive immunity [116]. pDCs express different cell-surface markers than cDCs such as the B cell marker B220 and low levels of MHC-II and CD11c. pDCs have also been shown to express Siglec-H, which are sialic acid-binding immunoglobulin-like lectins, and bone marrow stromal antigen 2, Bst2 [119]. cDCs refer to all other DCs and can be compartmentalized into two broad classifications, cDC1s and cDC2s.

### 2.21 Functional specialization of CD8⁺ and CD103⁺ (cDC1) Dendritic Cell Subsets

Lymphoid tissue CD8⁺ and nonlymphoid tissue CD103⁺ DCs share the same origin and similar phenotype and transcriptional profile [117]. In nonlymphoid tissue, CD103⁺ cDCs are specifically enriched at the interface with the environment and are constantly migrating to the T cell zone of draining LN charged with antigen [120]. In the spleen, CD8⁺ cDCs are located in the marginal zone to constantly filter blood antigens, and in LNs, they are located in the subcapular sinus, the site of entry of afferent lymphatic vessels that drain nonlymphoid tissues [121, 122]. CD8α and CD103 expressing DCs represent a subset of DCs that are specialized in mediating CD8⁺ T cell responses through MHC-I. cDC1s express X-C motif chemokine receptor 1 (XCR1), a receptor that binds its ligand X-C Motif Chemokine Ligand 1 (XCL1) and has been shown to promote cross-talk between cDC1 DCs and CD8⁺ T cells. cDC1s also express Clec9a, a receptor for necrotic material which plays an important role in cross-presentation of exogenous antigen onto MHC-I for stimulation of CD8⁺ T cells, a hallmark trait of cDC1s. cDC1s also express Toll-like receptors 3, 4, 11, and 13. [123]. In the periphery, CD8 and CD103 cDCs are thought to participate in deletional tolerance of self-reactive T cells and the induction of antigen-specific TREG [122, 124-127]. The basic leucine zipper transcription factor ATF-like 3 (BATF3) and
Interferon regulatory factor 8 (IRF8) are two essential transcription factors that regulate development of cDC1 DCs from bone marrow to tissue [123]. Recent studies have shown that transcriptional autoactivation circuits can stabilize lineage-fate decisions. For example, it has recently been shown that cDCs are committed to either the pre-cDC1 or pre-cDC2 lineage based on the dynamic expression of various cell surface factors. Grajales-Reyes et al., recently showed that commitment, but not specification to the cDC1 lineage, is BATF3-dependent. BATF3 expression is induced in pre-cDC1 cells in the bone marrow and is needed to sustain the activation of IRF8. Without BATF3, IRF8 expression decayed in the progeny of pre-cDC1 DCs, which did not commit to the CD24+ (cDC1) lineage but diverted toward the CD172a+ (cDC2) lineage [128].

The role of cDC1s in the activation of CD4+ T cells has been conflicting and likely depends on the tissue and the type of ligand encountered. For example, dermal CD103+ cDCs control the induction of pathogen-specific CD4+IFN-γ+ T cells upon cutaneous infection with Candida albicans [129], and the ablation of dermal CD103+ DCs in langerin-DTR transgenic mice abrogates the induction of encephalitogenic CD4+ Th1 responses and the development of experimental autoimmune encephalitis [130]. Conversely, BATF3-deficient mice that lack CD103+ and CD8+cDCs can mount efficient CD4+ T cell responses to immunogenic fibrosarcoma tumors and to West Nile virus [131] as well as the myelin oligodendrocyte glycoprotein-specific Th1 cells upon subcutaneous immunization, leading to severe experimental autoimmune encephalomyelitis [132]. Furthermore, ablation of dermal CD103+ cDCs in Langerin-DTR mice does not affect the development of a CD4+ T cell response to Leishmania major infection [133].
2.22 Functional specialization of CD11b+ (cDC2) Dendritic Cell Subsets

Similar to all cDCs, CD11b+ cDCs (cDC2) can also sense pathogens and migrate from nonlymphoid tissue to regional LNs charged with self and foreign antigens [134-137]. CD11b+ cDCs represent a subset of DCs that are specialized in mediating CD4+ T cell responses through MHC-II. cDC2s express heat stable antigen (CD24) and similar to cDC1s, rely on FLT3 for their maturation and survival. cDC2s express toll-like receptors 5, 6, 7, 8 and 13 as well as the intracellular RNA sensor RIG-1. Interferon regulatory factor 4 (IRF4) has been shown to be required for the development of mucosal CD11b+ compartment, CD11b+ compartment in the lung, and CD11b+CD103+ DC compartment in small and large intestine [117]. Heterogeneity within the cDC2 population has made it difficult to identify essential transcription factors for cDC2 development. Subsets of cDC2 DCs are differentially dependent on the transcription factors NOTCH2 and KLF4, indicating heterogeneity within IRF4+ cDCs. Production of Notch2-dependent IRF4+ cDCs is required for effective type 3 immune responses, whereas KLF4-dependent Irf4+ cDCs appear to be required for type 2 responses by an unknown mechanism [138-140]. At steady state and particularly during inflammation, CD11b+ DCs comprise of a heterogeneous subset of cells that arise from bone marrow DC precursors and monocytes. Absence of cell-surface markers distinguishing bona-fide and monocyte-derived CD11b+ cDCs complicates the analysis of the contribution of tissue-resident CD11b+ cDCs to tissue immunity [117].

The two branches of cDCs can be distinguished by mutually exclusive expression of XCR1 and Sirpα (CD172). XCR1 is expressed by IRF8+ cDCs (cDC1), and CD172 is expressed by IRF4+ cDCs (cDC2), independent of activation status and location [141].
2.23 Dendritic cells during inflammation

During inflammation, a distinct population of DCs is transiently formed and accumulates in injured tissue until inflammation is resolved. These inflammatory DCs are thought to arise from blood monocytes, which express high levels of Ly6C and the integrin CD11b and intermediate levels of the integrin CD11c [142]. The differentiation of monocytes into DCs during inflammation is likely propagated by several cytokines, including GM-CSF, TNF-α, IL-4 and Fms related tyrosine kinase 3 ligand (Flt3L). Secretion of Flt3L by activated T cells and stroma cells increases monocytes numbers in blood, and likely helps drive the differentiation of monocytes to DCs in inflamed tissue [143]. A recent study has identified two subsets of naïve monocytes, which are functionally different during inflammation. They have identified one subset of monocytes which turn into DCs, while another subset turns into MFs in tissue. Monocytes identified as NR4A1- and Flt3L-independent, CCR2-dependent, Flt3⁺CD11c⁻MHCII⁺PU.1hi were shown to act as precursors for FcγRIII⁺PD-L2⁺CD209a⁺, GM-CSF-dependent monocyte-derived DCs but was distal from the DC lineage, as shown by fate mapping experiments using ZBTB46. Conversely, Flt3⁺CD11c⁻MHCII⁺PU.1lo monocytes differentiated into FcγRIII⁺PD-L2⁻CD209a⁻iNOS⁺ MFs upon microbial stimulation [144].

2.24 Bone marrow development of dendritic cells and homeostasis in tissue

cDC development starts in the bone marrow and is dependent on Flt3L. Its receptor Flt3 (CD135) is expressed on a continuum of Flt3⁺ DC precursors that gradually differentiate in the bone marrow (BM). The macrophage DC progenitor (MDP) is thought to be able to differentiate into monocytes and DCs. The differentiation of MDPs to common DC progenitor (CDP) restricts
it to only DCs, including plasmacytoid DCs and cDCs. The differentiation of CDPs to pre-cDCs restricts these cells to only the cDC1 and cDC DCs. Recent studies have shown that precursors to cDC1 and cDC2 DCs, pre-cDC1 and pre-cDC2 respectively, commit to the individual lineages in BM rather than using tissue signals to drive the differentiation into mature DCs. Pre-cDCs enter circulation from bone marrow and seed both secondary lymphoid organs and tissues [123].

The development of pre-cDCs formed in the BM to mature dendritic cells residing in tissue is an elusive process. The tissue environment likely has a major impact on guiding the differentiation of pre-cDCs entering from circulation. Furthermore, it has been shown that across different tissues, dependency of transcription factors that are known to have critical roles in generation of mature DC populations is variable. For example, the dependency of cDC1 and cDC2 DCs on the transcription factors BATF3 and IRF4 respectively is different amongst tissues such as the intestine, mesenteric lymph nodes, skin, lungs, spleen, liver and brain. These results were demonstrated using competitive bone marrow chimeras in which CD45.1+CD45.2+ WT mice were lethally irradiated and reconstituted with a 50:50 mixture of Batf3−/− or Irf4−/− CD45.2+ bone marrow with WT CD45.1+ bone marrow. For example, in liver, the dependency of cDC1 DCs on the BATF3 transcription factor is higher than the intestine, and this results in varying levels of chimerism when the ratio between CD45.1+ and CD45.2+ cDCs is calculated after full bone marrow reconstitution. Similarly, in cutaneous lymph nodes, the dependency of cDC2 development on the IRF4 transcription factor is much higher than the skin or intestine [145].

Variation in key transcription factors that drive the development of cDCs in tissues results in significant variation in proportion and different subsets of cells across tissues which make the understanding of general DC maturation in tissues difficult [145]. High dimensional throughput technologies such as mass cytometry (CyTOF), which is a variation of flow cytometry in which antibodies are labeled with heavy metal ion tags rather than fluorochromes, is a novel method of
understanding cell phenotypes and variation in cell populations [145]. Identification of novel cell-surface markers and transcription factors to complement already-known markers such as CD103, CD11b, MHC-II, CD11c and transcription factors such as IRF8, BATF3, Id2, IRF4, NOTCH2, and RELB2 is necessary to understand both general and tissue-specific maturation and differentiation. For example, using CyTOF, it has been recently discovered that CD26 can be used as a marker complementary to CD11c to identify cDCs [145]. Furthermore, a recent study has demonstrated that within specific population of cells, differential regulation by transcription factors alters DC function in tissue. Kruppel-like factor 4 (Klf4) is a potential candidate for regulating the development of the IRF4-expressing CD11b⁺ cDCs. Klf4 can act as a repressor or activator of transcription and regulates development in several epithelial tissues, including skin, lung, and intestine. Deletion of Klf4 in mice downregulated IRF4 expression in the pre-cDC stage in bone marrow and selectively compromised the development of particular subsets of IRF4-expressing cDCs. Deletion of Klf4 resulted in significant reduction of migratory cDC2 DCs, particularly the CD11b⁻CD24⁻ double negative cDCs in skin lymph node [140]. Although IRF4-expressing cDCs control Th17 and Th2 immune responses, deletion of Klf4 only influenced Th2 cell priming [140]. Th17 cell immunity has been shown to be attributed to the action of a Notch2-dependent subset of IRF4 expressing cDCs [138, 139]. These results indicate that distinct tissue-specific transcriptional programs regulate cDC function. In addition, these results show that within cell populations, significant heterogeneity with regards to development and function exists. Additional studies, particularly at the tissue-specific level, are required to elucidate cDC function.

2.25 Cytokines involved in dendritic cell homeostasis and survival

Four main cytokines involved in DC homeostasis and survival are Flt3L, GM-CSF,
macrophage colony-stimulating factor (M-CSF/CSF-1), and transforming growth factor-β1 (TGF-β1). Of these cytokines, Flt3 plays the most essential role in DC development in bone marrow and tissue homeostasis [146-150]. Flt3L is expressed by multiple tissue stroma cells and by activated T cells [151]. The role of GM-CSF in DC maturation is controversial. It has been shown that GM-CSF is a key cytokine for the differentiation of mouse and human hematopoietic progenitors and human monocytes into DCs in vitro, but in vivo studies have shown that DCs develop normally albeit with lower expression of CD103 in mice lacking GM-CSF or its receptor [152-155]. M-CSF is a key cytokine for MF development and has also been shown to be necessary for Langerhaans cell development [156, 157]. TGF-β1 has also been shown to be a nonredundant cytokine for Langerhaans cell development in vivo in mice [158].

3.0 Activation of T cells

Most tissue-resident cDCs are short-lived hematopoietic cells that are constantly replaced by blood-borne precursors [143]. At steady state and inflammation, cDCs are constantly migrating to lymph nodes via lymphatics charged with antigens. Once a DC is activated by DAMPS or PAMPS, it upregulates expression of MHC-II and CD11c, as well as co-stimulatory factors CD80/CD86 [159]. Enzymatic digestion of surrounding tissues is a critical step for the initiation of the complex process of DC relocation to lymph nodes. Matrix metalloproteinases (MMPs), such as MMP-9, is expressed on the surface of dendritic cells and helps participate in the migration of DCs to draining lymph nodes [160]. Chemokine receptors CCR7 and CXCR4 and their ligands CCL19, CCL21, and CXCL12 have been shown to play role in driving DC exit from tissue and migration to T-cell zones of draining lymph nodes. Furthermore, Rac1 and Rac2, which are in the
Rho family of triphosphatases, and the signaling adaptor Eps8, are critical in DC locomotion, polarization, and migration to lymph nodes [161].

Once the activated tissue-migratory DC is in lymph nodes, the process of naïve T cell activation is mediated through three signals. The first signal to initiate T cell activation is through the T-cell receptor (CD3) and MHC complex. The second signal is through co-stimulatory ligands. Naïve T cells express the co-stimulatory receptor CD28. CD28 binds to two co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86) expressed on APCs. Signal three is cytokine-mediated T cell activation [162]. cDC1 DCs are the main source of IL-12 and IL-15, the two main cytokines involved in the differentiation of cytotoxic CD8$^{+}$ T cells [117]. CD8$^{+}$ T cell activation also requires a signal induced by the interaction of IL-2 with the high-affinity IL-2 receptor, resulting in proliferation and differentiation of antigen-activation of cytotoxic T lymphocyte (CTL) precursor into effective CTLs. IL-2 can be generated by helper T cells as well as the CD8$^{+}$ T cell itself [163].

Type 1 IFNs are also potent mediators of T cell activation. Type 1 IFNs can be produced by almost any cell type in the body in the setting of viral infection and can have profound effects on T cell responses during infection. Type 1 IFN can directly augment CD8$^{+}$ T cell proliferation by binding the Type 1 IFN receptor on CD8$^{+}$ T cells [164]. Furthermore, Type 1 IFN upregulates expression of both MHC and costimulatory molecules which can greatly affect the initiation of T-cell responses [165]. Activation of CD4$^{+}$ T cells is mediated through various cytokines that direct differentiation of naïve CD4$^{+}$ T cells into different effector cells [162]. Absence of co-stimulation of T cells has been shown to induce T cell anergy, rather than activation. Once activated, T cells undergo a process called clonal expansion – rapid proliferation of antigen-specific T cells. In addition, activated T cells downregulate CD62L – a cell surface marker expressed on naïve T cells, and upregulate cell surface markers CD44 and CD69 [162].
3.1 T cell egression from lymph nodes into circulation

Trafficking and localization of T cells is mediated through interactions between selectins, chemokine receptors, and integrins. Naïve T cells circulate freely through blood and secondary lymphoid organs. During inflammation, inflammatory cytokines affect homing of T cells to site of injury through direct impact on cell-adhesion molecules on T cells and also on the vasculature [166].

Activation of naïve CD8\(^+\) T cells through T-cell receptor (TCR)-MHC-I interaction, co-stimulation, and inflammatory cytokines, results in rapid degradation of CD62L through a combination of protease-mediated cleavage and transcriptional repression. These recently activated CD8\(^+\) T cells lose the ability to enter lymph nodes through high endothelial venules. The newly activated CD8\(^+\) T cells produce daughter T cells that also express sphingosine 1 phosphate receptor 1 (S1RP1) – an important receptor involved in T cell egression from lymph nodes. Activated T cells exit the lymph nodes, enter the efferent lymphatic vessels, and then return to the blood stream either via the thoracic duct or the right lymphatic duct [166-168].

3.2 T cell migration into tissue

Migration of leukocytes into inflamed tissue is regulated by expression of various chemokines and receptors expressed on endothelium. Inflamed endothelium express P and E-selectins which bind P-selectin glycoprotein ligand 1 (PSGL-1), E Selectin Ligand-1, CD43 and CD44. Ligands for P and E-selectins are found exclusively on activated CD8 T cells, as opposed to naïve T cells to ensure infiltration of effector CD8\(^+\) T cells to site of injury [169, 170]. Activated CD8\(^+\) T cells also express chemokine receptors CCR4, CCR9, CCR6, CCR10, and CXCR3. These
are receptors for inflammatory cytokines CXCL9, CXCL10, and CXCL11 [171, 172]. In addition, activated CD8 T cells express a variety of integrins including $\alpha_4\beta_1$, $\alpha_1\beta_1$, and $\alpha_4\beta_7$. These integrins have been shown to direct migration and retention of CD8 T cells to inflamed tissue [173, 174].

3.3 Mechanisms of CTL-mediated cell death

Once activated CD8$^+$ T cells enter tissue, binding of the antigen with the T cell receptor (through TCR-MHC complex) results in activation of three distinct pathways that mediate cell death. The first pathway is through secretion of cytokines, primarily IFN-$\gamma$ and TNF-$\alpha$. TNF-$\alpha$ binds its receptor on the target cell and triggers caspase cascade, leading to apoptosis of the target cell. IFN-$\gamma$ increases infiltration and activation of other immune cells involved in apoptosis and antigen presentation, and it also stimulates presentation of endogenous peptides by MHC class I and increased expression of Fas receptor (CD95). The second mechanism of cell-mediated death is through binding of Fas ligand, expressed on activated CD8$^+$ T cells with Fas receptor on target cells. This binding triggers apoptosis through the classical caspase cascade. The last mechanism of cell death is through release of perforin and granzymes into the intercellular space. These are highly cytotoxic proteins that impact the target cell and cells in the surrounding space. There are two types of CTLs based on their cytokine expression profiles and method of cell-mediated death. Type 1 CTLs secrete IFN-$\gamma$, but no IL-4 and use perforin and Fas-mediated strategies, whereas type 2 CTLs express much more IL-4 and IL-5 and appear to only use perforin. Both CTL subtypes are potent killers and studies have shown that the majority of CTLs are biased toward the type 1 subset, whereas the type 2 subset is formed in the presence of IL-4 [175].
CTLs deploy various mechanisms to ensure death of the infected cell while protecting themselves and other neighbouring cells. Firstly, studies have shown that CTLs coat their cell surface with cathepsin-B post degranulation and exocytosis of perforins, which serves as a self-protective mechanism [176]. Secondly, CTLs use regulated secretory organelles to deploy targeted cell-mediated death. The regulated secretory organelles in which the lytic proteins are stored only mobilize to the cell surface and expose their content upon contact with a target triggering the TCR.

In addition, the secretory lysosomes do not exocytose their content randomly over the cell surface but are mobilized to a defined point on the plasma membrane that is immediately opposite the target cell, termed the secretory domain. Lastly, the secretory lysosomes release their content not into the general extracellular matrix milieu, which could kill other neighbouring bystander cells, but into a defined space, or “cleft” that forms between the tightly opposed CTL and target-cell membrane. This mechanism ensures that the cytotoxic proteins are concentrated for maximum impact and is confined to the environment of the target cell [177].

3.4 Role of CD4+ T cells in CTL-mediated death

Activation of naïve CD4+ T cells by APCs leads to effector T helper cell subsets that are defined by an array of features, including the polarizing cytokines that induce the expression of a master gene regulator that regulates expression of a signature set of effector cytokines the T-cell population produces once it is fully differentiated. T_{H1} cells are polarized by the cytokines IL-12, IFN-γ and IL-18 and these cells secrete IFN-γ and TNF. T_{H1} cells enhance APC activity, enhance CTL activation, and protect against intracellular pathogens [178]. T_{H2} cells are polarized by the cytokines IL-4 and these cells secrete IL-4, IL-5 and IL-13 [178]. T_{H2} cells protect against extracellular pathogens (particularly IgE responses) and are involved in allergy [178]. T_{H17} cells
are mainly polarized by TGF-β, IL-6 and IL-23 and these cells secrete IL-17A, IL-17F, and IL-22. The main function of T_{H17} are to protect against some fungal and bacterial infections, contribute to inflammation and autoimmunity [179]. T_{REG} T cells are often polarized by TGF-β and IL-2 and secrete IL-10 and TGF-β. The main function of T_{REG} T cells are to inhibit inflammation [180]. Lastly, T_{FH} T cells are polarized by IL-6 and IL-21 and secrete IL-4 and IL-21. These cells regulate B cell immunity in follicles and germinal centers [181].

A lesser known role for CD4 T cells is their ability to acquire cytotoxic activity and directly kill infected, transformed, or allogeneic MHC-II expressing cells. Unlike CD8^+ CTL that recognize cognate antigen in the context of ubiquitously expressed MHC class I molecules, the cytotoxic function of CD4^+ T cells is restricted to class II APCs such as the professional APCs that include dendritic cells, MFs, and B cells, as well as a number of infected tissue types. For example, it has been shown that cytotoxic CD4^+ T cells are generated against the Epstein Barr virus, a herpes virus typically harbored in latent form by B cells [182].

CD4^+ T cell activity is also required for the development of effective CD8^+ T cell memory and for optimal proliferative burst of functional T cells. Activated CD4^+ T cells express CD40L, a member of the TNF family of proteins, which is an important costimulatory signal to the APC. CD40L interacts with CD40, a TNF receptor family member expressed by activated APCs. Binding of CD40 with CD40L initiates a signalling cascade within the APC that increases the expression of costimulatory ligands (CD80 and CD86), chemokines, and cytokines, which significantly enhances the APC’s ability to activate CD8^+ T cells [183].
Rationale, Hypothesis and Objectives

Rationale:

Very little is known about cardiac DCs, due to limitations related to cell abundance and the challenge of distinguishing these cells from MFs, an abundant cell population in the heart that shares numerous cell surface markers with DCs. We sought to gain an in-depth understanding of cardiac DCs first in homeostatic/steady state conditions, and then study their functional role in the setting of viral myocarditis, a clinically important, yet poorly understood contributor to the development of HF.

One of the major difficulties in understanding why some patients develop HF, while others do not is the multi-factorial nature of disease. The cardiac immune landscape changes significantly both during viral myocarditis and development of HTN. We wondered if these two important risk factors could synergistically interact with each other to cause HF. Clinically, this would be analogous to a patient who has long standing high blood pressure, and then develops what would have been otherwise an innocuous viral infection. However, due to changes in immune cell composition, the patient deteriorates and develops cardiac dysfunction.

Hypothesis:

We hypothesize that cardiac cDCs are necessary to mount adaptive immune responses to EMCV-D infection. We hypothesize that deficiency in cardiac cDCs will lead to abrogated antigen-specific T-cell responses and worsening cardiac function following EMCV-D infection.
In addition, we hypothesize that EMCV-D infection during chronic HTN will lead to exacerbated immune infiltration into the myocardium leading to increased cardiac pathology.

**Objectives:**

1. Identification and characterization of cardiac dendritic cells.

2. Developing a model of viral myocarditis using EMCV-D and assessing the role of mononuclear phagocytes.

3. Does prior HTN predispose to the development of HF following subclinical viral myocarditis induced by EMCV-D infection.
Materials and Methods

Mice:

6-15 week old C57BL/6J mice (The Jackson Laboratory) were used for this study and were bred in our animal facility prior to use. \( Cx3cr1^{GFP/+} \) mice were kindly provided by Dr. Daniel Link [184]. \( Zbtb46^{GFP/+} \) mice were provided by Dr. Kenneth Murphy and have been previously described [185-189]. \( Flt3\)-cre mice were a kind gift from Dr. Thomas Boehm and were crossed with \( Rosa-mTmG \) C57BL/6 reporter mice (Jackson) [190]. \( Ccr2^{GFP/+} \) mice were generated by insertion of GFP under control of the native CCR2 promoter [191]. \( Csf1R-Mer-\text{iCre-Mer} \) mice were provided by Dr. Jeffry Pollard [192] and were crossed with the \( Rosa-mTmG \) reporter. Tamoxifen (2 mg) was gavaged in 100 µl of corn oil at E8.5 to label yolk sac MFs [73, 77]. \( Batf3^{-/-} \) and \( Zbtb46^{DTR/DTR} \) mice were purchased from The Jackson Laboratory. \( Irf8^{-/-} \) mice were a kind gift from Dr. Deepta Bhattacharya [193] and separately purchased from The Jackson Laboratory. \( Ccr7^{-/-} \) mice were kindly provided by Dr. Myron Cybulsky. \( Rag1^{-/-} \) mice were kindly provided by Dr. Daniel Winer. CD45.1 mice were purchased from The Jackson Laboratory. All mice were bred and maintained at University Health Network and experimental procedures were done in accordance with the animal-use oversight committees.

Tissue Isolation:

Mice were euthanized by \( \text{CO}_2 \) fixation. Hearts were then perfused with 20 ml of cold PBS. Hearts, lungs, and livers were minced finely and digested with shaking for 1 hour at 37°C in DMEM containing collagenase I (450 U/mL), DNase I (60 U/mL), and hyaluronidase (60 U/mL) enzymes (all Sigma). The digested material from heart, lungs and livers was filtered through 40 µm filters and pelleted by centrifugation (400xg for 5 minutes at 4°C) in HBSS supplemented with 2% FCS + 0.2% BSA. Liver was filtered and resuspended into 40% percoll. Brain was
filtered and resuspended into 40% Percoll mixed over top 80% percoll, and centrifuged for 30 min. Spleens were removed, minced and triturated through 40 µm filters in HBSS. Blood was collected into heparinized syringes. Red blood cells were lysed in ACK lysis buffer (Lonza) for 5 minutes at room temperature, and then resuspended in FACS buffer (PBS containing 2% FCS and 2 mM EDTA). Single-cell suspensions were then stained for cell-surface markers using antibodies listed in the Gating Strategy and Antibodies section.

**Flow cytometry, gating strategies and antibodies:**

Single cell suspensions were labelled with antibodies (0.2µl/sample) for 30 min at 4°C and washed in FACS buffer. All flow cytometric analysis was done on either an LSR Fortessa or FACS Canto II. After gating on CD45$^+$ cells, doublets were excluded and live cells were analyzed using FSC/SSC live-dead exclusion. Single cell analysis software (FlowJo) was used to analyze cell cytometric data. Full gating strategy of cardiac cDCs is shown in Figure 1A and S1A. Gating strategy for pre-cDCs is shown in Figure S3C. Gating strategy for DNs cDCs is shown in Figure 4A-B.

**Gating Strategy:**

Total cardiac and liver cDCs: CD45$^+$CD64$^-$CD11c$^+$MHC-II$^{hi}$ZBTB46$^+$

Cardiac MHC-II$^{hi}$ MFs: CD45$^+$CD64$^+$CD11b$^+$CD11c$^+$MHC-II$^{hi}$

Infiltrating monocytes: CD45$^+$CD64$^{lo-hi}$CD11b$^+$Ly6c$^+$

Lung cDCs: CD45$^+$CD64$^+$CD11c$^+$ MHC-II$^{hi}$SSC$^{Lo}$ZBTB46$^+$

Alveolar MFs: CD45$^+$CD64$^{hi}$CD11b$^{lo}$CD11c$^+$CD103$^-$. 

T cells: CD45$^+$B220$^-$CD3$^+$.

Double Negative cDCs: Above gates used for total cDCs and CD103^CD11b^- and either XCR1^{lo} or CD172a^{lo}.

Pre-cDC1 cells: Lin^−FLT3^+CD11c^+MHC-II^-/loCD172a^−Siglec-H^−Ly6c^-.

Pre-cDC2 cells: Lin^−FLT3^+CD11c^+MHC-II^-/loCD172a^−Siglec-H^−Ly6c^+.

*Antibodies used:*

CD45 (30-F11); CD64 (X54-5/7.1); XCR1 (ZET); CD103 (2E7); CD11c (N418); MHC-II (AF6-120.1); CD11b (M1/70); CD172a (P84); Ly6c (HK1.4); Ly6g (1A8); SiglecH (551); CD45.1 (A20); BrdU (Bu20a); Rat IgG1 (RTK2071); ESAM (1G8/ESAM); Clec9a (7H11); TNF-α (MP6-XT22); CD4 (RM4-5); CD8a (53-6.7); CD3e (145-2c11) CD49b (Dx5); TruStain CD16/CD32 FcBlock (93). All antibodies listed above were purchased from Biolegend. ZBTB46 (U4-1374) and B220 (RA3-6B2) were supplied by BD Biosciences. IFN-γ (XMG1.2) was supplied by eBiosciences. CCR2 (FAB5538N) was purchased from R&D Systems.

*Intracellular Staining:*

*Intracellular cytokines:* Cells in single-cell suspensions were stimulated with 500X Cell Stimulation Cocktail™ (plus protein transport inhibitors) from eBioscience for 5 hours at 37°C in DMEM with 10% FBS. Cells were then washed with FACS buffer and stained for surface markers for 30 minutes. Cells were washed again with FACS buffer and fixed for 45 minutes with FOXP3 Staining Kit™ (eBioscience). Cells were washed and left overnight with perm buffer. Cells were washed with perm buffer the next day, blocked with Fceblock™ for 15 minutes and stained for cytokines in perm buffer for 30 minutes.
**Transcription factors:** Cells in single-cell suspension were fixed for 45 minutes with the FOXP3 Staining Kit (eBioscience). Cells were washed with perm buffer blocked with Fcblock for 15 minutes and stained for cytokines in perm buffer for 30 minutes.

**BrdU:** For proliferation experiments, 2 mg of BrdU (Sigma) was injected i.p. 2 hours prior to harvest and for BrdU pulse-chase labeling experiments, 2 mg of BrdU was injected daily for four days for the indicated time points [73]. To detect intracellular BrdU, the BD Bioscience Cytofix/cytoperm technique was used. Briefly, cells were fixed after cell surface staining as above, DNA was digested for 1 hour with DNase (Sigma), and then samples were labeled with anti-BrdU antibody.

**RNA isolation and qPCR:**

RNA was isolated from homogenized tissue using Trizol Reagent (Ambion) and the RNeasy Micro Kit (Qiagen) as per manufacturer’s instructions. Samples were digested with DNase (Qiagen) prior to reverse transcription using the iScript cDNA synthesis kit (Bio-Rad) using 800ng of RNA. The resultant cDNA was analyzed by qPCR using LightCycler® 480 SYBR Green I Master Mix in a Roche LightCycler (Roche). [HRPT (forward: 5’-CAAGCTTGCTGGTGAAAAGGA -3’ ; reverse : 5’-TGAAGTACTCATTATAGTCAAGGGCATATC -3’) ; EMCV (forward 5’-GTCGTGAAGGAAGCAGTTCCCTC-3’ ; reverse 5’-CACGTTGGCTTTTGCCGCAGAGG-3’)]]. For absolute quantification, a standard curve of known concentration for EMCV was generated, as well as for HPRT to normalize for amount of RNA added to qPCR plate wells.
Cell sorting for microarray:

Cell sorting was performed using AriaII instrumentation (BD Bioscience). To sort cDCs and MFs, we altered our digestion protocol to minimize activation. Minced cardiac tissue was digested for only 45 minutes in Collagenase I (Roche) and DNase I (as described in Tissue Isolation section) RBCs were lysed for 3 minutes. Cells were gated on live (DAPI-) and gated as described in Gating strategy and antibodies section. All samples were sorted directly into either RPMI + 50% bovine serum for cell visualization studies or into RLT buffer for RNA extraction (Qiagen).

Cell sort and culturing for DN maturation:

Lungs from Rosa-mTmG mice were digested as explained in Tissue Isolation section and sorted for XCR1^+ and CD172^+ DNs. Sorted cells were separately co-cultured in 2x10^4 BM from C57Bl/6 mice in 96 well plates. CD11b and CD103 gMFI expression was analyzed immediately after sort (Day 0) and 24 hours with 100 ng Flt3 ligand treatment (Day 1).

Cell Visualization:

To visualize DC and MF morphology, hearts were harvested, digested, and sorted in MHC-II^hi MFs, CD103^+ DCs and CD11b^+ DCs. Cells were centrifuged in a Cytospin and stained with Hema3 (Fisher Scientific). Cells were imaged using an Olympus BX51 brightfield microscope with a 60x oil immersion lens (AOMF - Advanced Optical Microscopy Facility, Toronto, ON Canada). Approximately 18 cells were counted per cardiac DC and MF subset.
Parabiosis:

Briefly, after shaving the corresponding lateral aspects of each mouse, matching skin incisions were made from behind the ear to the tail of each mouse, and the subcutaneous fascia was bluntly dissected to create ~0.5 cm of free skin. The olecranon and knee joints were attached by a mono-nylon 5.0 (Ethicon), and the dorsal and ventral skins were approximated by continuous suture as previously described [73]. Mice were given Buprenorphine 0.1mg/kg after surgery. All animals received 3% neomycin antibiotics for 2 weeks. Fig. 2: CD45.1 and CD45.2 mice were surgically joined to create parabiotic mice and were analyzed after 2 and 6 weeks as described previously [73]. The percentage chimerism for each cardiac cDC and MF subset was normalized to blood monocytes in the recipient mouse (that originated from the donor) and expressed as a percentage. (% Normalized chimerism = % Donor cell in recipient / % Ly6c$^{hi}$ monocytes donor cells in recipient x 100).

Echocardiography:

Mouse echocardiography was performed using the VisualSonics Vevo® 2100 System. 2D and M-mode images were obtained in the long and short axis views. Wall motion index score was calculated using the following scaling: 0 = normal contraction, 1 = hypokinesia, 2 = akinesia, 3 = dyskinesia) with 10 segments of each heart analyzed. Total number of abnormal segments calculated using standard techniques. Some mice developed heart block (second degree), which was given a score of either 0 or 1, which a score of 0 no arrhythmia, and a score of one 1 indicating a degree of heart block, which made the final number of segments = 11.
**Bone marrow transplantation:**

Naive wild-type mice (CD45.1) were lethally irradiated twice with 628cGy with 3 hours in between. Chimeras were made with CD45.1 C57BL/6 hosts receiving ~8x10^6 BM cells from CD45.2 C57BL/6 mice (WT chimera) or CD45.2 Zbtb46^{DTR/DTR} mice (Zbtb46^{DTR/DTR} chimera). Animals were allowed to recover for a minimum 12 weeks. All animals received 3% neomycin antibiotics for 2 weeks.

**EMCV-D Culturing and Viral Infection.**

EMCV-D was added to L929 cells in a 90% confluent T-150 flask. Cells were infected for 24 hours. Cells were disrupted by putting them through 1-3 freeze-thaw cycles to release additional virus. Aliquots were stored at -80°C. For experiments shown in Figures 1-25 mice were infected *i.p* with 1x10^4 PFU EMCV-D (producing subclinical infection), unless otherwise specified in the figure legends. For experiments 26-35 mice were infected *i.p* with 2x10^4 PFU EMCV-D.

**Histology:**

Hearts of infected mice were harvested 14 days or 28 days after infection. After fixation in 10% buffered formalin overnight, hearts were paraffin embedded and then sectioned at 8 µm-thickness. Sections were then stained with H&E, Picrosirius Red/Fast Green (Chondrex) for fibrosis, or WGA (Vector Labs) and mounted with SlowFade® Diamond Antifade Mountant with DAPI, (ThermoFisher) for hypertrophy. Slides were imaged using the Aperio AT2 slide scanner or the Olympus Fluorescence Upright microscope at 20x (AOMF - Advanced Optical Microscopy Facility, Toronto, ON Canada). Fibrosis images were processed using Aperio ImageScope software and WGA images were processed using Metamorph software; both sets were analyzed using ImageJ software.
**Transcriptional Array:**

RNA from sorted cardiac cDCs and MFs was extracted using the Qiagen micro-kit (Qiagen). cDNA was prepared using pre-IVT Amplification Master Mix (Affymetrix). A total of 600 pg of RNA per subset was amplified using GeneChip WT Pico Reagent Kit (Affymetrix). Hybridization was carried out for 16 hours at 45°C. Microarray analysis was performed using GeneChip® Mouse Gene 2.0 ST Array (Affymetrix). Gene expression was analyzed as previously described using Partek Genomics Software [73]. For some experiments, GEO datasets were downloaded for additional cell subsets (also performed on Affymetrix 2.0 ST platform). Batch effect was removed prior to analysis.

**Angiotensin-II pumps:**

HTN was induced in Flt3-cre Rosa-mTmG or C57Bl/6 mice for 6 weeks using Miniosmotic pumps (Alzet Model 2006) or 4 days (Alzet Model 1003) surgically implanted subcutaneously. Angiotensin-II (Bachem) was administered 2.0mg/kg/day.

**Quantification and statistical analysis:**

All data are presented as mean ± SEM. Two-tailed Student’s t test was used for comparisons between experimental groups. Significant differences were defined at P < 0.05.

*Methods section has been altered from a manuscript currently in revision titled “A BATF3-Dependent CD103+ cDC Surveillance System Prevents Progression of Subclinical Viral Myocarditis to Overt Heart Failure”. Siyavash Hossein Zadeh is co-first author of this manuscript.*
Results

AUTHOR CONTRIBUTIONS: Experiments outlined in Figures 1-25 were completed in conjunction with Xavier Clemente-Casares. Xavier designed the MHC-I tetramer. Iulia Barbu performed histology quantification and qPCR for viral RNA. Figures 1-25 are based on a manuscript currently in revision, titled “A BATF3-Dependent CD103+ cDC Surveillance System Prevents Progression of Subclinical Viral Myocarditis to Overt Heart Failure”. Figures 26-35 were experiments designed and completed by Siyavash with assistance from other lab members.

OBJECTIVE 1: IDENTIFICATION AND CHARACTERIZATION OF CARDIAC DCS

1.1 Utilizing cell surface marker expression, transcriptional identity and genetic fate mapping to definitively characterize cardiac DCs (Figures 1-3).

An accurate characterization of DCs has been difficult because of common cell-surface markers with MFs in most tissues, including the heart, such as CD11c and MHC-II. We devised a gating strategy to identify and clearly separate MFs from DCs using a variety of complementary approaches. First, we performed flow cytometric analysis from a mouse cardiac single cell suspension. Total CD45+ leukocytes were identified and dead cells removed using either DAPI staining (labels the nucleus of dead cells) OR SSA-A and FCS-A gating as shown, which similarly removes dead cells by their smaller size (Fig 1A). Representative DAPI+ flow cytometric plot outlines location of dead cells/debris enabling proper identification of dead cells using our SSC-A/FCS-A gating strategy. Next, we excluded doublets using FSC-W/FSC-A and SSC-W/SSC-A gates as shown in (Fig 1A). CD64 has recently been described as a distinct cell-surface marker to identify mature tissue MFs [194]. We gated CD64- cells to exclude CD64+...
cardiac MF subsets (MHC-II\textsuperscript{hi}CCR2\textsuperscript{-} - R1, MHC-II\textsuperscript{lo}CCR2\textsuperscript{-} - R2, MHC-II\textsuperscript{hi}CCR2\textsuperscript{+} - R3), as previously described (Fig 1A)[73]. Following MF exclusion, we gated MHC-II\textsuperscript{hi}CD11c\textsuperscript{+} cells and identified two presumptive DC populations, CD103\textsuperscript{+} DCs and CD11b\textsuperscript{+} DCs (Fig 1A).

Integrin alpha X, CD11c, has been a hallmark trait of DCs. Our results show that CD11c is an inadequate sole marker to identify DCs because of its inability to parse CCR2\textsuperscript{+} MHC-II\textsuperscript{hi} MFs from DCs (Fig 1A). To definitively identify conventional cardiac DCs, we studied expression of ZBTB46 in our cardiac DCs. ZBTB46 is a transcription factor expressed in all conventional DCs, but not in pDCs or monocyte-derived DCs [110, 116, 144]. CD103\textsuperscript{+} and CD11b\textsuperscript{+} DCs expressed high levels of ZBTB46, whereas the cardiac MF populations did not (Fig 2A).

To ensure cardiac DCs were extravascular, we injected anti-CD45 antibody intravenously (i.v) and sacrificed mice 5 minutes post-injection. Cardiac DCs were not labelled while blood neutrophils were, indicating the cardiac DCs were extravascular (Fig 2B).

To assess cellular morphology and transcriptional identity of cardiac DCs, we sorted the two main cardiac DC populations and MHC-II\textsuperscript{hi} MFs (R1 – Fig 1A). Subsequent analyses of cardiac DCs include MHC-II\textsuperscript{hi} MFs as a comparator since this subset is a major MF population within the myocardium, and shares some potential functions with DCs, such as the ability to process and present antigen to T cells [73, 74, 87]. Visually, both cardiac DC populations contained dendrites and contained cytoplasmic vacuoles. (Fig 2C). We observed heterogeneity in the CD11b\textsuperscript{+} DCs in terms of size and number of dendrites (Fig 2C).

Principle component analysis, a statistical technique used to emphasize variation and bring out strong patterns in a data set, demonstrated that at the level of global gene expression,
each cardiac cDC subset and MHC-II\textsuperscript{hi} subset had its own global transcriptional identity (Fig 3A). Gene expression analysis revealed differentially expressed and shared genes amongst cardiac cDC and MF populations. CD103\textsuperscript{+} cDCs expressed \textit{Clec9a}, \textit{Xcr1}, and \textit{Id2}; CD11b\textsuperscript{+} cDCs expressed \textit{Irf4}, \textit{Sirp1a}, and \textit{Sirp1b} (Fig 3B). These are genes that have been shown in literature to be differentially expressed in CD103\textsuperscript{+} and CD11b\textsuperscript{+} DCs, respectively [117]. In addition, we compared our gene expression profiles with published data on spleen cDCs. Cardiac CD103\textsuperscript{+} DCs grouped with their respective CD24\textsuperscript{+} DCs in the spleen, and cardiac CD11b\textsuperscript{+} DCs grouped with their respective CD172\textsuperscript{+} DCs, confirming their DC identity (Fig 3C). We also ensured that the gene expression profiles we observed in our array correlated with protein expression. CD103\textsuperscript{+}, CD11b\textsuperscript{+}, and MHC-II\textsuperscript{hi} MFs were analyzed for various transcription factors and cell-surface markers (ZBTB46, XCR1, FLT3, CD24, CLEC9a, CD172a, DEC205, CX3CR1, CCR2), which demonstrated that their protein expression was similar to gene expression profiles (Fig 3D).
Figure 1. Gating strategy for cardiac cDCs and MFs. A) Extended gating strategy of cardiac cDCs and MHC-II\textsuperscript{hi} MFs is shown. Single cell suspension of cardiac tissue was labeled with antibodies and analyzed via FlowJo software. Cells were gated for CD45\textsuperscript{+} to identify leukocytes. Live/dead exclusion was performed through both DAPI staining (of dead cells) and also FSC-A/SSC-A exclusion - demonstrating similar results between the two. We utilized FSC/SSC for the all experiments except cell sorting studies. After live / dead exclusion, doublets were excluded using FSC/SSC (R1 and R2) gates as shown. Cardiac cDCs were then gated for CD64\textsuperscript{-} (R3) and MerTK\textsuperscript{-}. Subsequent analyses exclude MerTK and include only CD64 to exclude cardiac MFs. Cells were then gated on CD11c\textsuperscript{+}MHC-II\textsuperscript{hi} (R4) cells. Two major subsets of cardiac DCs are shown (CD103\textsuperscript{+} and CD11b\textsuperscript{+}). Cardiac MFs were CD64\textsuperscript{-}MerTK\textsuperscript{+} and stratified by MHC-II and CCR2 revealing three subsets (R1-R3) as previously described (Epelman et al., 2014a). Expression of CD11c in cardiac MF subsets using Cd11c\textsuperscript{YFP\textsuperscript{+}} mice.
Figure 2. ZBTB expression, location, and imaging of cardiac cDCs. A) Relative ZBTB46 fluorescence ratio in DC and MHC-II$^{\text{hi}}$ MF subsets within the myocardium. The geometric mean fluorescence intensity (gMFI) in each subset in Zbtb46$^{\text{GFP/+}}$ mice was divided by the gMFI of that subset in WT mice. The MHC-II$^{\text{hi}}$ MF population was further stratified by CD11c expression. B) Mice were injected i.v. with anti-CD45 and sacrificed 5 minutes later to label intravascular leukocytes. Neutrophils were identified as Ly6g$^{+}$CD11b$^{-}$CD64$^{-}$. C) Sorted cardiac DCs and MHC-II$^{\text{hi}}$ MFs were stained with Hema3 solution and the percent of cells expressing dendrites is shown. Error bars represent mean ± SEM. *p < 0.05, **p < 0.01, ****p < 0.0001. Experiments repeated at least twice, 3-8 per group.
Figure 3. PCA, gene-expression analysis and cell-surface marker expression of cardiac cDCs and MFs. A-C) Cardiac DCs and MHC-II$^{hi}$ MFs were sorted, RNA was extracted (8-9 pooled animals per group, n=3) for array-based gene expression analyses. A) Principle component analysis is shown. B) Hierarchical clustering of common DC and MF genes. C) Gene expression data from splenic cDC subsets was retrieved (Grajales-Reyes et al., 2015), batch effect was removed and hierarchical clustering performed with cardiac DCs and MFs. D) Expression of ZBTB46 and cell surface markers on cardiac DCs and MFs. Error bars represent mean ± SEM. *p < 0.05, **p < 0.01, ****p < 0.0001.
1.2 Differential life cycle of CD103+ and CD11b+ cDCs is driven by a balance between tissue level proliferation and replacement by circulating precursors (Figures 4-6).

To elucidate the temporal innate and adaptive immune response to a cardiac pathogen, it was first necessary to understand the lifecycle and kinetics of cardiac cDCs. To study cardiac cDC turnover in situ, we performed a bromodeoxyuridine (BrdU) pulse-chase experiment. BrdU is a synthetic nucleoside that is an analogue of thymidine. When injected i.p, BrdU is rapidly taken up by cells that are in S-phase of cell cycle. Subsequent intracellular staining for BrdU enables tracking of cells which have taken up BrdU and accurately identifies proliferating cells in tissue. We pulsed mice with BrdU for 4 consecutive days and allowed a chase period of 1, 3, and 17 days. Results are shown as percentage of BrdU labelled cells relative to day 1 frequency. By 3 days, nearly 50% of CD103+ cDCs lost their BrdU labelling highlighting their fast turnover rate in tissue. CD11b+ cDCs were labelled approximately 75% after 3 days. By 17 days, BrdU labelling was completely absent indicating full turnover. Similar results were observed in lung cDCs (Fig 4A, 4B).

We then assessed steady state proliferation of cardiac DCs using a BrdU pulse for 2 hours. Cardiac CD103+ cDCs were the most proliferative cells (~9%) compared to CD11b+ cDCs (~5%) and MHC-IIhi MFs (~2%). Similar results were observed in lung cDCs; except CD11b+ and CD103 DCs proliferation were similar (7-8)%, although still statistically less than CD103+ cDCs (Fig 5A, 5B). Using our gene expression profiles, we examined genes involved in activating and inhibiting proliferation. CD103+ cDCs expressed higher levels of proliferation-activating genes such as Tbk1 and Traf2, and lower levels of genes involved in inhibition of proliferation, such as Chek2 and Cdkn1a; compared to CD11b+ cDCs and MHC-IIhi MFs (Fig 6A).
To understand recruitment of cells through blood circulation, we performed parabiotic studies. Parabiosis is when the lateral sections of two animals are attached via skin incisions to share a circulation. For these studies, we combined female *Rosa*-mTmG mice in which all cells are red (TD tomato; Td+) with C57Bl/6 mice. When a uniform blood circulation is achieved between the two animals, this enables tracking of the TD+ cells in the recipient (C57Bl/6) mouse. To account for blood chimerism variability amongst parabionts, we normalized percentages of TD+ DCs within the myocardium to a general measure of chimerism achieved in the animals (the percentage of TD+ monocytes in the same animal). We performed parabiotic studies to elucidate contribution of blood precursors to tissue-resident cDCs. By two weeks, CD11b+ cardiac DCs had significant chimerism (~70%) whereas CD103+ cDC chimerism was similar to cardiac MFs (~10-15%). By 6 weeks, both cardiac cDC subsets had significant levels of chimerism (>80%). A plausible explanation for this is the higher proliferative capacity of CD103+ cDCs in tissue at steady state, while CD11b+ DCs are more dependent on recruitment of precursors (Fig 6B).

Lastly, we studied cDC activation/migration using a Toll-like receptor 3 (TLR3) agonist, Poly (I:C). Our gene expression profile revealed that TLR3 is present on cardiac cDCs and its activation with Poly I:C, which is a synthetic analogue of double-stranded RNA, enabled us to better understand cardiac cDC activation and migration (Fig 3C). Three days post Poly (I:C) administration, both CD11b+ and CD103+ cDCs decreased in numbers and by day 14 they returned to steady state numbers (Fig 6C, 6D).
Figure 4. Assessing cardiac cDC turnover using a BrdU pulse-chase system. A) Total cardiac cDC turnover was assessed by a BrdU pulse chase system. Animals were injected with BrdU for 4 consecutive days and sacrificed after a chase period of 1, 3, or 17 days. Frequency of BrdU+ cDCs was expressed as a percentage of initial day 1 label. B) Total lung DC turnover was assessed by BrdU Pulse chase system as described in Figure 2A. Labeled BrdU+ cDCs are expressed as a percentage of initial day 1 label and washout assessed over time to define total turnover kinetics. Error bars represent mean ± SEM. *p < 0.05, **p < 0.01, ****p <0.0001. Experiments repeated at least twice, 3-6 per group.

Figure 5. Assessing cardiac cDC in situ proliferation using a 2 hour BrdU pulse system. A-B) Mice were injected with BrdU 2 hours prior to sacrifice to label proliferating cells. Percentage of proliferating cardiac (A) and lung (B) cDCs and MFs at steady state shown as representative flow cytometric plots (left) and averaged data (right). Error bars represent mean ± SEM. *p < 0.05, **p < 0.01, ****p <0.0001. Experiments repeated three times, 4-8 per group.
Figure 6. Gene expression profiling of genes involved in proliferation, assessing cardiac cDC turnover using parabiosis, and cDC migration using a TLR3 agonist. A) Cardiac cDCs and MHC-II^{hi} MFs were sorted as described in Fig 2A. Hierarchical clustering of genes involved in proliferation. B) CD45.1 and CD45.2 mice were surgically paired and chimerism was analyzed after 2 and 6 weeks. The percent tissue chimerism was normalized to blood monocyte chimerism. (C, D) Mice were injected with 100 μg Poly (I:C) and sacrificed 3 or 14 days after injection. Number of cDCs were normalized to weight of tissue for hearts (C) or lung (D) and then expressed as a fraction of untreated control. Percentage of cells in S-phase at steady state in cardiac cDCs and MHC-II^{hi} MFs (right). Error bars represent mean ± SEM. *p < 0.05, **p < 0.01, ****p < 0.0001. Experiments repeated at least twice, 4-7 per group.
1.3 Identification of tissue pre-cDCs that differ from bone marrow pre-cDCs (Figures 7-10).

Analysis of cardiac cDCs during steady state consistently revealed a population of double negative (DN) cDCs lacking the CD103 and CD11b cell surface markers. These DN cDCs fell into all our previous gates (CD45\(^+\)CD64\(^-\)MHC-II\(^{hi}\)CD11c\(^+\)) (Fig 7A). Further analysis showed that these cells differentially express cell-surface markers XCR1 and CD172, which are differentially expressed on CD103\(^+\) and CD11b\(^+\) cDCs, respectively (Fig 7B, 7C). XCR1\(^+\) DNcs expressed intermediate levels of XCR1, while CD103\(^+\) cDCs expressed high levels of XCR1, these results are similar with CD172 expression in CD172\(^+\) DNcs and CD11b\(^+\) cDCs (Fig 7B, 7C). We sought to decipher whether these cells were committed, less mature counterparts to their respective cDC populations.

Recent studies have shown that tissue cDC precursors are committed to the cDC1 (CD103\(^+\)) and cDC2 (CD11b\(^+\)) lineage in bone marrow prior to entering tissue [123]. These precursors have been well investigated, including at the single cell level, and key factor in their identification was low / absent expression of MHC-II [123]. However cardiac DN cDCs we gated on expressed high levels of MHC-II, and were likely more mature, than their respective bone marrow and blood precursors. Anti-CD45 injection i.v ensured that DN cDCs were extravascular and not in blood (Figure 7C).

To ensure conventional DC identity of our DN cDCs, we stained for ZBTB46. DN cDCS express high levels of ZBTB46, significantly more than MHC-II\(^{hi}\) MFs. Similar results were observed in lung (Fig 8A). XCR1\(^+\) and CD172\(^+\) DNcs express similar cell surface markers as their counterpart CD103\(^+\) and CD11b\(^+\) cDCs in heart and lung (Fig 8B). In addition, steady state proliferation of DN cDCs showed proliferative levels similar to CD103\(^+\) and CD11b\(^+\) cDCs in
the respective populations (Fig 8C).

To show that DN cDCs mature into their respective counterpart DCs, we cell sorted female *Rosa*-mTmG mice where all cells are red (TD+). We digested lungs of these mice and sorted for XCR1+ and CD172+ DN cDCs. Lung tissue was used rather than cardiac tissue because numerically, lung tissue contain sufficient numbers of DN cDCs to perform the experiments, whereas cardiac tissue may only have 20-50 cells per heart. We co-cultured these sorted cells with bone marrow feeder cells from C57Bl/6 mice to create an environment conducive for DC differentiation. We also supplemented our TD+ sorted, bone marrow co-culture with Flt3L to drive their differentiation since Flt3L has been shown to be a critical growth factor for the survival and differentiation of cDCs [147, 149]. Immediately after the sort (d0) we measured CD103 and CD11b expression in our sorted cells to obtain baseline fluorescence. After 24 hours treatment of FLT3-ligand, significant increase in CD103 and CD11b expression in XCR1+ and CD172+ sorted cells was observed (Fig 9A). Similarly, administration of Flt3L intraperitoneally (*i.p*) to mice resulted in significant increase in DNs, CD11b+, and CD103+ cDCs (Fig 9B).

BATF3 and IRF8 are two transcription factors essential for the development of tissue-resident CD103+ cDCs [109, 128]. Absence of BATF3 or IRF8 leads to complete loss of CD103+ cDCs and a modest reduction in CD11b+ cDCs in *Batf3−/−* mice with no difference in *Irf8−/−* mice (Fig 10A). In both *Batf3−/−* and *Irf8−/−* mice we observed a significant reduction in XCR1+ DNs and a slight reduction in CD172+ DNs compared to WT mice (Fig 10B). These results indicate that XCR1+ and CD172+ DNs are under similar transcriptional factor dependency as their mature cDC counterparts.
Figure 7. Identification of DN cDCs. A-B) Flow cytometric plots of CD103⁺CD11b⁻ cardiac cDCs (A) and expression of CD172a and XCR1 (B) in CD11b⁺ (left), CD103⁺ (middle) and DNs cDCs (right) in cardiac tissue (top) and lung (bottom). C) Mice were injected i.v. with anti-CD45 and sacrificed 5 minutes later to label intravascular leukocytes. Error bars represent mean ± SEM. *p < 0.05, **p < 0.01, ****p <0.0001. Experiments repeated at least twice, 3-6 per group.
Figure 8. ZBTB46 and cell-surface expression of DN cDCs. A) Expression of ZBTB46 in the MHC-II^{hi} MFs, cDCs and DNs in heart (top) and lung (bottom). ZBTB46 expression was normalized in the heart to MHC-II^{hi} MFs (left) and alveolar MFs in the lung (right). B) Cell surface expression of CD24, FLT3, and CD8a in CD172a^{+} and XCR1^{+} DN cDCs in heart (top), and lung (bottom). C) Percentage of cells in S-phase at steady state in cardiac MHC-II^{hi} MFs and DNs. Error bars represent mean ± SEM. *p < 0.05, **p < 0.01, ****p < 0.0001. Experiments repeated at least twice, 4-8 per group.
Figure 9. Maturation of DN cDCs in vitro and in vivo using Flt3L. A) Lungs from Rosa-mTmG mice were and sorted for XCR1+ and CD172+ DNs. Sorted cells were separately co-cultured in 2x10⁴ BM cells from C57Bl/6 mice in 96 well plates. CD11b and CD103 gMFI expression was analyzed immediately after sort (Day 0) and 24 hours with 100 ng Flt3 ligand treatment (Day 1). B) Total number of CD11b+ and CD103+ cDCs (top) and CD172a+ and XCR1+ DN cDCs (bottom) per mg cardiac tissue. Mice were treated with 10 or 20 μg Flt3 ligand i.p. for 3 days and compared to untreated control. Error bars represent mean ± SEM. *p < 0.05, **p < 0.01, ****p <0.0001. Experiments repeated three times, 3-4 per group.
Figure 10. Number of cDCs in cardiac tissue in Batf3<sup>−/−</sup> and Irf8<sup>−/−</sup> mice. A) Number of cells/mg of cardiac tissue of cardiac cDCs in WT, Irf8<sup>−/−</sup> and Batf3<sup>−/−</sup> mice with representative flow cytometric plots (left) and averaged data (right). B) Number of cardiac XCR1<sup>+</sup> (top) and CD172a<sup>+</sup> (bottom) DN cDCs in per mg tissue in WT, Batf3<sup>−/−</sup>, and Irf8<sup>−/−</sup> mice. Error bars represent mean ± SEM. *p < 0.05, **p < 0.01, ****p < 0.0001. Experiments repeated at least twice, 4-8 per group.
Objective 1 Summary:

In summary, we devised a gating strategy to accurately identify cardiac cDCs using flow cytometry. Using BrdU pulse-chase, 2 hour BrdU pulse, TLR3 activation, and parabiotic experiments we identified the lifecycle and activation of cardiac cDCs. Global gene expression analyses identified genes commonly and differentially expressed amongst cardiac cDCs and MFs. Comparison of our gene array data with published data confirmed our cardiac cDC identify. Lastly, we identified tissue pre-cDCs, which we term DN cDCs, that are commonly found in major tissues and we believe are less mature tissue precursors. Based on similar cell-surface markers, in situ proliferation, and dependency on transcription factors, our initial assumption is that XCR1+ DN s are tissue precursors to CD103+ cDCs, and CD172+ DN s are tissue precursors to CD11b+ cDCs.
OBJECTIVE 2: DEVELOPING A MODEL OF VIRAL MYOCARDITIS USING EMCV-D AND ASSESSING THE ROLE OF MONONUCLEAR PHAGOCYTES

2.1 The innate immune response during viral myocarditis (Figure 11-13).

Viral myocarditis is a prevalent and understudied disease in the field of cardiovascular disease. Patients with viral myocarditis are often asymptomatic but can have serious cardiac complications later in life such as dilated cardiomyopathy and HF [5, 7, 10, 11]. Importantly, immune cells have been shown to play a major role in modulating cardiac repair and inflammation following injury [73, 87]. An in-depth understanding of the specific immune cells involved in the pathogenesis of viral myocarditis is lacking. More specifically, the functional role of cardiac cDCs, which are potent APCs and mediate both innate and adaptive immune responses to viral pathogens, has not been studied in the context of viral myocarditis. In this objective, we sought to understand the immune cells involved in the initiation and progression of viral myocarditis. We generated mouse models to specifically assess cDC function, such as ZBTB46DTR/DTR bone marrow chimeras, which add an additional level of specificity over CD11c-DTR mice which depletes MFs, NK cells and other lymphocytes in addition to DCs, and we generated MHC-I tetramers to understand the role of antigen-specific CD8+ T cells in viral myocarditis [34, 114-116].

We first sought to elucidate how the innate cardiac immune cell composition changes during EMCV-D infection using C57Bl/6 mice. When mice were infected with EMCV-D, CD103+ and CD11b+ cDCs numerically expanded in cardiac tissue, peaking at 7 d.p.i and decreasing at 28 d.p.i (Fig 11A). In CD103+ cDCs, this correlated with a significant increase in proliferation 7 d.p.i while in CD11b+ cDCs we did not see significant changes in proliferation
ZBTB46 expression in DCs at day 7 post infection confirmed the DC identity in expanded CD103⁺ cDCs while CD11b⁺ cDCs showed decreased expression of ZBTB46 compared to uninfected control CD11b⁺ cDCs. Expansion of CD103⁺ cDCs in the myocardium is likely due to both increase in proliferation and an increase in mobilization of pre-cDCs from bone marrow. Since pre-cDCs also express high levels of ZBTB46, this would be congruent with our findings. Recent studies have shown that monocyte derived DCs, which primarily express CD11b, during inflammation do not express ZBTB46 [144]. Our results, however, show that during infection, the majority of CD11b⁺ cDCs still express ZBTB46 although in lower levels than at steady state (Fig 11C). Although the infiltration of monocyte-derived DCs is definitely possible, and could explain the lower levels of ZBTB46, our finding that CD11b⁺ cDCs still express high levels of ZBTB46 confirms they are bona fide cDCs and likely expand from pre-cDCs exiting the bone marrow.

To define the time course of viral infection, we measured viral RNA within the myocardium. Viral RNA also peaked at day 7 d.p.i and was completely cleared after 28 days (Fig 11D). Although amount of viral RNA is not necessarily a predictor of cardiac outcome or damage, this time course of viral RNA enabled us to assess long-term changes in immune cell composition with relation to stages of viral infection (for example acute 1-7 days, long-term ~28 days) [195].

There are two main subsets of circulating monocytes in mice, Ly6c^hi and Ly6c^low monocytes. Ly6c^low monocytes adhere to and move along the endothelium, clearing damaged cells and triggering inflammatory responses without entering tissue [78, 79]. Ly6c^+ monocytes progenitors give rise to Ly6c^hi monocytes and through a nuclear receptor subfamily 4 group A
member 1 (NR4A1)-dependent transcriptional program, Ly6c<sup>hi</sup> monocytes differentiate into Ly6c<sup>low</sup> monocytes [76, 80, 81]. During cardiac stress, Ly6c<sup>hi</sup> monocytes are recruited from blood and enter tissue. During EMCV-D infection, we observed infiltration of monocytes into cardiac tissue which peaked at day 7 and returned to steady state numbers 28 d.p.i. Using monocyte and MF-specific markers, we were able to track the transition from newly-recruited monocytes to their differentiation into cardiac MFs. At day 4 post-infection, infiltrating monocytes were Ly6c<sup>+</sup>MHC-II<sup>low</sup> and expressed low levels of CD64 (Fig 12A). Between day 4 to day 7 post-infection, we observed downregulation of Ly6C with subsequent upregulation of MHC-II and CD64, indicating they are differentiating into cardiac MFs (Fig 12A). By day 28 post-infection, when the viral infection is cleared, we see similar numbers of cardiac monocytes and MFs as observed during steady state, indicating cardiac inflammation has subsided (Fig 12A, 12B).

Interestingly, we observed long-term changes in cardiac MF composition. We infected Flt3-Cre x Rosa-mTmG mice which enable tracking of cells that have or have not passed a transient Flt3 stage in hematopoiesis. In these mice, all cells are red (TD<sup>+</sup>), including MFs which were formed during embryonic development. Conversely, MFs which arise from hematopoietic precursors – and transiently pass through a Flt3 stage – become green, GFP<sup>+</sup> [73]. At 7 d.p.i we saw significant infiltrate of CCR2<sup>+</sup>GFP<sup>+</sup> (hematopoietic derived) monocytes/MFs in cardiac tissue and significant reduction of embryonically derived/TD<sup>+</sup> MFs. By day 28, TD<sup>+</sup> MFs partially recover to steady state numbers while GFP<sup>+</sup> MFs remained elevated 2 fold compared to steady state – thus decreasing the ratio of embryonic : hematopoietic cardiac MFs (Fig 13 A-D). Our previous results have shown that embryonic MFs self-renew in situ and are the predominant subset of cardiac MFs at steady state in adult mice [73]. These data provide a phenotypic and ontogeny-based map of the evolving innate immune response to viral myocarditis and show that
embryonic-derived MFs, which have been shown to be reparative and promote regeneration, are decreased during viral myocarditis [87].

Figure 11. Change in cardiac cDC composition following EMCV-D infection. A) Change in cardiac cDC composition following EMCV-D infection over time. B) Flow cytometric plots of cardiac cDCs in S-phase (BrdU+, 2 hr pulse) 7 d.p.i. (left, bottom) compared to uninfected control (left, top) with averaged data graphed (right). C) ZBTB46 expression in cardiac cDCs and MHC-IIhi MFs during EMCV-D. D) fold change expression of EMCV-D RNA relative to HPRT RNA in cardiac tissue 2, 4, 7, 14 and 28 d.p.i. Error bars represent mean ± SEM. *p < 0.05, **p < 0.01, ****p <0.0001. Experiments repeated at least twice, 4-8 per group.
Figure 12. Change in cardiac innate immune cell composition following EMCV-D infection. 
A) Flow cytometric plots showing total leukocytes gated on MF populations (CD11b+CD64+), and then further stratified into recently recruited monocytes (Ly6c+MHC-II-) which then differentiated into MFs (Ly6c-MHC-II+). B) Total number of cells per heart is shown following EMCV-D infection over time, expressed as a fold change of uninfected controls. Error bars represent mean ± SEM. *p < 0.05, **p < 0.01, ****p < 0.0001. Experiments repeated at least twice, 3-6 per group.

Figure 13. Change in cardiac MF composition following EMCV-D infection. A-C) Flow cytometric plots of Flt3-Cre x Rosa-mTmG mice analyzed for reporter expression in blood monocytes and cardiac MFs following EMCV-D (A) with changes in cardiac GFP+ (EmbryoD) and GFP+ (HSCD) normalized as total cell number per heart as a fold change over uninfected controls (B). C) Ratio of EmbryoD:HSCD cardiac MFs. D) Flow cytometric plots of cardiac MFs (CD11b+CD64+) in Ccr2GFP/+ mice. Error bars represent mean ± SEM. *p < 0.05, **p < 0.01, ****p < 0.0001. Experiments repeated at least twice, 4-8 per group.
2.2 Generation of EMCV-D specific CD8+ T cells activation during viral myocarditis (Figures 14-17).

The field of viral myocarditis, unlike other fields in immunology, lacks in-depth understanding of antigen-specific T cells in disease manifestation and pathogenesis. A tool to track T-cells via flow cytometry is through generation of antigen-specific tetramers. An MHC tetramer is a fluorochrome-conjugated peptide-MHC multimer that enables tracking, quantifying, and analyzing antigen-specific T cells using flow cytometry. In order to study antigen-specific anti-viral CD8+ T cells, which we believe are necessary for viral clearance, we generated MHC class I tetramers specific to the VP2 protein of the EMCV-D virus. To control for tetramer avidity when staining our samples, we also generated MHC class I control tetramers for the MCMV virus (Fig 14A). At day 7 post-infection – which we know is the peak of viral infection in the myocardium, EMCV-specific CD8+ (VP2+) T cells were approximately 10% of all CD8+ T cells in the myocardium, compared to our control tetramer which binds less than 2% of all CD8+ T cells (Fig 14A). This non-specific binding is taken into account when calculating VP2+ CD8+ T cell responses in organs.

CD4+ and CD8+ T cell response peaked 7 d.p.i and numbers returned to steady state numbers 28 d.p.i (Fig 14B). During EMCV-D infection, infiltrating CD8+ and CD4+ T cells produced large amounts of IFN-γ and TNF-α proving they were functional and in an inflammatory state (Fig 14C). In addition, injection of anti-CD45 antibody confirmed infiltrating CD8+ T cells were extravascular (Fig 14D).

Clonal expansion of VP2+ CD8+ T cells occurred at day 4 post-infection and peaked 7 d.p.i in mediastinal/thoracic lymph nodes with the majority of T cells in cell-cycle. Percentage of proliferating CD8+ T cells were highest in VP2+ CD8+ T cells and the highest proportion of
CD8+ T cells were tetramer-labelled; indicating that the VP2\textsubscript{121-130} peptide sequence is immunodominant (Fig 15A, 15B). Furthermore, LN regionality was observed 5 d.p.i, with the highest proportion of VP2+ CD8+ T cells in thoracic/mediastinal LN (which are closest to the heart), compared to mesenteric and popliteal LNs (Fig 15C). Accordingly, peak of VP2+ CD8+ T cell response in blood and heart was observed 7 d.p.i (Fig 15 D, 16A-B). Similar to LNs, the proportion of CD8+ T cells in cell-cycle was highest in VP2+ CD8+ T cells 7 d.p.i (Fig 16 E, 16F). In addition, CD4+ T cell response also peaked at 7 d.p.i both in cell numbers and proportion of cells in cell-cycle (Fig 16C).

After a viral infection, tissue-resident CD8+ T cells are formed and retained at the site of infection in numerous tissues, including the lungs, brain and skin [196], but this has not been assessed in myocarditis. On day 56, after viral clearance and resolution of cardiac inflammation, we observed maintenance of EMCV-D-specific memory CD8+ T cells in the heart, spleen and bone marrow (Fig 17A-C). Tissue-resident memory CD8+ T cells in the heart were almost exclusively VP2-specific (~70-80%), as compared to the spleen (~2%) and BM (~6%), with the vast majority (~80%) expressing effector memory markers (CD62L+ CD44+) (Fig 17A-C).
Figure 14. Identification of functional EMCV-D specific CD8\(^+\) T cells within the myocardium during viral myocarditis. 

A) Representative flow cytometry plots for VP2\(^+\) and VP2\(^-\) CD8\(^+\) T cells in WT mice 7 d.p.i using the EMCV-D specific and MCMV specific tetramers. 

B) Flow cytometry plots of CD4\(^+\) and CD8\(^+\) T cells in the heart 4, 7, and 28 d.p.i (left). Fold change in CD4\(^+\) and CD8\(^+\) T cells in the heart 4, 7, and 28 d.p.i compared to uninfected control (right). 

C) Representative flow cytometric plots for the expression of intracellular TNF-α and IFN-γ in CD4\(^+\) and CD8\(^+\) T cells 7 d.p.i compared to uninfected control is shown. 

D) To label intravascular leukocytes, mice were injected i.v. 1μg anti-CD45 and sacrificed 5 minutes later. Intravascular CD45 fluorescence is shown in CD3\(^+\) T cells. Error bars represent mean ± SEM. *p < 0.05, **p < 0.01, ****p < 0.0001. Experiments repeated at least twice, 4-8 per group.
Figure 15. Generation of EMCV-D specific CD8\(^+\) T cell activation during viral myocarditis.  

A-B) Flow cytometry plots (A) of infiltrating (top) and proliferating (BrdU\(^+\), S-phase) VP2\(^+\) CD8\(^+\) T cells 4 and 7 \(d.p.i.\) in thoracic LNs following EMCV-D infection, (B) graphed as percentage of infiltrating CD8\(^+\) T cells (left) and percentage of cells in S-phase in VP2\(^+\) and VP2\(^-\) CD8\(^+\) T cells (right) compared to uninfected control.  

C) Percentage of VP2\(^+\) CD8\(^+\) T cells in mediastinal, popliteal, and mesenteric LNs 5 \(d.p.i.\)  

D) Percentage of VP2\(^+\) CD8\(^+\) T cells in blood 4 and 7 \(d.p.i.\) compared to uninfected control. Error bars represent mean \(\pm\) SEM. *\(p < 0.05\), **\(p < 0.01\), ****\(p < 0.0001\). Experiments repeated at least twice, 3-5 per group.
Figure 16. Generation of EMCV-D specific CD8+ T cells in cardiac tissue during viral myocarditis.  A) Flow cytometric plots (left) of VP2+ CD8+ T cells in cardiac tissue over time course of EMCV-D infection and averaged data (right).  B-C) Flow cytometric plots of proliferating VP2+ and VP2- CD8+ T cells (B) and CD4+ T cells (C) in cardiac tissue 7 d.p.i. (left) and averaged data after 4 and 7 d.p.i (right). Error bars represent mean ± SEM. *p < 0.05, **p < 0.01, ****p <0.0001. Experiments repeated at least twice, 4-8 per group.
Figure 17. Generation of EMCV-D specific CD8\(^+\) Memory T cell activation during viral myocarditis. A) Percentage of VP2\(^+\) CD8\(^+\) T cells in the blood 7 d.p.i compared to uninfected control after infection with EMCV-D (10\(^4\) versus 10\(^5\) PFU). B) Number of CD8\(^+\) T cells in the heart 7 d.p.i expressed as a fold change based on EMCV-D dose (left). Percentage of VP2\(^+\) CD8\(^+\) T cells in the heart 7 d.p.i compared to uninfected control (right). C-E) Percentage of memory VP2\(^+\) CD8\(^+\) T cells in heart, spleen, and BM 56 d.p.i. Percentage of VP2\(^+\) and VP2\(^-\) CD8\(^+\) Naive, effector, and central memory T cells in heart (top), spleen (bottom, left) and bone marrow (bottom, right) 56 d.p.i. with representative flow cytometry plots and averaged data. Naive (N, CD44\(^-\)CD62L\(^+\)), central memory (CM, CD44\(^+\)CD62L\(^+\)) and effector memory (EM CD44\(^-\)CD62L\(^-\)) were identified. Error bars represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. A-C) N=5 per group.
2.3 During subclinical infection, BATF3-dependent CD103+ cDCs are required to generate Ag-specific CD8+ T cell responses and prevent cardiac dysfunction (Figures 18-25).

ZBTB46 is a transcription factor expressed on endothelial cells, erythroid progenitors, and cDC1 and cDC2 DCs [117]. Zbtb46<sup>DTR/DTR</sup> chimera mouse models have become a novel tool to target specific depletion of cDCs without affecting monocytes, MFs and natural killer (NK) cells – a limitation of the cDC11c-DTR mouse model [110]. In these mice, diphtheria toxin (DT) receptor (DTR) is inserted into the 3’UTR of the ZBTB locus as a means to specifically deplete cDCs. Mice bearing this knock-in express DTR in cDCs but not other immune cell populations, and DT injection into Zbtb46<sup>DTR/DTR</sup> bone marrow chimeras results in cDC depletion [116]. Bone marrow chimeras are generated to ensure other ZBTB46-expressing cells, such as endothelial cells, are spared following DT administration. We first assessed whether number of cardiac cDCs was altered in ZBTB46<sup>DTR/DTR</sup> (not chimeras) by the insertion of DTR into the 3’UTR of the ZBTB locus. Cardiac CD103<sup>+</sup> and CD11b<sup>+</sup> cDC numbers were similar in WT and ZBTB46<sup>DTR/DTR</sup> (not chimeras) (Fig 18A). Furthermore, infection of ZBTB46<sup>DTR/DTR</sup> (not chimeras) and WT mice with EMCV-D showed similar antigen-specific CD8<sup>+</sup> T cell responses and similar percentage of fibrosis in cardiac tissue (Fig 18 B-C). These results indicate that without DT administration and generation of chimeras, ZBTB46<sup>DTR/DTR</sup> numbers of cardiac cDC and their ability to generate CD8<sup>+</sup> T cell responses is not affected. These data are supported by published data which prove that although ZBTB46 is expressed on cDCs, deficiency of ZBTB46 does not affect cDC development [110].

In order to elucidate the functional role of cardiac cDCs in the setting of EMCV-D infection, we generated ZBTB46<sup>DTR/DTR</sup> bone marrow chimeras and B6 bone marrow chimeras which are control mice to account for DT toxicity. (Fig 19A). At steady state, administration of
DT to ZBTB46\textsuperscript{DTR/DTR} chimeras resulted in significant reduction in CD103\textsuperscript{+} and CD11b\textsuperscript{+} cDCs, but not in cardiac MFs (Fig 19B-C). Infection of ZBTB46\textsuperscript{DTR/DTR} chimeras with EMCV-D along with constant administration of DT to deplete cDCs resulted in significant reduction of CD103\textsuperscript{+} cDCs 7 \(d.p.i\); meaning infiltrating CD103 DCs were effectively depleted. CD11b\textsuperscript{+} cDCs were reduced almost 50\% following DT administration, but this was not statistically different compared to B6 chimeras (this was likely due to low n values) (Fig 20A). Two plausible explanations for why we see a greater reduction in CD103\textsuperscript{+} cDCs than in CD11b\textsuperscript{+} cDCs following depletion in ZBTB46\textsuperscript{DTR/DTR} chimeras during EMCV-D infection could be that infiltrating CD11b\textsuperscript{+} cDCs have lower expression of ZBTB46, making them less susceptible to depletion or that a subset of ZBTB46\textsuperscript{-}, monocyte-derived DCs was present in this gate, which were not depleted at all.

ZBTB46\textsuperscript{DTR/DTR} chimeras given DT had significantly reduced VP2\textsuperscript{+} CD8\textsuperscript{+} T cells in blood and heart 7 \(d.p.i\), indicating that cardiac cDCs are necessary for the generation of antigen-specific CD8\textsuperscript{+} T cell responses (Fig 20B). To understand the effect of cDC depletion on cardiac function, we measured LV function using a wall motion index score as explained in Fig 22. A wall motion index score is calculated for EMCV-D infections because a common cardiac outcome we have observed after viral infection is not a change in global LV function, rather abnormalities in contraction in interspersed sections of the myocardium. Depletion of cDCs led to significantly more wall-motion abnormalities of the LV in ZBTB46\textsuperscript{DTR/DTR} chimeras, and thus decreased cardiac function (Fig 20C).

Since ZBTB46\textsuperscript{DTR/DTR} chimeras showed a complete loss of CD103\textsuperscript{+} cDCs with only partial depletion of CD11b\textsuperscript{+} cDCs post-DT administration, we postulated that CD103\textsuperscript{+} cDCs play a major role in modulating the CD8\textsuperscript{+} T cell response and subsequent cardiac injury following EMCV-D infection. In order to study the role of CD103\textsuperscript{+} cDCs, we used Batf3\textsuperscript{-/-} mice. These
mice are deficient in the BATF3, a transcription factor essential for the development of CD103+ cDCs during steady state (Fig 10A). Infection of Batf3−/− mice with EMCV-D resulted in significantly reduced VP2+ CD8+ T cell response in blood and heart (Fig 22A, 22B).

Interestingly, while antigen-specific CD8+ T cells numbers were reduced in cardiac tissue, percentage of proliferation of these cells in cardiac tissue remained similar to WT infected mice, suggesting proliferative expansion of antigen-specific CD8+ T cells is preserved despite lower numbers – likely a tissue-mediated process (Fig 22C). Infiltration of Ly6c+ monocytes were also significantly reduced in Batf3−/− mice, and a slight reduction of neutrophils 7 d.p.i (Fig 23C) was observed, suggesting that compared to WT mice, Batf3−/− mice had a general blunting of both the innate and adaptive response to infection.

Batf3−/− mice showed significant cardiac fibrosis as quantified by PicroSirius Red staining (Figure 23A). PicroSirius Red is a method of histological visualization of collagen I and III fibers in addition to muscle in tissue sections. Collagen appears red in light microscopy whereas other cells have a distinct green autofluorescence [197]. In addition, Batf3−/− had significant wall motion abnormalities as measured by the WMI score compared to WT infected mice (Fig 20B) at 14 d.p.i. Interestingly, Batf3−/− mice showed no difference in viral RNA in the myocardium compared to WT infected mice (Fig 23D).

Next, we sought to replicate our findings that the adaptive immune response is necessary to prevent cardiac dysfunction following EMCV-D infection in another mouse model. We infected Rag1−/− mice, which are deficient in CD4+, CD8+, and B cells. Rag1−/− mice had more than 70% mortality 10 d.p.i while WT infected mice all survived, significant wall motion abnormality, and more viral RNA in the myocardium compared to WT infected mice. These results indicate that the adaptive immune response is necessary to prevent the transition from
subtle cardiac injury – as observed in our WT mice – to significant cardiac dysfunction and mortality (Fig 24A-C).

Lastly, we tried to replicate our findings using a CD8\(^+\) T cell neutralizing antibody. Interestingly, when mice were infected with EMCV-D and received CD8\(^+\) T cell neutralizing antibody, we did not observe any changes in cardiac function compared to WT infected mice or any difference in viral load in the myocardium 14 days post infection (Figure 25A). One possible explanation for this might be that CD8\(^+\) T cells were not completely depleted, with residual CD8\(^+\) T cells in the myocardium of mice 14 d.p.i.

Figure 18. Adaptive immune response in Zbtb46\(^{DTR/DTR}\) (not chimeras). A) Number of cardiac cDCs cells / mg tissue in WT mice and Zbtb46\(^{DTR/DTR}\) mice. B) Percentage of antigen-specific CD8\(^+\) T cells in the blood 7 d.p.i in WT mice and Zbtb46\(^{DTR/DTR}\) mice. C) Percentage of fibrotic cardiac tissue 14 d.p.i in WT mice and Zbtb46\(^{DTR/DTR}\) mice. Experiments repeated twice, 3-5 per group.
Figure 19. Generation of Zbtb46<sup>DTR/DTR</sup> chimeras enables specific depletion of cardiac cDCs. A) Representative schematic outlining generation of Zbtb46<sup>DTR/DTR</sup> and WT chimeras in mice receiving DT injection. B-C) Total number of cardiac cDCs (B) and MFs (C) per heart after DT administration in Zbtb46<sup>DTR/DTR</sup> chimeras. Error bars represent mean ± SEM. *p < 0.05, **p < 0.01, ****p < 0.0001. Experiments repeated at least twice, 4-8 per group.

Figure 20. Composition of cardiac cDCs and cardiac function following EMCV-D infection in Zbtb46<sup>DTR/DTR</sup> chimeras. A) Number of cDCs per mg of cardiac tissue 7 d.p.i. with EMCV-D (10<sup>4</sup> PFU) in Zbtb46<sup>DTR/DTR</sup> and WT chimeras B) Percentage of VP2<sup>+</sup> CD8<sup>+</sup> T cells in blood C) Cardiac Function Day 7.
(left) and heart (right) 7 d.p.i. (10^4 PFU) in Zbtb46^{DTR/DTR} and WT chimeras. C) Wall motion index score (left) and total number of abnormal cardiac segments (right) 7 d.p.i. (10^5 PFU) in Zbtb46^{DTR/DTR} and WT chimeras. Error bars represent mean ± SEM. *p < 0.05, **p < 0.01, ****p <0.0001. Experiments repeated at least twice, 4-8 per group.

Figure 21. cDC1 DCs are required to generate an antigen-specific CD8^+ T cell response. A) Time course of VP2^+ CD8^+ T cell generation over time post EMCV-D infection in Batf3^{-/-} and WT mice (10^4 PFU). B) Flow cytometric plots of cardiac CD4^+ and CD8^+ T cells (left-top) and percentage of VP2^+ CD8^+ T cells (left-bottom) and absolute numbers of CD8^+ and VP2^+ CD8^+ T cells (right) 7 d.p.i. (10^4 PFU) Batf3^{-/-} and WT mice. C) Flow cytometric plots (left), absolute numbers (middle) and percentage of cells in S-phase (right) in VP2^+ CD8^+ T cells in cardiac tissue 7 d.p.i. (10^4 PFU) in Batf3^{-/-} and WT mice. Error bars represent mean ± SEM. *p < 0.05, **p < 0.01, ****p <0.0001. Experiments repeated at least twice, 4-8 per group.
Figure 22. Calculation of the wall-motion index score. Representative echocardiographic videos are observed in short and long-axis views and the myocardium is divided into 10 segments, with segments 2 and 5 being repeated in both views. Each segment of the myocardium is given a score from 0-3. Wall motion index score was calculated using the following scaling: 0 = normal contraction, 1 = hypokinesia, 2 = akinesia, 3 = dyskinesia) with 10 segments of each heart analyzed. For each animal, all 10 scores were added to give a final wall motion index (WMI) score.
Figure 23. During subclinical infection, BATF3-dependent CD103+ cDCs are required to prevent cardiac dysfunction. A) Cardiac tissue fibrosis was detected using Fast Green staining.
Percentage fibrotic area was quantified with ImageJ analysis 14 d.p.i. (10^4 PFU) Batf3^−/− and WT mice. B) Representative echocardiographic images outlining the cardiac cycle during contraction (systole) and relaxation (diastole), and averaged data of the wall motion index score in Batf3^−/− WT and uninfected control mice 14 d.p.i. (104 PFU). C) Flow cytometric plots of cardiac MF composition (CD45^+ CD64^+ CD11b^+), gated on Ly6c^− monocytes/MFs. Averaged data of Ly6c^− monocytes/MFs and Ly6g^+ neutrophil 7 d.p.i. with EMCV-D. D) Fold change expression of EMCV-D RNA relative to HPRT RNA in cardiac tissue 14 d.p.i. Error bars represent mean ± SEM. *p < 0.05, **p < 0.01, ****p < 0.0001. Experiments repeated at least twice, 4-8 per group.

Figure 24. The adaptive immune system is necessary to prevent lethal myocarditis. A-C) Survival curve (A), wall motion index score (B), and fold change expression of EMCV-D RNA relative to HPRT RNA in cardiac tissue (C) in WT and Rag1^−/− mice. Mice were infected with 4x10^4 PFU EMCV-D. Experiments repeated once, n = 7.
Figure 25. Depletion of CD8+ T cells with neutralizing antibody during viral myocarditis. 
A) WMI score of WT and CD8+ T cell depleted mice 14 d.p.i. B) Representative flow cytometric plots of CD4+ and CD8+ T cells in WT and CD8+ T cell depleted mice 14 d.p.i. Mice were administered 200 µg CD8 neutralizing antibody i.p on days -1, 1, 4, 6, 8, 11, 13 and infected with EMCV-D on day 0. Echocardiography and tissue harvest was performed on day 14 post infection. Experiments repeated once, n = 10.

Objective 2 Summary:

In objective 2 we sought to understand the innate and adaptive immune response to viral myocarditis. In the early stages of viral infection, we observed significant infiltration of innate immune cells followed by adaptive immune cells. Generation of MHC-I tetramers enabled us to efficiently track antigen-specific CD8+ T cells post EMCV-D infection. We showed that cardiac cDCs, particularly CD103+ cDCs, are necessary to mount a CD8+ T cell response to EMCV-D. CD103+ cDC deficient mice showed significant reduction in antigen-specific CD8+ T cells and severe cardiac injury post EMCV-D infection. To corroborate that the adaptive immune response is required for prevention of cardiac damage, we infected Rag1-/- mice which lack B and T cells. By 10 d.p.i, we observed >70% mortality in Rag1-/- mice, significant cardiac dysfunction, and higher viral RNA compared to WT infected mice. These findings outline the functional role of CD103+ cDCs and implicate an important role for the adaptive immune response to prevent transition from subtle viral myocarditis to overt HF.
OBJECTIVE 3: DOES PRIOR HTN PREDISPOSE TO THE DEVELOPMENT OF HF FOLLOWING SUBCLINICAL VIRAL MYOCARDITIS INDUCED BY EMCV-D INFECTION.

3.1 Identification of immune cells involved in HTN (Figures 26-28).

Hypertension (HTN) is the leading cause of heart failure (HF) with preserved ejection fraction (HFpEF), which can lead to NICM, and is also the leading cause of NICM itself [49, 50]. There are currently no approved treatment options for HFpEF, even though these patients account for ~50% of all HF hospital admissions [49, 50]. Interestingly, not all patients with poorly controlled HTN develop HFpEF or NICM. Traditional risk factors account for only 50% of the risk for HF development, indicating that additional mechanisms promote susceptibility [49]. In large, well characterized cohort studies, multivariable models adjusted for known risk factors, including systolic blood pressure, explained only 50% of the variability of LV mass as assessed by echocardiography and 60% to 68% by cardiac magnetic resonance imaging. These data suggest the presence of other unmeasured risk factors [51-53].

The renin-angiotensin system (RAS) is a major regulator of blood pressure homeostasis. Cleavage of angiotensin-I by ACE yields Ang-II. The main effects of circulating Ang-II are to increase vasoconstriction, release of aldosterone, sodium and fluid retention, increase in inflammation, oxidative stress, and fibrosis. These effects are primarily mediated through the binding of the angiotensin type 1 (AT1) receptor [38]. Administration of Ang-II is a commonly used method to induce HTN in mice [41-45, 82, 102, 106, 107].

Our results from Objectives 1 and 2 implicate that the adaptive immune system response
is necessary for viral clearance and to prevent myocardial dysfunction. Since both viral myocarditis and HTN are two contributors to HF, and studies have shown that the development of HF is often a multifaceted process, comprising multiple risk factors, our next focus was to understand the immune system response to viral infection in a more clinically-relevant setting: chronic HTN.

To begin to define the impact of Ang-II administration on cardiac tissue alone, a panel of immune cells was designed to understand the immune system response during the initial manifestation of HTN (day 4) and chronic HTN (6 weeks). To ensure the efficacy of our model of HTN, we measured systolic and diastolic blood pressure using a tail-cuff sphygmomanometer. We saw significantly increase in systolic and diastolic blood pressure in mice receiving Ang-II compared to mice which underwent sham surgery (Fig 26) after 4 days. 4 days post-Ang-II administration, we observed a significant infiltration of innate and adaptive immune cells in cardiac tissue (Fig 27A-B). In addition, CD4+ and CD8+ T cells had cell-surface markers indicative of effector functions (downregulation of CD62L and upregulation of CD44) (Fig 27C-D). At the 6 week timepoint, no significant differences compared to steady state control mice were observed in cardiac immune cells other than CCR2+ MFs. These results indicate that in addition to blood pressure effects, Ang-II mediated cardiac inflammation through HTN is partially through an initial robust inflammatory response which subsides at the 6 week time point (Fig 28A-D).
Figure 26. Systolic and Diastolic Blood pressure following Ang-II administration Mini-osmotic pumps were surgically implanted into mice. Mice received 2.0mg/kg/day of Ang-II. Systolic and diastolic blood pressure were recorded day 4 post Ang-II administration using a tail-cuff sphygmomanometer.
**D4 HTN**

**A**  

**Innate Immune Response**

- **CD4 CD62L+ CD44-**
  - **Control**
  - **Hypertension**
  - Control: 0, Hypertension: 40
  - Control: 0, Hypertension: 60
  - **P = 0.073**

**B**  

**Adaptive Immune Response**

- CD4 T cells
- CD8 T cells
- B cells

**C**  

**CD4 CD62L+ CD44-**

- **Percent**
  - 4 days Sham: 0, 4 days HTN: 60
  - **P = 0.073**

**D**  

**CD8 CD62L+ CD44-**

- **Percent**
  - 4 days Sham: 0, 4 days HTN: 60
  - **P = 0.073**
Figure 27. Short-term Ang-II administration results in significant infiltration of immune cells in cardiac tissue. A-B) Number of cells in cardiac tissue expressed as fraction of WT control mice receiving sham surgery and WT mice 4 days post Ang-II administration. C-D) Percentage of CD44 and CD62L expressing cells in CD4$^+$ and CD8$^+$ T cells in WT control mice receiving sham surgery and WT mice 4 days post Ang-II administration. Error bars represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001. A-D). Experiments repeated 2 times, N=10.
Innate Immune Response

- **total ly6c+ monocytes**
- **ly6c+ CD64+ monocytes**
- **ly6c+ CD64- monocytes**
- **neutrophils**
- **total MFs**
- **CCR2+ MFs**
- **NK Cells**

Adaptive Immune Response

- **CD4 T cells**
- **CD8 T cells**
- **B cells**

**CD4 CD62L+ CD44-**
- Percent: 60% for 6 Weeks Sham, 40% for 6 Weeks HTN

**CD4 CD62L- CD44+**
- Percent: 15% for 6 Weeks Sham, 20% for 6 Weeks HTN

**CD8 CD62L+ CD44-**
- Percent: 40% for 6 Weeks Sham, 60% for 6 Weeks HTN

**CD8 CD62L- CD44+**
- Percent: 10% for 6 Weeks Sham, 15% for 6 Weeks HTN
Figure 28. Composition of immune cells in chronically hypertensive mice. A-B) Number of cells in cardiac tissue expressed as fraction of WT control mice receiving sham surgery and WT mice 4 days post Ang-II administration. C-D) Percentage of CD44 and CD62L expressing cells in CD4^+ and CD8^+ T cells in WT control mice receiving sham surgery and WT mice 4 days post Ang-II administration. Error bars represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001. A-D). Experiments repeated 2 times, N=10.
3.2 Viral infection during chronic HTN results in a dampened immune response and increased cardiac damage (Figures 29-35).

Poorly controlled and undiagnosed HTN are very prevalent diseases worldwide. Based on data collected by the National Health and Nutrition Examination Survey, the estimated prevalence of HTN in the US in the year 2000 was 28.7%. Among these participants with HTN, 68.9% were aware of the problem and only 58.4% were under pharmacological treatment. Overall, only 31% of individuals had HTN controlled to a blood pressure of <140 mmHg systolic and 90 mmHG diastolic. These data imply that more than 40 million adults have uncontrolled HTN in the US [198].

Our previous results indicate that cardiac immune cells play a major role in tissue repair, tissue inflammation, and modulating innate and adaptive immune responses to viral pathogens and in the setting of HTN [73]. We sought to mimic a clinically-relevant and prevalent setting, chronic HTN, by administrating Ang-II via miniosmotic pumps for 6 weeks. Furthermore, we wanted to understand whether chronic HTN had an effect on the immune response to a viral pathogen. In these sets of experiments, chronically hypertensive mice were infected with EMCV-D at 4 weeks of HTN and remained hypertensive until the 6 week timepoint. We studied the innate and adaptive immune responses, and cardiac function when chronically hypertensive mice were infected with EMCV-D. All sets of experiments had uninfected normotensive mice, hypertensive mice, and infected normotensive mice as controls.

In our first set of experiments, we wanted to understand whether there was a difference in cardiac function when chronically hypertensive become infected with EMCV-D. Based on our previous results, day 14 post-infection represents a timepoint where wall-motion abnormalities in the LV are discernable via echocardiography. First, we measured the heart weight to body weight
ratio at the 6 week time point – a common measure of cardiac hypertrophy – and confirmed significant heart weight to body weight ratio in mice receiving Ang-II, regardless of co-infection with EMCV-D. Virally infected mice alone did not show significant hypertrophy compared to control mice (Fig 29B). Chronically hypertensive mice which were infected with EMCV-D showed reduction in body weight 11 d.p.i. Normotensive uninfected mice, normotensive mice with infection, and hypertensive mice, all increased in body weight (Fig 29C). We examined cardiac function, and the combination of chronic HTN and EMCV-D infection led to a profound decrease in cardiac function (LV ejection fraction declined by -22% compared to WT control). HTN alone did not induce a change, while EMCV-D infection induced a smaller decline in LV ejection fraction (-10.5% compared to WT control) (Fig 29D). Ejection fraction refers to the amount, or percentage, of blood that is pumped (or ejected) out of the ventricles with each contraction. Generally, a higher ejection fraction is indicative of a better functioning heart. Since HTN generally impacts global LV function, rather than interspersed areas of the myocardium like EMCV-D infection, we had to use LV ejection fraction as a measure of cardiac function, rather than the WMI score.

Furthermore, we also investigated whether viral myocarditis prior to chronic HTN affects cardiac function (Fig 30A). In these experiments, mice were infected with EMCV-D first. After 14 d.p.i, a timepoint in which our previous results show that cardiac inflammation and viral RNA is resolved, we administered Ang-II for 6 weeks. Our results show that mice which had prior viral myocarditis and subsequent chronic HTN had the lowest ejection fraction (-17.2% compared to 8 weeks EMCV-D, -27.4% compared to WT control, Fig 30B). To understand whether the immune system is involved in the transition to HF in chronically hypertensive mice, our subsequent experiments focused on mice which are infected during chronic HTN (Fig 29A schematic) rather than viral infection prior to chronic HTN (Fig 30A schematic).
We next sought to investigate a possible reason why chronically hypertensive mice which received viral infection had significant cardiac dysfunction compared to normotensive infected mice, and hypertensive mice. Our previous results implicate that the adaptive immune response is essential for preventing significant cardiac damage post-EMCV-D infection and the peak of adaptive immune response in cardiac tissue is seen at day 7 post infection. At 7 d.p.i., chronically hypertensive mice showed significant reduction in total cardiac MFs and a reduction in ly6C+ monocytes with no differences observed in neutrophils and Ly6c+CD64- monocytes (Fig 31B-D). Interestingly, chronically hypertensive mice also showed significant reduction in CD4+ T cells, CD8+ T cells, and antigen-specific CD8+ T cells compared to normotensive-infected mice (Fig 32 B, C, F). Percentage of antigen-specific CD8+ T cells in blood and heart, and number of B cells in heart were similar in chronically hypertensive mice compared to control infected mice (Fig 32 D, E, G). These results were seen either when cells were normalized to mg of tissue or total cells per heart (which takes into account the effect that HTN increased cardiac mass). These results indicate that in the setting of chronic HTN, the immune response, particularly the adaptive immune response, is significantly impaired following cardiac viral infection and this may be a reason why infection of chronically hypertensive mice with EMCV-D demonstrated more cardiac injury.

To exclude the possibility that the dampened immune response was a result of inability to infect chronically hypertensive mice to the same magnitude as normotensive-infected mice, we performed qPCR on EMCV-D RNA in cardiac tissue 7 d.p.i. In both experiments that were performed, chronically hypertensive mice had subtly more viral RNA compared to control mice, although this was not statistically significant (Fig 33B-C).

Although chronically hypertensive mice have similar numbers of cardiac cDCs as mice
receiving sham surgery (Fig 34), since we observed that adaptive responses were blunted in chronically HTN mice post ECMV-D infection, we wondered if initial generation of antigen-specific CD8 T cells was affected in the thoracic LNs.

We infected chronically hypertensive mice and sacrificed them 4 d.p.i. This represents a time-point where CD8 T cell priming in mediastinal lymph nodes occurs without detectable emigration of CD8 T cells into the blood or myocardium. We measured proliferation of CD4+ and CD8+ and we observed no difference in proliferation in chronically hypertensive mice compared to normotensive control (Fig 35E, F). In addition, we observed a slight reduction, although not significant, in Ly6c+CD64− monocytes and total MFs with no difference observed in total Ly6c+ monocytes and neutrophils (Fig 35B-D) in cardiac tissue 4 d.p.i.

Since we observed no differences in T cell priming in lymph nodes 4 d.p.i and no differences in percentage of antigen-specific T cells in blood 7 d.p.i, it is likely that the mechanism of the dampened adaptive immune response in chronically hypertensive mice is due to an infiltration issue and not to aberrant T cell priming in lymph nodes. Ongoing experiments in the laboratory are to elucidate the various chemokines and cell surface adhesion molecules involved in T cell trafficking, and whether T cell survival in the myocardium is impaired in chronically hypertensive mice. This is discussed in greater detail in the Discussion and Future Experiments sections.
Figure 29. Infection of chronically hypertensive mice results in impaired cardiac function. A) Schematic representation of experiments. B) Heart weight to body weight ratio. C) Change in
body weight in grams of chronically hypertensive mice, 11 days EMCV-D infection, and 11 days EMCV-D infection in chronically hypertensive mice. **D** Ejection fraction of mice as measured by LV trace in long-axis view echocardiography. Error bars represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001. B-D). Experiments repeated 2 times, N=10-11.

### D14 EMCV + Chronic HTN

**A**

[Diagram showing the timeline of experiments: 14 days EMCV, 6 Weeks Chronic HTN, Viral Myocarditis, Ang-II pumps, Sacrifice Mice]

**B**

Cardiac Function - Echocardiography

![Graph showing ejection fraction (%) with error bars](image)

**Figure 30. Administration of chronic HTN to mice with prior viral myocarditis results in impaired cardiac function.** A) Schematic representation of experiments. B) Ejection fraction of mice as measured by LV trace in long-axis view echocardiography. Error bars represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001. B Experiments repeated 2 times, N=10-11.
**Chronic HTN + D7 EMCV**

**Figure 31.** Infection of chronically hypertensive mice results in dampened innate immune response 7 days post EMCV-D infection. **A)** Schematic representation of experiments. **B-D)** Number of cells/mg of total MFs (B), neutrophils (C), monocytes (D) in cardiac tissue 7 d.p.i in WT mice which received sham surgery and chronically hypertensive mice. Error bars represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001. **B-D**). Experiments repeated 3 times, N=14-16.
Figure 32. Infection of chronically hypertensive mice results in dampened adaptive immune response 7 days post EMCV-D infection. A) Schematic representation of experiments. B-C) Number of cells/mg of CD8\(^+\) T cells (B) and VP2\(^+\) CD8\(^+\) T cells (C) in cardiac tissue 7 d.p.i in WT mice which received sham surgery and chronically hypertensive mice. D-G) Percentage of VP2\(^+\) CD8\(^+\) T cells in heart (D) and blood (E) and number of cells/mg of CD4\(^+\) T cells (F) and B cells (G) 7 d.p.i in WT mice which received sham surgery and chronically hypertensive mice. Error bars represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001. B-G). Experiments repeated 3 times, N=14-16.
Figure 33. Viral load in cardiac tissue 7 days post EMCV-D infection in chronically hypertensive mice. A) Schematic representation of experiments. B-C) Fold change expression of EMCV-D RNA relative to HPRT RNA in cardiac tissue (B) and represented as a fraction of WT mice (C) which received sham surgery and chronically hypertensive mice. Error bars represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001. B-C). Experiments repeated 2 times, N=10-14.
Figure 34. Quantification of cardiac cDCs in chronic HTN (not infected). Number of cardiac cDCs per/mg of tissue in mice which received sham surgery and hypertensive mice at 6 weeks. Error bars represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001. Experiments repeated 2 times, N=10. HTN = 6 weeks Ang-II. Sham = 6 weeks post sham surgery.
Figure 35. Innate immune response in cardiac tissue and T cell priming in chronically hypertensive mice. A) Schematic representation of experiments. B-D) Number of cells/mg of Chronic HTN + D4 EMCV

Innate Immune Response

B) Total Cardiac MFs

C) Cardiac Neutrophils

D) Total Cardiac Ly6c+ Monocytes

T cell priming in Mediastinal Lymph Nodes

E) LN CD8 T cell Proliferation

F) LN CD4 T cell proliferation
Total MFs (B), neutrophils (C) and monocytes (D) in cardiac tissue 4 d.p.i in WT mice which received sham surgery and chronically hypertensive mice. E-F) Percentage of CD8$^+$ T cells (E) and CD4$^+$ T cells (F) in S-phase in mediastinal LNs 4 d.p.i in WT mice which received sham surgery and chronically hypertensive mice. Error bars represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001. B-F). Experiments repeated 2 times, N=5-10.

Objective 3 Summary

In this objective, we sought to apply our understanding of the immune cells involved in viral myocarditis pathogenesis in a clinically relevant and prevalent condition, HTN. We used Ang-II to induce HTN in mice. By 4 days post Ang-II administration, we observed significant infiltrate of immune cells into the myocardium which expressed high levels of activation markers. By 6 weeks post Ang-II administration, the majority of cardiac immune cells were in similar numbers as steady state, normotensive control mice. We then infected chronically hypertensive (6 weeks) mice with EMCV-D to characterize the innate and adaptive immune response and cardiac function in mice. After infection of chronically hypertensive mice with EMCV-D we observed significant cardiac dysfunction as measured by echocardiography at 14 d.p.i. Since the results from our objective 2 demonstrated that the adaptive immune response is crucial to prevent cardiac dysfunction following EMCV-D infection, we infected chronically hypertensive mice and assessed B and T cell responses at 7 d.p.i. We observed significant reduction in antigen specific CD8$^+$ T cells and CD4$^+$ T cells in the myocardium of hypertensive mice compared to WT-infected mice. Since priming of T cells in mediastinal lymph nodes in chronically hypertensive mice was similar to WT mice post infection, and percentage of antigen-specific CD8$^+$ T cells in blood is similar between the two groups, we ruled out the possibility the deficiency in CD4$^+$ and CD8$^+$ T cells in cardiac tissue is because of impaired T cell priming. We
believe this dampened immune response in hypertensive mice following EMCV-D infection is either due to impaired infiltration of T cells, or impaired survival of T cells inside the myocardium. Further studies are required to understand the mechanism behind the dampened immune response in chronically hypertensive mice.
Discussion

HTN is one of the major risk factors for cardiovascular disease, yet it is unclear why some patients with HTN progress to HF. Recent studies have shown that the transition to HF is mediated by multiple risk factors in addition to common risk factors assessed in clinic. It has now been established that a significant proportion of HF patients have viral genomes in their myocardium, indicating a high prevalence of infection. Similar to HTN, many subjects with normal cardiac function also have viral genomes detected within the myocardium – thus it is unclear how prior cardiac infection impacts future development of HF. To gain a more comprehensive understanding of how viral myocarditis and HTN could interact, and synergistically promote the development of HF, it was necessary to understand the immune cells involved in the pathogenesis of viral myocarditis first – specifically, cardiac DCs – a subset of mononuclear phagocytes that have been poorly characterized.

Our studies on cardiac cDCs revealed two main cDC populations: CD103+ and CD11b+ cDCs along with a rare population of DN cDCs. Our preliminary studies on DN cDCs indicate that they are a rare population of cells that are found in all major tissues of the mouse, including the heart, lung and liver. These cells lack the CD103 and CD11b markers but express intermediate levels of XCR1 and CD172; which we believe are less mature precursors to their respective tissue-resident CD103+ and CD11b+ cDCs. These findings postulate an additional maturation step that has not yet been characterized, and that is what signals pre-cDCs in the bone marrow and blood in their transition to tissue pre-cDCs, and then more mature cDCs.

Recent studies have also identified “double negative” DCs. The study of Tussiwand et al identified a double negative population of DCs in the skin lymph nodes that mediate Th2 immune responses. These authors defined these cells as CD24CD11b- DCs, which are dependent
on the transcription factor KLF4 and IRF4 [140]. Furthermore, another study has identified a double negative population of DCs in the spleen, labelled CD24^−CD172a^− [199]. These two studies, however, do not identify the same tissue pre-cDCs we describe, since our DN cDC population is characterized as CD103^−CD11b^− and XCR1^+ OR CD172^+. The study that comes closest to identifying a precursor “double negative” population similar to our findings is the recent work by Miriam Merad’s group. In this paper, Salmon et al focused on CD103^+ cDCs as a therapeutic target to enhance melanoma immunogenicity. It was discovered that FLT3L administration for 9 days increased both CD103^+ DC numbers in tumor, as well as a “double negative” (CD103^−CD11b^−) population in bone marrow, blood, and tumor. The “double negative” population expressed high levels of IRF8 [200]. A major difference in this study performed by Salmon et al is that these “double negative” were identified in the tumor microenvironment, whereas we focus on tissue pre-cDCs at steady state conditions. The functional role of our DN cDCs, their lifecycle, and specific maturation steps driving their differentiation to mature tissue cDCs is not yet fully understood.

Our previous work on cardiac MFs have shown that they play a critical role in modulating tissue repair and damage following cardiac injury [73]. Furthermore, we showed that as a result of cardiac injury, the immune cell composition of the heart significantly changes, with specific immune cells playing both reparative functions and inflammatory functions. In the context of EMCV-D infection, a common pathogen used to mimic viral myocarditis, the role of immune cells in mediating these processes has been conflicting. We sought to perform an in-depth analysis of the major immune cells involved in the initiation and pathogenesis of viral myocarditis, which would be used as framework in future studies to elucidate the functional role of specific immune cells. One important finding was that we observed long-term changes in the composition of cardiac MFs during EMCV-D infection. In the adult mouse, cardiac MFs
comprise of the majority of immune cells and are mostly derived from MFs made during embryonic development [73]. The use of genetic-fate mapping mouse lines has enabled us to not only track these embryonically-derived MFs, but also deduce their functional role. Recent work from our lab and others have shown embryonically derived cardiac MFs play reparative roles in cardiac injury, whereas MFs originating from blood monocytes, are enriched in inflammatory genes and have been shown to play a pathological role in injury [73, 87, 90, 91, 107, 144, 192]. The finding that cardiac MF composition is shifted to a more pro-inflammatory, CCR2⁺ subset, has important clinical applicability because the CCR2-CCL2 axis has emerged as the central monocyte recruitment pathway that is being evaluated for therapeutic utility in all cardiovascular diseases [56, 201, 202].

The role of the adaptive immune response, particularly T cells, has been controversial with regards to the extent that they mediate protective effects following viral infection of the heart. For example, in a study performed by Opavsky et al., mice deficient in CD8⁺ T cells infected with Coxsackievirus B3 virus had higher mortality compared to control mice, whereas CD4⁺ T cell deficient mice had similar survival rates compared to control. Mice deficient in both CD4⁺ and CD8⁺ T cells (double knock out) had higher survival rates compared to control mice [195]. Conversely, in a study performed by Sriram et al., depletion of CD4⁺ or CD8⁺ T cells prior to EMCV infection was sufficient to limit the development of paralytic syndrome by 79% in BALB/c mice [25]. These studies imply that T cell subsets can play both a protective and pathological role in the setting of viral infection; host susceptibility is often determined by both the pathogen and the tissues impacted.

One major limitation to these studies was their inability to track antigen-specific T cells. The generation of MHC-I tetramers specific for the EMCV-D virus enabled us to study the
temporal adaptive immune response in great detail – the first time a tetramer has been used in the setting of viral myocarditis. To study the functional role of cardiac cDCs in the setting of viral infection we used Batf3−/− mice. In these mice, deficiency of CD103+ cDCs resulted in significant reduction in antigen-specific CD8+ T cells and significant cardiac injury as measured by wall-motion abnormalities and fibrosis following EMCV-D infection. WT mice showed very little cardiac dysfunction following EMCV-D infection, highlighting the importance of the adaptive immune response in preventing transition of subtle viral myocarditis to overt HF. These results were further demonstrated with Rag1−/− mice which lack B and T cells. Rag1−/− mice had even more exacerbated injury than Batf3−/− with more than 70% mortality by 10 d.p.i. These results indicate that in addition to CD8+ T cells, CD4+ T cells and B cells also play a role in preventing cardiac injury following EMCV-D infection. Surprisingly, when we infected WT mice with EMCV-D and depleted CD8+ T cells with neutralizing antibody, we did not see the any major differences in wall motion abnormalities compared to WT infected mice. This difference we observed in WT infected mice receiving neutralizing antibody and Batf3−/− could be due to the incomplete depletion of CD8+ T cells. Therefore, since we are using a dose of EMCV-D which normally induces slight cardiac dysfunction in WT mice, the presence of antigen-specific CD8+ T cells – although they were numerically less than WT-infected mice – could likely have been enough to mediate protective effects against the virus and prevent the transition to severe cardiac dysfunction. Specific depletion of CD8+ T cells, CD4+ T cells, and B cells are some ongoing experiments to elucidate the specific contribution of these cells in viral myocarditis. These data also help address an unresolved question of why some patients transition to severe cardiac dysfunction and HF following viral myocarditis while others fully recover. A prior viral infection which may have permanently altered embryonic:hematopoietic MF composition, or the inability of some individuals to mount sufficient adaptive immune responses – either through cDC
malfunction/deficiency or in immunocompromised individuals which are deficient in B and T cells, are all plausible explanations to account for this dichotomy.

Our focus on cardiac cDCs in the setting of viral myocarditis revealed that the initiation of an adaptive response is important for protecting against EMCV-D induced cardiac damage. Recent studies have shown that the adaptive immune response, specifically T cells, also play a major role in maintaining HTN. *Rag1*−/− mice have a reduction in blood pressure in Ang-II-infused mice, and this blood pressure is recovered through adoptive transfer of T cells [203]. Furthermore, CTLA4 inhibition also reduces blood pressure [204]. Mice depleted of CD8+ T cells also had reduced blood pressure and reduced cardiac injury in hypertensive mice [42]. These studies indicate that T cells play a key role in the maintenance of HTN and the subsequent cardiac injury in Ang-II, transverse aortic constriction, and Doca-salt induced models [42, 44, 203, 204].

Our results indicate that the initial stages of Ang-II-induced HTN (day 4) cardiac tissue is infiltrated with a large number of immune cells, including, but not limited to CCR2+Ly6c+ monocytes, cardiac MFs, B cells, CD4+ T cells, CD8+ T cells, CD103+ cDCs, CD11b+ cDCs, and neutrophils. These immune cells also had a higher activation state compared to normotensive control mice. Conversely, when we studied chronically hypertensive mice after 6 weeks Ang-II administration, we did not see significant changes in total numbers of immune cells other than CCR2+ MFs, and the activation state of CD4+ and CD8+ T cells was similar to normotensive mice after 6 weeks sham surgery. These results indicate that during hypertensive challenge, inflammation and subsequent cardiac injury is mediated by a brief inflammatory response which subsided by 6 weeks. These data enabled us to understand the immune response to HTN alone, both in acute and chronic HTN. We next investigated how chronically hypertensive mice
responded to EMCV-D infection.

As it is becoming clear that the development of HF is multifaceted, given the high prevalence of both HTN, and viral genomes found in patients with dilated cardiomyopathy, we believed co-infection of chronically hypertensive mice might mimic a clinically-probable scenario. Based on our findings in Objectives 1 and 2, we hypothesized that a viral infection during chronic HTN also induces a major inflammatory response. As a result of this exacerbated immune-cell driven inflammation, we thought chronically hypertensive mice would have increased cardiac pathology compared to WT infected mice.

We initially sought to investigate how cardiac function is affected in chronically hypertensive mice. By day 14, a timepoint where viral infection is almost completely resolved, our echocardiographic data revealed that chronically hypertensive mice had the lowest ejection fraction compared to WT mice, hypertensive mice, and infected mice. Infected mice had a slight reduction in ejection fraction but nevertheless comparable levels to control mice.

To understand why hypertensive mice had the lowest ejection fraction in response to viral infection, we shifted our focus on day 7 post-infection since our previous results indicated that this is the peak of the adaptive immune response. We saw remarkable reduction in total CD8+ T cells, antigen-specific CD8+ T cells, and CD4+ T cells 7 d.p.i compared to normotensive infected mice. Although not significant, we also saw reduction in monocytes and neutrophils in hypertensive mice after infection.

We next focused on the initial T-cell priming steps during EMCV-D infection. Our previous results showed that initial priming of CD8+ and CD4+ T cells occurred at day 4 post infection in draining LNs near the heart. Day 4 post infection also represents a timepoint where
only innate immune cells infiltrate cardiac tissue. Our results indicate that infiltration of innate immune cells – neutrophils and Ly6c+ monocytes were not different between chronically hypertensive mice and normotensive infected mice 4 d.p.i. In addition, there was no difference in T cell proliferation, and percentage of antigen-specific CD8+ T cells in LNs. Percentage of antigen-specific CD8+ T cells in blood at day 7 post-infection was also the same in both groups. These results eliminate the possibility that the dampened adaptive-immune response to viral infection in chronically hypertensive mice is a result of defective T-cell priming in LNs. These results implicate that the adaptive immune response is sufficiently activated in hypertensive mice following viral myocarditis, but CD4+ and CD8+ T cells are not present in the myocardium to the same magnitude as normotensive infected mice. Therefore, two plausible explanations are that either T cells cannot enter the myocardium during chronic HTN, or that their survival inside the myocardium is hindered. These results are intriguing because they do not align with what has been shown in literature. For example, studies have shown that Ang-II induces, rather than downregulates, the expression of CXCL10, a chemokine involved in T cell recruitment into tissue [205, 206]. Furthermore, our own results and other studies show that Ang-II-induced HTN favours the activation of T cells, rather than suppressing them [42, 44, 203]. Elucidating the molecular signal that is impaired in chronically hypertensive mice responsible for T cell migration is an ongoing study in our lab. Alternatively, impaired T cell survival in the chronically hypertensive myocardium is another possibility. Another focus of the lab is to understand whether these results can be replicated using another form of pathogen, such as the LCMV virus, in the setting of chronic HTN.

The findings that chronically hypertensive mice have a lowered immune response to EMCV-D infection raises many interesting questions. In the setting of sterile injury, such as HTN, the role of immune cells, such as T cells, is often pathological. Studies have shown that in
hypertensive mice, depletion of CD8⁺ T cells by neutralizing antibodies show a significantly reduced cardiac inflammatory response and fibrosis. Furthermore, it has been shown that these CD8⁺ T cells are required for MF infiltration in myocardium and subsequent activation to express proinflammatory cytokines and chemokines [42]. Conversely, in the setting of non-sterile injury, such as viral-induced myocarditis, we show that the adaptive immune response is mainly beneficial to prevent the transition from subtle viral myocarditis to overt HF and death. While studies have shown that the prevalence of viral genomes cannot necessarily predict a worse prognosis than in individuals who successfully clear cardiac pathogens, studies have shown that individuals who experience recurrent cardiac viral infections generally have worse prognosis and often transition to dilated cardiomyopathy and/or HF [7-11]. Taken together, the findings of a dampened immune response to EMCV-D during chronic HTN might be a host defense mechanism that ultimately results in worse cardiac outcomes. A reasonable hypothesis is that in chronic HTN, the host, which has already sustained some sort of cardiac injury, disables entry of immune cells (in this case it could be Ang-II mediated, or through general tissue mechanisms) to prevent further injury, which is something that aligns with our results: at day 4 HTN we see massive infiltration of activated immune cells but by 6 weeks HTN, immune cell composition resembles steady state conditions, even though these mice are still hypertensive and have elevated circulating Ang-II. Although this might initially serve as a defense mechanism in sterile injury, once the host is faced with another cardiac challenge, such as a pathogen, this defense mechanism might be deleterious in that the subsequent dampened immune response might be insufficient to protect the host from the invading pathogen. As a result, higher viral loads and worse cardiac function as measured by echocardiography is observed.

Our findings have important clinical implications. Patients who are chronically hypertensive and have been diagnosed with viral myocarditis might benefit from immunotherapy
to enhance viral clearance and prevent exacerbated cardiac injury. More importantly, our findings highlight that the transition to HF is likely multifaceted, with multiple risk factors playing a role in the transition. This “multiple-hit” cardiac damage might be the reason why some patients with HTN transition to HF, while others do not.
Conclusions

In summary, this thesis focused on several concepts. The first objective was to extensively characterize cardiac DCs. Using flow cytometry, microarray, parabiosis and BrdU pulse-chase experiments we elucidated their cell surface markers, transcriptional identities, and lifecycle. The second objective was to study the role of DCs and MFs in viral myocarditis. We studied the innate and adaptive immune responses to EMCV-D, a cardiotropic virus, and how cDC deficiency leads to impaired T cell responses to EMCV-D infection and subsequent cardiac injury. In the third objective, we applied our understanding of viral myocarditis in the setting of a prevalent and well-studied contributor of HF, HTN. We discovered that chronic HTN predisposed mice to have a blunted immune response to EMCV-D, without a major effect on viral titers, but led to markedly increased, virus-induced cardiac dysfunction. Collectively, these studies outlined in great detail the function of cardiac DCs and the immune response to viral myocarditis, and is the first study, to our knowledge, to extensively study the immune response to viral infection in the setting of chronic HTN. Further studies are required to define the mechanisms by which chronic HTN predisposes to HF development following viral challenge, the answers to which could help identify patients at risk for HF development, or may yield novel directions for therapeutic advancement.
Future Directions

A major limitation in our understanding of tissue cDCs is regarding the heterogeneity of CD11b+ cDCs. Heterogeneity in this population has made it difficult to create mouse models to appropriately identify the functional role of these cells. The first step would be to identify cell surface markers to help parse the different populations. Using high-dimensional analyses, such as CyTOF and single-cell RNA sequencing, we are currently investigating this issue. These experiments need to be performed both in steady state, and during inflammation, in multiple tissues, to identify the different populations. The next step would be to generate appropriate mouse models which are deficient in specific CD11b+ cDC subsets and understand their role in steady state and in inflammation, particularly in Th2/Th17-mediated immunity.

Additional experiments are required to fully elucidate the mechanism underlying the dampened immune response in chronically hypertensive mice following viral infection. If recruitment of T cells is impaired, expression levels of CXCL9, CXCL10, CXCL11, P-selectin, and E-selectin should be quantified. In addition, expression levels of chemokine receptors CCR4, CCR9, CCR6, CCR10, and CXCR3 and integrins including α4β1, α1β1, and α4β7 should be studied on activated T cells to elucidate whether impaired migration is a result of downregulation of receptors on the myocardium/endothelial cells or on the T cells. Another possibility is that activated T cells enter the myocardium but are unable to survive because of the microenvironment. To rule out this possibility, BrdU analysis for proliferating cells and DAPI staining for dead cells at day 7 post-EMCV-D infection would be appropriate.

Furthermore, it would be interesting to see if our results could be replicated in other forms of infection. Infection of chronically hypertensive mice with LCMV or Coxsackievirus B3
would be beneficial to further our understanding of whether the dampened immune response is common to all pathogens or just EMCV-D.

Lastly, we plan to replicate these experiments in other models of HTN such as transverse aortic constriction or DOCA-salt induced HTN. These experiments would clarify whether our results are specific to HTN or a combination of HTN and Ang-II effect. To discern whether our results are a hypertensive effect or a combination of HTN and Ang-II, we can also administer hydralazine, which is a smooth muscle relaxant to decrease blood pressure, to Ang-II-infused hypertensive mice.
References


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