

The Role of Fractalkine and CX3CR1- expressing lymphocytes during myocardial ischaemia/reperfusion injury

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Abstract

Introduction: Ischaemia/reperfusion (I/R) injury contributes up to 50% of the final infarct size following myocardial infarction (MI). Lymphocytes expressing the fractalkine receptor (CX3CR1) are associated with poor long-term survival. This project aims to investigate the role of fractalkine in lymphocyte-mediated myocardial I/R injury and evaluate whether this damage can be reduced by inhibiting fractalkine/receptor (CX3CL1/CX3CR1) interaction.

Methods: Flow cytometry, microscopy, qPCR, mass cytometry and MRI are used to study lymphocyte infiltration following MI in a surgical mouse model of I/R injury.

Results: CX3CR1 knockout leads to over 30% reduction in leukocyte infiltration at the MI site 2h after reperfusion, both in heterozygous ($p \leq 0.01$) and knockout ($p \leq 0.001$) mice. This returns to WT levels by 24h I/R. Despite the 20% increase in leukocyte infiltration at 24h, CX3CR1 knockout mice show a 2-fold decrease in T cell infiltrate, compared to WT ($p \leq 0.01$) and heterozygous animals ($p \leq 0.001$). Knockout mice show 30% reduction in the number of CX3CR1-expressing T cells and 50% in total CX3CR1⁺ cells ($p \leq 0.01$) that infiltrate the injured myocardium. MRI shows a trend towards more detrimental cardiac function in CX3CR1 heterozygous and knockout mice. Moreover, RAG2 knockout mice show non-significant 35% increase ($p = 0.067$) in leukocyte infiltrate. Finally, significant T cell maturation and increase in CX3CR1 expression is detected in mouse models of ageing and infection.

Conclusions: These findings suggest that knockout of CX3CR1 leads to delayed recruitment of leukocytes following cardiac I/R. This results in an altered immune cell infiltrate including reduced number of T cells. In addition, the role for CX3CR1-expressing T cells in myocardial I/R is supported by results showing that the number of CX3CR1⁺ CD3⁺ cells is significantly reduced from heterozygous to knockout animals following injury. However, more clinically representative mouse models may be required in order to elucidate the role of CX3CR1⁺ T cells in myocardial I/R.

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List of Abbreviations

AAR	Area at risk
ACS	Acute coronary syndrome
ADAM	A Disintegrin And Metalloprotease
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
ART	Antiretroviral therapy
ATP	Adenosine triphosphate
BCL	B cell lymphoma
BCR	B cell receptor
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary DNA
CHD	Coronary heart disease
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocytes
cTn	Cardiac troponin
CVD	Cardiovascular disease
CyTOF	Mass cytometry / cytometry by time of flight
DAMP	Damage-associate molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
DPBS	Dulbecco's Phosphate-Buffered Saline
ECG	Electrocardiogram
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EDV	End diastolic volume
EF	Ejection fraction
EGFP	Enhanced green fluorescent protein
Eomes	Eomesodermin
ESV	End systolic volume
FBS	Foetal bovine serum
FFPE	Formalin fixed paraffin embedded
FGF	Fibroblast growth factor
FMO	Fluorescence minus one
FSC	Forward scatter
FSC-A	Forward scatter area
FSC-H	Forward scatter height
GC	Germinal centre
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
HCMV	Human cytomegalovirus

HET	Heterozygous
HIV	Human immunodeficiency virus
I/R	Ischemia-reperfusion
IBD	Inflammatory bowel disease
ICAM	Intercellular adhesion molecule
ICP	Inductively coupled plasma
ICP-MS	ICP mass spectrometer
IF	Immunofluorescence
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMC	Imaging mass cytometry
INF	Infarct
iTreg	Induced Treg
JAMA	Junctional adhesion molecule A
KO	Knockout
LAD	Left anterior descending
LFA1	Lymphocyte function-associated antigen 1
Lt α	Lymphotoxin α
LV	Left ventricle
MAC1	Macrophage antigen 1
MCMV	Murine cytomegalovirus
MHC	Major histocompatibility complex
MI	Myocardial infarction
MMP	Matrix metalloproteinase
MPTP	Mitochondrial permeability transition pore
MRI	Magnetic resonance imaging
MuLV	Murine leukaemia virus
MVO	Microvascular obstruction
NF κ B	Nuclear factor κ B
NK	Natural killer cell
NLR	Nucleotide-binding domain, leucine-rich repeat
NSTEMI	Non-ST elevation MI
nTreg	Natural Treg
OCT	Optimal cutting temperature
PAMP	pathogen-associated molecular pattern
PAR-1	protease-activated receptor 1
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death 1
PECAM1	Platelet/endothelial cell adhesion molecule 1
PFA	Paraformaldehyde
PPCI	Primary percutaneous coronary intervention
PSGL1	P-selectin glycoprotein ligand 1
QC	Quality control
qPCR	Quantitative PCR
RAG	Recombination-activating gene
RNA	Ribonucleic acid

ROI	Region of interest
ROR	Retinoid orphan receptor
ROS	Reactive oxygen species
RV	Right ventricle
SEM	Standard error of the mean
sCX3CL1	Soluble fractalkine
SP	Single positive
SPF	Specific pathogen free
SSC	Side scatter
SSC-A	Side scatter area
STAT	Signal transducer and activation of transcription family
STEMI	ST elevation MI
TACE	TNF α converting enzyme
TAE	Tris-acetate EDTA
Tc	T cytotoxic cell
TCEP	Tris(2-carboxyethyl)phosphine
Tcm	T central memory
TCR	T cell receptor
TEM	Transendothelial migration
Tem	T effector memory
tCX3CL1	Transmembrane fractalkine
TGF	Transforming growth factor
Th	T helper cell
TLR	Toll-like receptors
TNF	Tumour necrosis factor
TOF	Time of flight
Treg	T regulatory cell
TRIS	Trisaminomethane
Trm	T tissue resident memory
Tscm	T stem cell memory
t-SNE	t-distributed stochastic neighbour embedding
TTC	Triphenyl tetrazolium chloride
UV	Ultraviolet
VCAM	Vascular cell adhesion molecule
VLA4	Very late antigen 4
WT	Wild type

Chapter 1. Introduction

1.1 Myocardial Infarction

Coronary heart disease (CHD) is the single leading cause of mortality worldwide, responsible for 12.8% of all deaths (Task Force on the management of ST-segment elevation acute myocardial infarction of the European Society of Cardiology (ESC), Steg et al. 2012). Although, its rate has decreased over the past four decades, about one-third of middle-aged individuals will develop some manifestation of fatal CHD (Lloyd-Jones, Adams et al. 2010, Nichols, Townsend et al. 2014). The most serious and acute form of CHD is acute myocardial infarction (MI), which is characterised by myocardial necrosis and cardiomyocyte death, as a result of prolonged myocardial ischaemia (Thygesen, Alpert et al. 2012, Montecucco, Carbone et al. 2015).

1.1.1 Pathogenesis

The most common underlying condition for development of MI is the acute rupture of a coronary atherosclerotic plaque, resulting in thrombus formation. This leads to occlusion of an epicardial artery and subsequent loss of blood flow distal from the thrombus (Montecucco, Carbone et al. 2015, Thygesen, Alpert et al. 2012). Other causes include vascular smooth muscle cell calcification, which is a highly prevalent age-associated pathology conferring CHD susceptibility to seemingly healthy individuals (Thijssen, Carter et al. 2016, Ruparelia, Chai et al. 2016).

1.1.2 Diagnosis

MI can be diagnosed by clinical features such as symptoms of ischaemia (chest pain, upper extremity or epigastric discomfort, dyspnea or fatigue), electrocardiographic (ECG) abnormalities and imaging evidence of loss of viable myocardium (Thygesen, Alpert et al. 2012). However, the guidelines for diagnosis were recently updated, focusing on serum-derived tissue-specific cardiac biomarkers of myocardial necrosis (Thygesen, Alpert et al. 2012, Montecucco, Carbone et al. 2015). The preferred biomarkers are cardiac troponin (cTn) I and T, components of the myocyte contractile apparatus, because of their high cardiac tissue specificity and clinical sensitivity (Torre, Jarolim 2015, Thygesen, Alpert et al. 2012). A detection of an increase and/or a decrease of plasma cTn levels with at least one value above the 99th percentile of the upper normal reference signifies MI (Montecucco, Carbone et al. 2015, Thygesen, Alpert et al. 2012).

1.1.3 Clinical classification of MI

It is critical for optimal and rapid diagnosis and clinical triage to designate patients presenting with acute coronary syndrome (ACS) into one of three groups. Those who develop ST elevation in two contiguous ECG leads are classed as ST elevation (formerly Q-wave) MI (STEMI) patients. Cases that present with no ST elevation are usually diagnosed as non-ST elevation (formerly non-Q wave) MI (NSTEMI) (Reeder, Kennedy 2017, Thygesen, Alpert et al. 2012, Montecucco, Carbone et al. 2015). Patients who have ischemic symptoms but have no biomarker elevation, regardless of ECG abnormalities, may be diagnosed as having unstable angina (Thygesen, Alpert et al. 2012, Reeder, Kennedy 2017).

In addition to these categories, the recently updated third definition of MI classifies MI into several types based on the clinical and pathophysiological presentation (Thygesen, Alpert et al. 2012). Type 1 (Spontaneous) MI is due to atherosclerotic plaque rupture, ulceration, fissuring, erosion or dissection. This leads to thrombus formation in a coronary artery, causing loss of blood flow distal from the thrombus which ultimately results in cardiac myocyte necrosis (Simons 2017, Thygesen, Alpert et al. 2012). Type 2 MI occurs secondary to an ischemic imbalance. In these cases, a condition other than coronary artery disease causes imbalance between oxygen supply and demand, such as coronary vasospasm, endothelial dysfunction, tachy-/bradyarrhythmias, respiratory failure and hypo-/hypertension (Figure 1.1) (Montecucco, Carbone et al. 2015, Thygesen, Alpert et al. 2012, Simons 2017). Sudden cardiac death with symptoms suggestive of myocardial ischaemia which occurs before blood sampling and biomarkers assessment is classed as Type 3 MI (Montecucco, Carbone et al. 2015, Thygesen, Alpert et al. 2012, Simons 2017). Furthermore, novel entities of MI are identified due to revascularisation procedures (Type 4a – Percutaneous coronary intervention (PCI), Type 4b – stent thrombosis, Type 5 – MI associated with coronary artery bypass graft surgery). In these cases, various insults may occur as a result of the revascularisation procedure, causing increase in cTn values (Montecucco, Carbone et al. 2015, Thygesen, Alpert et al. 2012, Simons 2017).

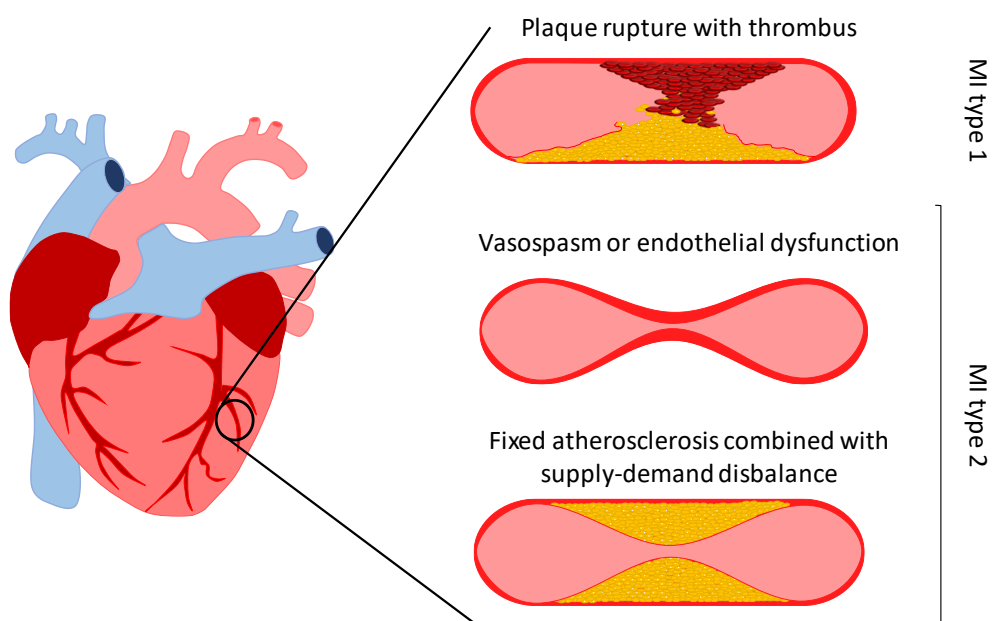


Figure 1.1 Type 1 and type 2 myocardial infarction (MI). Occlusion of a coronary artery can occur due to atherosclerotic plaque rupture leading to thrombus formation (type 1) or secondary to an ischemic imbalance without plaque rupture (type 2).

1.1.4 Treatment

The survival rate following MI markedly increased with the pioneering development of percutaneous transluminal coronary angioplasty in 1979 (Santos-Zas, Lemarié et al. 2019, Nabel, Braunwald 2012). The technique involves a catheter being introduced over a guidewire, passed across the coronary lesion, followed by the inflation of a balloon that dilates the blocked coronary artery (Nabel, Braunwald 2012). The method was further advanced with the insertion of metal and drug-eluting stents to prevent coronary restenosis (Nabel, Braunwald 2012). The development of primary percutaneous coronary intervention (PPCI) represents one of the major therapeutic advances of the last century (Libby, Theroux 2005). It is the preferred therapy for MI when performed by a skilled interventionalist, shown to be superior to fibrinolytic therapy which confers a greater risk of minor or major bleeding (Gibson, Pinto et al. 2017). Multiple randomised trials have shown that the use of PPCI results in improved short-term survival, as well as reduced risk of stroke or non-fatal MI when compared to thrombolytic therapy (Gibson, Pinto et al. 2017, Keeley, Boura et al. 2003). However, although the mortality rate following MI has decreased with the development of these interventions, an increase in the long-term incidence of heart failure has been reported (Velagaleti, Pencina et al. 2008). This trend is partially explained by the fact that myocardial reperfusion therapy itself causes damage to the myocardium, a phenomenon termed ischaemia-reperfusion (I/R) injury (Hausenloy, Yellon 2013).

1.2 Ischaemia reperfusion injury

I/R injury is now recognised as having an increasingly important role in a number of clinical conditions including organ transplantation, stroke and myocardial injury (Zuidema, Zhang 2010, Linfert, Chowdhry et al. 2009). It has highly complex pathogenesis, represented by an interplay between vascular endothelium, biochemical, cellular and tissue specific factors (Zuidema, Zhang 2010, Linfert, Chowdhry et al. 2009). Furthermore, inflammation has been implicated as a key mediator of I/R injury, with both innate and adaptive immune cells playing a role (Linfert, Chowdhry et al. 2009, Huang, Y., Rabb et al. 2007). Acute myocardial ischaemia leads to oxygen and nutrient deprivation, causing biochemical and metabolic changes in the affected area (Hausenloy, Yellon 2013). The hypoxia causes an abrupt stop in oxygen phosphorylation, mitochondrial membrane depolarisation and subsequent adenosine triphosphate (ATP) depletion (Hausenloy, Yellon 2013, Zuidema, Zhang 2010, Linfert, Chowdhry et al. 2009). Within seconds of ischaemia, cellular metabolism switches to anaerobic glycolysis due to the lack of oxygen (Hausenloy, Yellon 2013, Zuidema, Zhang 2010). This leads to accumulation of byproducts like lactic acid, which reduces the intracellular pH (Hausenloy, Yellon 2013, Zuidema, Zhang 2010). The loss of ATP generation and the high intracellular proton concentration results in an intracellular sodium overload and cellular swelling (Hausenloy, Yellon 2013, Zuidema, Zhang 2010). As the cells try to extrude sodium ions, the sodium-calcium exchanger is activated, leading to an increase in intracellular calcium ions (Hausenloy, Yellon 2013). A further increase in Ca^{2+} also occurs as a result of changes in the ion transport system in the sarcolemma and sarcoplasmic reticulum (Buja 2005). Ultimately, this leads to reversible loss of myocardial contraction within minutes of ischaemia (Zuidema, Zhang 2010, Hausenloy, Yellon 2013, Turer, Hill 2010). If the ischaemia is sustained, irreversible cardiomyocyte injury occurs after 20-40 minutes, resulting in myocardial tissue necrosis (Zuidema, Zhang 2010). Acute ischaemia also triggers vascular endothelial dysfunction with an increased permeability and elevated expression of adhesion molecules (Zuidema, Zhang 2010, Linfert, Chowdhry et al. 2009). The activated endothelial cells are highly adhesive to inflammatory cells, aiding their recruitment and migration into the cardiac tissue (Zuidema, Zhang 2010, Linfert, Chowdhry et al. 2009).

Prompt restoration of blood flow to the occluded epicardial artery, using mechanical or pharmacological reperfusion therapy, can save the myocardial tissue and limit the infarct size (Pinto, Gibson 2017, Montecucco, Carbone et al. 2015). However, paradoxically, the

reperfusion itself (spontaneous or induced) can cause additional tissue damage and complications, some of which are irreversible (Figure 1.2) (Pinto, Gibson 2017, Montecucco, Carbone et al. 2015, Hausenloy, Yellon 2013).

1.2.1 Reperfusion-induced arrhythmias

Reperfusion-induced arrhythmias occur within seconds after sudden restoration of blood flow and their incidence is associated with the severity and duration of ischaemia in STEMI patients (Podrid, Ganz 2017). They develop due to mitochondrial dysfunction and electrolyte imbalance following ischaemia and are mediated by the sudden restoration of extracellular calcium and oxygen supply (Podrid, Ganz 2017, Pinto, Gibson 2017). Although reperfusion arrhythmias are common in patients treated with PPCI or thrombolytic therapy, they are usually easily treated or self-terminating and therefore do not present a major complication (Hausenloy, Yellon 2013, Pinto, Gibson 2017, Podrid, Ganz 2017).

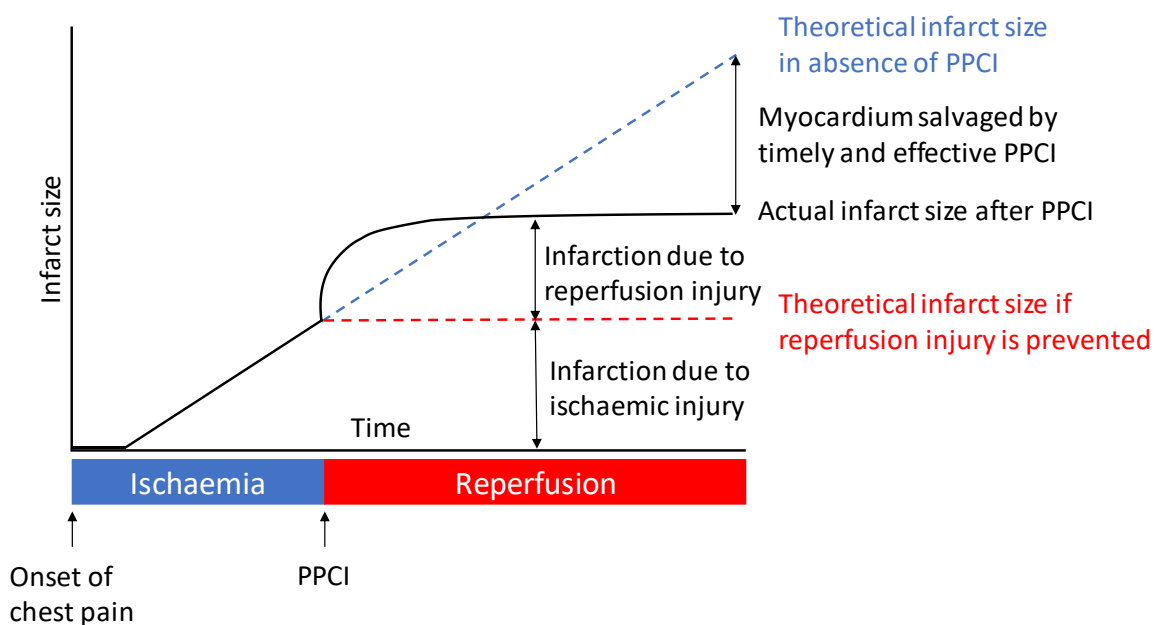


Figure 1.2 Individual contribution of myocardial ischaemia and reperfusion injury to final infarct size. Adapted from (Hausenloy, Yellon 2013).

1.2.2 Myocardial stunning

Another reversible complication is myocardial stunning, which refers to the transient post-ischaemic contractile dysfunction (Hausenloy, Yellon 2013, Pinto, Gibson 2017). It occurs even after a short period of total coronary artery occlusion, lasting only 5 to 15 minutes, which is insufficient to cause myocardial necrosis (Buja 2005, Shavelle 2017). Mediated by the effects of reactive oxygen species (ROS) and intracellular calcium overload that continues after

reperfusion, myocardial stunning is not associated with cell death and therefore myocytes ultimately recover their contractile function (Buja 2005, Pinto, Gibson 2017, Hausenloy, Yellon 2013).

1.2.3 Microvascular obstruction

Microvascular obstruction (MVO) is another form of myocardial reperfusion injury for which there is currently no effective treatment (Hausenloy, Yellon 2013). MVO, also known as the “no reflow” phenomenon, refers to a severe reduction in antegrade coronary blood flow within the postischaemic vasculature, distal to the initial site of occlusion (Hausenloy, Yellon 2013, Gibson, Carrozza et al. 2017, Pinto, Gibson 2017). Multiple factors contribute to MVO, including external compression of the capillaries due to endothelial cell and cardiomyocyte oedema, distal embolization of plaque material or a clot, the formation of a new thrombus as a result of platelet and immune cell activation, as well as release of soluble vasoconstricting and thrombogenic factors (Hausenloy, Yellon 2013, Gibson, Carrozza et al. 2017, Pinto, Gibson 2017). MVO has been shown to be present in up to 55% of PPCI treated patients and appears to have an increased incidence among diabetic patients (Gibson, Carrozza et al. 2017, Bulluck, Hausenloy 2015). Its occurrence is associated with a larger infarct size, lower left ventricle (LV) ejection fraction, adverse LV remodelling and is an independent marker of cardiac death and worse clinical outcome (Hausenloy, Yellon 2013, Bulluck, Hausenloy 2015, Kidambi, Mather et al. 2013, Pinto, Gibson 2017).

1.2.4 Lethal myocardial reperfusion injury

The most serious consequence of myocardial reperfusion is lethal myocardial reperfusion injury, characterised by death of cardiomyocytes that were viable at the time of reperfusion (Pinto, Gibson 2017). This irreversible loss of cardiomyocytes accounts for up to 50% of the final infarct size, as suggested by an increasing number of studies in animal MI models and STEMI patients (Hausenloy, Yellon 2013). Several important mediators of lethal myocardial reperfusion injury have been identified, the major ones of which are briefly described below.

1.2.4.1 Hypercontracture of myocytes and calcium overload

Intracellular and mitochondrial calcium overload during the ischaemic period is exacerbated by reperfusion, resulting in myocyte hypercontracture (Hausenloy, Yellon 2013, Ibanez, Fuster et al. 2011). The increase in Ca^{2+} due to disruption of the plasma membrane and the sarcoplasmic reticulum, and the re-energisation of myocytes as a result of re-established

blood flow and oxygen supply, leads to uncontrolled cardiomyocyte contraction and subsequent opening of the mitochondrial permeability transition pore (MPTP) (Hausenloy, Yellon 2013, Ibanez, Fuster et al. 2011, Pinto, Gibson 2017). Furthermore, hypercontracture can result in disruption of tight junctions and chemical signalling through gap junctions to adjacent myocytes, therefore causing damage to neighbouring cardiomyocytes (Ibanez, Fuster et al. 2011, Pinto, Gibson 2017). Some studies have shown that attenuation of calcium overload by pharmacological antagonists at the onset of reperfusion can reduce infarct size by up to 50% (Hausenloy, Yellon 2013, Ibanez, Fuster et al. 2011). However, translating this beneficial effect in patient studies has proven challenging (Hausenloy, Yellon 2013, Ibanez, Fuster et al. 2011).

1.2.4.2 Oxidative stress

Within minutes of myocardial reperfusion, a burst of oxidative stress is produced and free radicals (in particular superoxide anion (O_2^-) and hydroxyl radical ($OH^\cdot$)) continue to be generated for hours after restoration of blood flow to the ischaemic area (Pinto, Gibson 2017, Hausenloy, Yellon 2013, Ibanez, Fuster et al. 2011). They can develop via several mechanisms including electron leakage from ischaemic mitochondria and from activated neutrophils that infiltrate the injured myocardium (Pinto, Gibson 2017). ROS mediate direct myocyte injury and death by disrupting cellular membrane proteins and phospholipids, thereby affecting the function of various receptors, ion channels and enzymes (Pinto, Gibson 2017). However, there has been conflicting evidence of the effect of antioxidant therapy to prevent reperfusion injury (Ibanez, Fuster et al. 2011, Hausenloy, Yellon 2013). Mixed results have been reported following superoxide dismutase administration at the onset of reperfusion, with some studies showing beneficial effect when co-administered with thrombolytic therapy (Pinto, Gibson 2017, Hausenloy, Yellon 2013, Ibanez, Fuster et al. 2011).

1.2.4.3 Rapid restoration of physiological pH

Physiological pH is rapidly restored following reperfusion by the washout of lactic acid and the activation of the Na^+-H^+ exchanger (Hausenloy, Yellon 2013). This quick restoration of normal acid-base status is an important contributor to cardiomyocyte necrosis and lethal myocardial reperfusion injury, mediated by mitochondrial permeability transition pore (MPTP) opening and myofibril hypercontracture (Pinto, Gibson 2017, Hausenloy, Yellon 2013). Studies have shown that the reperfusion injury can be reduced by prolonging acidosis during myocardial reperfusion using an acidic buffer (Ibanez, Fuster et al. 2011, Hausenloy, Yellon 2013).

Alternatively, this may be achieved by pharmacological inhibition of the $\text{Na}^+\text{-H}^+$ exchanger or by using ischaemic postconditioning (repeated cycles of brief reperfusion and occlusion of coronary blood flow at the time of reperfusion) (Ibanez, Fuster et al. 2011, Hausenloy, Yellon 2013).

1.2.4.4 MPTP opening

The MPTP is acknowledged as a key mediator in the development of lethal myocardial reperfusion injury as multiple injury mediators converge on its opening (Ibanez, Fuster et al. 2011, Hausenloy, Yellon 2013). This is a nonselective channel located on the inner mitochondrial membrane, the opening of which induces depolarisation of the mitochondrial membrane, subsequent ATP depletion and cell death (Ibanez, Fuster et al. 2011, Hausenloy, Yellon 2013). The MPTP remains closed during ischaemia but opens following reperfusion in response to restoration of physiological pH, calcium overload and oxidative stress (Ibanez, Fuster et al. 2011, Hausenloy, Yellon 2013). Consequently, drugs inhibiting MPTP opening, such as the immunosuppressant cyclosporine A, have been tested both in experimental and clinical setting (Ibanez, Fuster et al. 2011, Hausenloy, Yellon 2013). According to these studies, administering the drug at the onset of reperfusion results in significant 40-50% reduction in final MI size in animal studies (Ibanez, Fuster et al. 2011, Hausenloy, Yellon 2013). Unfortunately, these results were not replicated in large phase 3 STEMI patient trials (Cung, Morel et al. 2015).

1.3 The immune system

The traditional view of the immune system is that it has evolved as a mechanism to protect organisms from infectious diseases caused by pathogens such as bacteria and viruses, as well as toxic or allergenic substances. To achieve this, the system is able to distinguish between self and non-self in order to ensure that only foreign pathogenic microorganisms are eliminated, while it tolerates beneficial commensal microorganisms and avoids excessive response that damages self-tissues (Sattler 2017, Chaplin 2010). Therefore, this evolutionary conserved system provides a survival advantage to the host organism. While this is the classic understanding of the immune system, and in fact its primary function, this system plays a key role in organism development, maintenance of tissue homeostasis, wound healing and reproduction. Furthermore, our growing understanding of the immune system has elucidated

complex interplay with different physiological systems and contribution to diseases of the nervous and cardiovascular systems (Sattler 2017).

The mechanisms of the immune system, consisting of a collection of cells (cellular immunity) and proteins (humoral immunity), can broadly be divided into two categories: rapid, antigen-independent innate immunity; and highly specific, antigen-dependent adaptive immunity. The major immune cell types involved in innate and adaptive immunity are shown on Figure 1.3. These two arms of the immune system are complementary to each other and their complex cross-talk is crucial for the host's ability to mount a rapid and effective immune response to both exogenous or endogenous insults (Chaplin 2010, Warrington, Watson et al. 2011).

1.3.1 Innate immunity

Innate immunity is the first line of defence of the host and includes multiple cell types and mechanisms that are encoded by germ-line genes. It relies on the recognition of molecules expressed by a large number of cells and is poised for a very rapid response – immediately or within hours of encountering the antigen (Warrington, Watson et al. 2011). While the innate immune response has no immunological memory, it is crucial for activating the adaptive immune response and inducing its memory.

The first line of defence of the innate immune system are the physical barriers of an organism, such as the epithelial cell layer of the skin, the gastrointestinal track and its cilia, as well as the mucus layer overlaying the epithelial barrier in the respiratory system (Chaplin 2010). These barriers act to prevent entry of pathogens into the organism and/or contain bioactive substances against microorganism. Another major part of the innate immunity is its humoral response. Soluble proteins and small molecules can either be constitutively expressed, such as complement proteins, or can be induced upon immune cell activation, such as cytokines, chemokines and reactive oxygen species (ROS) (Chaplin 2010). They have different effector functions but their overall role is to inactivate pathogens, opsonise pathogens to enhance their phagocytosis, and to recruit and activate different types of immune cells. They do this by recognising pathogen-associated molecular patterns (PAMPs) and damage-associated molecular pathogens (DAMPs) using Toll-like receptors (TLRs) and nucleotide-binding domain, leucine-rich repeat (NLR) proteins (Chaplin 2010, Cronkite, Strutt 2018).

A number of different cell types (primarily granulocytes) are involved in the innate immune response. These include phagocytes such as macrophages and neutrophils, as well as

monocytes, dendritic cells (DCs), basophils, eosinophils and natural killer (NK) cells (Warrington, Watson et al. 2011). Neutrophils are the first to respond to an insult and release granules containing ROS and enzymes which are cytotoxic to pathogens. Although they are short lived, they are recruited in high numbers and are potent phagocytes, allowing them to engulf bacteria and damaged cells, safely degrading them. Ultimately, they play a key role in stopping the spread of infections and aiding repair of injured tissues (Chaplin 2010, Warrington, Watson et al. 2011). In addition to this, neutrophils are potent immunoregulators as they secrete cytokines such as Tumour Necrosis Factor (TNF) and interleukin (IL)-12 (Chaplin 2010). Other granulocytes such as basophils and eosinophils play important roles in response to allergens and parasites by release of cytoplasmic granules containing enzymes and histamine (Warrington, Watson et al. 2011, Chaplin 2010). Similar to neutrophils, monocytes and macrophages are key phagocytes. However, they are more long-lived and persist for longer at sites of injury and infection (Warrington, Watson et al. 2011). They are also potent immunoregulators due to their secretion of large amounts of cytokines such as IFN γ and IL-12 (Chaplin 2010). However, in addition to these functions, monocytes and macrophages provide a crucial link between innate and adaptive immunity. They do this by not only engulfing pathogens, but by also then presenting antigens to T cells that are required for their activation and for subsequent mounting of an effective adaptive immune response. DCs are another major type of antigen presenting cells (APCs) which reside in most tissues. They can potently present antigens on both class I and class II major histocompatibility complex (MHC) molecules and are concentrated in the secondary lymphoid tissues such as lymph nodes and the spleen (Chaplin 2010). Unlike phagocytes that engulf pathogens and destroy them in intracellular vacuoles filled with nitric oxide, ROS and degradative enzymes, NK cells exert their cytotoxic properties by releasing perforin and granzyme containing granules (Chaplin 2010, Warrington, Watson et al. 2011). Although they develop from lymphoid progenitors, they have no antigen-specific receptors and are therefore part of the innate immune response. They recognise self and non-self via MHC molecules. This gives them a key anti-tumour and anti-viral cytotoxic role as they recognise and induce apoptosis of cells that have downregulated MHC class I expression (Warrington, Watson et al. 2011, Chaplin 2010). This is of particular importance as cancerous and some virally infected cells avoid clearance by cytotoxic CD8 T cells that require MHC class I expression for recognition (Chaplin 2010).

1.3.2 Adaptive immunity

In addition to innate immunity, the adaptive immune response can take over to aid in the effective elimination of infections. The unique features of adaptive immunity are its ability to distinguish non-self antigens in the presence of self antigens; its exquisite antigen-specific effector function; and the development of immunological memory that allows rapid elimination of pathogens on subsequent re-infection (Warrington, Watson et al. 2011). T and B lymphocytes are the effector cells of the adaptive immune response and APCs like DCs, monocytes and macrophages provide the essential bridge between the innate and adaptive immune response by contributing to lymphocyte activation. In contrast to innate immune receptors that are encoded by germ-line genes that recognise PAMPs, the genes encoding T and B cell receptors are assembled by somatic rearrangements of multiple genes, resulting in millions of different random gene combinations (Chaplin 2010). Ultimately, this allows for the formation of millions of different unique antigen receptors.

Both B and T cells arise from a common lymphoid progenitor and they are responsible for the humoral and cell-mediated adaptive immune response, respectively. As T cells are the focus of this project, their development, activation and effector function are described in detail in section 1.4. B cells develop and mature in the bone marrow and their principle function is the production of antigen-specific antibodies. Unlike T cells, B cells do not require their cognate antigen to be presented by an APC on an MHC molecule, but instead can directly recognise free antigens (Warrington, Watson et al. 2011). Following antigen binding to their B cell receptor (BCR), B lymphocytes undergo rapid proliferation in the spleen and/or lymph nodes and differentiate into antibody generating plasma cells or memory B cells (Warrington, Watson et al. 2011). It is these long-lived memory B cells that provide immunological memory and can be quickly re-activated to produce antibodies following repeated encounter with their cognate antigen. Plasma cells on the other hand, are short-lived cells, producing large amounts of antigen-specific antibodies, that undergo apoptosis once the insult that activated them is inactivated (Warrington, Watson et al. 2011). B cells produce five types of antibodies – immunoglobulin A (IgA), IgD, IgE, IgG and IgM. Each antibody type has a different function and together with the innate complement cascade, they neutralise, opsonise pathogens and enhance their clearance by phagocytes (Warrington, Watson et al. 2011).

While innate and adaptive immunity have distinct and even contrasting roles in host defence, they are complementary mechanisms. The rapid innate immune response contributes to the

activation of the antigen-specific adaptive response which then amplifies the response by further recruiting innate immune cells (Chaplin 2010, Warrington, Watson et al. 2011). Ultimate, this synergy between the two arms of the immune system allows for effective and timely host defence from both endogenous and exogenous insults.

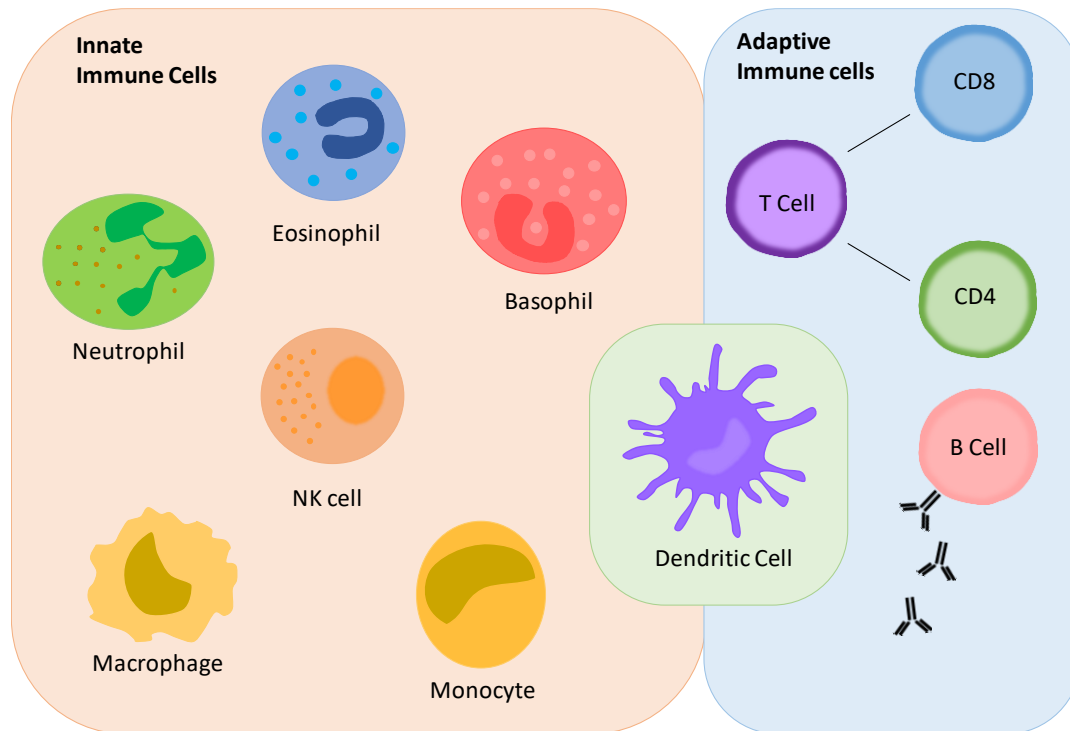


Figure 1.3 Types of leukocytes in the innate and adaptive immune system. Dendritic cells provide a bridge between the two arms of the immune system by presenting antigens to T cells.

1.3.3 Inflammation

Inflammation is an essential immune response that protects the host from harmful stimuli such as toxic substances, infection, damaged cells or irradiation. It is a complex biological process requiring an interplay between the innate and adaptive immune systems in order to restore tissue homeostasis following disruption by infection or injury (Taams 2018, Medzhitov 2008). While inflammation is an effective survival mechanism, its distinct feature is that damage to self is unavoidable and it comes at the cost of transient decline in tissue function (Ashley, Weil et al. 2012). The role of inflammation in response to wounds and infections has been known to humankind for thousands of years with its five cardinal signs: calor, rubor, tumor, dolor, function laesa (heat, redness, swelling, pain, loss of function) (Medzhitov 2010). These occur within minutes to hours and are the result of vasodilation and exudate production due to plasma leakage and leukocyte migration into the surrounding tissue (Medzhitov 2010, Leick, Azcutia et al. 2014). However, over the last two decades, our understanding of the

immune system has elucidated a role for inflammation in response to nonmicrobial activators. This process is known as sterile inflammation and one of the acute conditions that causes it is ischaemia reperfusion injury (Shen, Kreisel et al. 2013). Although the exact response depends on the nature of the trigger, overall the inflammatory pathway consists of four components – inducers of inflammation, sensors that detect them, inflammatory mediators and the tissues targeted by the mediators (Medzhitov 2010).

Depending on the type of inducer (bacterial, viral or parasitic infection, or injury), different sensors are triggered, resulting in a different response. The inducers can be classified into two groups – exogenous and endogenous. The former can be further divided into microbial and non-microbial inducers (Medzhitov 2008). Microbial inducers include pathogen-associated molecular patterns (PAMPs) which are conserved molecular patterns carried by all microorganisms (Ashley, Weil et al. 2012). Immune cells have a limited number of extracellular and intracellular receptors, such as TLRs, that detect these common pathogen patterns (Medzhitov 2010, Ashley, Weil et al. 2012). Non-microbial exogenous inducers include allergens, toxic compounds and foreign bodies. Allergens induce inflammation following recognition by circulating IgE but the precise sensors of foreign particles such as asbestos are less well understood (Medzhitov 2008). Endogenous inducers are those produced by stressed, damaged or necrotic host cells (Medzhitov 2008). They are also less well understood and can vary greatly depending on the tissue affected but like PAMPs, they can be recognised by the innate immune system via damage-associated molecular patterns (DAMPs) (Ashley, Weil et al. 2012). Examples of endogenous inducers include intracellular components such as DNA, ATP and K^+ ions being released into the interstitium, as well as hypoxia and elevated ROS levels. Vascular damage can also induce inflammation by platelet and Hageman factor activation upon contact with components of the extracellular matrix (ECM) (Medzhitov 2008). Following ligand binding to TLRs on tissue resident macrophages and DCs, common signalling pathways are engaged, leading to activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and its translocation into the nucleus (Ashley, Weil et al. 2012). Once activated, NF- κ B rapidly upregulates transcription of genes encoding pro-inflammatory cytokines such as TNF, IL-1, IL-6, as well as chemokines like CCL2 (Medzhitov 2010, Ashley, Weil et al. 2012, Chen, L., Deng et al. 2017).

Inducers of inflammation activate various inflammatory mediators that in turn exert an effect on different organs and tissues. These mediators are produced by leukocytes (macrophages,

mast cells) or by non-immune cells resident to the injured tissue and can be classified into seven groups based on their properties (Medzhitov 2008). The first group consists of vasoactive amines such as histamine and serotonin. Histamine is produced rapidly by mast cells and can cause increased vascular permeability and vasodilation or vasoconstriction (Medzhitov 2008). The second group is formed by vasoactive peptides such as Hageman factors and thrombin which need to undergo proteolytic activation before causing vasodilation and enhancing vascular permeability. Furthermore, they can activate bradykinin which is a potent pro-algesic (Medzhitov 2008). Pain receptor (nociceptor) activation is an important function of the inflammatory response as it alerts the host to the abnormal state of the tissue (Medzhitov 2010, Medzhitov 2008). The third group of inflammatory mediators consists of anapylatoxins of the complement cascade – C3a, C4a and C5a. They play a role in granulocyte recruitment to site of infection and promote degranulation of mast cells (Medzhitov 2008). Leukocytes are also recruited by certain lipids such as platelet-activating factors, which are members of the fourth class of inflammatory mediators. The fifth group consists of inflammatory cytokines that are produced by many different cell types and can affect a wide number of tissues (Medzhitov 2008). Similarly, chemokines are the sixth class of mediators that are produced by multiple different cell types. Their role consists of recruiting leukocytes to the inflamed area by controlling their extravasation (Medzhitov 2008). And finally, the seventh type of inflammatory mediator is represented by proteolytic enzymes such as elastin and matrix metalloproteinases. They play a key role in degrading ECM that is required for leukocyte migration and tissue remodelling allowing inflammation resolution (Medzhitov 2008).

The effectors of the inflammatory response are tissue specific, although certain mediators such as TNF- α and IL-1 induce a response across most organ systems (Medzhitov 2008). Ultimately, the role of the acute inflammatory response is to restore tissue homeostasis by removing the cause of its disruption (infection, injury, foreign body). Once this is achieved the inflammatory response terminates in a process known as resolution of inflammation (Medzhitov 2010). This is a crucial step as timely resolution allows for the collateral damage to the host to be reduced by diluting the inflammatory chemokine gradient (Chen, L., Deng et al. 2017). It is mainly controlled by macrophages that switch from a pro-inflammatory to an anti-inflammatory phenotype (Ashley, Weil et al. 2012). In doing so, neutrophil chemotaxis is ceased, apoptosis of spent neutrophils is induced and recruitment of wound healing

monocytes is initiated (Ashley, Weil et al. 2012, Chen, L., Deng et al. 2017). If however, the insult persists and cannot be removed, then the inflammatory response continues, leading to chronic inflammation (Medzhitov 2008).

1.3.4 Leukocyte recruitment and extravasation

For any immune response to take place, innate or adaptive, leukocytes need to be recruited to the site of inflammation, leave the bloodstream by transmigrating across postcapillary venules and enter the inflamed tissue (Muller 2013). This is triggered by inflammatory mediators and it is a highly controlled process in order to prevent unnecessary host tissue destruction (Herter, Zarbock 2013). The mechanism behind this involves coordinated adhesive interaction between leukocytes and endothelial cells that lead to a cascade of leukocyte capture, rolling, arrest and crawling on endothelial cells, before they extravasate through the endothelial barrier in a process termed diapedesis or transendothelial migration (TEM) (Vestweber 2015, Leick, Azcutia et al. 2014).

The first step of leukocyte recruitment is that leukocytes need to be able to distinguish and interact with “activated” vascular endothelial cells at sites of inflammation. This is enabled by two major mechanisms where the endothelial cells play a critical role (Vestweber 2015). The first one is the release of chemokines and other chemoattractants, such as complement fragment C5a, that guide and activate leukocytes. These chemoattractants are either produced by endothelial cells or by other inflammatory cells in the tissue and are then transcytosed through the endothelium and expressed on their surface (Vestweber 2015). The chemoattractants cause activation of the immune cells and subsequent upregulation of adhesion molecule expression such as integrins. The second mechanism involves the upregulated expression of selectins and other leukocyte adhesion molecules on the surface of vascular endothelium (Vestweber 2015). Their expression is induced following NF- κ B activation by pro-inflammatory cytokines such as TNF- α and IL-1 β , inflammatory mediators like histamine or bacterial lipopolysaccharides (Leick, Azcutia et al. 2014). Local inflammation additionally promotes interaction of adhesion molecules between leukocytes and endothelial cells by changes in the local haemodynamics resulting in reduced blood flow rate (Muller 2013).

After leukocytes have been recruited to the inflamed vascular endothelium, the multistep cascade of leukocyte extravasation begins. As shown on Figure 1.4, the first step is leukocyte

capture. This is mediated by P-selectin, expressed on the surface of endothelial cells, interacting with selectin ligands on leukocytes, such as P-selectin glycoprotein ligand 1 (PSGL1) (Vestweber 2015, Muller 2013). Binding between the receptor and its ligand has very fast on/off rates, which initially allows capture of leukocytes in the bloodstream (Muller 2013). The resulting tentative binding due to the low-affinity receptor-ligand interaction, results in leukocytes rolling along the surface of the vascular endothelium in the direction of blood flow. Continuous activation of endothelial cells causes upregulation of an additional selectin, E-selectin, which slows down leukocyte rolling (Muller 2013). This is achieved by the activation of leukocyte integrins following signalling downstream of PSGL1 (Vestweber 2015). The major integrins involved in leukocyte TEM are lymphocyte function-associated antigen 1 (LFA1) that is expressed on all immune cells, as well as myeloid specific macrophage antigen 1 (MAC1) and effector lymphocyte and monocyte specific very late antigen 4 (VLA4) (Vestweber 2015). The integrin receptor family consists of $\alpha\beta$ -heterodimers that can have a low-affinity “bent” structure or an “upright” intermediate-affinity and “upright” high-affinity conformation (Vestweber 2015). Intermediate-affinity binding is required for the slow rolling stage of leukocyte extravasation and it is mediated by interaction with intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1) on the surface of endothelial cells (Vestweber 2015, Muller 2013). Despite being slowed down, receptor-ligand binding during leukocyte rolling is still only transient (Hordijk 2016, Herter, Zarbock 2013). However, at this stage leukocytes are exposed to locally accumulated chemokines which activate G protein-coupled receptors (GPCRs) on their surface (Herter, Zarbock 2013). Downstream signalling causes further activation of integrins into their high-affinity conformation (Herter, Zarbock 2013). This results in leukocyte arrest, characterised by tight binding of integrins to ICAM1 and VCAM1 on the surface of endothelial cells (Hordijk 2016, Herter, Zarbock 2013, Muller 2013, Vestweber 2015). The next stage of this mechanism is crawling (or locomotion) of adherent leukocytes along the vessel wall in search of a suitable exit site for extravasation (Hordijk 2016, Muller 2013, Vestweber 2015). These sites are usually transiently opened endothelial cell junctions (paracellular diapedesis) or occasionally TEM can occur directly through the body of an endothelial cell (transcellular diapedesis) (Vestweber 2015). Although crawling is usually in the direction of blood flow, some leukocyte crawling against blood flow has been described (Muller 2013). This process is again dependent on the binding of leukocyte integrins to endothelial ICAM1 and VCAM1, as well as interactions between myeloid-specific MAC1 to ICAM2 (Vestweber 2015, Muller 2013). In the stages leading up to TEM, an additional role for

endothelial cells has been described in that they can extend actin-rich “docking structures” or “transmigratory cups” that promote leukocyte transmigration into the inflamed tissue. These structures surround crawling leukocytes and are characterised as ICAM1- and VCAM1-enriched pseudopod-like apical membrane extensions (Hordijk 2016, Muller 2013, Vestweber 2015). Once the leukocytes find a suitable exit site, they commit to diapedesis where they protrude and squeeze in amoeboid fashion across the endothelial monolayer and then crawl through the dense 3D ECM that contains pericytes and smooth muscle cells, until they reach the site of inflammation (Hordijk 2016, Muller 2013).

Diapedesis is a multistep sequential process that requires loosening of endothelial cell junctions (except for transcellular migration) in order for leukocytes to migrate while preventing plasma leakage, and subsequently rapidly resealing the junction after the leukocyte has transmigrated and crossed the basement membrane (Vestweber 2015). While leukocyte capture, rolling, arrest and crawling involve heterophilic adhesion interactions, diapedesis depends primarily on homophilic adhesion where the same molecule is expressed both by the endothelial cell and the leukocyte (Muller 2013). In addition to ICAM1 and VCAM1, diapedesis requires engagement of junctional adhesion molecules A, B and C (JAMA, JAMB, JAMC), platelet/endothelial cell adhesion molecule 1 (PECAM1 or CD31), CD99 and CD99L2 (Muller 2013). Although the exact molecular mechanisms behind TEM are not well understood, the different receptors and ligands appear to play differential roles, depending on the inflammatory stimulus. For example, ICAM2, JAMA and PECAM1 respond to IL-1 β stimulation but not to TNF α (Vestweber 2015). Moreover, a mechanism of membrane constitutive internalisation and recycling has been described to occur at endothelial cell borders during leukocyte TEM. This has been termed lateral border recycling compartment (LBRC) and it appears that this is a mechanism to concentrate unligated adhesion molecules required for transmigration such as CD99, JAMA while excluding VE-cadherin. The latter has a role in loosening the endothelial cell junctions, allowing migration of leukocytes (Leick, Azcutia et al. 2014, Vestweber 2015). Once the leukocyte detaches from the endothelial cells, it starts migrating through the ECM. The process behind this are not well understood as they require study of cell movement in a 3D environment, unlike the 2D migration along the vascular lumen. Current methods of doing this successfully are limited but studies on DC migration through 3D microchannels show that myosin contractility and formin-dependent actin polymerisation are required (Huse 2017).

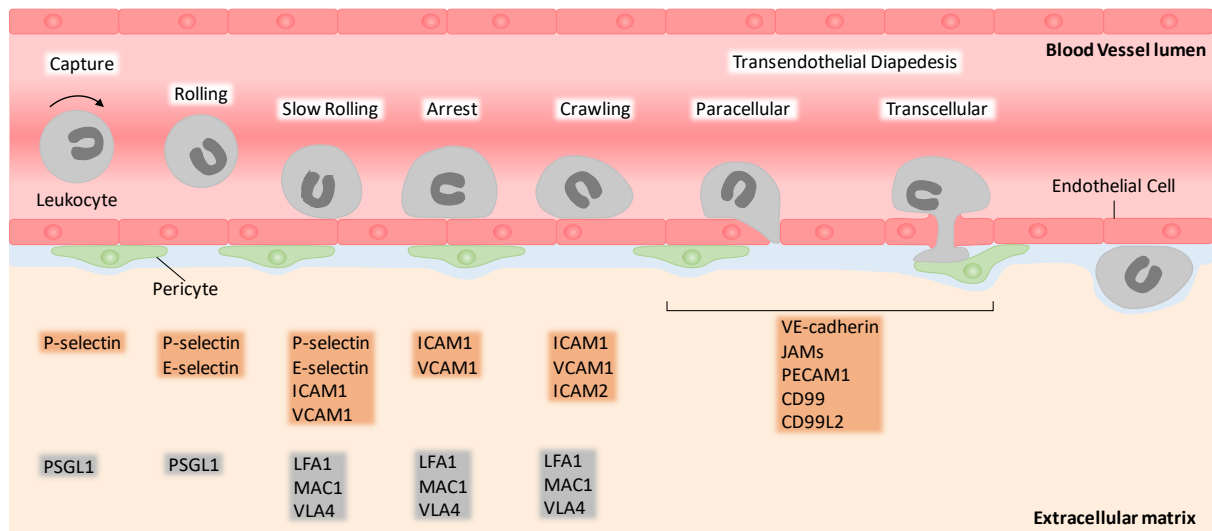


Figure 1.4 Multi-step mechanism of leukocyte transendothelial migration. Leukocytes are recruited to activated endothelium by increased local levels of chemokines. This is followed by successive steps of leukocyte capture, rolling, arrest and crawling before migrating through the endothelium membrane. These processes are controlled by the adhesion molecules expressed on the surface of endothelial cells (orange boxes) and leukocytes (grey boxes). PSGL1 (P-selectin glycoprotein ligand 1); LFA1 (lymphocyte function-associated antigen 1); MAC1 (macrophage antigen 1); VLA4 (very late antigen 4); ICAM (intercellular adhesion molecule); VCAM1 (vascular cell adhesion molecule 1); JAMs (junctional adhesion molecule); PECAM1 (platelet endothelial cell adhesion molecule 1); CD99L2 (CD99 antigen-like protein 2). Adapted from (Vestweber 2015).

1.4 T cells

T lymphocytes are key immune cells, providing the powerful cellular adaptive immune response as well as enhancing and modulating the B cell humoral response. They coordinate responses not only to infections by recognising diverse antigens, but also play a key role in allergy and tumour protection. Moreover, T cells are responsible for maintaining tissue homeostasis throughout the lifetime of an organism by providing immunological memory and self-tolerance (Kumar, Connors et al. 2018). These diverse T cell functions are the result of an increasing number of distinct T cell subtypes that have remarkable plasticity and specific roles depending on their environment, tissue localisation and age of organism. Their development, function and major subtypes are described in the sections below.

1.4.1 T cell development

T cells develop in the thymus from a common lymphoid progenitor, which previously differentiated from a bone marrow-derived hematopoietic stem cell (Figure 1.5) (Germain 2002, Jin, Sun et al. 2012, Zhang, Yafeng, Li et al. 2009). Upon emigration from the blood into

the thymus, the lymphoid progenitor loses its ability to give rise to B cells and natural killer (NK) cells, therefore committing to a T cell fate (Germain 2002). These cells are referred to as double negative (DN) because they do not yet express the co-receptors cluster of differentiation (CD)4 or CD8 (Germain 2002, Jin, Sun et al. 2012). They migrate from the corticomedullary junction to the subcapsular region of the thymic cortex, while sequentially differentiating into four DN developmental stages (Jin, Sun et al. 2012). Each stage is characterised by the surface expression of CD44 and CD25 – DN1 (CD44⁺CD25⁻), DN2 (CD44⁺CD25⁺), DN3 (CD44⁻CD25⁺) and DN4 (CD44⁻CD25⁻) (Germain 2002, Jin, Sun et al. 2012). DN3 cells can commit to either express an $\alpha\beta$ or a $\gamma\delta$ TCR, the latter having a low frequency in peripheral blood but constituting a major population in the intestinal intraepithelium (Holtmeier, Kabelitz 2005, Taghon, Yui et al. 2006). For the cells that follow the $\alpha\beta$ TCR pathway, expression of recombination-activating gene 1 (RAG1) and RAG2 genes results in somatic recombination of the TCR β -chain (Germain 2002). The β -chain forms a heterodimer with the non-rearranged pre-TCR- α , leading to the expression of the pre-TCR- $\alpha\beta$ on the surface of DN3 (Germain 2002). The pre-TCR is functionally coupled to CD3 and the ζ -chain on the cell surface, involved in active signalling required for further T cell maturation (van Oers, von Boehmer et al. 1995, Germain 2002). After β -chain selection, cells in late DN3 and DN4 undergo 6-8 rounds of cell division, followed by α -chain recombination (Germain 2002). At this stage, the cells lose expression of the pre-TCR and instead start expressing low levels of the mature $\alpha\beta$ TCR (Germain 2002). Following this, expression of the co-receptors begins, ultimately forming a large population of CD4⁺CD8⁺ double positive (DP) thymocytes in the thymus cortex (Germain 2002, Jin, Sun et al. 2012). This is followed by TCR-mediated DP thymocytes interaction with cortical thymic epithelial cells (cTECs) which present self-peptide-MHC complexes (Germain 2002, Jin, Sun et al. 2012). Depending on the avidity and aggregation of TCRs, DP cells undergo selection that only around 5% of DP cells survive (Germain 2002). Around 90% of them have poor response to TCR/co-receptor engagement (Germain 2002). These cells cannot receive the necessary survival signals which results in death by neglect (Germain 2002). The TCRs of around 5% of the DP thymocytes have very high avidity for self-antigens (Germain 2002). These autoreactive cells would potentially give rise to autoimmunity and therefore undergo negative selection, characterised by TCR:MHC-induced apoptosis (Germain 2002). The remaining DP cells have intermediate self-antigen recognition, therefore receive survival signals and are positively selected (Germain 2002). This leads to the differentiation of single positive CD8⁺ or CD4⁺ mature T cells, depending on

whether they bind peptides presented on MHC class I and MHC class II, respectively (Germain 2002, Jin, Sun et al. 2012).

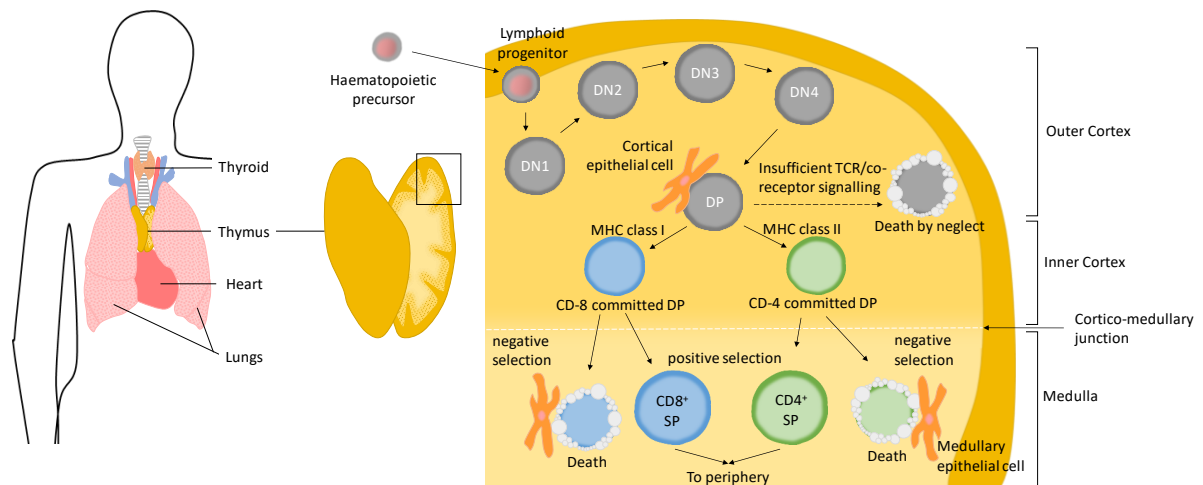


Figure 1.5 T cell development in the thymus. Lymphoid progenitors develop in the bone marrow and migrate to the thymus. A series of T cell receptor (TCR) editing events occur while the cells move from the double negative (DN)1 to DN4 stage. Cells differentiate into double positive (DP) cells when the mature TCR is formed and both CD4 and CD8 are expressed. They then undergo a series of positive and negative selection, as well as death by neglect, mediated by the strength and quality of the TCR/co-receptor signalling. The cells become single positive (SP) CD4 or CD8 T cells depending on whether they recognise MHC class II or class I molecules, followed by emigration to the periphery. Adapted from (Germain 2002).

The mature T lymphocytes then leave the thymus and migrate towards to peripheral lymphoid organs, where they are subject to peripheral tolerance (Jin, Sun et al. 2012). In steady state, without inflammation, professional antigen presenting cells (APCs) in the lymph nodes, such as dendritic cells (DCs), B cells and monocytes, present various self-antigens to induce tolerance in autoreactive naïve T cells that escaped negative selection in the thymus (Hofmann, Frantz 2016, Hofmann, Frantz 2015). Naïve T cells recirculate between blood and lymphatics and are continuously presented with antigens from APCs that collect them in the peripheral tissues and present them to T cells in the lymph nodes (Hofmann, Frantz 2016). Furthermore, it is in the peripheral lymph organs, mainly the spleen and the draining lymph nodes, that naïve T cells become activated in inflammatory condition (Hofmann, Frantz 2016). For this process to occur T cells require three signals from an activated APC (Caza, Landas 2015). The first one is the recognition of their cognate peptide presented on an MHC molecule that primes T cells (Hofmann, Frantz 2016, Hofmann, Frantz 2015). They then need a second co-stimulatory signal, in the form of T cell CD28 and CD80/CD86 upregulated on the surface of activated APCs, in order to exert their effector or regulatory function (Hofmann, Frantz

2016, Hofmann, Frantz 2015). The third signal is cytokine signalling that drives lineage-specific transcription factors, downstream of signal transducer and activation of transcription family (STATs) activation (Caza, Landas 2015). This directs naïve T cells to differentiate into specific helper (for CD4 T cells) or cytotoxic (for CD8 T cells) subtypes (Caza, Landas 2015). Activation of naïve T cells results in IL-2 secretion, clonal expansion and homing to the site of inflammation, mediated by chemokine signalling (Hofmann, Frantz 2015). Depending on the specific cytokine environment during T cell activation, different subtypes of CD4 and CD8 lymphocytes differentiate, having distinct functions (Jin, Sun et al. 2012). The different CD4 and CD8 effector subtypes are described in sections 1.4.3 and 1.4.4. respectively.

1.4.2 Function of T cell subtypes

Maintaining immunity and homeostasis throughout the lifetime of an organism is a key function of mature T cells that are exported in the periphery following thymic development. Naïve T cells that have not yet encountered their cognate antigen do this in secondary lymphoid organs, causing them to activate and proliferate in a process termed clonal expansion (Kumar, Connors et al. 2018). They differentiate into short-lived effector cells, 90-95% of which die by apoptosis following pathogen eradication (contraction phase), and long-lived memory cells (Mueller, Gebhardt et al. 2013). The latter is termed memory phase and this cell population provides immunological memory and allows rapid response upon re-infection with the same pathogen (Kumar, Connors et al. 2018).

Naïve T cells in humans have a long lifespan and can respond to new antigens throughout life. They have immense TCR diversity, allowing the naïve T cell pool to respond to up to 100 million different antigens (Kumar, Connors et al. 2018). Naïve T cells are characterised by their high expression of CD62L (L-selectin). CD62L is a lymph node homing receptor, allowing naïve T cells to migrate into secondary lymphoid organs where they encounter their cognate antigens (Mahnke, Brodie et al. 2013).

Upon activation, naïve T cells differentiate into effector cells (described in more detail in the sections 1.4.3 and 1.4.4). These cells lose expression of CD62L which allows them leave the lymph nodes and migrate to the sites of inflammation where they exert their effector function (Golubovskaya, Wu 2016). Although the majority of these T effector cells die rapidly after inflammation resolution, a population of them persists in circulation. These cells are known as terminal effector cells (Temra cells), are mostly from the CD8 T cell lineage and are

identified as CD45RA⁺ CCR7⁻ cells (Kumar, Connors et al. 2018). CD8⁺ Temra cells are associated with cytomegalovirus (CMV) and Dengue virus infections in humans but so far there is no known mouse counterpart (Kumar, Connors et al. 2018).

Memory T cells are a heterogeneous population with several different subtypes which have specific localisation and function. They all share several features in that they have been previously activated, they persist even after their antigen is no longer present and they have increased activity upon subsequent encounter with the same antigen (Golubovskaya, Wu 2016). Naïve T cells differentiate into circulating central memory (Tcm), effector memory (Tem), stem cell memory (Tscm) and tissue resident memory (Trm) T cells (Kumar, Connors et al. 2018). Tcm cells are CD45RA⁻ CCR7⁺ CD44^{high} CD62L⁺ and this allows them to traffic into the spleen and lymph nodes where they scan for antigens for 6-18 hours before returning to the circulation (Mueller, Gebhardt et al. 2013). They have the potential to both self-renew and generate more differentiated daughter cells, giving them the ability to provide long-lived T cell memory (Mahnke, Brodie et al. 2013). Tem cells are CD45RA⁻ CCR7⁻ CD44^{high} CD62L⁻ and they primarily home to the spleen with fewer numbers present in lymph nodes (Kumar, Connors et al. 2018, Mueller, Gebhardt et al. 2013). While both Tcm and Tem cells secrete effector cytokines, Tem do so in larger quantities and are more cytolytic (Martin, Badovinac 2018, Kumar, Connors et al. 2018). In addition to their downregulation of CD62L, they also express chemokine receptors such as CCR4, which allow them to migrate into the inflamed tissues where they provide superior protection to some viral infections (Martin, Badovinac 2018, Mueller, Gebhardt et al. 2013, Kumar, Connors et al. 2018). Tscm cells are a relatively rare population of CD45RA⁺ CCR7⁺ CD95⁺ CD122⁺ cells. In contrast to Tcm and Tem, they do not have any effector function. However Tscm cells have some stem cell-like properties, including being highly proliferative (Kumar, Connors et al. 2018, Mahnke, Brodie et al. 2013). Trm cells are a recently identified T cell subset that is retained in diverse types of tissues and does not recirculate (Kumar, Connors et al. 2018, Mahnke, Brodie et al. 2013). They are CCR7⁻ CD44^{high} CD62L⁻ CD69⁺ and they are responsible for the rapid and effective protection to tissue infections (Mueller, Gebhardt et al. 2013). They achieve this by secreting IFN γ and promoting recruitment of both innate and adaptive immune cells (Martin, Badovinac 2018).

1.4.3 CD4 T cells

CD4 T cells are also known as T helper (Th) cells as they play a role in B cell activation and enhance their ability to secrete antibodies (Zhu, Jinfang, Paul 2008). Moreover, different

subtypes of CD4 T cells modulate B cell response by inducing Ig class switching according to the tissue microenvironment and the nature of the infectious stimuli. CD4 T lymphocytes can also promote leukocyte recruitment to sites of inflammation and increase macrophages' effector function (Zhu, Jinfang, Paul 2008). All of this is achieved by the production of different cytokines and chemokines which characterise the known CD4 T cell subtypes – Th1, Th2, Th3, Th9, Th17, Th22, Tfh (follicular helper T cells), Treg (regulatory T cells) (Figure 1.6) (Golubovskaya, Wu 2016, Zhu, Jinfang, Paul 2008). These subtypes differentiate from mature naïve CD4 T cells that leave the thymus and are functionally and phenotypically heterogeneous (Caza, Landas 2015). However, they possess remarkable plasticity and can “dedifferentiate” in the appropriate conditions. Growing evidence suggests that epigenetic imprinting is behind this ability (Murphy, Reiner 2002, Caza, Landas 2015). This allows them to maintain immunocompetence and be able to adapt to different microenvironments even in adulthood, after thymic involution when the development of new naïve T cells is greatly reduced (Caza, Landas 2015). CD4 T cell plasticity is also maintained as a result of activated naïve T cells undergoing rounds of clonal expansion, before lineage specification begins (Caza, Landas 2015).

Th1 cells are produced in response to intracellular pathogens, such as viruses and intracellular bacteria, and therefore they are involved in cell-mediated immunity. They are induced by IFN γ , IL-12 and IL-27 stimulation that activate T-bet via STAT1 and STAT4 (Caza, Landas 2015). T-bet is the lineage-specific transcription factor driving production of IFN γ , TNF α and lymphotoxin α (LT α) that make up the characteristic Th1 effector cytokine phenotype (Caza, Landas 2015, Zhu, Jinfang, Paul 2008). These cytokines are chemotactic to neutrophils and enhance macrophage activation, therefore providing a cross talk between adaptive and innate immunity (Caza, Landas 2015, Zhu, Jinfang, Paul 2008, Golubovskaya, Wu 2016). Additionally, IFN γ secretion results in a positive feedback loop, further polarising the CD4 T cell response towards cell-mediated immunity (Caza, Landas 2015). Although, the main role of Th1 cells is in cell-mediated immunity, their secretion of IFN γ promotes IgG2a class switching in B cells (Smith, Pottage et al. 2000).

In contrast to Th1 cell, Th2 CD4 lymphocytes mediate defence against extracellular pathogens, such as bacteria and parasites, by providing help for B cells to mount an effective humoral response (Caza, Landas 2015, Zhu, Jinfang, Paul 2008). Th2 cells play a role in allergic reactions by recruiting mast cells and eosinophils and upregulating mucus production of epithelial cells

(Yagi, Zhu et al. 2011). DCs that release IL-4, cause activation of STAT6 in naïve CD4 cells, leading to activation of Th2 lineage-specific factor GATA-3 (Caza, Landas 2015). In turn, GATA-3 binds to regulatory elements in the Th2 locus, inducing production of IL-4, IL-5 and IL-13. IL-4 provides positive feedback for Th2 cells and promotes B cell class switching to secrete IgE (Caza, Landas 2015, Yagi, Zhu et al. 2011). IL-5 and IL-13 are critical in the recruitment of eosinophils and the expulsion of helminths respectively (Zhu, Jinfang, Paul 2008). GATA-3 not only skews the CD4 response to a Th2 phenotype via the IL-4 positive feedback loop, but by also suppressing Th1 differentiation (Caza, Landas 2015, Yagi, Zhu et al. 2011). This is achieved by IL-12-STAT4 signalling suppression, blocking Runx3-mediated IFN γ production (Yagi, Zhu et al. 2011) and by inducing secretion of anti-inflammatory IL-10 in Th1 cells (Mitchell, Hassan et al. 2017).

Th3 cells are a population of Foxp3⁺ regulatory T cells that are characterised by TGF β production (Chien, Chiang 2017, Gol-Ara, Jadidi-Niaragh et al. 2012, Carrier, Yuan et al. 2007). The specific transcription factors that lead to their differentiation are not yet elucidated (Chien, Chiang 2017) but Th3 cells are known to be induced following oral antigen ingestion (Gol-Ara, Jadidi-Niaragh et al. 2012, Zhu, Jinfang, Paul 2008). They enhance IgA secretion by B cells and suppress both Th1 and Th2 phenotypes (Gol-Ara, Jadidi-Niaragh et al. 2012).

Th9 are a population of IL-9 secreting CD4 cells that have only been identified as a distinct subset in the past decade (Caza, Landas 2015). They play a role in mucosal helminth protection and anti-tumour immunity, as well as aggravating autoimmune diseases such as asthma, colitis and inflammatory bowel disease (IBD) (Vyas, Goswami 2018, Malik, Awasthi 2018). Their differentiation is induced by IL-4 and TGF β via the activation of STAT6 (Caza, Landas 2015, Srivastava, Dar et al. 2018). The exact lineage-specific transcription factor for Th9 cells is not yet determined, as they appear to have a complex regulatory network of transcription factors (Malik, Awasthi 2018). However, recent evidence has identified a cooperative interaction between IRF4, BATF and PU.1 that promote Th9 development (Malik, Awasthi 2018, Russ, Prier et al. 2013, Caza, Landas 2015).

Th17 cells have a role in extracellular bacterial and fungal protection at mucosal and epithelial surfaces (Guéry, Hugues 2015). They contribute to many organ-specific autoimmune diseases such as IBD as well as the immune response to key pathogens like *Mycobacterium tuberculosis* (Caza, Landas 2015, Guéry, Hugues 2015). Th17 differentiation is induced by IL-6, IL-21 and TGF β (Yasuda, Takeuchi et al. 2019, Caza, Landas 2015). These cytokines cause STAT3

activation, inducing the Th17 lineage-specific transcription factors retinoid orphan receptor γ T (ROR γ T) and ROR α (Yasuda, Takeuchi et al. 2019, Caza, Landas 2015). This leads to production of IL-17A, IL-17F, GM-SCF, IL-21 and IL-22 (Yasuda, Takeuchi et al. 2019, Zhu, Jinfang, Paul 2008, Caza, Landas 2015, Guéry, Hugues 2015). These cytokines cause the induction of multiple pro-inflammatory cytokines and chemokines such as IL-6 and CXCL8. This leads to neutrophil chemotaxis and secretion of anti-microbial peptides from epithelial cells (Yasuda, Takeuchi et al. 2019, Zhu, Jinfang, Paul 2008). Furthermore, IL-21 secreted by Th17 cells serves as a positive feedback cytokine, promoting further Th17 differentiation (Zhu, Jinfang, Paul 2008).

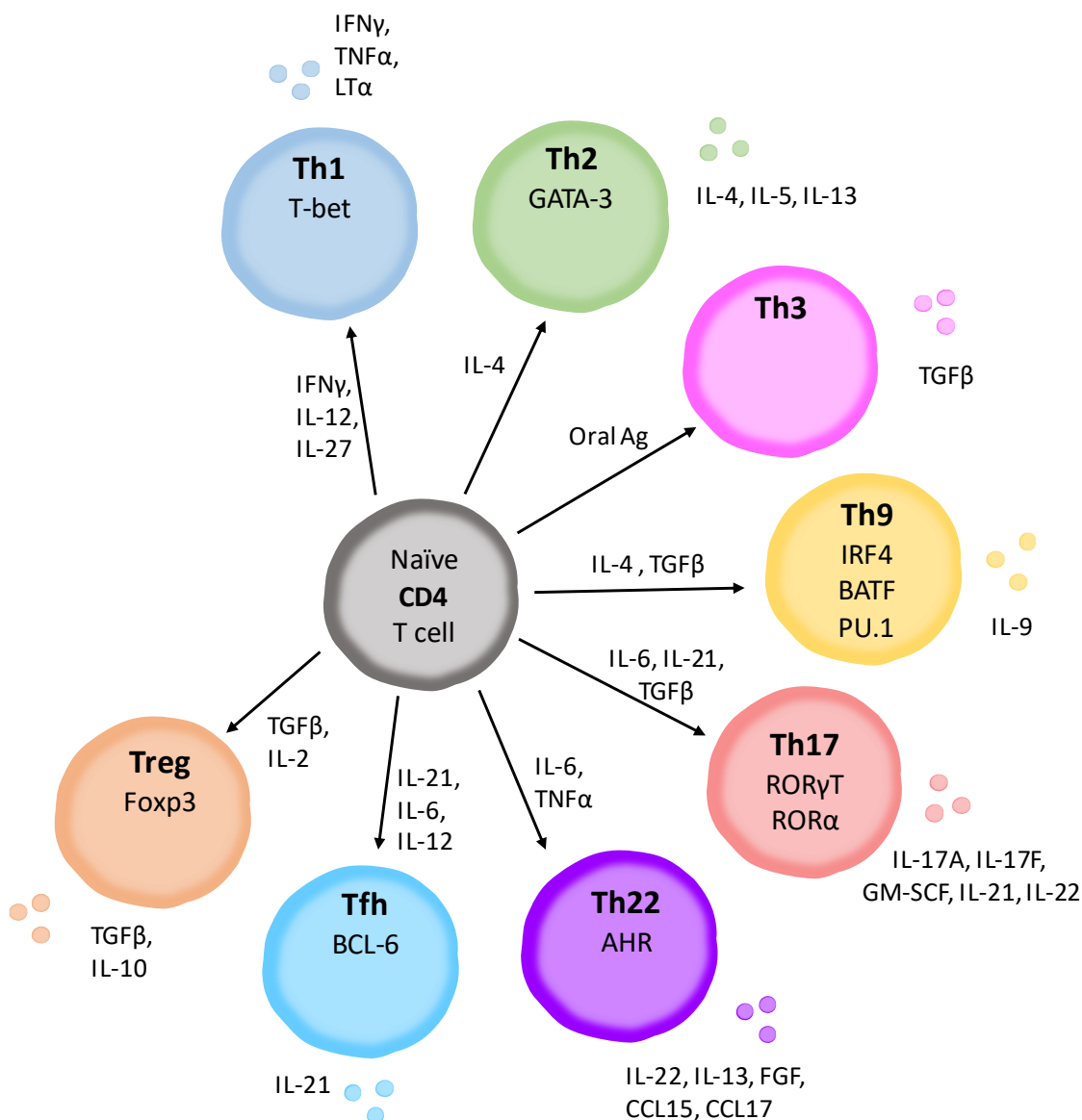


Figure 1.6 Summary of CD4 T cell effector subsets. CD4 subsets are generated by naïve T cell stimulation with different factors, resulting in activation of lineage-specific transcription factors leading to secretion of characteristic cytokines.

Th22 cells are functionally and phenotypically similar to Th17 cells. They have a role in wound repair, as well as protection at epithelial and mucosal surfaces from bacteria, viruses and fungi (Caza, Landas 2015). They have a particular role in skin infections and autoimmunity due to their expression of skin-homing receptors CCR4 and CCR10 (Jia, Wu 2014). Their differentiation is induced by IL-6 and TNF α which causes STAT3 mediated upregulation of aryl hydrocarbon receptor (AHR) (Caza, Landas 2015, Jia, Wu 2014, Tamasauskiene, Sitkauskiene 2018). This causes Th22 cells to express a number of molecules including IL-22, IL-13, fibroblast growth factor (FGF), CCL15, CCL17 and CCR10 (Caza, Landas 2015, Jia, Wu 2014).

Tfh cells are a specialised subset of CD4 T cells that provide essential help for B cell development and differentiation into antibody producing plasma cells (Le Saos-Patrinis, Loizon et al. 2020). They are critical for germinal centre (GC) formation where they can effectively help B cells (Jogdand, Mohanty et al. 2016). Their hallmark is expression of CXCR5 which allows them to enter the CXCL13⁺ GC follicle (Crotty 2015, Jogdand, Mohanty et al. 2016, Le Saos-Patrinis, Loizon et al. 2020). Tfh differentiation requires strong TCR stimulation, signalling by IL-21, IL-6 and IL-12, and STAT3 activation (Jogdand, Mohanty et al. 2016, Caza, Landas 2015). This leads to induction of the Tfh lineage-specifying transcription factor B cell lymphoma 6 (BCL-6), resulting in production of CD40L, programmed cell death 1 (PD-1), ICOS and IL-21 secretion (Crotty 2015, Crotty 2011, Le Saos-Patrinis, Loizon et al. 2020, Caza, Landas 2015).

Tregs are specialised for immune suppression and maintenance of peripheral tolerance. They play a crucial role in preventing host damage by regulating excessive immune responses as well as maintaining tolerance to self-antigens (Sakaguchi, Yamaguchi et al. 2008). Therefore, defects in the Treg population are associated with autoimmune diseases. They can either develop in the thymus, known as natural T regs (nTregs), or in the periphery, where they are known as induced Tregs (iTregs) (Sakaguchi, Yamaguchi et al. 2008, Caza, Landas 2015, Zhu, Jinfang, Paul 2008, Yesillik, Agrawal et al. 2019). The latter consists of two known types – Th3 cells described above and Tr1 which are Foxp3⁻ and secrete IL-10 and TGF β upon IFN α induction (Sakaguchi, Yamaguchi et al. 2008, Caza, Landas 2015, Zhu, Jinfang, Paul 2008). Differentiation of nTregs require a strong TCR signal which leads to STAT5 activation and Foxp3 expression (Lee, G. R. 2018). TGF β , IL-2 and CD25 (component of IL-2 receptor) expression are also essential for Treg development and population maintenance (Sakaguchi, Yamaguchi et al. 2008, Kraj, Ignatowicz 2018, Russ, Prier et al. 2013). Treg suppressor function is achieved by

cell-cell contact and/or anti-inflammatory cytokine secretion such as TGF β and IL-10 (Caza, Landas 2015, Zhu, Jinfang, Paul 2008).

1.4.4 CD8 T cells

CD8 T cells are well known for their important role in clearing cells infected with intracellular pathogens and secretion of IFN γ and TNF α . This is why they are canonically known as cytotoxic T lymphocytes (CTLs) (Mittrücker, Visekruna et al. 2014). However, more recent research has shed light on the functional diversity that CD8 T cells have depending on the environmental cues during their activation. Therefore, it is now known that like CD4 T cells, CD8 T cells can differentiate into multiple subtypes, T cytotoxic cell (Tc) 1, Tc2, Tc9, Tc17 and CD8 Treg cells (Valentine, Hoyer 2019, Mittrücker, Visekruna et al. 2014). As described below and shown on Figure 1.7, many of the signals that induce their differentiation, the lineage-specifying (master regulator) transcription factors and effector cytokines are shared with their CD4 Th counterparts (Mousset, Hobo et al. 2019).

Tc1 cells are the best characterised effector CD8 subtype, as these are the classical CTLs that were renamed after the discovery of alternative subsets (Annunziato, Romagnani et al. 2015). Like Th1 CD4 T cells, Tc1 differentiation is induced by IFN γ and IL-12 (Mittrücker, Visekruna et al. 2014, Annunziato, Romagnani et al. 2015, Valentine, Hoyer 2019). This leads to STAT4 phosphorylation and subsequent T-bet and eomesodermin (Eomes) activation that is required for their cytolytic functions (Annunziato, Romagnani et al. 2015, Mittrücker, Visekruna et al. 2014). Upon activation, Tc1 cells kill their target cells by releasing cytotoxic granzymes and perforin into the immunological synapse (Valentine, Hoyer 2019, Mittrücker, Visekruna et al. 2014). Their secretory signature consists of highly proinflammatory IFN γ , TNF α and LT α that activate innate immune cells like macrophages and neutrophils (Kumaresan, da Silva et al. 2018, Annunziato, Romagnani et al. 2015). And similar to Th1 cells, they are characterised by CCR5 and CXCR3 chemokine receptor expression (Annunziato, Romagnani et al. 2015, Mousset, Hobo et al. 2019). Although their main role is in anti-viral and intracellular infection response, they are also implicated in some autoimmune diseases (Valentine, Hoyer 2019).

Tc2 cells have an overlapping cytokine profile as Th2 cells. They secrete IL-4, IL-5 and IL-13 (Mittrücker, Visekruna et al. 2014, Annunziato, Romagnani et al. 2015). Therefore, they are also implicated in allergic reactions and aggravate allergic airway inflammation (Valentine, Hoyer 2019, Annunziato, Romagnani et al. 2015). Tc2 have even been reported to promote B

cell class switching to IgE (Maggi, Giudizi et al. 1994). Although they express perforin, they exhibit significantly reduced cytotoxicity compared to Tc1 (Annunziato, Romagnani et al. 2015, Mittrücker, Visekruna et al. 2014). As with Th2 cells, Tc2 development is dependent on IL4 and results in STAT6 mediated GATA-3 activation (Mittrücker, Visekruna et al. 2014, Annunziato, Romagnani et al. 2015, Valentine, Hoyer 2019, Mousset, Hobo et al. 2019).

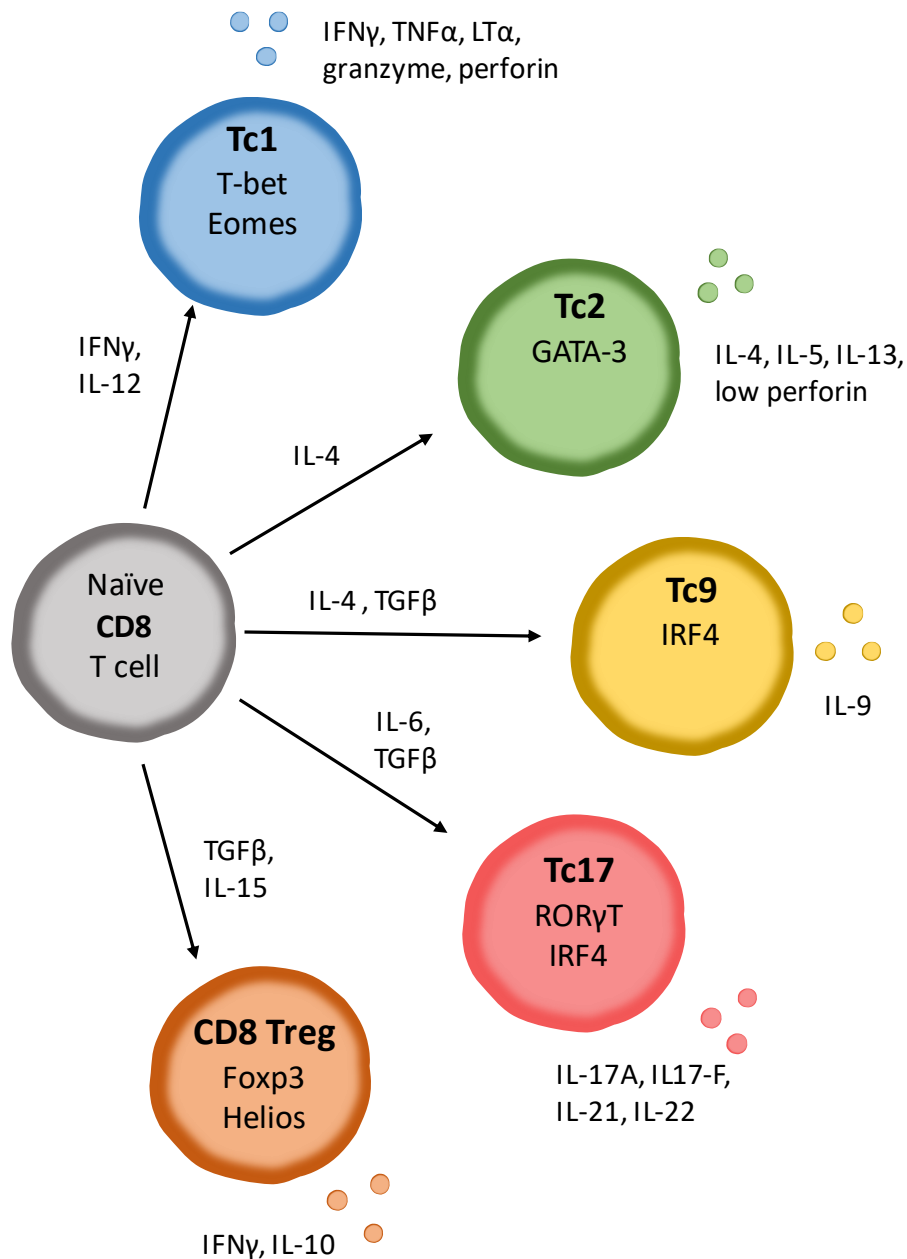


Figure 1.7 Summary of CD8 T cell effector subsets. CD8 subsets are generated by naïve T cell stimulation with different factors, resulting in activation of lineage-specific transcription factors leading to secretion of characteristic cytokines.

Tc9 are one of the latest CD8 T cell subpopulations to be identified, first described less than a decade ago (Visekruna, Ritter et al. 2013). They are very similar to their CD4 counterpart, Th9,

in that their differentiation relies on STAT6 mediated IRF4 activation following TGF β and IL-4 stimulation (Viserkruna, Ritter et al. 2013, Huber, Lohoff 2015, Mittrücker, Viserkruna et al. 2014)s. They can also differentiate in a Th9-induced environment and like Th9 cells, Tc9 lymphocytes secrete cytokines IL-9 and IL-10 (Huber, Lohoff 2014, He, Dong et al. 2020, Wan, Wu et al. 2020, Mittrücker, Viserkruna et al. 2014, Mousset, Hobo et al. 2019). In contrast to Tc1 cells, this population has low expression of T-bet, Eomes, IFN γ and granzyme, and therefore reduced cytotoxic properties (Viserkruna, Ritter et al. 2013, Huber, Lohoff 2014, Wan, Wu et al. 2020). However, they have been described to have a potent anti-tumour effect which is primarily due to IL-9 secretion (He, Dong et al. 2020, Wan, Wu et al. 2020). Additionally, together with Th2 cells, they play a role in promoting airway inflammation (Viserkruna, Ritter et al. 2013, Huber, Lohoff 2014, Mittrücker, Viserkruna et al. 2014).

Tc17 are a population of IL-17 expressing CD8 T cells that are involved in response to viral infections, autoimmunity and cancer (Liang, Pan et al. 2015b, Liang, Pan et al. 2015a, Huber, Lohoff 2014, Mittrücker, Viserkruna et al. 2014). Similar to their CD4 counterpart, Th17, their differentiation is induced by IL-6 and TGF β that lead to STAT3 phosphorylation (Liang, Pan et al. 2015b, Liang, Pan et al. 2015a, Huber, Lohoff 2014). This then leads to activation of the master regulators of Tc17 development – ROR γ T and IRF4 (Liang, Pan et al. 2015b, Liang, Pan et al. 2015a, Huber, Lohoff 2014, Mittrücker, Viserkruna et al. 2014). The resulting cytokine hallmark of Tc17 cells consists of IL-17A, IL17-F, IL-21, IL-22, as well as expression of chemokine receptors CCR6 and CCR7 (Liang, Pan et al. 2015b, Mittrücker, Viserkruna et al. 2014, Annunziato, Romagnani et al. 2015). In contrast to Tc1 and Tc9 cells, Tc17 lymphocytes possess low cytotoxic activity as they have reduced production of perforin, granzyme and IFN γ (Huber, Lohoff 2014, Mittrücker, Viserkruna et al. 2014). However, Tc17 cells express low levels of T-bet and Eomes and have a strong plasticity to develop into a Tc1 phenotype in response to an IL-12 environment (Liang, Pan et al. 2015b, Liang, Pan et al. 2015a, Mittrücker, Viserkruna et al. 2014, Annunziato, Romagnani et al. 2015).

In addition to the more well known CD4 Tregs, a population of CD8 T cells with an immunoregulatory function has also been described. These CD8 Tregs are known to have a function in autoimmunity, cancer and transplantation (Nakagawa, Wang et al. 2018, Zhang, Shuping, Wu et al. 2018, Yesillik, Agrawal et al. 2019, Mittrücker, Viserkruna et al. 2014, Bézie, Anegon et al. 2018). They are restricted by MHC class Ib Qa-1 or HLA-E molecules for mouse and human respectively (Mittrücker, Viserkruna et al. 2014). These are unique non-classical

MHC molecules that have limited polymorphism and they are expressed at low levels but can be upregulated in response to cell stress and activation (Wasnik, Baylink et al. 2019). CD8 Tregs can recognise their target cells expressing these receptors, including CD4 effector cells, and eliminate them by secreting perforin (Mittrücker, Visekruna et al. 2014). Although studies on the inducers of CD8 Treg differentiation are limited, TGF β and IL-15 signalling is believed to be involved (Zhang, Shuping, Wu et al. 2018, Mittrücker, Visekruna et al. 2014). As with CD4 Tregs, CD8 Tregs require signalling by the master regulator Foxp3, although they express it at a lower level than their CD4 counterpart (Mittrücker, Visekruna et al. 2014, Flippe, Bézie et al. 2019, Bézie, Anegon et al. 2018). In addition to Foxp3 expression, Helios-dependent STAT5 activation is essential for the survival and maintenance and CD8 Tregs (Nakagawa, Wang et al. 2018, Flippe, Bézie et al. 2019, Kim, H., Barnitz et al. 2015, Bézie, Anegon et al. 2018). Following activation, CD8 Tregs exert their immunoregulatory function by IFN γ and IL-10 secretion (Mittrücker, Visekruna et al. 2014, Flippe, Bézie et al. 2019).

1.5 Inflammation in MI

The sudden restoration of blood flow to previously ischaemic myocardium triggers an intense inflammatory response, characterised by abundant leukocyte infiltrate in the infarcted region (Ibanez, Fuster et al. 2011, Saxena, Russo et al. 2016). Post MI I/R is a form of sterile inflammation and the role of the immune system in I/R injury has been increasingly recognised and studied in the past few decades (Swirski, Nahrendorf 2018, Rieckmann, Delgobo et al. 2019). It appears that the immune response can serve both a detrimental and a beneficial role by exacerbating injury following recruitment of circulating inflammatory cells, as well as clearing the wound from necrotic debris and contributing to tissue healing (Ibanez, Fuster et al. 2011, Montecucco, Carbone et al. 2015, Saxena, Russo et al. 2016). Despite leukocytes playing a crucial role in myocardial healing, insufficient resolution of inflammation causes chronic adverse left ventricular (LV) remodelling due to severe pro-inflammatory reaction (Ramos, Hofmann et al. 2019, Andreadou, Cabrera-Fuentes et al. 2019). Multiple studies have investigated the role of various immunosuppressive and immunomodulating therapies to reduce I/R injury following MI, however, clinical results have been inconclusive and largely negative (Ibanez, Fuster et al. 2011, Hausenloy, Yellon 2013).

1.5.1 Role of immune system in MI

Although, historically, the sole function of the immune system was considered to be protection from infectious pathogens, it is now known that immunity also plays a key role in wound healing (Hofmann, Frantz 2015). And since the adult human heart has negligible regenerative potential, innate and adaptive immunity mediate the healing process of a post-MI heart, aiding the formation of a collagenous scar at the injured site (Hofmann, Frantz 2015, Saxena, Russo et al. 2016). Repair of the infarcted heart is divided into three phases: the inflammatory phase, the proliferative phase and the maturation phase (Chen, B., Frangogiannis 2017, Saxena, Russo et al. 2016).

1.5.2 Inflammatory phase

The first phase is initiated by the recruitment and activation of immune cells, primarily innate immune cells (Saxena, Russo et al. 2016, Chen, B., Frangogiannis 2017). This occurs as a result of the release of alarmins and danger signals from dying cardiomyocytes, triggering pro-inflammatory responses in vascular endothelial cells and tissue resident fibroblasts (Saxena, Russo et al. 2016, Chen, B., Frangogiannis 2017). It has been suggested that cardiomyocyte-derived high mobility group box 1 (HMGB1), heat shock proteins, extracellular RNAs and interleukin (IL)-1 α are important early stimuli of the innate immune response (Saxena, Russo et al. 2016, Chen, B., Frangogiannis 2017). Released danger signals can activate Toll-like receptor (TLR) pathways in multiple tissue resident cells types, as well as infiltrating leukocytes (Saxena, Russo et al. 2016, Chen, B., Frangogiannis 2017). The non-antigen-specific activation of TLRs induces nuclear translocation of the nuclear factor (NF)- κ B, which is a key transcription factor promoting pro-inflammatory and co-stimulatory molecule gene expression (Tak, Firestein 2001, Zuidema, Zhang 2010, Montecucco, Carbone et al. 2015). Although several TLRs have been shown to be expressed in cardiac myocytes, their exact role in the MI pathology remains poorly understood (Zuidema, Zhang 2010). However, TLR4 appears to be involved in the MI immune response, as its expression is markedly increased in the hearts of mice and rats following infarct and TLR4 deficiency results in reduced inflammation and smaller infarct size (Zuidema, Zhang 2010). Similarly, a role for TLR2 has been suggested in modulating post-infarction ventricular remodelling, resulting in accentuated injury (Zuidema, Zhang 2010, Chen, B., Frangogiannis 2017). Activation of the complement cascade in the MI heart has also been described (Zuidema, Zhang 2010, Saxena, Russo et al. 2016). Subcellular membrane fragments, released from necrotic cells, trigger upregulation of components of the

classical complement pathway (Zuidema, Zhang 2010). Complement activation may further enhance the pro-inflammatory microenvironment by triggering degranulation of tissue resident mast cells, causing the release of pro-inflammatory mediators (Chen, B., Frangogiannis 2017). In addition to this, the role of complement in MI has been confirmed in animal studies showing beneficial effect of complement depletion, although these results have not been repeated in humans (Zuidema, Zhang 2010).

Activation of the local inflammatory response in the infarcted heart causes a systemic immune response, resulting in recruitment of leukocytes from the primary immune organs (Chen, B., Frangogiannis 2017). This occurs due to upregulated secretion of pro-inflammatory cytokines and chemokines in the systemic circulation from fibroblasts, vascular endothelial cells, cardiomyocytes, as well as leukocytes already resident in the infarcted area (Chen, B., Frangogiannis 2017, Saxena, Russo et al. 2016). IL-1 signalling plays a critical role in regulating the post-MI inflammatory response by stimulating leukocyte activation and infiltration, as well as chemokine expression by fibroblasts (Bujak, Dobaczewski et al. 2008, Saxena, Russo et al. 2016, Chen, B., Frangogiannis 2017). Its release is dependent on the action of the inflammasome which is a key molecular platform required for caspase-1 activation and subsequent generation of bioactive IL-1 β from pro- IL-1 β (Chen, B., Frangogiannis 2017, Saxena, Russo et al. 2016, Montecucco, Carbone et al. 2015). Moreover, pro-inflammatory cytokines, such as tumour necrosis factor (TNF)- α and IL-6, stimulate expression of adhesion molecules and chemokines, therefore further promoting leukocyte infiltration (Saxena, Russo et al. 2016).

In addition to complement activation and cytokine secretion, chemokine upregulation is a hallmark of the immune response following MI (Saxena, Russo et al. 2016). It is a critical step in the recruitment of leukocytes, mediated by chemokine immobilisation on glycosaminoglycans (GAGs) on the surface of vascular endothelial cells, followed by leukocyte adhesion upon chemokine:chemokine receptor engagement and ultimately leukocyte extravasation into the injured myocardium (Chen, B., Frangogiannis 2017). There are four, structurally and functionally distinct families of chemokines (C, CC, CXC and CX3C, where X is any amino acid) depending on the pattern of cysteine residues at the amino terminus of the mature protein (Kufareva, Salanga et al. 2015). Chemokines from CXC family, such as IL-8 are potent neutrophil chemoattractants, while CC chemokines, such as CCL7 and CCL2 (monocyte chemoattractant protein (MCP)-1) are involved in monocyte recruitment (Saxena, Russo et al.

2016, Chen, B., Frangogiannis 2017). Neutrophils are the first cells to be recruited to the post-MI myocardium (within 30 minutes), secreting various proteolytic enzymes and phagocytosing dead cells and extracellular matrix debris (Chen, B., Frangogiannis 2017, Montecucco, Carbone et al. 2015). ROS generation in the injured heart causes a positive feedback loop that recruits additional neutrophils and prolongs their otherwise short life-span (Montecucco, Carbone et al. 2015). Neutrophils are required for successful repair and scar formation of the myocardium, however they may also exert cytotoxic effect and perpetuate myocardial injury (Montecucco, Carbone et al. 2015). Monocytes and tissue resident macrophages also have a key role in clearing the infarcted heart from necrotic debris (Chen, B., Frangogiannis 2017). Two different subsets of monocytes infiltrate the myocardium at temporally distinct points, differentiating into M1 (inflammatory) or M2 (reparative) macrophages (Chen, B., Frangogiannis 2017, Zuidema, Zhang 2010, Heppner, Ransohoff et al. 2015). During the early inflammatory phase (1-4 days following MI) highly pro-inflammatory and phagocytic Ly-6C^{high} monocytes are recruited via their CCR2 receptor (the main receptor for CCL2) (Chen, B., Frangogiannis 2017, Zuidema, Zhang 2010). During the reparative phase (4-8 days following MI) Ly-6C^{low} monocytes assist with the repair of the injured myocardium (Chen, B., Frangogiannis 2017, Zuidema, Zhang 2010).

The end of the inflammatory phase is marked by apoptosis of the short-lived phagocytic neutrophils and is followed by suppression of the immune response and resolution of the inflammatory infiltrate (Saxena, Russo et al. 2016). Macrophages clear the myocardium from apoptotic neutrophils which triggers the release of anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)- β (Saxena, Russo et al. 2016, Chen, B., Frangogiannis 2017). Lymphocytes, recruited to the myocardium along with monocytes, have various roles in MI inflammation, depending on the particular lymphocyte subset (Chen, B., Frangogiannis 2017). For example, regulatory T cells (Tregs) contribute to the negative regulation of the MI immune response and although only a small number of Tregs is found in the myocardium, their ablation results in impaired repair (Chen, B., Frangogiannis 2017).

1.5.3 Proliferative phase

The proliferative phase of infarct repair is marked by the suppression of the pro-inflammatory response and the expansion of activated cardiac myofibroblasts (Saxena, Russo et al. 2016, Chen, B., Frangogiannis 2017). These fibroblasts express high levels of contractile proteins like α -smooth muscle actin which help reduce the injured area as a result of strong contraction

forces (Saxena, Russo et al. 2016, Chen, B., Frangogiannis 2017, van Amerongen, Bou-Gharios et al. 2008). They are the main source of structural and extracellular matrix proteins and therefore play a major role in the repair of the infarcted heart (Saxena, Russo et al. 2016, Chen, B., Frangogiannis 2017). Cross-talk between myofibroblasts and immune cells by the secretion of cytokines and growth factors allows modulation of fibroblast function and recruitment of anti-inflammatory leukocytes (Saxena, Russo et al. 2016, Chen, B., Frangogiannis 2017). In this respect, macrophages have been shown to be critical in the proliferative phase as they are a major source of TGF- β and vascular endothelial growth factor (VEGF) (Chen, B., Frangogiannis 2017). These growth factors are important mediators of cardiac repair by stimulating angiogenesis (Chen, B., Frangogiannis 2017). Therefore, macrophage depletion results in reduction of their levels and subsequent impaired myocardial healing (Chen, B., Frangogiannis 2017). In addition to this, mast cells, secreting a wide range of growth factors and enzymes (including tryptase and chymase), are markedly increased during the proliferative phase of cardiac healing and are important mediators of fibroblast activation (Saxena, Russo et al. 2016, Chen, B., Frangogiannis 2017). Mast cells also release TNF- α and histamine, causing upregulation of the adhesion molecules Intercellular adhesion molecule (ICAM)-1 and P-selectin, respectively (Zuidema, Zhang 2010). The role of lymphocytes in this phase is not as well understood, although experimental evidence suggests that they can affect myofibroblast phenotype both directly and indirectly by modulating macrophage-driven response (Chen, B., Frangogiannis 2017).

1.5.4 Maturation phase

The maturation phase is characterised by suppression of myofibroblast activity and proliferation, and subsequent inhibition of collagen-based matrix deposition (Saxena, Russo et al. 2016, Chen, B., Frangogiannis 2017). The timely quiescence and apoptosis of fibroblasts and other reparative cells is critical in preventing unrestrained fibrosis and remodelling of non-infarcted myocardium (Saxena, Russo et al. 2016, Chen, B., Frangogiannis 2017). The exact mechanisms and effector cells remain unknown, however cross-linking of the collagenous matrix results in the formation of a mature stable scar, marking the end of post-MI myocardial repair (Saxena, Russo et al. 2016, Chen, B., Frangogiannis 2017).

1.5.5 T cells in myocardial I/R injury

Although human myocardial lymphocytes in steady state and post-MI are not well characterised, mouse MI models have shown the sterile inflammation induced by the

infarction contains considerable number of leukocytes (Hofmann, Frantz 2015). In fact, B and T cells are heavily recruited to the injured heart, showing a lymphocyte increase of 5- to 10-fold, peaking at 7 days following MI (Hofmann, Frantz 2015, Yan, Anzai et al. 2013a). The predominant CD4 T cell subsets are Th1 and Foxp3⁺ Tregs, while Th2 and Th17 are only minor populations (Hofmann, Frantz 2015, Yan, Anzai et al. 2013a). Furthermore, interferon (IFN)- γ secretion by both Th1 CD4 and CD8 T lymphocytes is considerably increased (Hofmann, Frantz 2015, Yan, Anzai et al. 2013a).

While multiple studies have described a role for T cells in a variety of organ systems following I/R injury (Linfert, Chowdhry et al. 2009, Huang, Y., Rabb et al. 2007), there are relatively few studies to date investigating their contribution to myocardial I/R injury. However, a major study in a mouse MI model has specifically evaluated the role of T lymphocytes in myocardial I/R and has clearly shown that CD4 T cells contribute to the injury. Using RAG1 knock out (KO) mice, which lack mature lymphocytes, and wild type (WT) controls, Yang et al. examined myocardial infarct size after 45 minutes of ischaemia (Yang, Day et al. 2006). They showed that RAG1 KO mice had significantly lower infarct size, as well as reduced neutrophil infiltration 24 hours after reperfusion (Yang, Day et al. 2006). Furthermore, they confirmed that this protective effect is mediated by depletion of CD4 T cells but not CD8 T cells (Yang, Day et al. 2006, Hofmann, Frantz 2015, Hofmann, Frantz 2016). This was done by adoptive transfer of CD4 T cells which resulted in reversing the protection observed in RAG1 KO mice (Yang, Day et al. 2006, Hofmann, Frantz 2015, Hofmann, Frantz 2016). This is also supported by similar myocardial protection following CD4 T cell depletion using a neutralising anti-CD4 monoclonal antibody (Yang, Day et al. 2006). In addition to this, reconstituting RAG1 KO mice with CD4 T cells from IFN- γ KO mice did not result in an increased infarct size (Yang, Day et al. 2006). Therefore, this study strongly suggests a critical role for CD4 T cells secreting IFN- γ in myocardial I/R injury, possibly due to cross-talk with innate immune cells (neutrophils and monocytes) mediated by pro-inflammatory cytokines.

However, how these CD4 T cells become activated during I/R injury remains poorly understood. Although there is some evidence for the presence of auto-antigens that may activate self-reactive T cells, currently it seems more likely that a TCR independent pathway is used (Hofmann, Frantz 2015, Hofmann, Frantz 2016). This may include pattern recognition receptors like the TLRs and the alarmin mediated recognition described above (Hofmann, Frantz 2015, Hofmann, Frantz 2016). This is supported by studies showing that mouse Th1

cells have elevated IFN- γ production in response to TLR2 stimulation, independently of TCR engagement (Imanishi, Hara et al. 2007).

Further cross-talk between the adaptive and the innate immune response in MI has been suggested by *in vitro* studies describing direct Treg regulation of neutrophil activity (Lewkowicz, Klink et al. 2013). This effect on the neutrophils may influence I/R injury as neutrophils are the predominant leukocyte population recruited immediately after MI (Hofmann, Frantz 2015). Tregs, characterised by broad anti-inflammatory properties, have also been implicated in the I/R immune response due to their rapid accumulation in post MI murine hearts (Hofmann, Frantz 2016, Hofmann, Frantz 2015, Xia, Jiao et al. 2015). In addition to this, it has been reported that transfer of *in vitro*-activated Tregs attenuates cardiomyocyte apoptosis, inhibits neutrophil infiltration and ultimately leads to reduced infarct size (Xia, Jiao et al. 2015). Activation of the Tregs appears to be key for this protective role as transfer of freshly isolated non-activated T cells had no beneficial effect (Xia, Jiao et al. 2015).

In addition to the classical $\alpha\beta$ CD4 T cells, the post MI myocardium also contains low number of $\gamma\delta$ T cells (Hofmann, Frantz 2015). The cells form a distinct subset of adaptive immune cells in that they can rapidly respond following tissue injury, similar to the innate immune response (Hofmann, Frantz 2015). The exact mechanism of activation and contribution to I/R injury mediated by $\gamma\delta$ T cells is not well studied (Hofmann, Frantz 2015). Nonetheless, Liao et al. have demonstrated that IL-17A, secreted from $\gamma\delta$ T cells but not CD4 helper T cells, is elevated after MI and plays a pathogenic role in I/R injury (Liao, Xia et al. 2012). They have used IL-17A KO mice, as well as anti-IL-17A monoclonal antibody treatment to show reduced neutrophil infiltration, cardiomyocyte death and ultimately smaller infarct size (Liao, Xia et al. 2012).

Clinical data on the exact lymphocyte subsets, their trafficking and role in MI myocardium is very limited due to the lack of non-invasive imaging techniques to monitor leukocytes (Hofmann, Frantz 2016, Hofmann, Frantz 2015). Recent work from our group has assessed the kinetics of T cell subsets in blood of STEMI patients during early PPCI reperfusion (Boag, Das et al. 2015). This study reported that lymphopenia following MI is associated with poor long-term outcome. The drop in lymphocytes is shown to be primarily due to effector T cell loss, with up to 40% reduction during the first two hours of reperfusion (Boag, Das et al. 2015). However, the loss of T cells is only transient, with almost complete recovery within 24 hours post reperfusion. This study also reports a specific loss of more differentiated effector T cells, CD8 effector memory (T_{EM}) and terminally differentiated CD8 effector memory (T_{EMRA}) cells,

which are known to have potent effector function and cytokine secretion (Boag, Das et al. 2015). Furthermore, loss of CD4 naïve, central memory and Tregs, expressing CCR7⁺, has been reported (Boag, Das et al. 2015, Hofmann, Frantz 2016). As the chemokine receptor CCR7 controls migration to and within secondary lymphoid organs, these results suggest a possible mechanism of CCR7-mediated redistribution of T cells and homing to the infarcted heart (Hofmann, Frantz 2016). In addition to this, the ligands for CCR7, CCL19 and CCL21, have been shown to have elevated levels in the plasma of CHD patients and in human atherosclerotic plaques (Damås, Smith et al. 2007), further supporting a role of CCR7 in recruitment of T cells in the reperfused myocardium. The loss of CD4 T cells from the circulation has been shown to be associated with patients who subsequently develop MVO (Boag, Das et al. 2015). Transcoronary gradient (aortic root to coronary sinus) of STEMI patients showed a drop in T cell numbers, reflecting T cell “trapping” in the infarcted area, possibly causing further injury and contributing to MVO (Boag, Das et al. 2015). Moreover, Boag et al. showed that depletion of circulating T cells has a striking correlation with their expression of a chemokine receptor CX3CR1 (Boag, Das et al. 2015). By simultaneously measuring T cells in the aortic root and draining coronary sinus the data indicated that T cells fell in number across this transcoronary route. Thus, T cells may be rapidly recruited to the injured heart immediately following reperfusion. However, without access to heart tissue this hypothesis cannot be tested in STEMI patients. Furthermore, as CX3CR1 expressing T cells showed a striking reduction in the circulation immediately following reperfusion (Boag, Das et al. 2015), it is possible that key interactions between CX3CR1 and its ligand fractalkine (also known as CX3CL1) play a role here.

1.6 Fractalkine in myocardial I/R

1.6.1 Structure of fractalkine and fractalkine receptor

Chemokines are a family of small (8-10 kDa) chemoattracting proteins that recruit leukocytes to sites of inflammation (Liu, W., Jiang et al. 2016, Jones, B. A., Beamer et al. 2010). They can be broadly divided into two types: 1) inflammatory chemokines, which are inducibly synthesised and recruit immune cells in response to inflammation, and 2) homeostatic chemokines, that are constitutively expressed and regulate basal leukocyte trafficking and formation of secondary lymph organ architecture (Jones, B. A., Beamer et al. 2010, Ferretti, Pistoia et al. 2014). The four subfamilies of chemokines, described above, mediate their function by binding to their seven transmembrane G-protein-coupled receptors, expressed on

the surface of immune cells (Jones, B. A., Beamer et al. 2010, Ferretti, Pistoia et al. 2014). The interaction between chemokines, expressed in endothelial cells, and their respective chemokine receptors on leukocytes, have a key role in immune cell development, homing to inflamed tissue and ultimately their effector function (Jones, B. A., Beamer et al. 2010).

The human body has over 50 different chemokines and in the late 1990s, a novel chemokine subset was discovered with a single unique member, termed fractalkine (CX3CL1) (Jones, B. A., Beamer et al. 2010). Fractalkine is 373 amino acids long, synthesised as an intracellular precursor, before being glycosylated to form a mature 100 kDa protein and transported to the cell membrane (Liu, W., Jiang et al. 2016, Jones, B. A., Beamer et al. 2010). Its structure, shown in Figure 1.8, consist of an extracellular N-terminal chemokine domain (76 amino acids), an extended mucin-like stalk (241 amino acids), a transmembrane α -helix (21 amino acids), and a short cytoplasmic tail (37 amino acids) (Jones, B. A., Beamer et al. 2010). Fractalkine is also unique in that it exists both as a transmembrane and soluble protein (Liu, W., Jiang et al. 2016, Jones, B. A., Beamer et al. 2010, Ferretti, Pistoia et al. 2014). The membrane-bound protein can have its extracellular domain cleaved from the cell surface by metalloproteinases, forming a 76 amino acid long 85 kDa soluble protein (Liu, W., Jiang et al. 2016). The soluble form is generated by the action of A Disintegrin And Metalloprotease (ADAM)10 and ADAM17/TNF- α -converting enzyme (TACE) (Liu, W., Jiang et al. 2016, Jones, B. A., Beamer et al. 2010, Ferretti, Pistoia et al. 2014). ADAM10 is responsible for constitutive cleavage under normal conditions, whereas ADAM17/TACE mediates accelerated cleavage in response to inflammatory agent stimuli (Liu, W., Jiang et al. 2016, Jones, B. A., Beamer et al. 2010, Ferretti, Pistoia et al. 2014). Fractalkine has a number of positively charged amino acids on its chemokine domain which mediate binding to the negatively charged CX3CR1 (Liu, W., Jiang et al. 2016). The soluble form of fractalkine serves as a chemoattractant to monocytes, T cells and NK cells, expressing fractalkine receptor, while the transmembrane form supports their integrin-independent firm adhesion (Jones, B. A., Beamer et al. 2010, Ferretti, Pistoia et al. 2014). In addition to fractalkine expression on endothelial cells, during inflammation it is also found on circulating monocytes (Jones, B. A., Beamer et al. 2010). Furthermore, CX3CR1 is highly expressed on cytotoxic effector lymphocytes, such as cytotoxic T lymphocytes, $\gamma\delta$ T cells and NK cells (Jones, B. A., Beamer et al. 2010). Therefore, the interaction between fractalkine and CX3CR1 may contribute to the rapid capture and migration of cytotoxic cells into tissue

(Jones, B. A., Beamer et al. 2010). This results in release of toxic granules, ultimately enhancing endothelial cell damage.

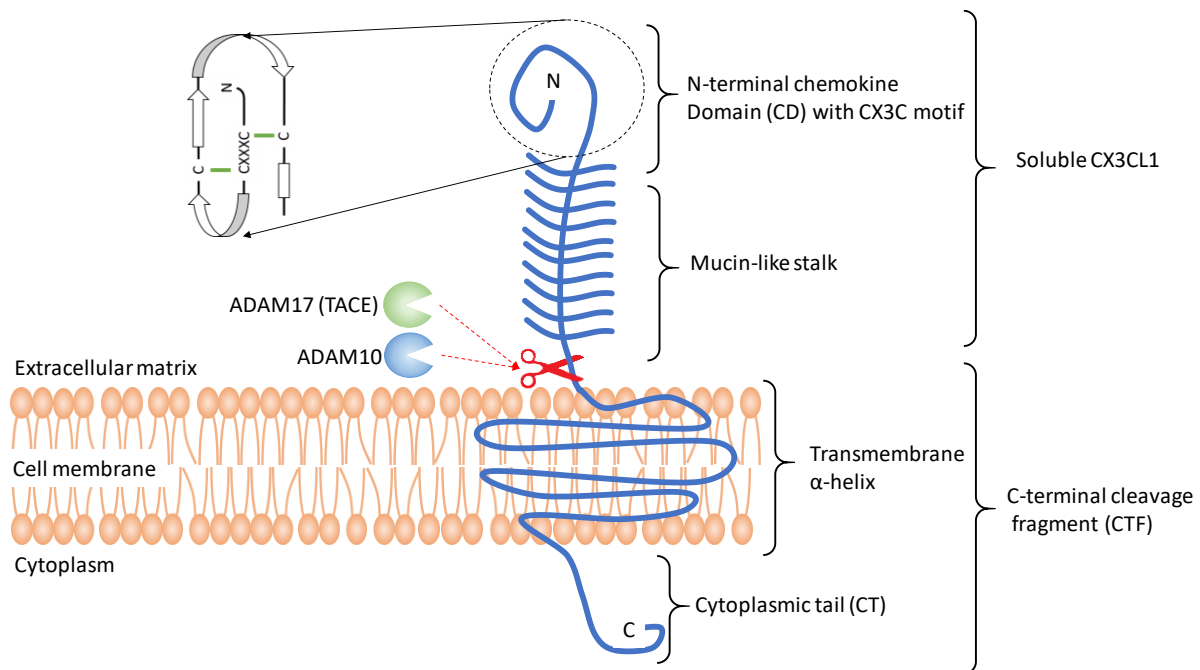


Figure 1.8 Structure of transmembrane fractalkine. It consists of four domains – N-terminal chemokine domain, mucin-like stalk, transmembrane α -helix and short cytoplasmic tail. The membrane-bound protein can be cleaved by ADAM17/TACE to release a soluble peptide.

1.6.2 Role of CX3CL1/CX3CR1 in MI

There is an increasing body of evidence describing a role for the CX3CL1/CX3CR1 axis in a number of inflammatory conditions, including rheumatoid arthritis, asthma, vasculitis, as well as CHD (Liu, W., Jiang et al. 2016, Jones, B. A., Beamer et al. 2010, Ferretti, Pistoia et al. 2014). Atherosclerosis in the coronary arteries, leading to primarily macrophages and T cell infiltration of the vessel wall, is the pathological cause of MI (Hofmann, Frantz 2015, Jones, B. A., Beamer et al. 2010). Injury to the arterial endothelium initiates interactions between cell components of the vascular wall and blood-derived cells (Liu, W., Jiang et al. 2016, Jones, B. A., Beamer et al. 2010). Although basal levels of fractalkine are expressed in endothelial cells under normal conditions, its expression is markedly increased by a number of pro-inflammatory factors, such as TNF- α , IFN- γ and IL-1 (Liu, W., Jiang et al. 2016, Jones, B. A., Beamer et al. 2010). This elevated fractalkine expression may contribute to atherogenesis and plaque rupture by causing enhanced recruitment of CX3CR1 expressing monocytes and T cells (Liu, W., Jiang et al. 2016, Jones, B. A., Beamer et al. 2010). Indeed, increased fractalkine expression has been reported in a proatherogenic apolipoprotein E-deficient (*ApoE*) mouse model, with subsequent reduction in lesion development and reduced macrophage

accumulation in an *ApoE/Cx3cr1* double KO mice (Combadière, Potteaux et al. 2003, Lesnik, Haskell et al. 2003). Furthermore, similar beneficial effect on atherosclerotic lesion formation has been reported following treatment with a CX3CR1 antagonist (Poupel, Boissonnas et al. 2013). In addition to this, genetic studies have identified two non-synonymous single nucleotide polymorphisms (SNPs) of CX3CR1 (V249I and T280M) that are associated with reduced incidence of CHD (Pucci, Mazzarelli et al. 2013, Jones, B. A., Beamer et al. 2010, Liu, W., Jiang et al. 2016). Ikejima et al. found that unstable angina pectoris patients with plaque rupture had significantly increased levels of fractalkine, as well elevated number of circulating CX3CR1⁺ T cells, NK cells and monocytes. The high levels of plasma fractalkine suggest increased recruitment of CX3CR1-expressing immune cells, which may result in T cell and NK cell mediated activation of macrophages, ultimately leading to plaque rupture (Ikejima, Imanishi et al. 2010). Similar to atherosclerosis, the CX3CL1/CX3CR1 axis has been associated with type 2 diabetes, another MI risk factor. Diabetic patients have increased levels of plasma fractalkine (Shah, Hinkle et al. 2011), as well as high CX3CL1 expression in the deep intima of coronary arteries (Wong, Wong et al. 2002). A recent study has shown that treatment with neutralising anti-fractalkine antibody improves MI survival, cardiac function and reduces infarct size in a mouse MI model (Gu, Xu et al. 2015). Furthermore, Gu et al. have also shown that anti-fractalkine treatment leads to reduction in macrophage activation and infiltration into the injured myocardium (Gu, Xu et al. 2015).

Levels of soluble fractalkine in MI patients have been shown to increase following PPCI, although Njerve et al. failed to find a correlation to infarct size (Njerve, Solheim et al. 2014, Boag, Das et al. 2015). Studies from our group have also shown that before this peak in fractalkine levels, there is an early drop at 15 minutes post reperfusion (Boag, Das et al. 2015).

1.6.3 T cells and the CX3CL1/CX3CR1 axis

CX3CR1 expression on T cells has started being elucidated only recently. Expression of fractalkine receptor on CD8 T cells correlates with the degree of effector function and has been shown to identify three functionally distinct memory and effector populations – CX3CR1⁻, CX3CR1^{int} and CX3CR1^{high} (Gerlach, Moseman et al. 2016). CX3CR1⁻ CD8 T cells display characteristics of central memory cells (T_{cm}) in that they are found in the lymph nodes and have high proliferative capacity. They have lower cytotoxic potential than CX3CR1^{int} or CX3CR1^{high} cells and express CD62L (Gerlach, Moseman et al. 2016, Martin, Badovinac 2018). CX3CR1^{high} CD8 T cells have overlapping characteristics with effector memory cells (T_{em}) as

they do not express CD62L and are absent from the lymph nodes. Instead, they are abundant in the blood and peripheral tissues where they have potent cytotoxic function (Gerlach, Moseman et al. 2016, Martin, Badovinac 2018). CX3CR1^{int} cells present a novel population termed peripheral memory T cells (Tpm). As their name suggests, they are found in the peripheral tissues, blood and lymph nodes (Gerlach, Moseman et al. 2016, Martin, Badovinac 2018). Their distinct function is trafficking from the circulation to the peripheral tissues and into the lymph nodes (Gerlach, Moseman et al. 2016, Martin, Badovinac 2018, Jameson, Masopust 2018, Böttcher, Beyer et al. 2015). Although numbers of CX3CR1^{int} CD8 T cells are lower than their CX3CR1⁻ and CX3CR1^{high} counterparts, viral infection induces an increase in CX3CR1^{int} levels, representing around 15% of memory cells in the blood (Gerlach, Moseman et al. 2016). In addition to viral infections, these three subsets of CX3CR1 expressing T cells have also recently been described in tumour microenvironments (Yamauchi, Hoki et al. 2020).

While some studies on the role of fractalkine in I/R injury exist, they are primarily focused on ischaemia of the brain (Tang, Z., Gan et al. 2014, Briones, Woods et al. 2014) and kidneys (Zou, Zhang et al. 2014, Gunay, Inal et al. 2013). However, fractalkine and its receptor are known to be involved in atherosclerosis, a main risk factor for development of MI. Fractalkine is chemotactic to CX3CR1 expressing monocytes. Although some subsets of monocytes are pro-angiogenic, monocytes and macrophages are crucial for development of atherosclerotic plaques (Lee, M., Lee et al. 2018). In fact, CX3CR1 signalling may be directly contributing to this role as downstream signalling activates anti-apoptotic B cell lymphoma 2 (Bcl2), promoting survival of deleterious monocytes (Landsman, Bar-On et al. 2009).

In addition to this, as mentioned above, CX3CR1 in STEMI patients has strong correlation between expression in T cell subsets and their depletion from the circulation following reperfusion (Boag, Das et al. 2015). And *in vitro* evidence shows binding of soluble fractalkine to CX3CR1 on the surface of T cells (Boag, Das et al. 2015). This may suggest that following reperfusion, the elevated circulating fractalkine is rapidly bound by CX3CR1 expressing leukocytes, resulting in their margination and eventually migration into the tissue. Furthermore, fractalkine-mediated binding of leukocytes to the inflamed endothelium could contribute to the development of MVO by contributing to immune cell plugging, therefore exacerbating myocardial injury. These emerging evidence of the role of CX3CL1/CX3CR1 in MI make this an important potential therapeutic target to reduce myocardial I/R injury.

1.7 Hypothesis

- 1) Myocardial ischaemia and reperfusion (I/R) cause activation of vascular endothelial cells and subsequent enhancement in expression of transmembrane fractalkine (tCX3CL1), as well as release of soluble fractalkine (sCX3CL1). This leads to recruitment and accumulation of cytotoxic CX3CR1⁺ T cells to injured region where they secrete pro-inflammatory cytokines such as IFN γ and promote neutrophil influx, further contributing to injury.
- 2) Inhibiting CX3CL1-CX3CR1 interaction will reduce T cell homing and transmigration into the injured myocardium. This will result in reduced immune cell-mediated damage to the heart and ultimately result in reduced infarct size (Figure 1.9).

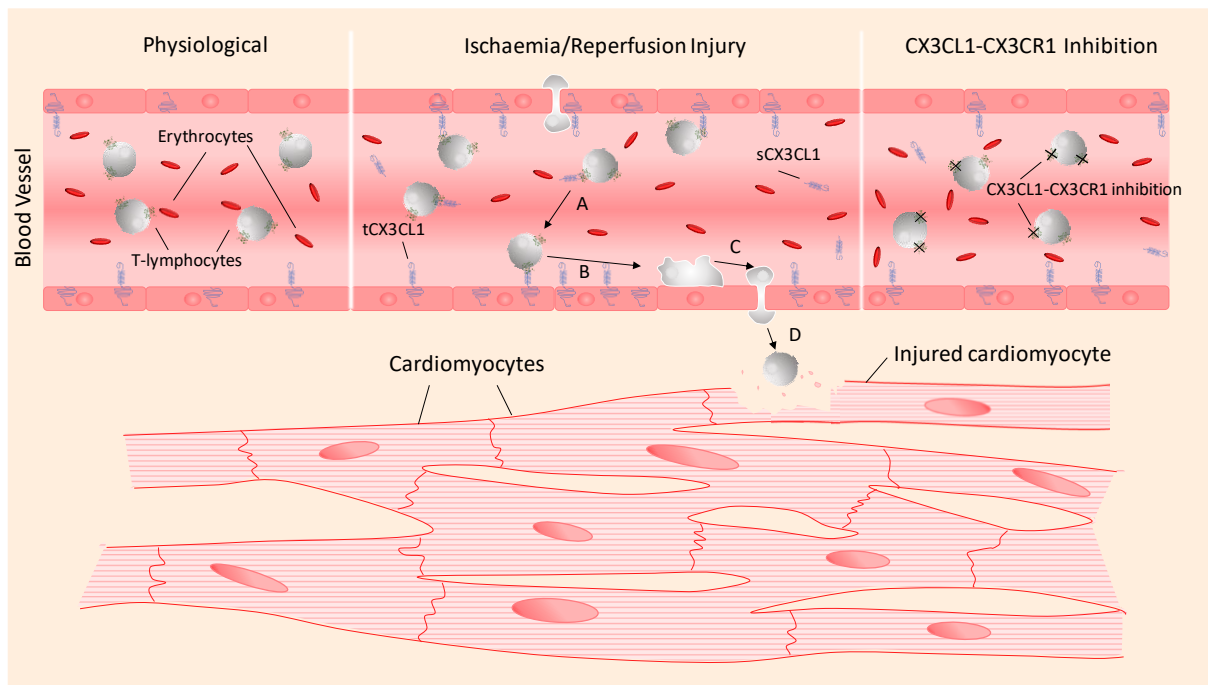


Figure 1.9 Project hypothesis. Cross-section through a coronary artery at physiological conditions, during I/R injury and following disruption of the CX3CL1-CX3CR1 axis. I/R injury causes endothelial cell activation which results in increased expression of transmembrane fractalkine (tCX3CL1) and release of soluble fractalkine (sCX3CL1). (A) sCX3CL1 is chemotactic to CX3CR1 expressing leukocytes, including T cells, which promotes their recruitment and (B) binding to the endothelium via tCX3CL1. CX3CR1⁺ T cells then (C) migrate through the endothelium, infiltrate the infarcted myocardium and (D) contribute to cardiomyocyte injury. Inhibiting the interaction between CX3CR1 and fractalkine (CX3CL1) would reduce T cell homing, transmigration and subsequent cardiac injury.

1.8 Aims

The overall aims of this project are to:

- 1) Investigate the effect of CX3CR1 knockout on myocardial I/R phenotype
- 2) Develop a method of deep immune profiling of myocardial I/R injury using imaging mass cytometry
- 3) Assess the effect of T cell phenotypes on inflammation following myocardial I/R and explore clinically relevant models for future research

Chapter 2. Methods

2.1 Mouse strains and Genotyping

2.1.1 Mouse strains

This project makes use of a CX3CR1 mutant mouse line (Jung, Aliberti et al. 2000). In the mutant strain, the CX3CR1 gene locus is replaced by an EGFP cassette, resulting in loss of CX3CR1 production and EGFP expression. Therefore, heterozygous CX3CR1^{+/EGFP} mice have reduced fractalkine receptor expression and also serve as a reporter line. Immunodeficient homozygous RAG2 knockout (KO) mice (Hao, Rajewsky 2001) were purchased from Charles River (Charles River Laboratory International). Both CX3CR1-EGFP and RAG2 KO mice are on a C57BL/6 background. C57BL/6 mice were purchased from Charles River (Charles River Laboratories) and colonies were bred in-house. Male C57BL/6 mice at 100 weeks (23 months) of age were used in a study described in Chapter 5. Tissues from these mice were obtained from Dr Gavin Richardson (Walaszczyk, Dookun et al. 2019).

Only the CX3CR1-EGFP mouse colony required genotyping (described below), as all others were maintained as homozygous crosses.

2.1.2 DNA extraction

Mouse DNA was extracted from ear clip tissue using an adapted HotSHOT protocol (Truett, Heeger et al. 2000). Briefly, the clip was collected in 1.5ml tube and 100µl of Alkaline Lysis buffer (25mM NaOH + 0.2mM EDTA, ~pH12) were added. Sample was incubated in thermo-mixer at 95°C, 350 rev for 50-60min. The reaction was terminated by addition of 100µl Neutralisation buffer (40mM Tris-HCL). Samples were then centrifuged for 5min at 13000xg.

2.1.3 Genotyping

Genotyping of CX3CR1-EGFP mice was performed using a combination of three primers specifically designed for this mouse line: 1) WT forward (GTCTTCACGTTCTGGTCTGGT) + 2) Common reverse (CCCAGACACTCGTTGTCCT) + 3) Transgene forward (CTCCCCCTGAACCTGAAAC). Reactions were prepared by adding 1x GoTaq Master Mix, 0.2µM of each primer, 25ng/ml DNA and topping up to 10µl with dH₂O. The following PCR reaction cycle was used – initial denaturation for 5min at 95°C, in cycle denaturation for 30sec at 95°C, 30sec annealing at 62°C for 35 cycles, extension for 90sec at 72°C, final extension for 5min at 72°C and 4°C until removed from thermocycler.

The samples were then loaded on a 2% agarose gel with 2 drops of ethidium bromide (500µg/ml) and electrophoresis was performed for 30min at a constant voltage of 150V in 1X Tris-acetate-EDTA (TAE) buffer. DNA was visualised by UV transillumination.

2.2 In vivo work

2.2.1 Mouse MI model

2.2.1.1 Open model

Acute transient MI was created in adult male C57BL/6 mice (12-14 weeks of age) by an experienced microsurgeon, Dr Rachael Redgrave, using an approved protocol (Redgrave, Tual-Chalot et al. 2016, van Laake, Passier et al. 2007), in accordance with the UK Home Office Scientific Procedures Act, 1986. In brief, mice were anaesthetised using 3% isoflurane/97% oxygen and general anaesthesia was maintained during the surgery following endotracheal intubation and mechanical ventilation. Following left-side thoracotomy through the fourth intercostal space, MI was created by loosely ligating the left anterior descending (LAD) coronary artery, using a 7-0 prolene suture, immediately below the left atria. A 1-2mm piece of PE-10 tubing was then inserted into the loosely tied knot so that when the knot is tightened, the tubing causes occlusion of the LAD. Ischaemia of the heart was confirmed by immediate blanching of the left ventricular myocardium. Mice in the sham control group underwent thoracotomy but no LAD ligation. The chest cavity of MI mice was closed for 60 minutes, followed by reopening to allow reperfusion, achieved by removing the PE tubing. Finally, the chest was closed and anaesthesia was discontinued. In addition to this, the mice were administered 0.05mg/kg buprenorphine subcutaneously to alleviate post-operative pain. To facilitate recovery, mice were placed in 33°C incubator and given soaked diet. The mice were culled by cervical dislocation at the designated time point and tissue was collected.

2.2.1.2 Closed model

This model was identical to the Open model with the following differences. Mice had the PE-10 tubing placed on the LAD with a loosely tied knot, the thoracic cavity was closed with the suture strings remaining on the outside and mice were left to recover for 7 days. Following recovery, the sutures strings were pulled, the knot was tightened, MI was induced for 60 minutes and monitored using electrocardiogram (ECG). Following this, the suture was released and reperfusion was allowed for 2 hours.

This myocardial I/R model was only used for one study described in Chapter 5 (Section 5.2.2). All other results in this thesis are obtained using the Open model.

2.2.2 Adoptive transfer of T cells

Total splenocytes from female heterozygous (n=2) and knockout (n=5) CX3CR1-EGFP mice were isolated in a sterile environment as described below in Section 2.5.2.1. Mononuclear cells were isolated using Histopaque 1083 (Sigma, Cat# 1083-1), according to the manufacturer's instructions. Cells were resuspended in sterile PBS and counted using Tali Cellular Analysis Slides (Thermo Fisher Scientific, Cat# T10794) on a Tali Image-Based Cytometer (Thermo Fisher Scientific). Three male RAG2 KO mice (13 weeks of age) were injected with approximately 3.3×10^7 cells/mouse by cannulation of the tail vein by an experienced microsurgeon, Dr Rachael Redgrave. To facilitate recovery, mice were placed in 33°C incubator overnight and given soaked diet. Two days after the adoptive transfer, blood was collected by tail vein sampling as described below in Section 2.5.1.2, and T cell were analysed by flow cytometry. Twenty-two days after adoptive transfer, blood was collected by terminal cardiac puncture (Section 2.5.1.1), mice were culled by cervical dislocation and their spleens were collected for flow cytometry cell analysis.

2.2.3 MuLV infection

Blood and spleen cells from adult C57BL/6 mice infected with murine leukemia retrovirus (MuLV) were obtained from a collaborator, Dr Lei Huang (Huang, L., Ou et al. 2016). Tissues were collected 5 weeks post MuLV infection and T cell populations were analysed using flow cytometry, as described below.

2.3 Histological procedures

2.3.1 Tissue processing and sectioning

Immediately after the mice were culled, their hearts were dissected and atria removed, washed in PBS to remove blood clots and connective tissue and placed in 1% paraformaldehyde overnight at 4°C. On the next day they were transferred into 30% sucrose and left at 4°C overnight. The hearts were then embedded in Optimal Cutting Temperature compound (OCT) for 30min on dry ice and stored at -80°C.

The tissue was then sectioned using a cryostat to produce 10µm transverse sections, collected as sister sections on Superfrost plus slides (VWR). Sections were collected from the ischaemic heart region, identified by the area between the LAD ligature and the apex of the heart.

2.3.2 Immunofluorescence staining

Heart sections were fixed in 4% paraformaldehyde (PFA) for 10min at room temperature, followed by three 5min washes in PBS. Samples were then permeabilised using 0.3% Triton-X-100 in PBS for 30min at room temperature. Following this, sections were blocked in 1% BSA for 30min at room temperature, primary antibodies, diluted in blocking solution, were added in concentrations shown on Table 2.1 and left to incubate overnight at 4°C. On the next day, primary antibodies were removed and samples were washed three times in PBS. Secondary antibodies staining was performed for 2 hours at room temperature in the dark, according to Table 2.1. After three 5min PBS washes, coverslips were mounted using Prolong Gold antifade reagent with DAPI (Thermo Fisher Scientific).

	Antigen	Monoclonal/ Polyclonal	Host	Reactivity	Company	Catalogue number	Dilution
Primary antibodies	CD45	Monoclonal (30-F11)	Rat	Mouse	BioLegend	103102	1:100
	CD3z	Monoclonal (17A2)	Rat	Mouse	R&D	MAB4841	1:50
	Podocalyxin	Polyclonal	Goat	Mouse	R&D	AF1556	1:100
	GFP	Polyclonal	Chicken	Mouse	Abcam	ab13970	1:300
Secondary antibodies	Anti-Rat Alexa 488	Polyclonal	Goat	Rat	Thermo Fisher Scientific	A-11006	1:200
	Anti-Goat Alexa 568	Polyclonal	Donkey	Goat	Thermo Fisher Scientific	A-11057	1:200
	Anti-Rat CF 568	Polyclonal	Donkey	Rat	Sigma Aldrich	SAB4600077	1:200
	Anti- Chicken CF488	Polyclonal	Goat	Chicken	Sigma Aldrich	SAB4600031	1:200

Table 2.1. Details of antibodies used for immunostaining.

2.4 Microscopy and image analysis

Imaging data was acquired using M2 Axio Imager (Zeiss). Images were taken at x5 or x40 magnification and analysed using ZEN 2.3 (Zeiss).

Immunofluorescence staining was used to investigate the immune cell infiltration in the heart following myocardial I/R injury. Because of the inherent variability in the surgical MI induction,

heart dissection and tissue sectioning, CD45 staining of all hearts was used to identify areas of highest CD45 infiltration, consistently across all hearts. In order to do this, x5 tile images of CD45 staining were taken every 300µm throughout the heart (Figure 2.1). Signal from the red-568 channel was included in these images as loss of autofluorescence has been shown to correlate with infarct size (Lagarto, Dyer et al. 2015). The area of maximum infiltration was identified and used as guide for further, higher magnification (x40), analysis.

2.5 Tissue collection and immune cell isolation

2.5.1 Blood sampling

2.5.1.1 Cardiac puncture

Mice were anaesthetised using 3% isoflurane/97% oxygen and general anaesthesia was maintained during the procedure. Blood was collected using terminal cardiac puncture into EDTA treated tubes.

2.5.1.2 Tail vein sampling

Mice were anaesthetised using 3% isoflurane/97% oxygen and general anaesthesia was maintained during the procedure. Their tails were heated using a heat lamp. An incision was made on the tails using a scalpel and 30µl blood was collected into an EDTA treated tube.

2.5.2 Immune cell isolation

2.5.2.1 Spleen

Dissected spleens were ground on a pre-wet 70µm filter with a 2ml syringe plunger. The filters were washed with 10ml PBS and cell suspension centrifuged at 300xg for 5min at 4°C. The pellet was resuspended in 3ml Pharmlyse red blood cell lysis buffer (BD) and incubated for 5min at room temperature. 10ml Flow cytometry buffer (PBS + 1% BSA + 0.05% NaN₃) was used to stop the reaction, followed by another centrifugation step. Cell suspension was filtered through a 30µm filter and cells were counted using Tali Cellular Analysis Slides (Thermo Fisher Scientific, Cat# T10794) on a Tali Image-Based Cytometer (Thermo Fisher Scientific) before flow cytometry staining.

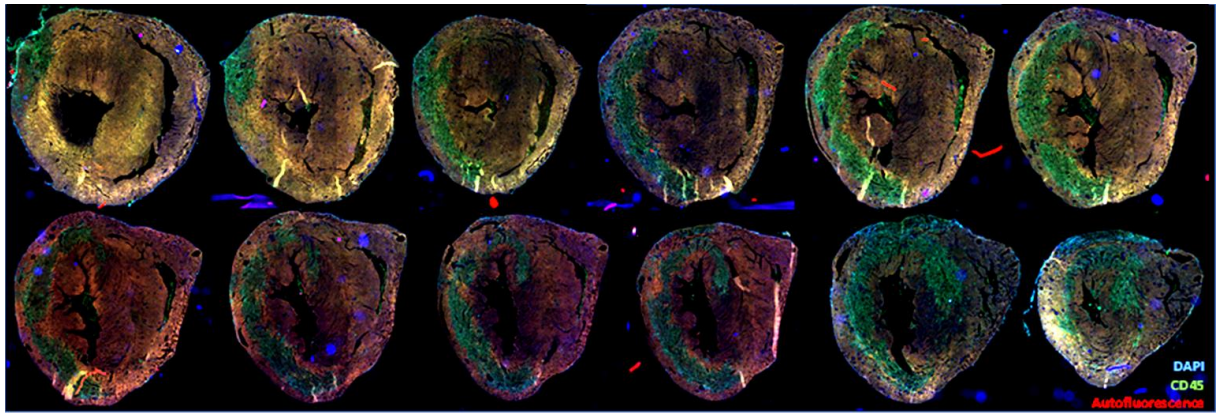


Figure 2.1 Tiled images of CD45 staining used as guide to area of maximum injury. Representative images showing immune cell infiltration at 24h I/R throughout the heart (CD45) that matches loss of autofluorescence (568nm channel), indicating infarct region. Images are taken every 300µm through the heart base to apex (going left to right and top to bottom). X5 magnification.

2.5.2.2 Lymph nodes

Popliteal and subiliac (peripheral) and cranial mediastinal (cardiac) lymph nodes were dissected and processed separately. Lymph nodes were put between two pieces of 70µm filter and were ground using a flat forceps in a petri dish. 500µl PBS were used to wash cell suspension from filters and transfer to a 1.5ml tube for centrifugation at 400xg for 5min. Cells were resuspended in the minimum volume of Flow buffer required for flow cytometry staining (1ml for peripheral and ~0.3ml for cardiac lymph nodes).

2.5.3 Cardiac digest

Hearts were dissected from ~12-14 week old mice, culled by cervical dislocation. The hearts were washed in PBS three times and any blood clots, visible vessels and connective tissue were removed. The left and right ventricles were separated and tissue was chopped into small pieces using a scalpel for 2min. Tissue was then digested in 1mg/ml Collagenase/Dispase (Roche/Sigma-Aldrich #10269638001) and 60U/ml DNase (Sigma Aldrich # D4527) in DPBS + Ca^{2+} + Mg^{2+} (Thermo Fisher Scientific) for a total of 60min at 37°C in a shaking water bath. After the first 30min incubation, the tissue suspension was triturated twice with a 10ml stripette, supernatant was removed and fresh digest buffer was added to the remaining tissue before returning it to the water bath. This process was repeated at the end of the 60min incubation. The digested cell suspension was filtered through a 70µm filter and washed with ice-cold DMEM+FBS. Following this the cells were centrifuged and the pellet was resuspended in Pharmlyse red blood cell lysis buffer (BD). Flow cytometry buffer (PBS + 1% BSA + 0.05%

NaN₃) was used to stop the reaction, followed by another centrifugation step. Finally, the cells were resuspended in 1ml of Flow cytometry buffer and filtered through a 30µm filter. It was not possible to confidently count the number of cells before antibody staining because of the high amount of debris.

2.6 Flow cytometry

2.6.1 Spleen and lymph node staining

Fc receptors of cells were blocked for 10min on ice using 1:50 anti-CD16/32 (clone 93, BioLegend). Without washing, 50µl of cell suspension at 2x10⁷ cells/ml was stained with a cocktail of antibodies for 60min, as shown on Table 2. The cells were incubated for 30min in the dark on ice and washed with 2ml (when stained in tubes) or 2x200µl (when stained in plates) Flow buffer and centrifugation at 400xg for 5min at 4°C. The cells were resuspended in 300µl of buffer and 1:10 DAPI was added immediately before acquisition to distinguish live and dead cells. For cardiac digest, DRAQ5 (eBioscience) was used to identify nucleated cells.

Fluorophore compensation was performed using UltraComp compensation beads (eBioscience), according to the manufacturer's instructions. Data was acquired on BD Fortessa II.

2.6.2 Whole blood staining

Blood collected by cardiac puncture was used for whole blood flow cytometry analysis. 100µl whole blood was stained using the method described in 2.6.1. Following the 60min incubation, 1ml of red blood cell lysis solution was added for 10min at room temperature. 2ml Flow cytometry buffer was used to stop the reaction, followed by another centrifugation step. Cells were resuspended in 300µl Flow buffer before proceeding to data acquisition.

2.6.3 Whole blood absolute cell count analysis

Blood collected by tail vein sampling was used for absolute cell count analysis. 25µl blood were reverse pipetted into an absolute counting Trucount tube (BD). A cocktail of antibodies was added as described on Table 2.2. The tube was mixed by vortexing briefly and left to incubate for 15min at room temperature, protected from light. 2ml of red blood lysis solution were added, the tube was mixed by vortexing and immediately following another 15min incubation, data was acquired on Fortessa II. Baseline blood sampling was done 7 days before surgery (for closed model, this was 7 days before the first surgery).

2.6.4 Antibody details

Flow cytometry antibody details are shown in Table 2.2. When two or more BV antibodies were used, antibody cocktail was made up in Brilliant Stain Buffer, as per the manufacturer's instructions. The gating strategy and specific panel used for each experiment are shown in the gating strategy in the results sections.

Antibody	Fluorochrome	Clone	Company	Catalogue number	Dilution/vol/conc. per test
CD3	BV605	17A2	BioLegend	100237	0.2µg
CD3	PE	17A2	BioLegend	100206	0.1µg
CD4	BV480	RM4-5	BD	565634	0.1µg
CD8	PerCP-Cy5.5	53-6.7	BioLegend	100733	0.1µg
CD44	BUV395	IM7	BD	740215	0.1µg
CD62L	PE	MEL-14	BioLegend	104407	0.1µg
CX3CR1	BV421	SA011F11	BioLegend	149023	0.1µg
CD11b	APC	M1/70	BioLegend	101211	0.1µg
CD45	BV786	30-F11	BioLegend	103149	0.1µg
Brilliant Stain Buffer			BD	563794	50µl
Fc Block - CD16/32	-	93	BioLegend	101302	1:50

Table 2.2. Details of antibodies used for flow cytometry.

2.6.5 Analysis and gating strategy

Flow cytometry data was analysed using FCS Express (V7, De Novo Software). Live cells are first gated based on forward side scatter area (FSC-A) and DAPI exclusion. Afterwards, intact lymphocytes are gated based on side scatter area (SSC-A) vs FSC-A, before gating for single cells based on forward scatter height (FSC-H) and FCS-A. Finally, fluorochrome gating is based on fluorescence minus one (FMO) controls.

2.7 Quantitative PCR (qPCR)

2.7.1 RNA isolation

Spleen and heart samples were used for gene expression analysis. Immediately following dissection, spleens were immersed in RNAlater stabilisation solution (Thermo Fisher Scientific, cat#AM7021). Hearts were dissected and rinsed in PBS. The left and right ventricle were separated using a scalpel and immediately immersed in RNAlater. Both spleen and heart samples in RNAlater were stored at -80°C until RNA isolation.

Before RNA isolation for the heart samples, tissue was thawed and finely chopped using a scalpel. Total RNA from both heart and spleen samples was extracted using RNeasy Fibrous Tissue Mini Kit (Qiagen, cat#74704), according to the manufacturer's instructions. TissueRuptor® (Qiagen, cat#9002757) was used to homogenise tissues in RLT buffer and samples were passed through QIAshredder columns (Qiagen, cat#79654) before running through the RNeasy Mini columns. Following RNA isolation, an extra DNase treatment step was performed according to manufacturer's instructions using DNA-free™ DNA Removal Kit (Thermo Fisher Scientific, cat#AM1906). The samples were then stored at -80°C until RNA was quality controlled.

2.7.2 RNA Quality Control (QC)

The quality and the concentration of the RNA samples was analysed using the Agilent RNA ScreenTape System (4200 TapeStation, Agilent Technologies) according to the manufacturer's instructions. RNA from heart samples with RIN^e ≥ 8.3 was used for downstream applications.

2.7.3 Reverse transcription

Reverse transcription of RNA samples was performed using Tetro cDNA Synthesis Kit (Bioline, cat#BIO-65043) using random hexamer primers.

2.7.4 TaqMan qPCR

Gene expression in spleen samples was analysed using TaqMan qPCR. Amplification of 50ng cDNA reactions was performed using TaqMan™ Gene Expression Master Mix (Thermo Fisher Scientific, cat# 4369016) and QuantStudio 7 Flex System (Thermo Fisher Scientific) for the following mouse probes Cx3cr1 (Mm00438354_m1), Gapdh (Mm99999915_g1) and Hprt (Mm03024075_m1). Housekeeping genes Gapdh and Hprt were used for normalisation of comparative $\Delta\Delta C_t$ analysis.

2.7.5 Mouse Cytokine microarray

Cytokine gene expression was assessed in left and right ventricle samples after 6h and 24h I/R. This was done using a 96 well plate microarray (Thermo Fisher Scientific, cat#4413255) with Table 2.3 showing the list of genes and probes used. Amplification reactions were performed using 50ng/well for left ventricle samples, 20ng/well for 6h right ventricle samples and 35ng/well for 24h right ventricle samples. The smaller amount of right ventricle tissue resulted in lower RNA yield and therefore this limited the amount of cDNA that can be used per experiment. Amplification was performed using TaqMan™ Fast Universal PCR Master Mix

(Thermo Fisher Scientific, cat#4352042) and QuantStudio 7 Flex System (Thermo Fisher Scientific). Gene expression for each sample was normalised to the following housekeeper genes: 18s, Actb, B2m, Gapdh, Hprt. Comparative $\Delta\Delta C_t$ analysis was performed using Expression Suite Software (v1.3, Thermo Fisher Scientific).

Gene name	TaqMan Probe	Protein encoded
18S	Hs99999901_s1	18S RNA
Adipoq	Mm00456425_m1	Adiponectin
Bmp2	Mm01340178_m1	Bone Morphogenetic Protein 2
Bmp4	Mm00432087_m1	Bone Morphogenetic Protein 4
Bmp6	Mm01332882_m1	Bone Morphogenetic Protein 6
Bmp7	Mm00432102_m1	Bone Morphogenetic Protein 7
Ccl1	Mm00441236_m1	CCL1
Ccl11	Mm00441238_m1	CCL11
Ccl12	Mm01617100_m1	CCL12
Ccl17	Mm01244826_g1	CCL17
Ccl19	Mm00839967_g1	CCL19
Ccl2	Mm00441242_m1	CCL2 /MCP-1 (monocyte chemoattractant protein 1)
Ccl20	Mm01268754_m1	CCL20
Ccl22	Mm00436439_m1	CCL22
Ccl24	Mm00444701_m1	CCL24
Ccl3	Mm00441259_g1	CCL3/MIP-1 alpha (macrophage inflammatory protein 1 alpha)
Ccl4	Mm00443111_m1	CCL4/MIP-1 beta (macrophage inflammatory protein 1 beta)
Ccl5	Mm01302427_m1	CCL5/RANTES
Ccl7	Mm00443113_m1	CCL7/MCP-3
Cd40lg	Mm00441911_m1	CD40L (CD40 ligand)
Cd70	Mm00441914_m1	CD70
Zfp91	Mm00446373_m1	ZFP91 Zinc Finger Protein/E3 Ubiquitin-Protein Ligase ZFP91
Csf1	Mm00432686_m1	Colony Stimulating Factor 1/ Macrophage Colony Stimulating Factor 1 (M-CSF)
Csf2	Mm01290062_m1	Colony Stimulating Factor 2/Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF)
Csf3	Mm00438335_g1	Colony Stimulating Factor 3/Granulocyte Colony Stimulating Factor (G-CSF)
Ctf1	Mm00432772_m1	Cardiotrophin 1
Cx3cl1	Mm00436454_m1	Fractalkine
Cxcl1	Mm04207460_m1	CXCL1
Cxcl10	Mm00445235_m1	CXCL10
Cxcl11	Mm00444662_m1	CXCL11
Cxcl12	Mm00445553_m1	CXCL12
Cxcl13	Mm04214185_s1	CXC13
Cxcl16	Mm00469712_m1	CXCL16
Cxcl3	Mm01701838_m1	CXCL3
Cxcl5	Mm00436451_g1	CXCL5
Cxcl9	Mm00434946_m1	CXCL9

Fasl	Mm00438864_m1	Fas Ligand
Gpi1	Mm01962484_u1	Glucose-6-phosphate isomerase
Hc	Mm00439275_m1	Hemolytic complement/C5
Ifna2	Mm00833961_s1	Interferon Alpha 2
Ifng	Mm01168134_m1	Interferon Gamma
Il10	Mm00439614_m1	Interleukin 10
Il11	Mm00434162_m1	Interleukin 11
Il12a	Mm00434165_m1	Interleukin 12a
Il12b	Mm00434174_m1	Interleukin 12b
Il13	Mm00434204_m1	Interleukin 13
Il15	Mm00434210_m1	Interleukin 15
Il16	Mm00516039_m1	Interleukin 16
Il17a	Mm00439618_m1	Interleukin 17A
Il17f	Mm00521423_m1	Interleukin 17F
Il18	Mm00434225_m1	Interleukin 18
Il1a	Mm00439620_m1	Interleukin 1 Alpha
Il1b	Mm00434228_m1	Interleukin 1 Beta
Il1rn	Mm00446186_m1	Interleukin 1 Receptor Antagonist
Il2	Mm00434256_m1	Interleukin 2
Il21	Mm00517640_m1	Interleukin 21
Il22	Mm01226722_g1	Interleukin 22
Il23a	Mm01160011_g1	Interleukin 23 Subunit Alpha
Il24	Mm00474102_m1	Interleukin 24
Il27	Mm00461162_m1	Interleukin 27
Il3	Mm00439631_m1	Interleukin 3
Il4	Mm00445259_m1	Interleukin 4
Il5	Mm00439646_m1	Interleukin 5
Il6	Mm00446190_m1	Interleukin 6
Il7	Mm01295803_m1	Interleukin 7
Il9	Mm00434305_m1	Interleukin 9
Lif	Mm00434762_g1	Leukemia inhibitory factor
Lta	Mm00440228_gH	Lymphotoxin Alpha
Ltb	Mm00434774_g1	Lymphotoxin Beta
Mif	Mm01611157_gH	Macrophage Migration Inhibitory Factor
Mstn	Mm01254559_m1	Myostatin
Nodal	Mm00443040_m1	Nodal Growth Differentiation Factor
Osm	Mm01193966_m1	Oncostatin M
Pf4	Mm00451315_g1	Platelet Factor 4
Ppbp	Mm01347901_g1	Pro-Platelet Basic Protein/CXCL7
Spp1	Mm00436767_m1	Secreted Phosphoprotein 1/Osteopontin
Tgfb2	Mm00436955_m1	Transforming Growth Factor Beta 2
Thpo	Mm00437040_m1	Thrombopoietin
Tnf	Mm00443258_m1	Tumor Necrosis Factor
Tnfrsf11b	Mm01205928_m1	TNF Receptor Superfamily Member 11b/Osteoprotegerin (OPG)
Tnfsf10	Mm01283606_m1	TNF Superfamily Member 10/TNF-related apoptosis-inducing ligand (TRAIL)

Tnfsf11	Mm00441906_m1	TNF Superfamily Member 11/RANKL
Tnfsf13b	Mm00446347_m1	TNF Superfamily Member 13b/B-Cell-Activating Factor (BAFF)
Vegfa	Mm01281449_m1	Vascular Endothelial Growth Factor A (VEGF)
Xcl1	Mm00434772_m1	XCL1/Lymphotactin
Gapdh	Mm99999915_g1	Glyceraldehyde-3-Phosphate Dehydrogenase
Hprt	Mm00446968_m1	Hypoxanthine Phosphoribosyltransferase 1
Gusb	Mm00446953_m1	Glucuronidase Beta
Actb	Mm00607939_s1	Actin Beta
B2m	Mm00437762_m1	Beta-2-Microglobulin
Hmbs	Mm00660262_g1	Hydroxymethylbilane Synthase
Ipo8	Mm01255158_m1	Importin 8
Pgk1	Mm00435617_m1	Phosphoglycerate Kinase 1
Rplp2	Mm00782638_s1	Ribosomal Protein Lateral Stalk Subunit P2
Tbp	Mm00446973_m1	TATA-Box Binding Protein
Tfrc	Mm00441941_m1	Transferrin Receptor

Table 2.3 Mouse Cytokine Microarray Gene List

2.8 Magnetic resonance imaging (MRI)

Cardiac function of mice following MI was monitored at 1 week and 6 weeks post reperfusion using MRI. Cardiac MRI was performed by Dr Simon Tual-Chalot and Dr Rachael Redgrave using the method that they previously described (Redgrave, Tual-Chalot et al. 2016, Redgrave, Tual-Chalot et al. 2017). Data was analysed by investigators blinded to the mouse genotype group. Short axis images were analysed using Image J (NIH) by manually tracing epicardial and endocardial borders on the end-diastolic and end-systolic image frames, as previously described (Schneider, Wiesmann et al. 2006, Redgrave, Tual-Chalot et al. 2016). This was performed for all slices, allowing the calculation of total myocardial volume (mm^3) as the sum of myocardial area per slice (mm^2) x number of slices x slice thickness (1mm/slice). LV mass was then calculated by multiplying this volume by the myocardial specific gravity ($1.05\text{mg}/\text{mm}^3$). The LV mass data presented in this thesis is shown as an average of mass measured from end diastolic and end systolic frames. Left ventricular end diastolic volume (EDV) and end systolic volume (ESV) were calculated by taking the sum of all slice measurements of the endocardial volume using the end-diastolic and end-systolic frames respectively. Finally, ejection fraction (%) was calculated as follows – $((\text{EDV}-\text{ESV})/\text{EDV})\times 100$.

2.9 Mass Cytometry (CyTOF)

All work involving suspension or imaging mass cytometry was performed using clean (if possible sterile) plastic labware and sterile filtered tips. Buffers and water of high-purity were

used and no glassware was used in order to avoid any metal contamination, such as barium in laboratory soap or lead in water piping which would be detected by the mass cytometer. Catalogue numbers of common laboratory reagents (e.g. 96 well plates, tubes and formaldehyde) are given in the sections below as they have been tested by Newcastle University Flow Cytometry Core Facility (FCCF) to be free of any metal contaminants. Furthermore, all tissue slide washing and blocking steps were performed in sterile 50ml Falcon tubes instead of reusable glass jars.

2.9.1 Panel design

Twenty intracellular and surface markers of interest were identified in order to detect all major immune cell populations, as shown on Table 2.4. Clone selection for each antibody was based on published literature and unpublished data shared by Fluidigm, showing successful use of the clones in formaldehyde fixed mouse cryosections. The panel was then designed by matching each antibody to lanthanide isotope using Fluidigm's Panel Designer, taking into account average expression levels for each marker for each cell type (allocating highly expressed markers to metal isotopes with lower signal intensity, and vice versa), signal intensity, signal spillover due to isotope impurities and oxidation, and marker expression patterns (unimodal or bimodal).

2.9.2 Antibody conjugation

The metal conjugation of the antibodies was done in-house using Maxpar Antibody Labelling kit (Fluidigm, Cat#201300) by Fluidigm, following the manufacturer's instructions. Firstly, metal-chelating polymers are loaded to saturation with each specific lanthanide metal (around 16-22 metal ions per polymer (Olsen, Leipold et al. 2019)). This is done by mixing 2.5mM lanthanide metal with L-Buffer in a 100µl reaction, followed by incubation at 37°C for 30-40 minutes. The lanthanide-loaded polymer is then purified using a 3kDa filter and centrifugation at 12,000xg for 25 minutes at room temperature. An additional wash step using 400µl C-Buffer and centrifugation at 12,000xg for 30 minutes at room temperature is performed. At the same time each antibody undergoes buffer exchange and partial reduction in preparation for the conjugation. The buffer is exchanged by loading 100µg of each antibody on a 30kDa filter with 400µl R-buffer and centrifugation at 12,000xg for 10 minutes at room temperature. This is followed by the partial reduction using 4mM TCEP in R-Buffer at 37°C for 30 minutes. The partially reduced antibody is then purified by two washes with C-Buffer and centrifugation at 12,000xg for 10 minutes at room temperature. At this stage, each partially

reduced antibody and its corresponding lanthanide-loaded polymer are mixed together with 100µl C-Buffer in the 30kDa filter. The conjugation is performed at 37°C for 90 minutes, followed by four wash steps with W-Buffer and centrifugation at 12,000xg for 10 minutes at room temperature. The now lanthanide conjugated antibodies are retrieved in 100µl W-Buffer by inverting the 30kDa filter and centrifugation at 1,00xg for 2 minutes. The antibody concentration is then measured using Nanodrop to measure 280nm absorbance before the addition of 1:1 volume stabilisation buffer (0.05% sodium azide) and storage at 4°C. Finally, the conjugation is tested by binding 1µl each antibody-metal conjugate to compensation beads. Following a 30 minute incubation at 37°C and two dH₂O washes, the antibody-bound beads are filtered through 30µm filters and ran through the suspension mass cytometer.

Antibody Target	Clone	Vendor	Catalogue number	Metal	Catalogue number
Gr-1 (Ly6G/C)	RB6-8C5	BioLegend	108449	141Pr	201141A
CD11c	N418	BioLegend	117341	142Nd	201142A
CX3CR1 (GFP)	FM264G	BioLegend	338002	145Nd	201145A
F4/80	BM8	BioLegend	123143	146Nd	201146A
CD45	30-F11	BioLegend	103141	147Sm	201147A
CD11b	M1/70	BioLegend	101249	148Nd	201148A
CD19	6D5	BioLegend	115547	149Sm	201149A
CD25	3C7	BioLegend	101913	151Eu	201151A
CD3	145-	BioLegend	100345	152Sm	201152A
TCRgd	GL3	BioLegend	118101	159Tb	201159A
CD62L	MEL-14	BioLegend	104443	160Gd	201160A
Ly6C	HK1.4	BioLegend	128039	162Dy	201162A
CD31	390	BioLegend	102425	165Ho	201165A
CD8	53-6.7	BioLegend	100755	168Er	201168A
TCRb	H57-597	BioLegend	109235	169Tm	201169A
NK1.1 (CD161)	PK136	BioLegend	108743	170Er	201170A
CD44	IM7	BioLegend	103051	171Yb	201171A
CD4	RM4-5	BioLegend	100561	172Yb	201172A
Pan-Actin	ACTN05	Thermo Fisher Scientific	MS-1295-P1ABX	175Lu	201175A
B220 (CD45R)	RA3-6B2	BioLegend	103249	176Lu	201176A

Table 2.4 Mass cytometry antibody and metal details.

2.9.3 Suspension CyTOF

2.9.3.1 Cell isolation and staining

Mouse splenocytes were isolated as described in Section 2.5.2.1. Cell viability was assessed by staining with 1:10 DAPI and running through a flow cytometer (Fortessa, BD). Staining of 3×10^6

live cells was performed in 96 well round bottomed plates (Greiner bio-one Cellstar #650 180). The cells were incubated with 2.5µM Cisplatin (Cat#201064, Fluidigm) in PBS for exactly 5 minutes at room temperature to identify dead cells. The reaction was quenched by the addition of Staining buffer (PBS + 2 % heat inactivated filtered (0.2µM) sterile FCS) and centrifugation at 400xg for 5 minutes at room temperature. Antibody master mix was prepared using 1µl of each antibody and topping up to 50µl with Staining buffer (per sample). Antibodies for 18 surface markers were used for suspension CyTOF experiments (as listed in Table 2.5, excluding intracellular markers GFP and actin). The antibody master mix and cells were mixed and incubated for 1 hour at room temperature, followed by two wash steps with PBS at 400xg for 5 minutes at room temperature. The cells were then fixed in 1:1 volume eBioscience working fix buffer (Cat#00-5523, Thermo Fisher Scientific) and 8% formaldehyde (Cat#F017/3, TAAB Laboratories Equipment), giving a final formaldehyde concentration of 4%. The cells were incubated for 30 minutes at room temperature, followed by centrifugation at 600xg for 5 minutes. Some samples were first fixed before staining, instead of surface staining prior to fixation. The same protocol was followed with the only difference that all centrifugation steps post fixation were at 600xg. The DNA of the cells was then stained with 250nM Iridium (Cat#201192A/B, Fluidigm) in PBS, alongside a final 3.2% formaldehyde fix. Following a 60 minute incubation at room temperature, the cells were centrifuged at 600xg for 5 minutes and stored overnight in PBS at 4°C or acquired on the same day.

Immediately before acquisition, the cells were washed once with PBS, followed by two washes with dH₂O at 800xg for 8 minutes. The cells were then counted using a Tali Slide (ThermoFisherScientific, Cat#T10795) and cell concentration was adjusted to 0.55x10⁶ cells/ml, allowing for the addition of 10% EQ (calibration) beads. The cell suspension was then filtered through a 30µm filter into a 5ml tube (Cat#352235, Falcon) and data was acquired on Helios (Fluidigm).

2.9.3.2 Data analysis

Suspension mass cytometry data was analysed both by manual gating and by using automated software. The manual gating analysis was performed using bivariate scatter plots on FCS Express (V6, De Novo), with pre-gating on live, intact, single cells using event length and DNA intercalators iridium and cisplatin. Automated analysis of pre-gated viable, intact total leukocyte (CD45+ cells) was performed using a mass cytometry analysis package called Cytokit (Chen, H., Lau et al. 2016). The data clustering algorithm chosen for our pipeline was

PhenoGraph, shown to be one of the highest performing unsupervised methods available (Weber, Robinson 2016). And dimensionality reduction t-Distributed Stochastic Neighbour Embedding (t-SNE) maps (van der Maaten L, Hinton G. 2008) were used for visualisation of the cell clusters. Cell populations were manually identified based on the median marker expression for each cluster.

Label	Target	µl/test
141Pr	Ly-6G/C (Gr-1)	0.57
142Nd	CD11c	0.57
145Nd	GFP	0.57
146Nd	F4/80	0.57
147Sm	CD45	0.57
148Nd	CD11b	0.57
149Sm	CD19	0.57
151Eu	CD25	1.14
152Sm	CD3e	0.57
159Tb	TCRgd	1.14
160Gd	CD62L	1.14
162Dy	Ly-6C	0.57
165Ho	CD31	1.14
168Er	CD8a	0.57
169Tm	TCRb	0.57
170Er	NK1.1 (CD161)	0.57
171Yb	CD44	0.57
172Yb	CD4	0.285
175Lu	Pan-Actin	0.57
176Yb	CD45R (B220)	0.57

Table 2.5 IMC antibody master mix. One test = 100µl.

2.9.4 Imaging mass cytometry (IMC)

2.9.4.1 Tissue processing and sectioning

Heart tissue has been processed and sectioned as described in Section 2.3.1.

Spleens were dissected and cut longitudinally into 3-4 pieces, discarding any visibly necrotic areas. The tissue was immediately immersed in 30% sucrose and left overnight at 4°C. On the following day, the separate spleen pieces were cryo-embedded in OCT over dry ice and stored at -80°C. For some experiments, two of the spleen pieces from one animal were also processed via additional methods using light overnight fixation at 4°C with 1% formaldehyde or 10% formalin prior to overnight sucrose immersion. Sister sections were then cut using a cryostat at 4µm, 7µm and 10µm thickness. Slides were stored at -80°C until staining.

2.9.4.2 Slide staining

Slides were thoroughly dried prior to 10 minute fixation at room temperature with 4% formaldehyde (TAAB laboratories). Following three 5 minute washes with DPBS (Thermo Cat#14190-094), slides were blocked for 30 minutes at room temperature with DPBS + 5% BSA + 1/100 anti-CD16/32. During this incubation, the antibody master mix was made up in staining buffer (DPBS + 0.5% BSA + 1/100 anti-CD16/32), according to Table 2.5. Sections were then covered with antibody master mix and were left to incubate at 4°C overnight. On the next day, slides were washed twice with DPBS-T (DPBS + 0.1% Triton X) for 8 minutes at room temperature, followed by two washes in DPBS. Slides were then covered with DPBS + 1/400 Iridium DNA intercalator (Cat#201192B, Fluidigm) to stain cell nuclei. Following 60 minutes incubation at room temperature, the slides were washed for 5 minutes in dH₂O and left to air dry for a minimum of 20 minutes at room temperature. Once the slides were fully dry, they were stored until acquisition in a sealed box (protecting from any dust particles contaminating the tissue sections) at room temperature (for short term storage for approximately 24-48h) or at 4°C (for longer term storage). Slides were then ready for IMC acquisition which was performed on Hyperion (Fluidigm).

One sister set for each sample was stained with 1/500 DAPI and mounted using Fluorescence Mounting Medium (Cat#S3023, DAKO). These slides were imaged using M2 Axio Imager (Carl Zeiss Microscopy) and visualised using ZEN (v. 2.3, Carl Zeiss Microscopy) to guide IMC ROI selection by identifying white pulp areas. Only one heart was analysed using IMC and CD45 IF staining of sister sections were used as guide to ROI selection (Figure 2.2).

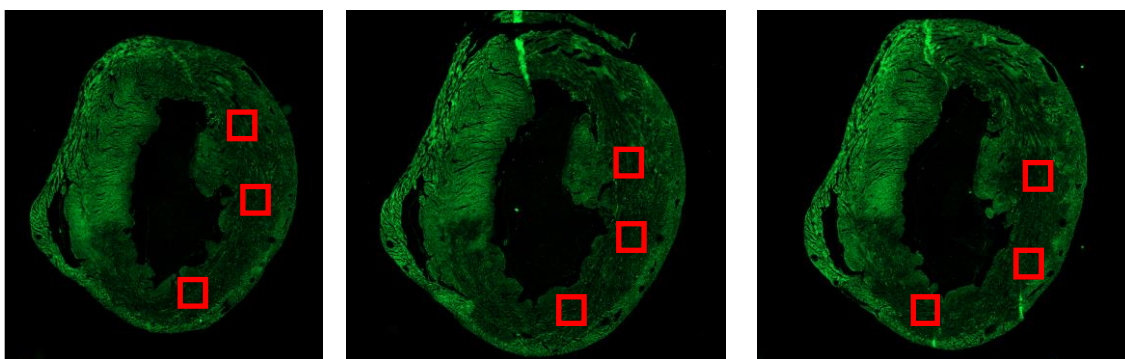


Figure 2.2 Tiled images of immune cell infiltrate in the heart used as guide to IMC ablation area. IF images showing CD45 (488nm channel) staining throughout the heart of a CX3CR1-EGFP KO mouse. Red squares indicate approximate area of ROIs that were selected for ablation from each of the three sections. Each ROI is approximately 1mm². X5 magnification.

2.9.4.3 Data Analysis

IMC raw data is presented in an .mcd file containing a stack of grey-scale images, each showing the abundance of one marker, measured by the per-pixel detection of each metal isotope. IMC data was visualised and false colour images were exported from the .mcd file using MCD Viewer (v. 1.0.560, Fluidigm). But in order to get single-cell quantitative data, a more complex analysis pipeline was used. Designed by the Bodenmiller group, this pipeline (<https://github.com/BodenmillerGroup/imctools>) utilises the Anaconda (v. 1.9.7) Python-based platform which converts raw IMC data into .tiff images. The files are then pre-processed using a CellProfiler (v. 3.1.5) pipeline (Jones, T. R., Kang et al. 2008) before image segmentation using the semi-supervised machine learning software, called Ilastik (v1.3.2) (C. Sommer, C. Straehle et al. 2011). Ilastik uses a Random Forest classifier which is trained by a user who annotates pixels as either background, nuclei or membrane, based on selected markers, creating a segmentation probability maps. The pixel classification is then exported as probability maps which are used to generate a segmentation mask using CellProfiler. Finally, single cell features were extracted from the masked .tif images using the computational Imaging Mass Cytometry analysis toolbox histoCAT (Schapiro, Jackson et al. 2017). Developed by the same group, histoCAT (v. 1.75) was used for PhenoGrpaph clustering, tSNE visualisation and phenotype identification (identical to suspension CyTOF analysis). Default tSNE and PhenoGraph (30 nearest neighbours) parameters were used for all images. Heatmaps, showing individual marker expression per cluster, were used for cluster identification.

2.10 Statistical Analysis

Results are shown as arithmetic mean values +/- standard errors of the mean (SEM). Statistical analysis was performed using GraphPad Prism (Version 6.01) and the specific statistical calculation used for each graph is described in the corresponding figure legend. Overall, statistical analysis was based on a two-sided t-test or, if multiple comparisons were required, a two-way ANOVA with post-hoc multiple comparisons test stated in the legend. Data significance was set at $p \leq 0.05$ and significance was indicated as: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$. Mouse cytokine gene expression data (Section 2.7.5) is analysed using Expression Suite Software (v1.3, Thermo Fisher Scientific) by t-test with p values adjustment for multiple testing using Benjamini-Hochberg false discovery rate.

Chapter 3. Effect of CX3CR1 knockout on myocardial I/R phenotype

3.1 Introduction

While the contribution of innate immunity in myocardial I/R injury has been the primary focus of researchers, the role of adaptive immunity, and more specifically T cells, has only recently been recognised (Santos-Zas, Lemarié et al. 2019, Rieckmann, Delgobo et al. 2019). Understanding the role of T cells in post MI I/R has been made ever more complex by the fact that multiple T cell subpopulations exist, with distinct and even contrasting functions. Indeed, this is in line with the existing reports that some populations of T cells, such as Tregs, have a beneficial effect to myocardial I/R, while others, such as Th1 CD4 and cytotoxic CD8 T cells, have a detrimental effect (Santos-Zas, Lemarié et al. 2019, Ramos, Hofmann et al. 2019, Boag, Andreano et al. 2017, Tang, T., Zhu et al. 2019). Furthermore, unlike cells of the innate immune system, T lymphocytes express a unique T cell receptor (TCR), capable of recognising 10^{15} different molecules (Ramos, Hofmann et al. 2019). This diverse antigen recognition presents an additional challenge to identifying the exact stimuli and mechanisms that drive T cell response in post MI I/R. In addition to this, very few studies have focused on the role of T cells in the early MI response. Work from our group has suggested that T cells are recruited to the infarcted myocardium during the acute MI I/R response and that this is associated with their expression of CX3CR1 (Boag, Das et al. 2015). Moreover, the lymphopenia described by Boag et al. is more prominent in patients with worse MI outcome, suggesting that CX3CR1 expressing T cells may contribute to myocardial I/R injury.

Less is known about the role of T cells and fractalkine in cardiovascular disease but it has been reported that endothelial cells secreting CX3CL1 can recruit CX3CR1⁺ T cells and upregulate their CX3CR1 expression (Ren, Hou et al. 2014). CX3CR1 expression on T cells correlates with their effector function, with highly cytotoxic T cells expressing the highest level of CX3CR1 (Gerlach, Moseman et al. 2016). These cells do not express CD62L, which is characteristic of differentiated effector T cells. In contrast, CD62L⁺ T cells do not express CX3CR1 and have a naïve phenotype (Gerlach, Moseman et al. 2016, Martin, Badovinac 2018). In addition to this, recent work shows that CX3CR1^{int} Tpm cells are the primary memory T cell subset circulating through the tissues (Gerlach, Moseman et al. 2016).

At the time of writing this thesis, no other research group has published work on the role of CX3CR1 expressing T cells in MI induced I/R. More than that, our group previously studied fractalkine and T cells in the clinical setting, not using a mouse model. Therefore, this is the first body of work investigating the role of CX3CR1 expressing T cells in the mouse I/R post

myocardial infarction. The first step was to use a model of CX3CR1 knockout described by Jung et al. (Jung, Aliberti et al. 2000). This mouse line has an EGFP knockin cassette, disrupting the CX3CR1 gene, meaning it can be used both as a reporter and as a knockout. Therefore, the first part of my PhD research project is focused on studying myocardial I/R injury in CX3CR1-EGFP wild type (WT), heterozygous (HET) and knockout (KO) mice. Specifically, this chapter aims to: (1) establish the baseline T cell phenotype of the CX3CR1-EGFP mouse line, (2) analyse the inflammatory response to acute myocardial I/R, (3) investigate T cell adhesion and infiltration in the first 24h post reperfusion and (4) assess the effect of loss of CX3CR1 expression on cardiac function.

3.2 Results

3.2.1 Baseline T cell phenotyping of the CX3CR1-EGFP mouse line

As this project focuses primarily on the CX3CR1-EGFP mouse line (Figure 3.1a), the first step was to characterise the baseline CX3CR1 gene and protein expression. Gene expression analysis on Figure 3.1b show that CX3CR1 heterozygous (HET) mice have 44% CX3CR1 transcript expression compared to wild type (WT) mice, while homozygous knockout (KO) mice have no detectable expression. This is confirmed for CX3CR1 and EGFP protein expression by flow cytometry. As shown on Figure 3.1c, circulating leukocytes from WT mice express CX3CR1 but no EGFP, HET cells are CX3CR1⁺ EGFP⁺ double positive and cells from KO mice are EGFP⁺ and CX3CR1⁻.

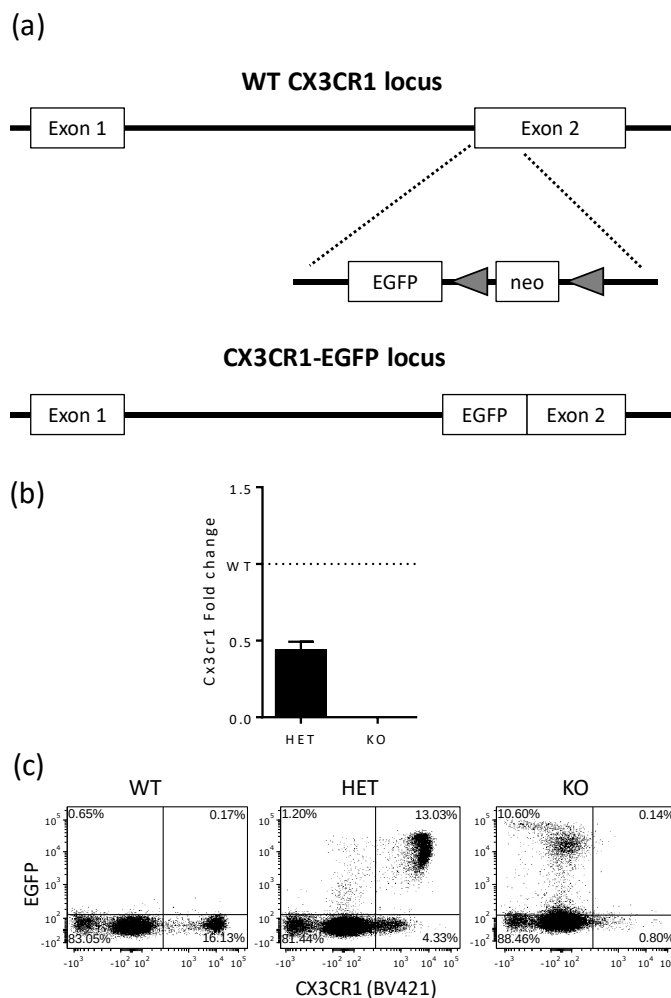
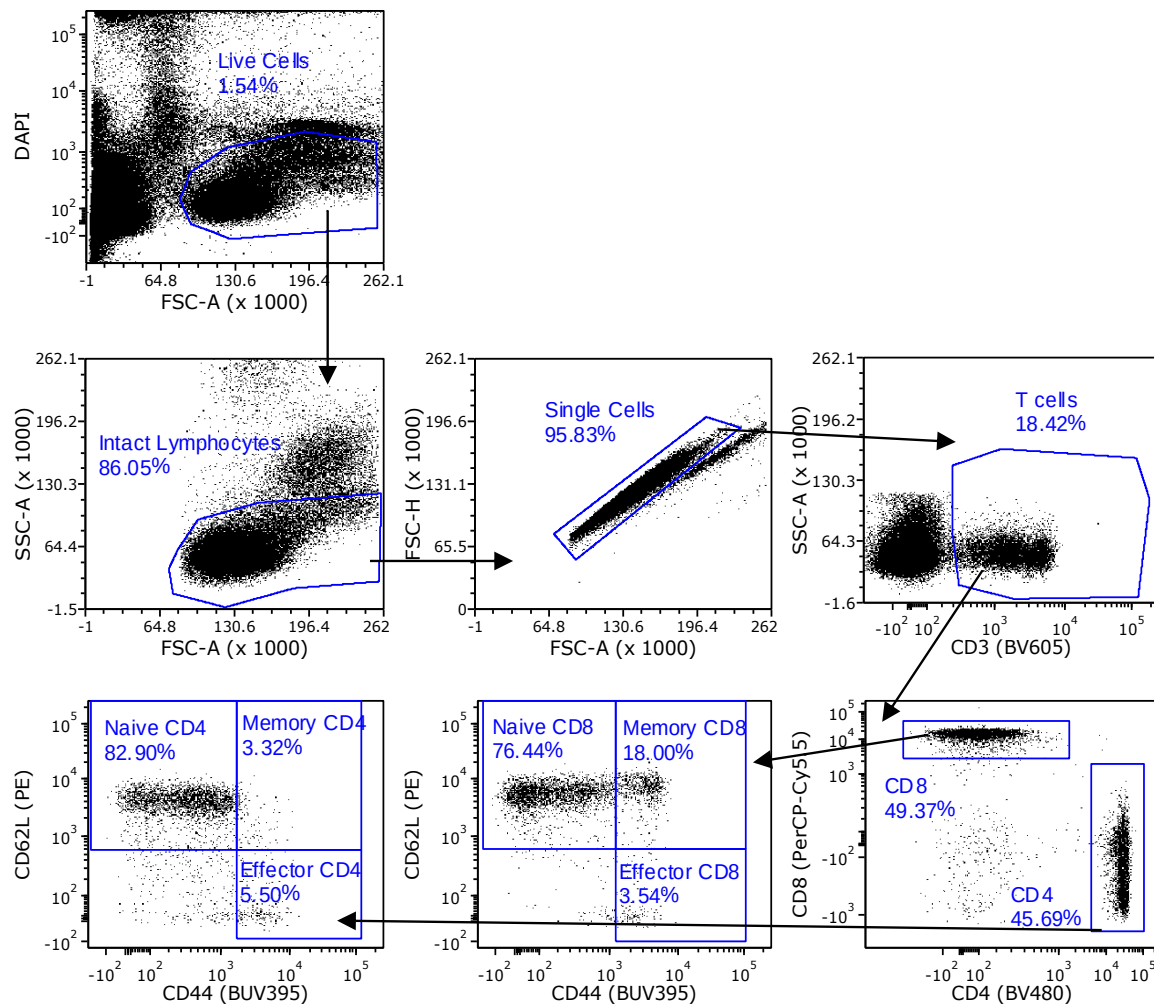


Figure 3.1 Characterisation of the CX3CR1-EGFP mouse line. (a) Cx3cr1 gene targeting strategy. In the transgenic mouse, the EGFP gene replaces the first 390bp of the second Cx3cr1 exon. (b) Cx3cr1 mRNA levels of total splenocytes. Error bars = SEM, n=6 (WT), n=5 (HET), n=3 (KO). Dotted line represents WT expression. (c) Representative flow cytometry dot plots of CX3CR1 and EGFP expression in whole blood. Gating based on all live leukocytes.

The next step was to characterise the baseline T cell phenotype in CX3CR1 WT, HET and KO mice. Several different lymphoid tissues (blood, spleen, peripheral lymph nodes and cardiac lymph nodes) were analysed using multicolour flow cytometry. The gating strategy used to phenotype T cell subsets is shown in on Figure 3.2a. In addition to this, CX3CR1 and EGFP expression of each cell type was assessed (Figure 3.2b). The graphs on Figure 3.3 show that there are no significant differences between the different T cell population numbers in the spleen, blood and lymph nodes between the different genotypes. The only exceptions are naïve and memory CD8 T cells in the two lymph node samples. Figure 3.3c shows that compared to WT controls, heterozygous mice have 7% reduction ($p \leq 0.05$) in the number of naïve CD8 T cells and 7% increase ($p \leq 0.01$) in the number of memory CD8 T cells. Similarly, the data for CD8 T cells in cardiac lymph nodes (Figure 3.3d) shows that HET mice have around 10% reduction in the number of naïve CD8 T cells and 7% increase in memory CD8 T cells (although no statistical analysis could be performed due to $n=1-2$ per genotype). The results show that overall all mice have predominantly naïve CD4 and CD8 T cells, compared to effector and memory cells, which is expected in young adult mice that have not been immunologically challenged. Moreover, the results on Figures 3.4 and 3.5 show that CX3CR1 and EGFP respectively are mostly expressed on effector T cell populations in blood, spleen and lymph nodes. The highest CX3CR1 expression was detected in effector CD8 cells, with 40% of effector CD8 T cells in the blood (Figure 3.4b) and 13% in the spleen being CX3CR1⁺ (Figure 3.4a). Lower CX3CR1 expression was detected in the lymph nodes (Figure 3.4c and d), however, peak expression there was also in effector and memory CD4 and CD8 T cells. Although the percentage EGFP⁺ cells is higher than that of CX3CR1⁺ cells, the expression pattern follows that of CX3CR1, with peak expression in effector T cells, especially CD8 effector cells (Figure 3.5). This difference is believed to be because EGFP is detected without an antibody, i.e. all intracellular EGFP signal is detected, whereas the CX3CR1 signal represents only surface staining with an antibody. As with CX3CR1 expression, EGFP levels were highest among the blood derived T cell populations (Figure 3.5b). Although CX3CR1/EGFP expression is largely consistent between WT/HET (for CX3CR1) and HET/KO (for EGFP), Figure 3.4c shows that heterozygous mice have around 30% increase ($p \leq 0.05$) in the number of CX3CR1⁺ CD8 T cells in their peripheral lymph nodes compared to WT controls. This elevated CX3CR1 expression appears to correspond to the naïve and memory CD8 T cell populations, although the small increase in CX3CR1⁺ CD8 naïve and memory T cells in HET mice compared to WT is not statistically significant (Figure 3.4). Similarly, the peripheral lymph nodes of KO mice show a

two-fold increase ($p \leq 0.05$) in the number of EGFP⁺ memory CD4 T cells compared to HET mice (Figure 3.5c).

(a)



(b)

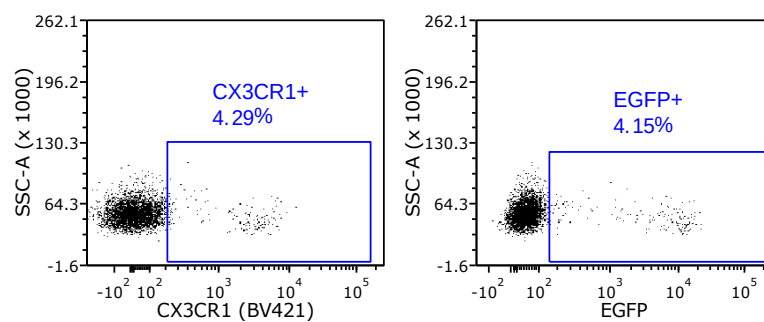
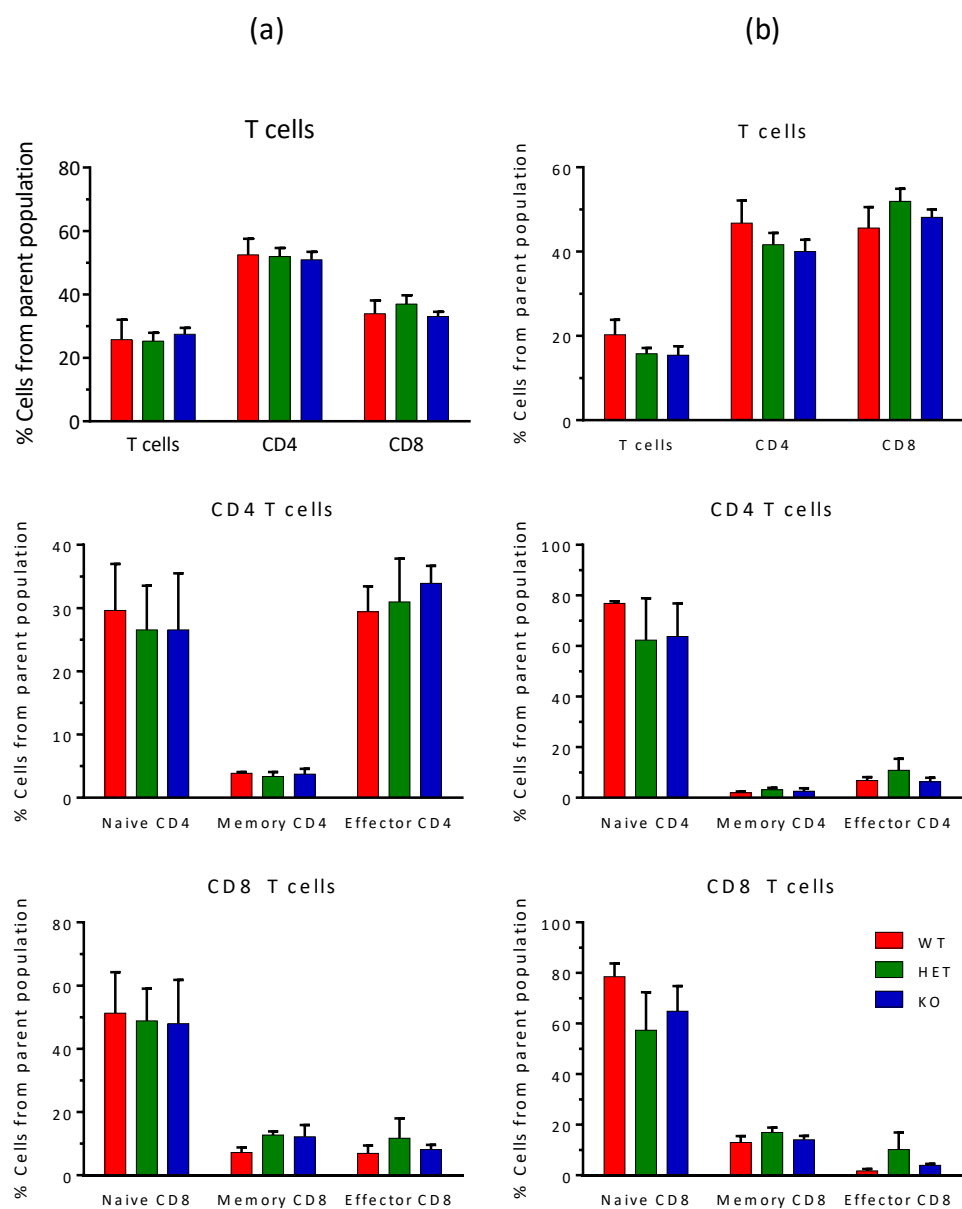


Figure 3.2 T cell phenotyping by flow cytometry. (a) Gating strategy, showing representative plots of a blood sample. (b) Expression of CX3CR1 and EGFP is measured for each T cell subset with representative plots showing circulating CD8 T cells. Representative plots of splenocyte gating.



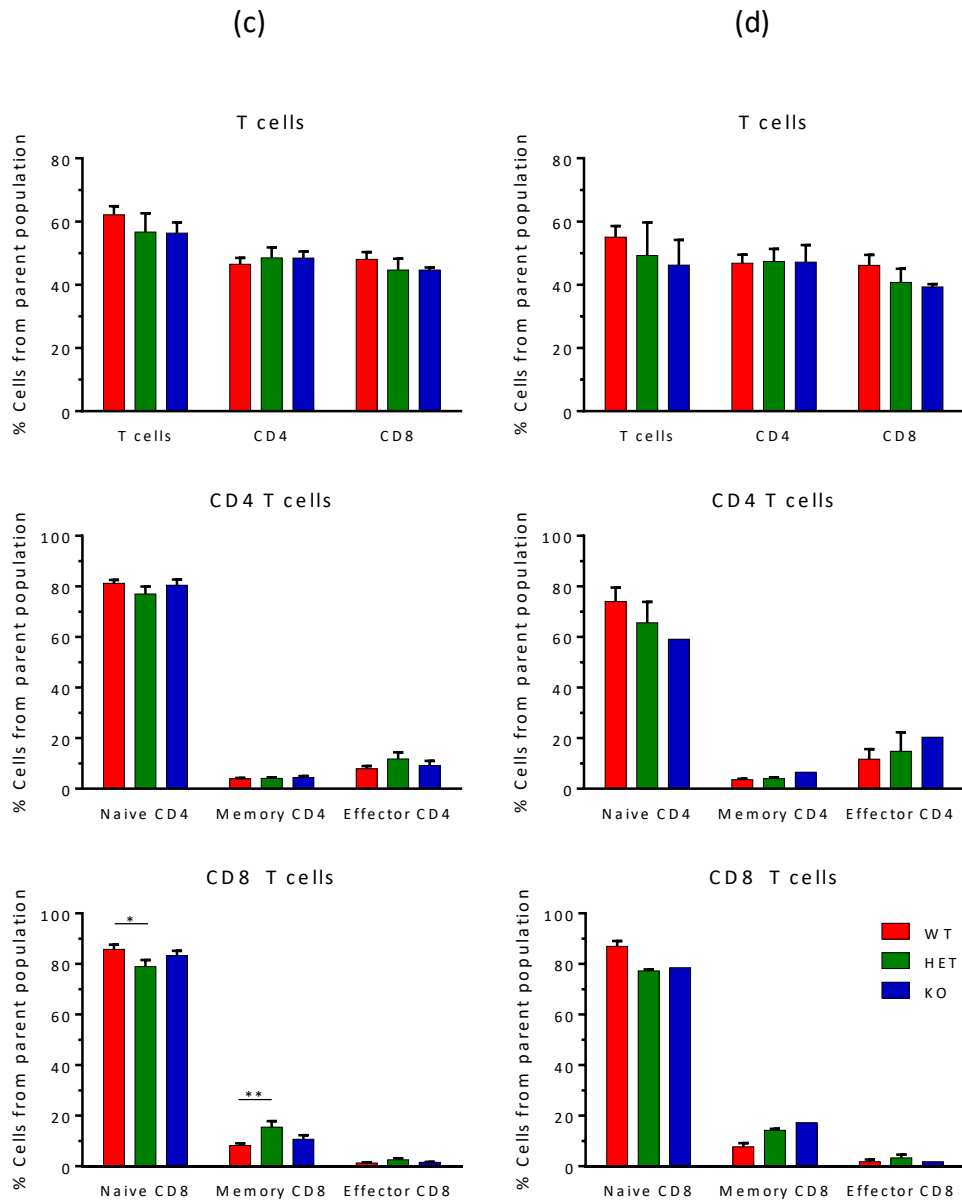
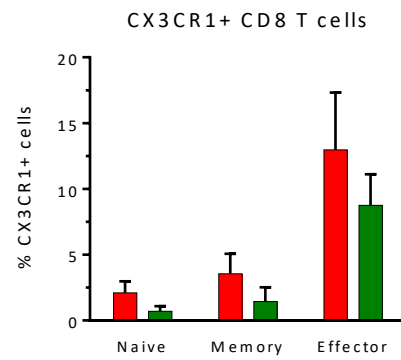
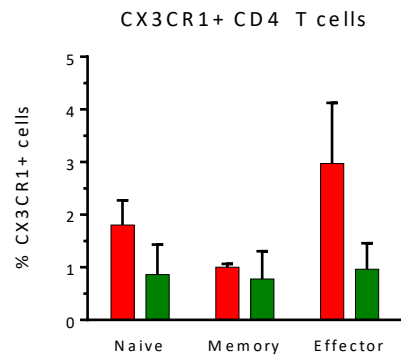
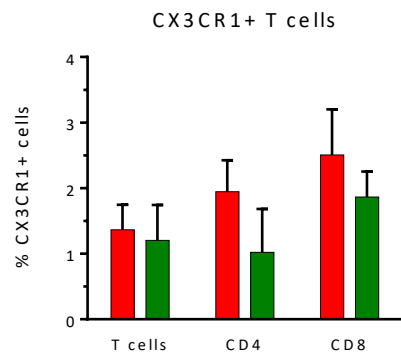
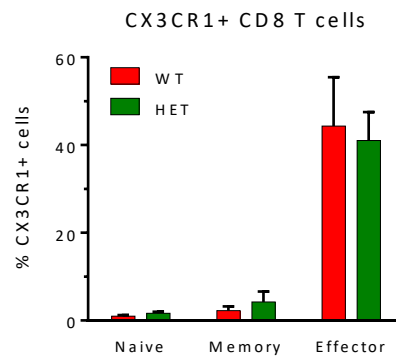
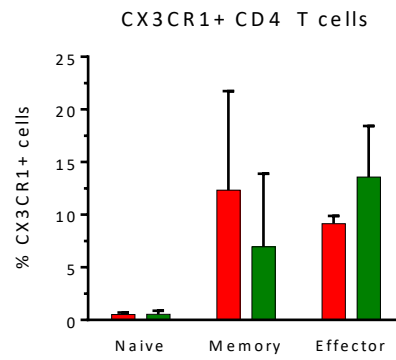
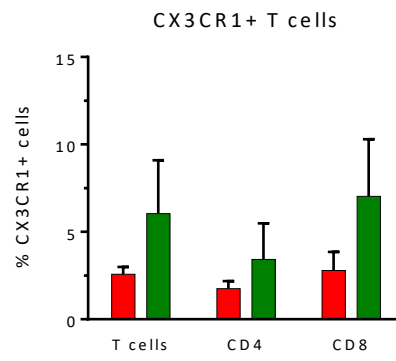


Figure 3.3 T cell phenotyping of CX3CR1-EGFP WT, HET and KO mice. Results from cells isolated from (a) spleen (n=3-4), (b) whole blood (n=3), (c) peripheral lymph nodes (n=4), (d) cardiac lymph nodes (1-2). Peripheral lymph nodes – popliteal and subiliac; cardiac lymph nodes – cranial mediastinal. Error bars = SEM; Two-way ANOVA with Tukey's multiple comparisons test. * $p \leq 0.05$; ** $p \leq 0.01$.

(a)



(b)



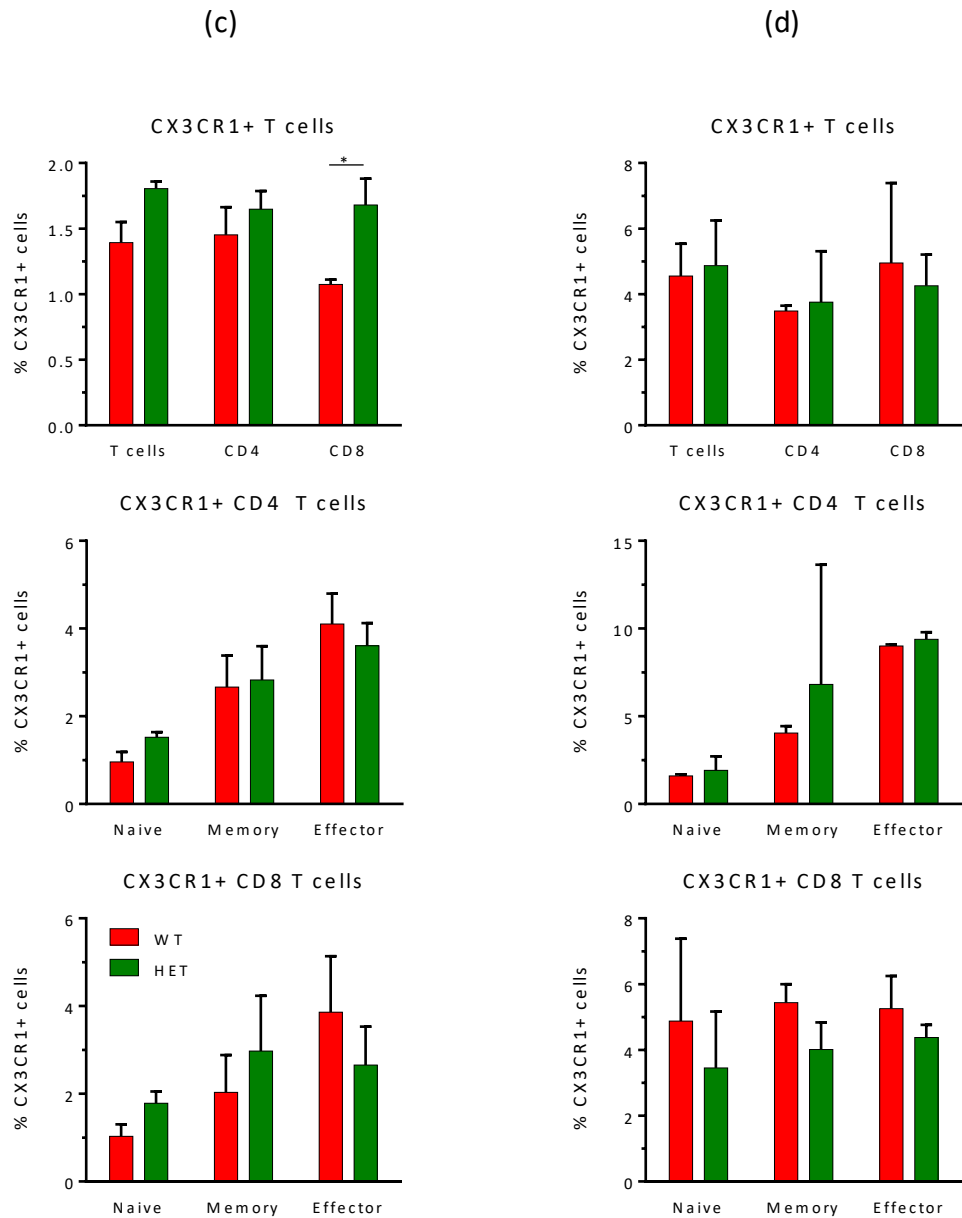
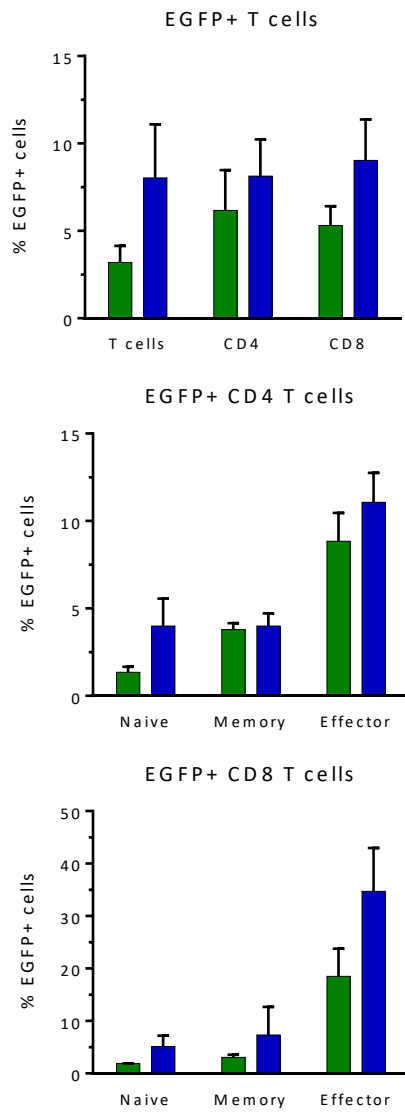
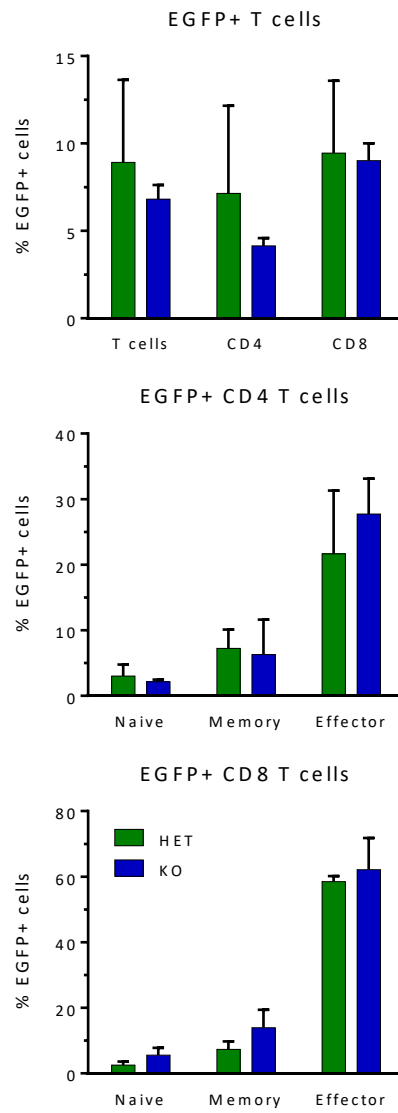


Figure 3.4 CX3CR1 expression in T cell populations of WT and HET mice. Results from cells isolated from (a) spleen (n=3-4), (b) whole blood (n=3), (c) peripheral lymph nodes (n=4), (d) cardiac lymph nodes (1-2). Peripheral lymph nodes – popliteal and subiliac; cardiac lymph nodes – cranial mediastinal. Error bars = SEM; Two-way ANOVA with Sidak's multiple comparisons test. * $p \leq 0.05$.

(a)



(b)



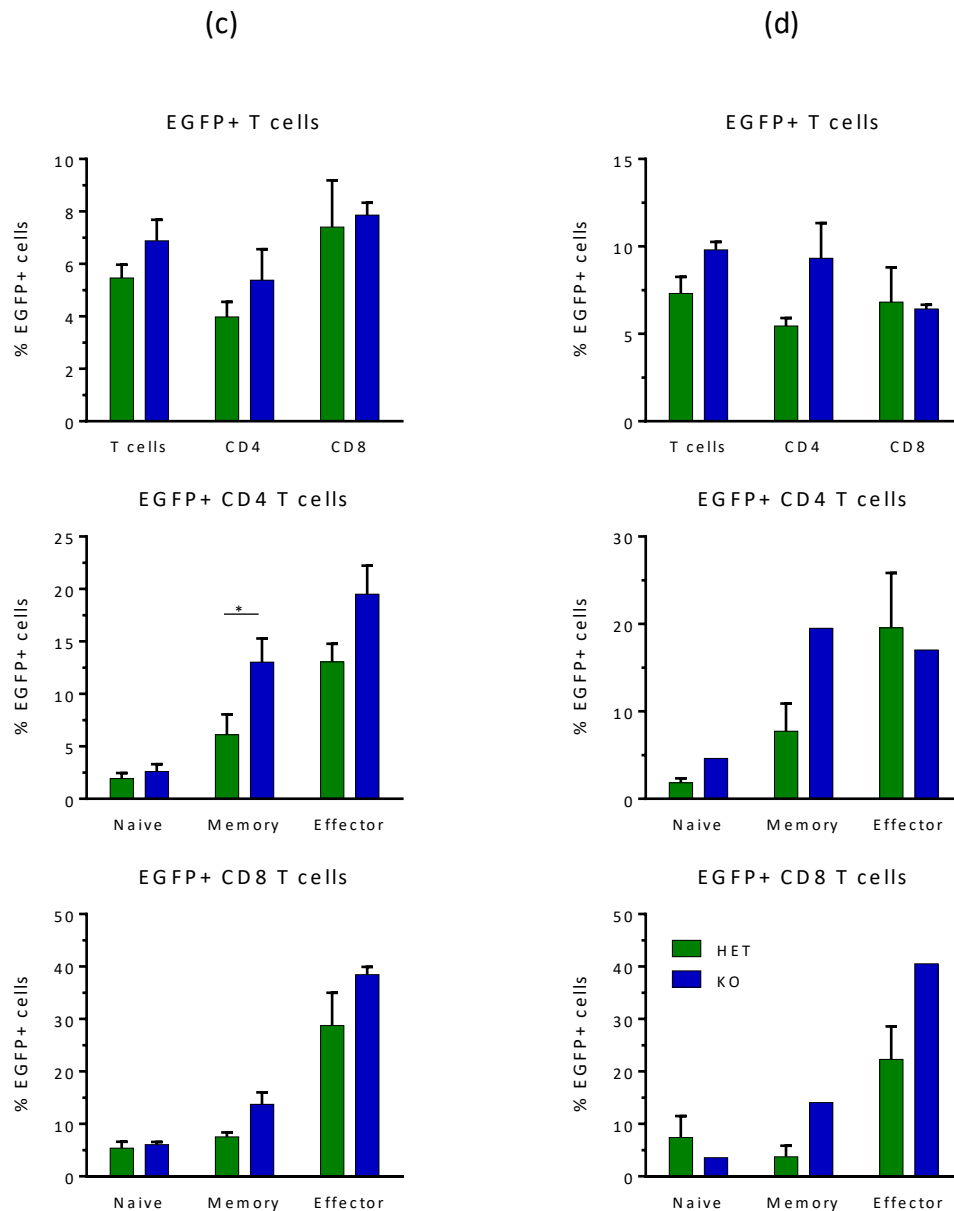


Figure 3.5 EGFP expression in T cell populations of HET and KO mice. Results from cells isolated from (a) spleen (n=3-4), (b) whole blood (n=3), (c) peripheral lymph nodes (n=4), (d) cardiac lymph nodes (1-2). Peripheral lymph nodes – popliteal and subiliac; cardiac lymph nodes – cranial mediastinal. Error bars = SEM; Two-way ANOVA with Sidak's multiple comparisons test. * $p \leq 0.05$.

3.2.2 Analysis of myocardial inflammation following I/R

Having established the baseline immunophenotype of the CX3CR1-EGFP mouse line, this study progressed to assessing the myocardial inflammatory response following I/R. This was done using three different complementary techniques as described below.

3.2.2.1 Epifluorescence microscopy

Immunofluorescence (IF) staining for CD45 was used to study the total leukocyte infiltration into the infarcted myocardium. Firstly, the area of immune cell infiltration in the left ventricle was measured for mice following 24h I/R (Figure 3.6a). This provided an indication of infarct size, as measured by the loss of autofluorescence in the red-568 channel (Lagarto, Dyer et al. 2015). As expected from an LAD ligation MI model, no significant CD45⁺ infiltrate was detected in the remote (right ventricle) area. As for the left ventricle, the results on Figure 3.6b show that the area of immune cell infiltration in heterozygous mice is 35% higher ($p \leq 0.05$) than the area in WT control mice. While KO mice also showed a small increase in the area of infiltration compared to WT animals, this was not shown to be significant.

(a)



(b)

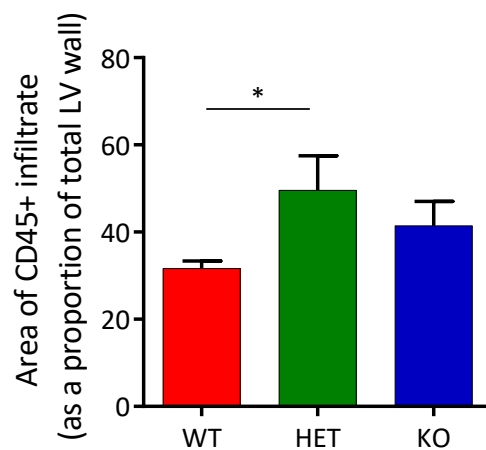


Figure 3.6 Area of leukocyte infiltrate in the injured myocardium following 24 hours reperfusion. (a) Representative image of immune cells (green) infiltrating the heart tissue over the infarcted area, indicated by the loss of autofluorescence (red) that is typical of viable myocardial tissue. Scale bar = 500 μ m; x5 magnification. (b) Area of CD45⁺ cell infiltration of the left ventricles in WT, HET and KO mice. n=4-8/group; One-Way ANOVA with Tukey's multiple comparisons test; * $p \leq 0.05$. Dr Rachael Redgrave has done the tissue processing and imaging of some WT 24h I/R hearts.

Following this, analysis of the density of leukocyte infiltration in the infarcted (left ventricle) area was performed. Five different mouse groups were analysed for each genotype, showing a consistent pattern of leukocyte accumulation (Figure 3.7). Both 2h and 24h sham hearts had very few numbers of CD45⁺ cells in their left ventricles, similar to naïve (no surgery) hearts. While hearts at 2h post reperfusion from WT, HET and KO mice showed a small increase in CD45⁺ cell density compared to naïve or sham hearts, this was not significant for any of the genotypes. However, all mice showed a highly significant infiltration of immune cells in the infarcted region following 24h I/R ($p \leq 0.0001$). Lastly, the differences of CD45⁺ cell density between the three genotypes was investigated (Figure 3.8). As shown on Figure 3.8b, no significant differences were detected in the naïve heart group between WT, HET and KO mice. However, following 2h I/R HET and KO mice showed a significant reduction of around 30% in leukocyte infiltration (Figure 3.8c). This appears to be a delay in cell recruitment, as CD45 infiltrate in both HET and KO mice has returned to WT levels by 24h I/R (Figure 3.8c).

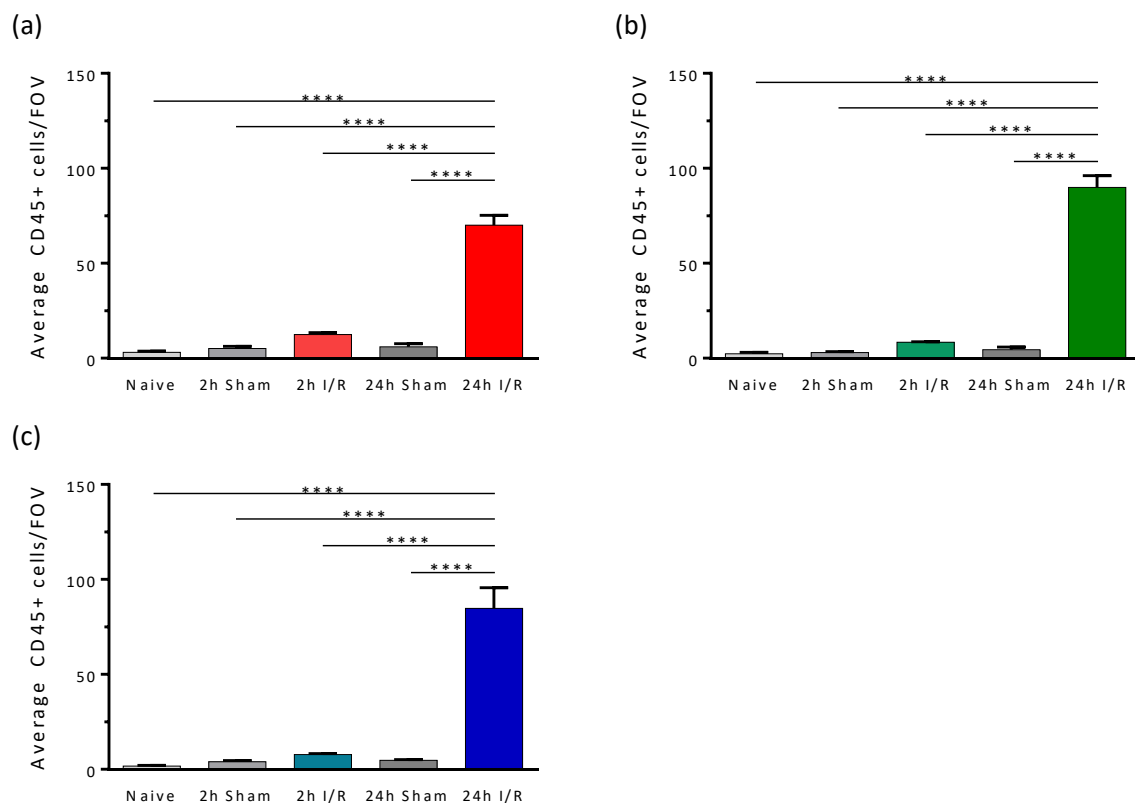
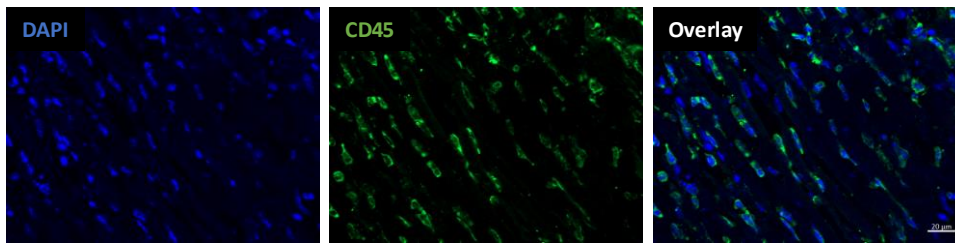
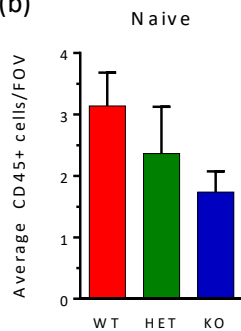


Figure 3.7 Leukocytes accumulate in the infarcted area after 24h reperfusion. Average number of immune cells infiltrating the left ventricles of (a) WT, (b) HET and (c) KO mice. Error bars = SEM; n=3-8/group; One-way ANOVA with Tukey's multiple comparisons test. **** $p \leq 0.0001$. Sarah Marsh has done the imaging and analysis of the 2h Sham and 2h I/R groups. Dr Rachael Redgrave has done the tissue processing and imaging of part of the WT 24h I/R hearts.

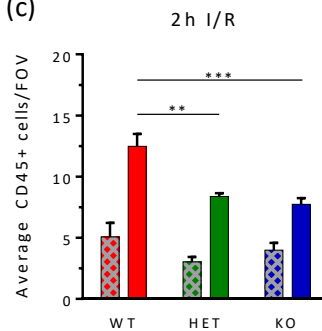
(a)



(b)



(c)



(d)

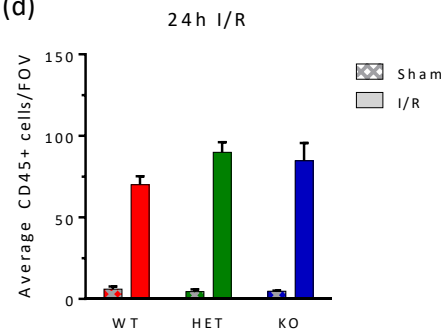


Figure 3.8 Loss of CX3CR1 expression leads to delayed leukocyte recruitment to the MI heart. (a) Representative image of CD45 IF staining. Scale bar = 20µm. Density of leukocyte recruitment in (a) naïve, (b) 2h and (c) 24h post surgery hearts. Error bars = SEM; n = 3-7/group. One-way (Naïve) and Two-Way (2h and 24h) ANOVA with Tukey's multiple comparisons test. ** $p \leq 0.01$; *** $p \leq 0.001$. Sarah Marsh has done the imaging and analysis of the 2h Sham and 2h I/R groups. Dr Rachael Redgrave has done the tissue processing and imaging of part of the WT 24h I/R hearts.

The same mouse hearts were also analysed for expression of EGFP by staining sister sections 100µm from the ones used for CD45 staining. Figure 3.9 shows the average number of EGFP⁺ cells infiltrating the injured myocardium of HET and KO mice. These data are not specific to leukocytes but show the total EGFP⁺. Similar to the infiltration of CD45 expressing cells, HET mice showed a highly significant ~75% increase in the number of infiltrating EGFP⁺ cells at 24h I/R (Figure 3.9a). In contrast, KO mice did not show such increase and instead the number of EGFP expressing cells remained at the level of naïve hearts (Figure 3.9b). Figure 3.10 shows the comparison of EGFP⁺ cell infiltration between HET and KO mice. Interestingly, KO mice have significantly more EGFP⁺ cells in the left ventricles of naïve hearts, as well as 2h post MI ($p \leq 0.05$). On the other hand, after 24h I/R HET mice had a 2-fold increase in EGFP⁺ infiltrate compared to KO animals ($p \leq 0.01$).

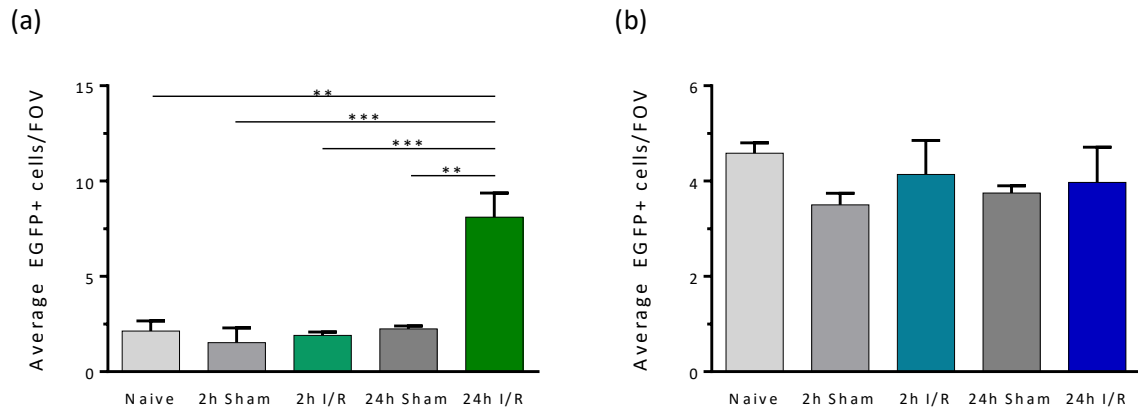


Figure 3.9 EGFP⁺ cell infiltration in the infarct area following myocardial reperfusion. Average number of EGFP⁺ cells accumulating in the left ventricles of (a) HET and (b) KO CX3CR1-EGFP mice. Error bars = SEM; n=3-6/group; One-way ANOVA with Tukey's multiple comparisons test. ** p ≤ 0.01; *** p ≤ 0.001.

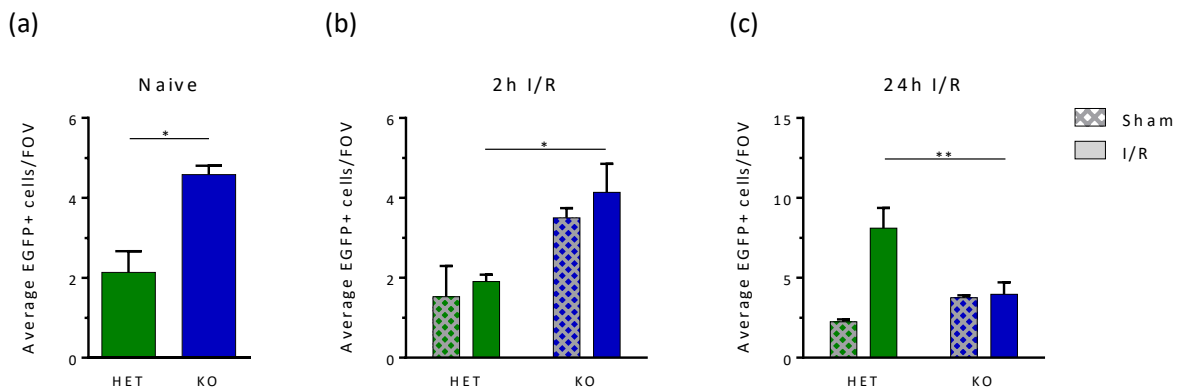


Figure 3.10 Loss of CX3CR1 expression leads to reduced EGFP⁺ cell recruitment to the left ventricles of MI heart. Density of total EGFP⁺ cell in (a) naïve, (b) 2h and (c) 24h post surgery hearts. Error bars = SEM; n = 3-6/group. Unpaired two-tailed t-test (Naive) or two-Way ANOVA with Sidak's multiple comparisons test (2h and 24h). * p ≤ 0.05 ; ** p ≤ 0.01.

3.2.2.2 Cardiac digest

IF studies of inflammatory infiltrate 24h post reperfusion were complemented by flow cytometry analysis of total ventricle cells isolated by cardiac digest. This is a technically challenging method as the enzymatic digestion causes the release of considerable amount of cellular debris, loss of some surface epitopes and cell death. Ultimately, this methodology is a balance between cell yield and cell death. No sham surgery controls were used for this study but instead cells from the right ventricle were used as control. IF analysis of leukocyte infiltrate in the sham left ventricle and I/R right ventricle shows that there is no significant difference between the two groups (Figure 3.11). As seen on the graph, both tissue groups are equally good controls for the large CD45⁺ cell accumulation in the 24h I/R left ventricle.

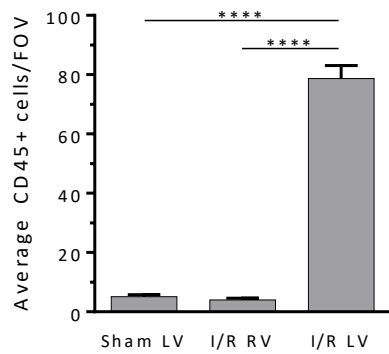
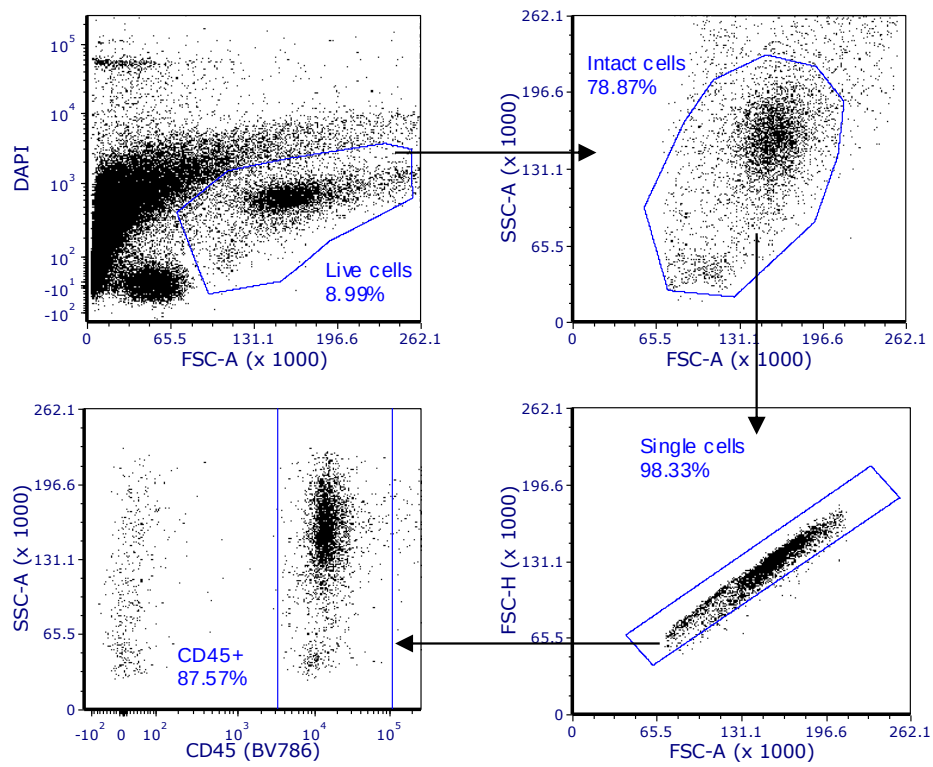


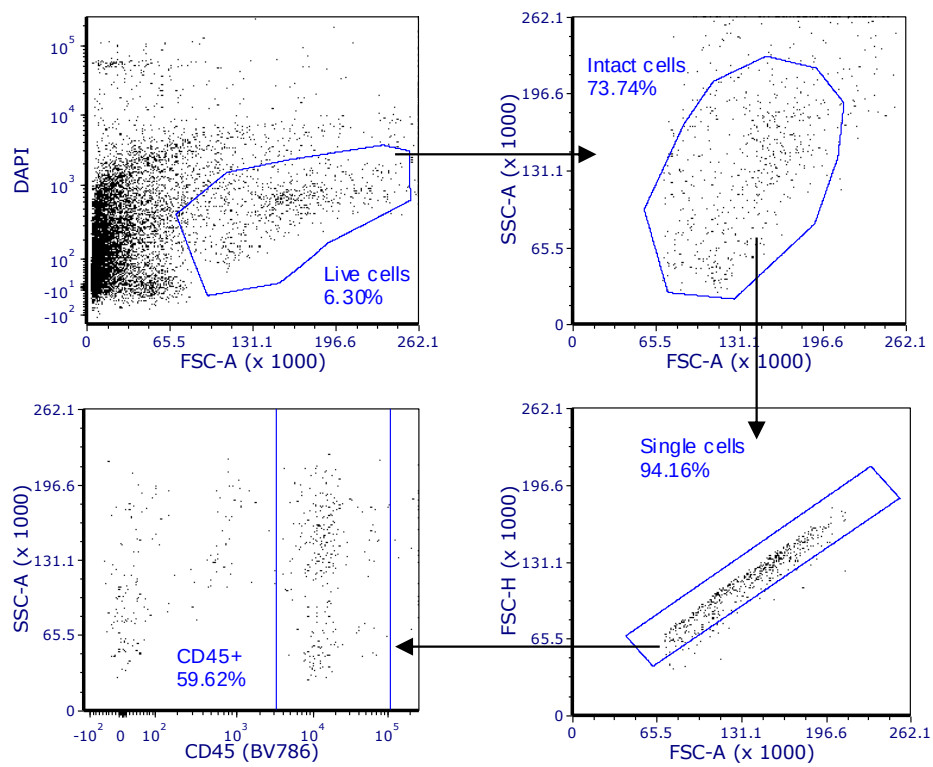
Figure 3.11 IF data of CD45 infiltration in the left ventricle of sham and right ventricle of I/R mice are both equally controls for I/R left ventricle. Pooled data for all CX3CR1-EGFP genotypes. Error bars = SEM; n=9-16/group. One-way ANOVA with Tukey's multiple comparisons test. **** $p \leq 0.0001$.

The flow cytometry gating strategy on Figure 3.12a and b shows that the right ventricle has fewer events which is due to the smaller amount of right ventricle tissue compared to the left ventricle. In addition to analysing CD45⁺ cell infiltration, the levels of CX3CR1 and EGFP were also assessed (Figure 3.12c and d). The results on Figure 3.13a show that mice from all genotypes appear to have higher percentage of leukocytes per mg tissue in their left ventricles compared to right, although this was not significant. However, it must be noted that these data do not show absolute number of CD45⁺ cells or even percentage of CD45⁺ cells from the total isolated cells. Instead the graph shows CD45⁺ cells as proportion of the total events collected. This is due to the fact that a cardiac digest sample contains a heterogenous population of cells, with different cell size and granularity (measured by the forward and side scatter (FCS and SSC respectively)). Therefore, it is not possible to gate on all Live Cells (the first gate on Figures 3.12a and b) and as the focus of the analysis is on leukocytes, the FSC and SSC voltage settings and gates are biased towards leukocytes. As a result, the lack of significant increase in leukocyte infiltration into the infarct area of HET and KO mice compared to the remote area, may reflect changes in other cell populations and/or the amount of debris in those samples (which are also included in All Events). This may also be the cause behind the significantly reduced CD45⁺ infiltrate in the infarct area of HET mice compared to WT controls (Figure 3.13a) that contrasts with the IF data on Figure 3.8d. In addition to this, the results on Figures 3.13b and c show no significant difference in the percentage of CX3CR1 and EGFP expressing leukocytes in the left ventricles of WT and HET (for CX3CR1) and HET and KO (for EGFP) mice. However, within each genotype, remote areas show a higher proportion of CX3CR1/EGFP expression which is significant for the CX3CR1⁺ and EGFP⁺ CD45⁺ HET, as well as the EGFP⁺ CD45⁺ KO groups. But this is again the result of fewer number of CD45⁺ events in the remote areas compared to the infarcted left ventricle.

(a)



(b)



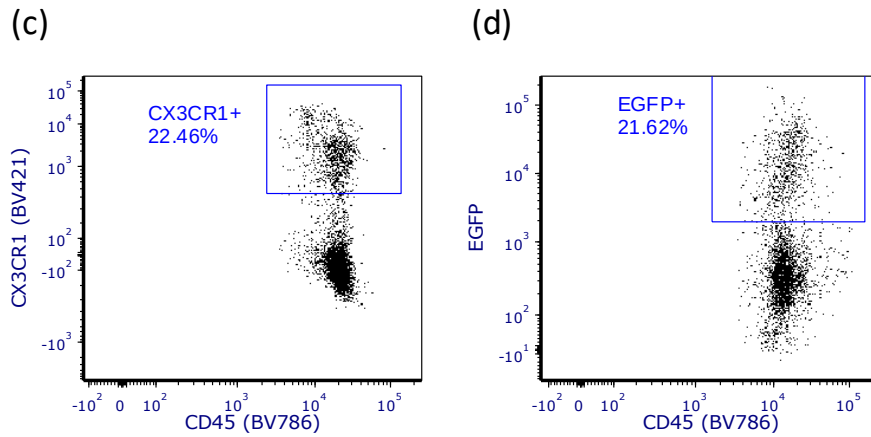


Figure 3.12 Flow cytometry gating strategy for digested cardiac tissue. Representative dot plots of (a) left ventricle and (b) right ventricle samples. Gating of (c) CX3CR1 and (d) EGFP expressing cells.

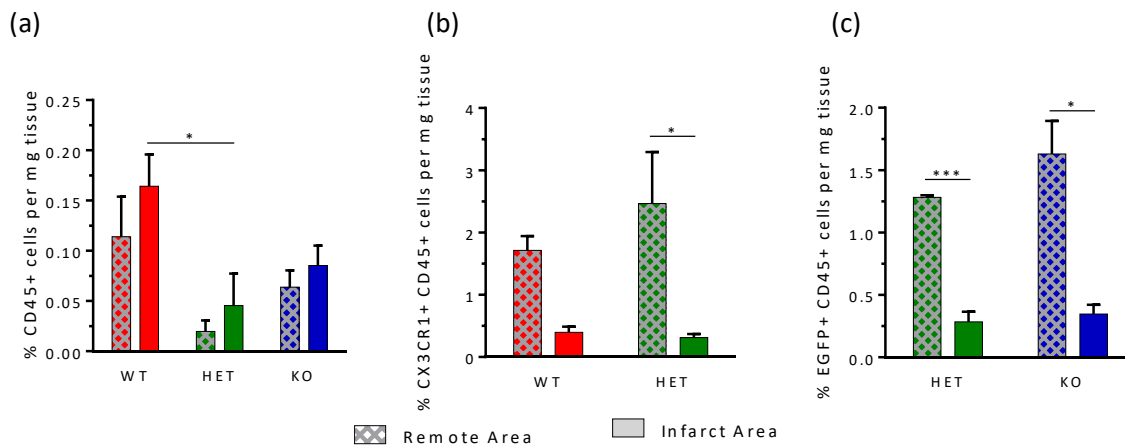


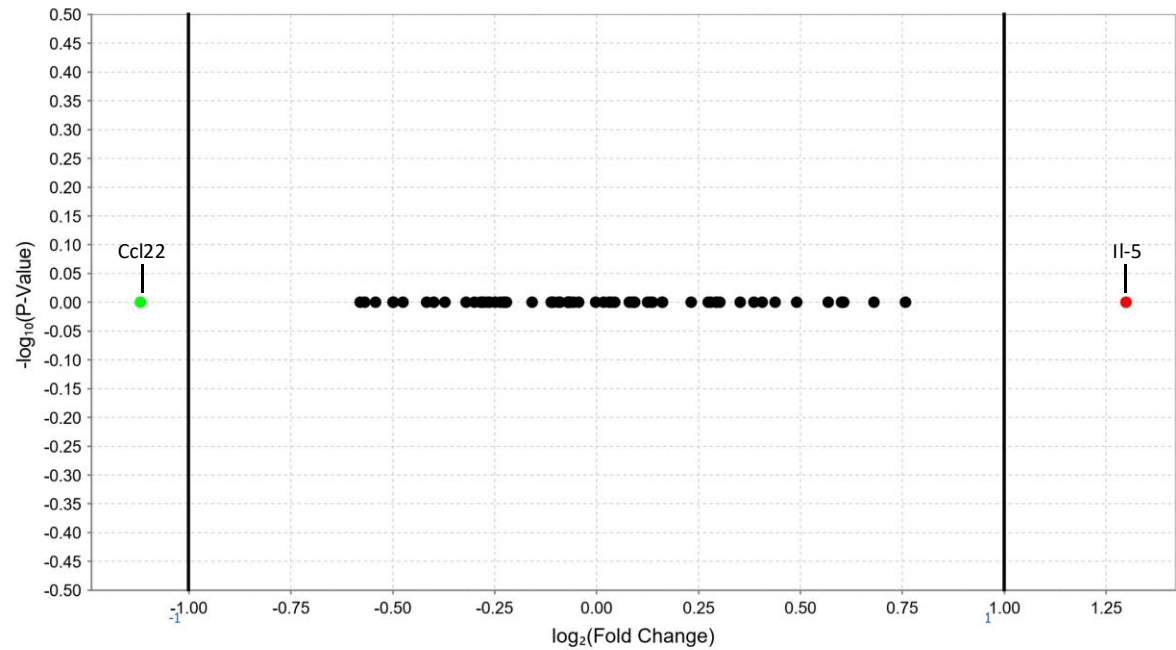
Figure 3.13 Flow cytometry analysis of digested cardiac tissue 24h post reperfusion in WT, HET and KO CX3CR1 mice. (a) Percentage leukocyte infiltration from total events per mg tissue. Two-way ANOVA with Tukey's (for comparing genotypes) or Sidak's (for comparing remote to infarct) multiple comparisons test. Proportion of leukocytes per mg tissue expressing (b) CX3CR1 and (c) EGFP. Two-way ANOVA with Sidak's multiple comparisons test. Infarct area – left ventricle; remote area – right ventricle. Error bars = SEM, n=3-6/group. * $p \leq 0.05$.

3.2.2.3 Gene Expression

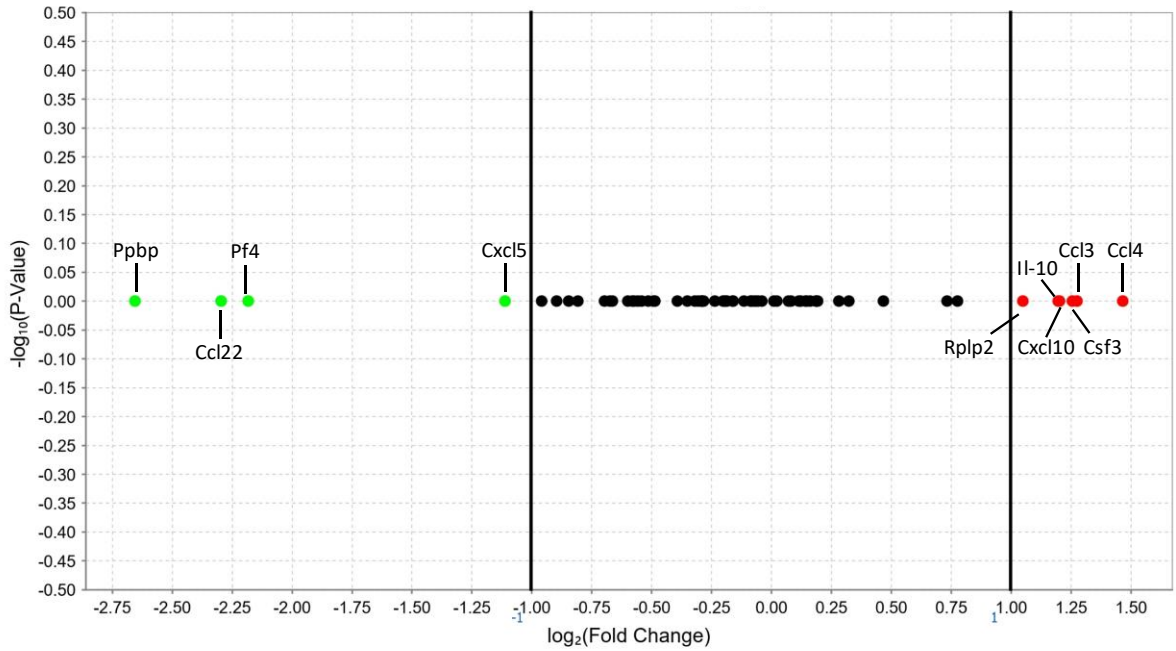
Finally, the inflammatory response to ischaemia/reperfusion injury following MI was studied using gene expression analysis. A qPCR microarray was used to assess the expression levels of 96 different genes (as described in section 2.7.5) in the left and right ventricles of WT and KO mice. Two I/R time points were investigated – 6 hours and 24 hours post reperfusion. Gene expression at 6h was studied instead of the 2h described above, because this has previously been reported as the peak chemokine expression time in a similar mouse I/R model (Dewald, Ren et al. 2004).

The volcano plots on Figure 3.14 show the results for 6h I/R mice. There is no significantly differential gene expression between the WT and KO mice in either the left or right ventricle (Figure 3.14a and b). Surprisingly, no significant difference is found between any of the genes when comparison is made between the left and right ventricle within a genotype. However, both the left ventricles in both WT and KO mice have a decrease of myostatin (Mstn) expression – 0.209-fold ($p=0.139$) and 0.176-fold ($p=0.526$) respectively (Figure 3.14c and d).

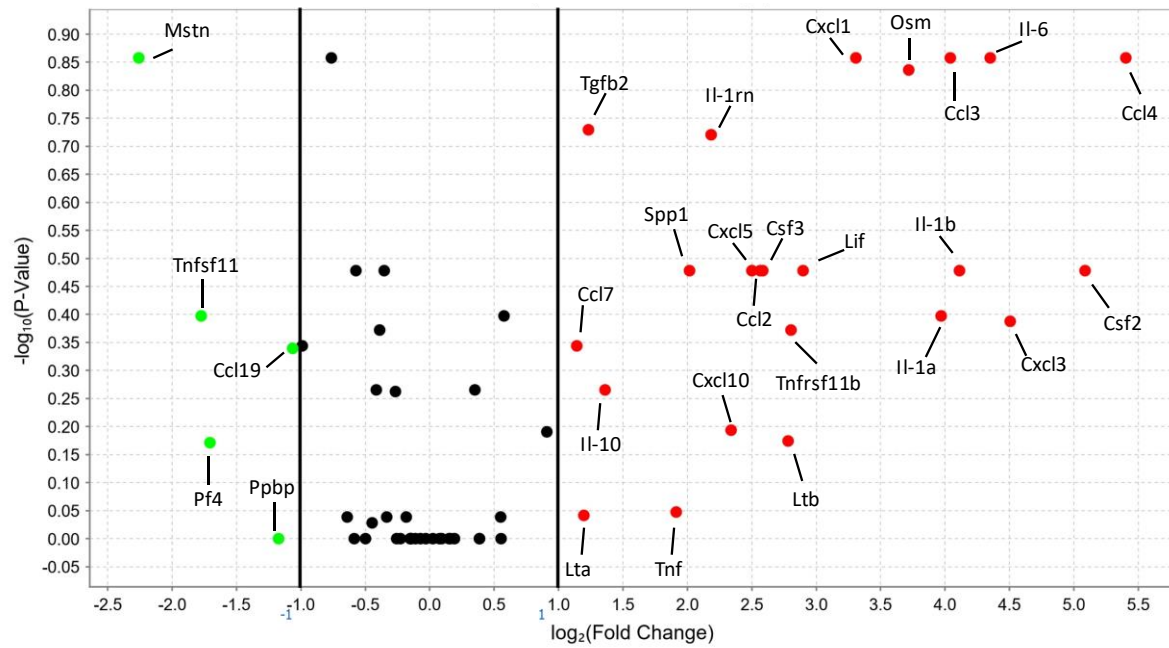
(a)



(b)



(c)



(d)

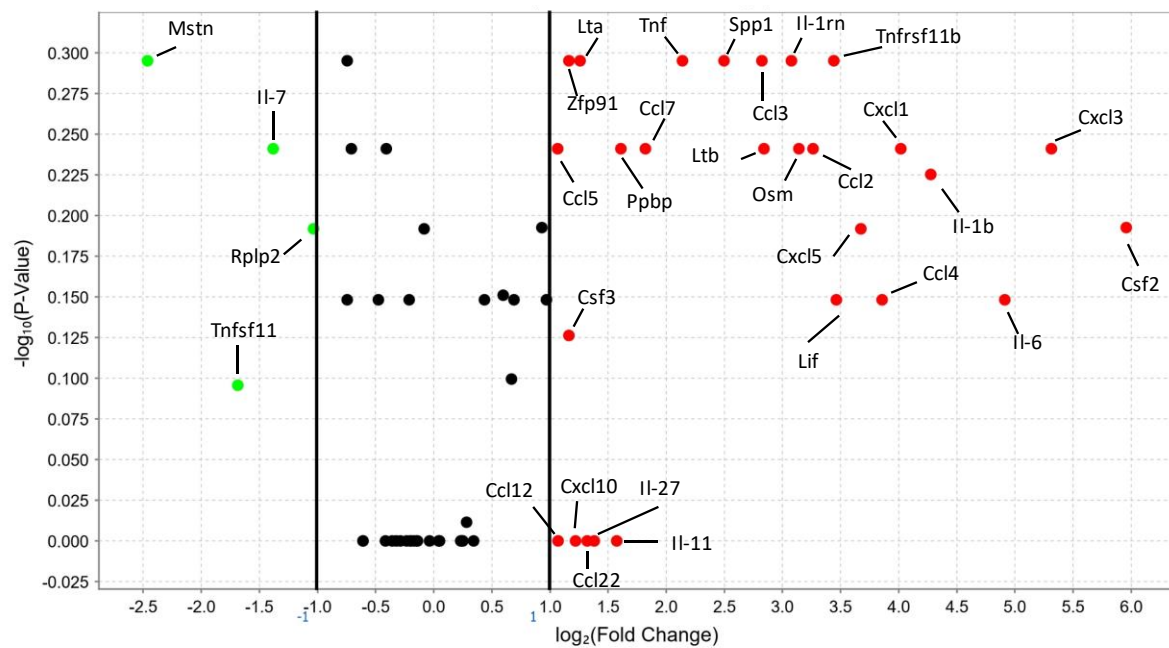
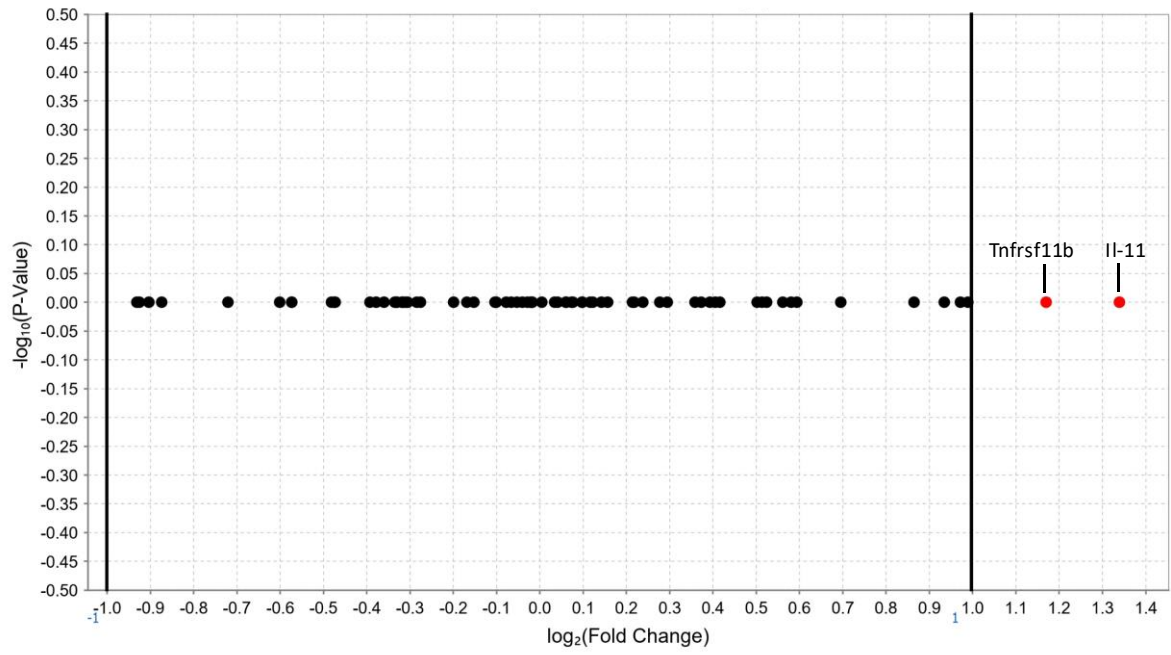


Figure 3.14 Cytokine gene expression 6h post reperfusion. Volcano plots showing differentially expressed genes in (a) left ventricles and (b) right ventricles of KO mice compared to WT, left ventricles of (c) WT and (d) KO mice compared to their right ventricles. Genes with over two-fold change in gene expression are labelled; $n = 3/\text{group}$. P values are adjusted using Benjamini-Hochberg false discovery rate.

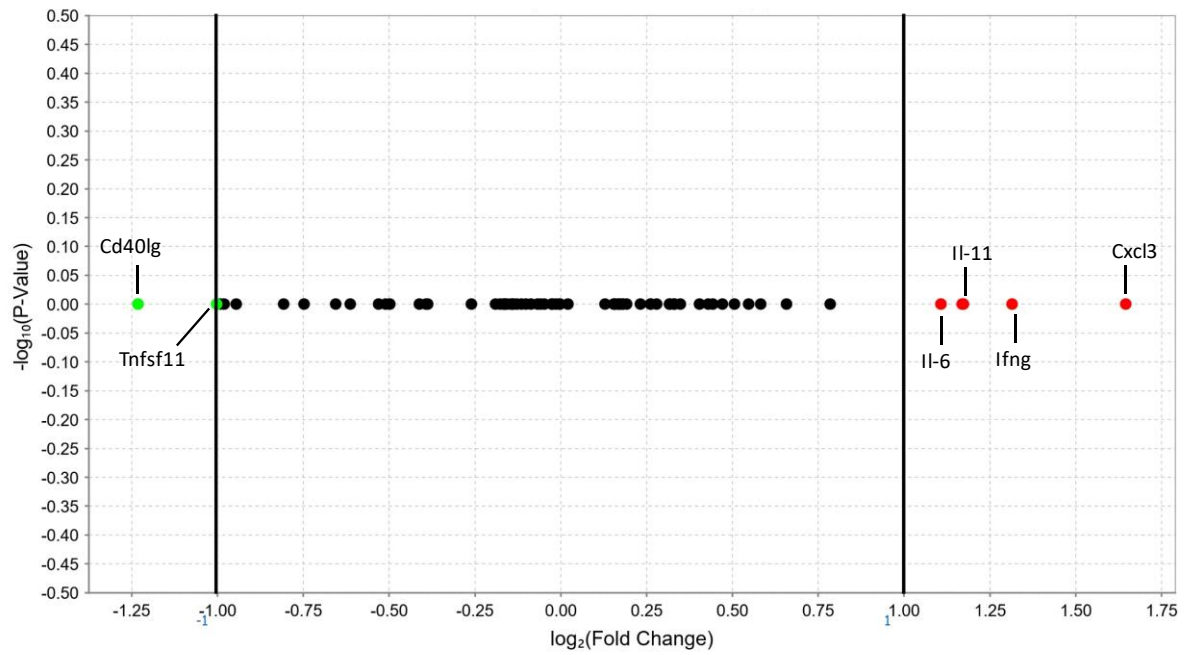
Similar to the 6h results, the data on Figure 3.15a and b shows no significant difference between WT and KO mice in either the left or right ventricle 24h after reperfusion. There is also no significant difference between the left and right ventricle in WT mice (Figure 3.15c). Although, some of the genes follow a similar pattern to the 6h group such as myostatin which has a 0.269-fold ($p=0.617$) change in the left ventricle. In contrast, four genes have significant differential expression between the left and right ventricles in KO mice (Figure 3.15d). A decrease of 0.202-fold ($p=0.007$) in myostatin (Mstn) and 0.295-fold ($p=0.007$) in Cardiotrophin 1 (Ctfr) is detected in the left ventricle of KO mice, as well as an increase of 2.747-fold ($p=0.007$) for CCL3 and 2.012-fold ($p=0.007$) for CXCL7 (Ppbb). In addition to this, Il-15 and Cxcl3 have fold change of 0.418 and 2.862 respectively that is borderline significant ($p=0.054$).

A comparison between the two time points was also performed (Figure 3.16). Although no significant changes are detected in the WT left and right ventricle (Figure 3.16a and b), expression of Osteopontin (Spp1) has over 20-fold increase in the 24h I/R hearts. Interestingly, the same gene is also increased in the 24h KO samples (Figure 3.16c and d), where there is a nearly 26-fold non-significant increase in the left ventricle ($p=0.129$) and a significant 36-fold increase in the right ventricle ($p=0.016$).

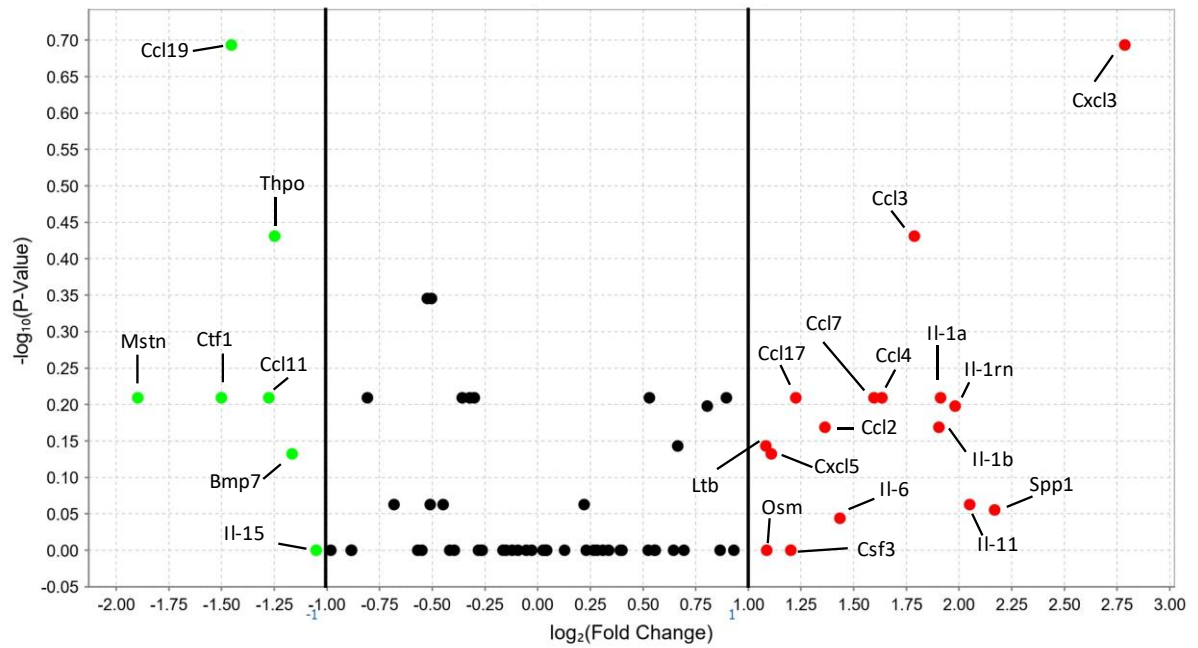
(a)



(b)



(c)



(d)

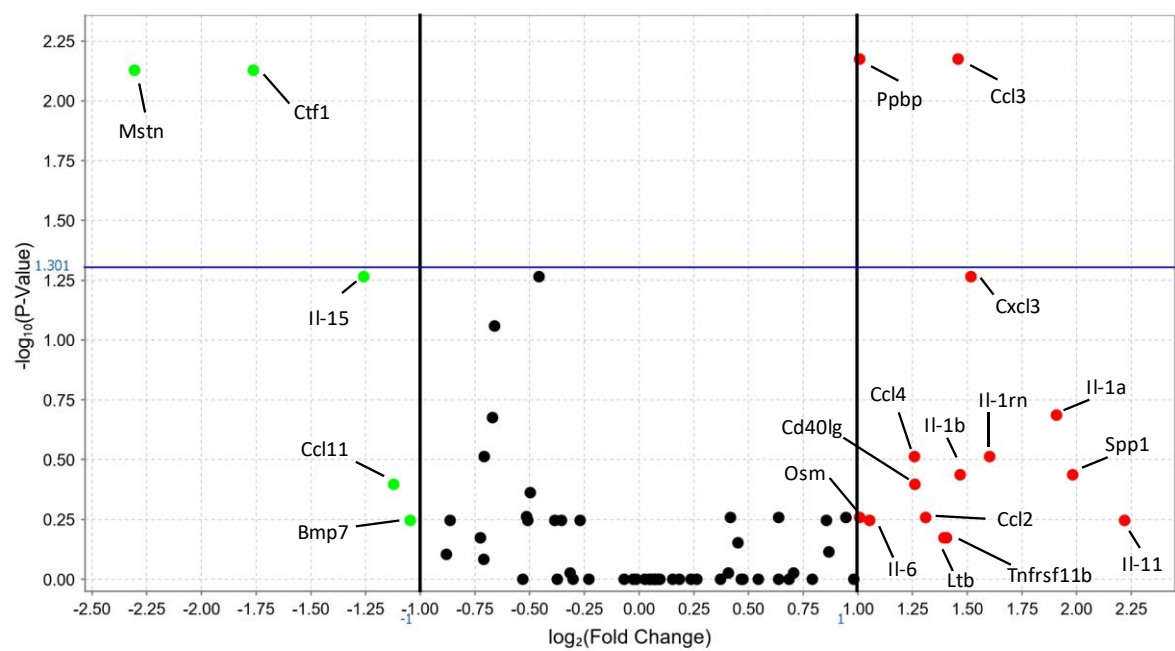
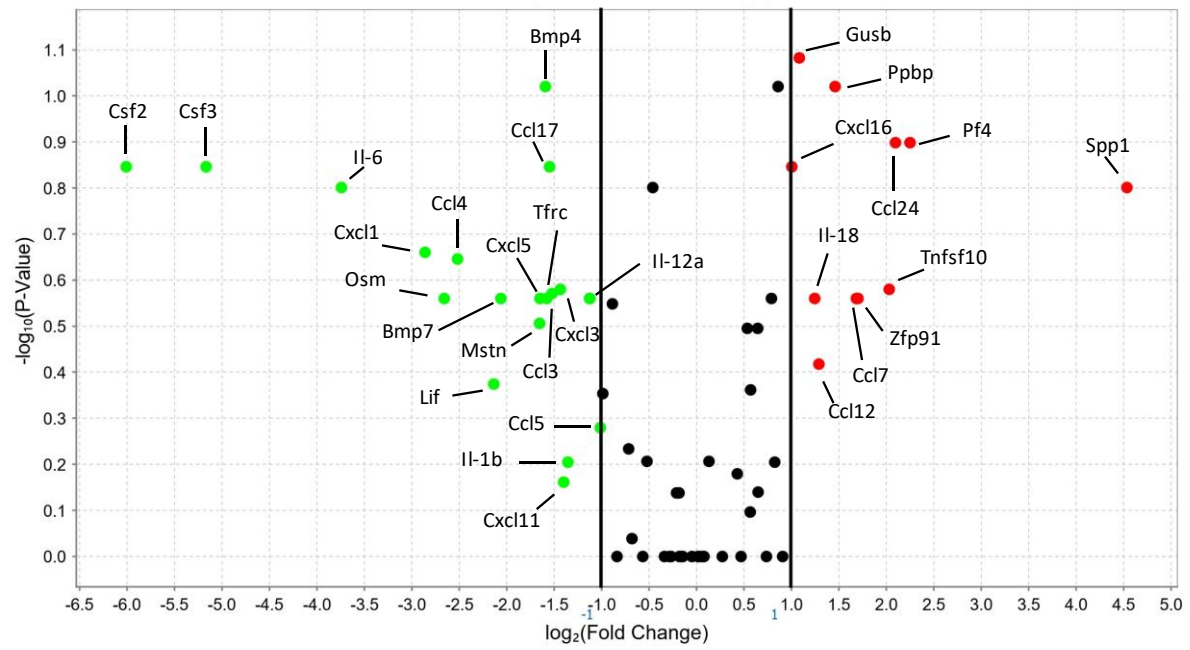
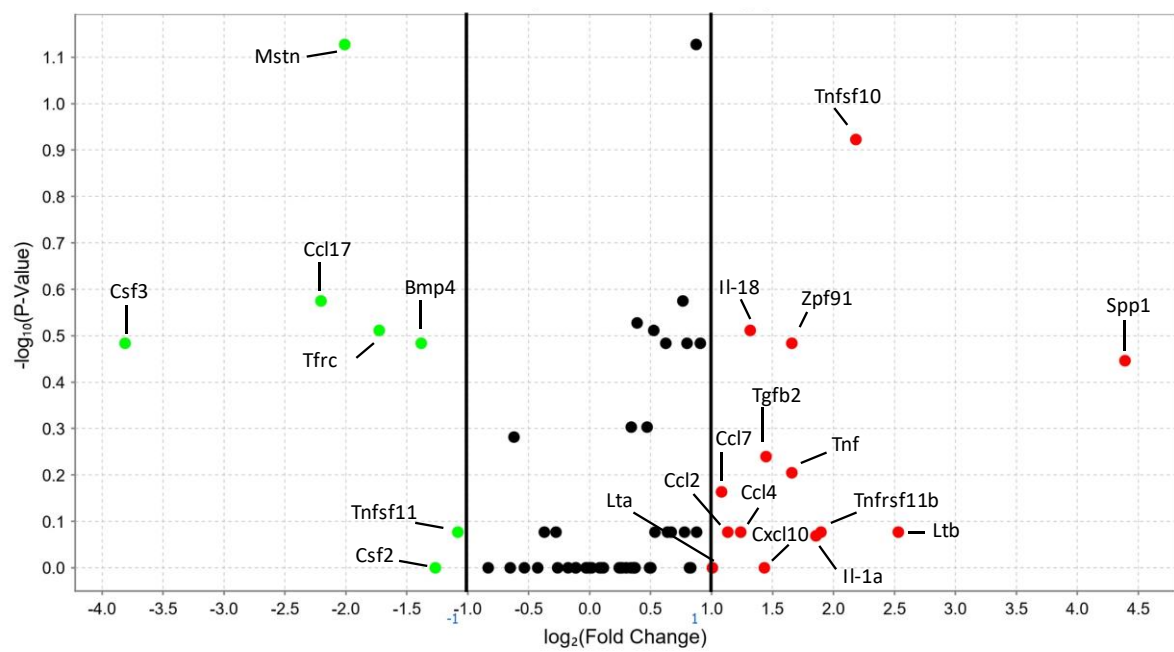


Figure 3.15 Cytokine gene expression 24h post reperfusion. Volcano plots showing differentially expressed genes in (a) left ventricles and (b) right ventricles of KO mice compared to WT, left ventricles of (c) WT and (d) KO mice compared to their right ventricles. Genes with over two-fold change in gene expression are labelled; blue line shows $-\log_{10}(0.05) = 1.301$, i.e. $p = 0.05$; $n = 3/\text{group}$. P values are adjusted using Benjamini-Hochberg false discovery rate.

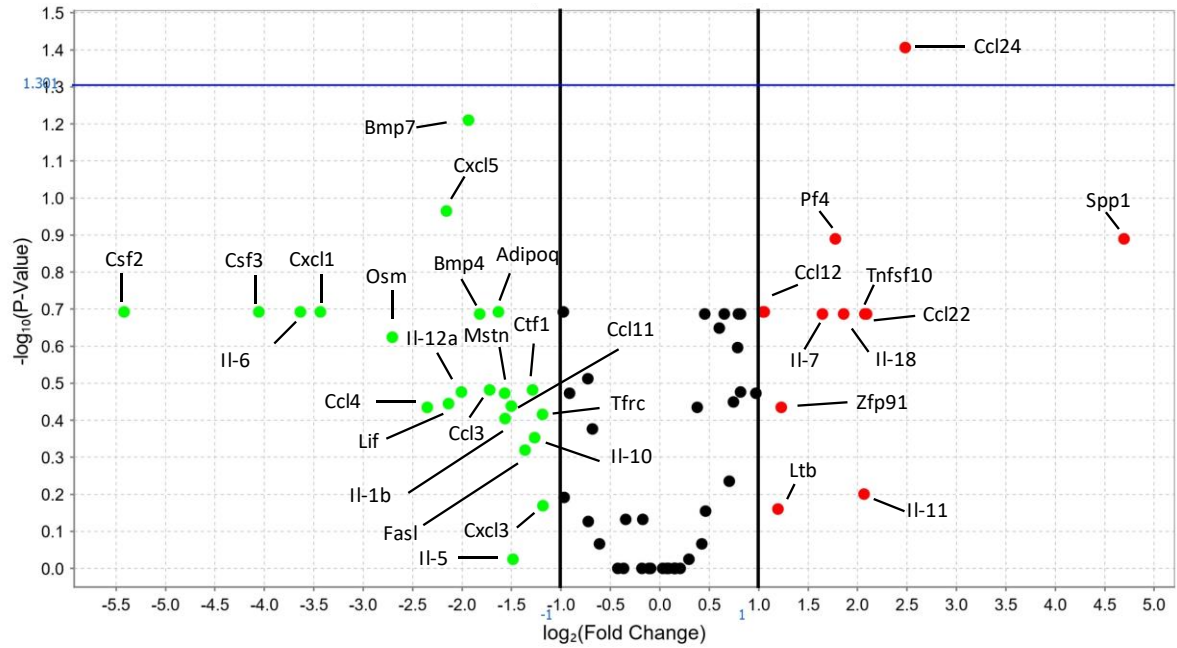
(a)



(b)



(c)



(d)

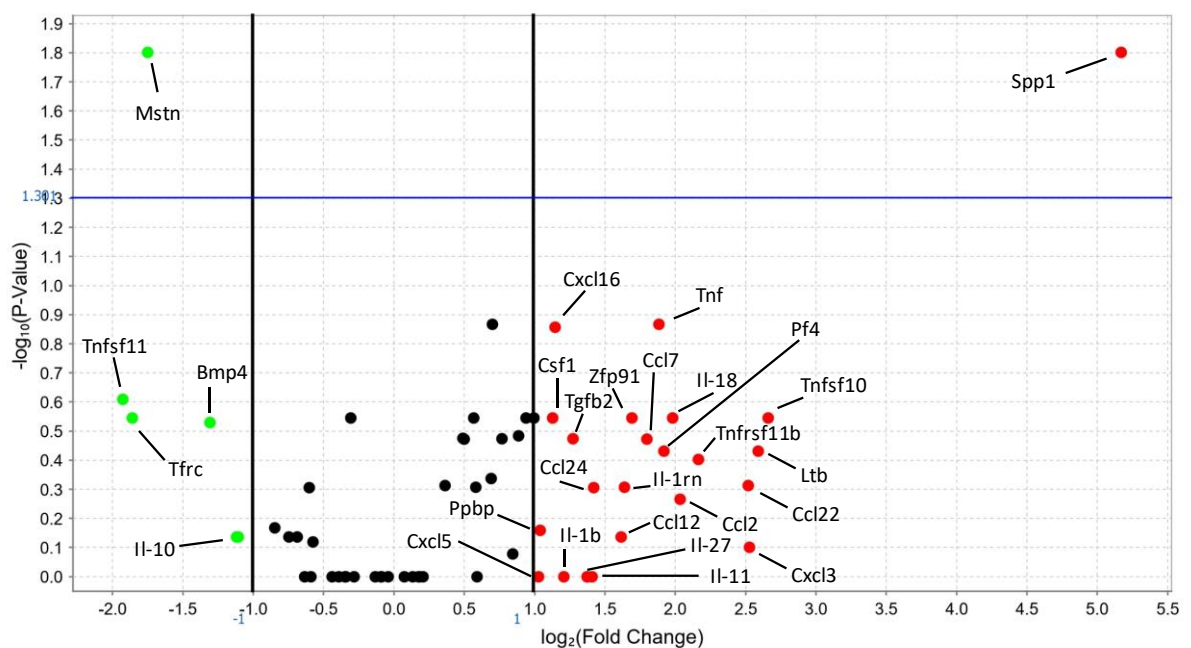


Figure 3.16 Analysis of change in cytokine gene expression between 6h and 24h I/R. Volcano plots showing differentially expressed genes in WT (a) left ventricles, (b) WT right ventricles, (c) KO left ventricles and (d) KO right ventricles at 6h compared to 24h. Genes with over two-fold change in gene expression are labelled; blue line shows $-\log_{10}(0.05) = 1.301$, i.e. $p = 0.05$; $n = 3/\text{group}$. P values are adjusted using Benjamini-Hochberg false discovery rate.

The lack of significant changes in gene expression in this pilot gene expression study is not surprising. While three hearts per group were tested, the amount of RNA that can be extracted

from a single ventricle and the number of genes per assay (96) did not allow for any technical replicates or repeated experiments per sample. Therefore, despite using RNA with very good quality ($RIN^e \geq 8.3$) and using custom pre-made assays which avoid TaqMan probe pipetting error, it is not unexpected that there will be increased variability in the data. Subsequently, it may be useful to study any patterns of changes in gene expression even if they are not statistically significant. The results on Table 3.1 are an attempt to do this. Genes that have more than two-fold change in expression and a $p < 1$ are shown as a “heatmap” across eight of the twelve comparison pairs. The four groups that are not included, looking at differences between WT and KO genotypes at 6h and 24h, are excluded because none of the differentially expressed genes have a fold change with $p < 1$.

As expected, there appears to be an overall increase in expression of inflammatory cytokines in the left ventricles of both WT and KO mice at 6h and 24h I/R. Expression levels of Ccl2 (MCP-1), Ccl3 (MIP-1), Ccl7 (MCP-3), Csf2 (GM-CSF), Csf3 (G-CSF), Cxcl1, Cxcl3, Cxcl5, Il-1, Il-6, Ita, Itb, Tnfrsf11b and Tnf appears to be increased. These cytokines are highly pro-inflammatory and chemotactic primarily to neutrophils and monocytes. This is in accordance with what is known about the acute inflammatory phase of post MI I/R injury where the predominant cell types involved are granulocytes and inflammatory monocytes. Interestingly, a number of genes, encoding cardioprotective factors, appear to show upregulated expression (although not significant) in the left ventricles of both WT and KO mice 6h post reperfusion (Lif and Osm), at 24h I/R (Il-11). TGF β ligands Bmp4 and Bmp7 appear to show increasing expression with progressive I/R injury. Mstn and Spp1, involved in muscle growth and cell matrix interactions respectively, show contrasting expression patterns across all samples with downregulation of Mstn and upregulation of Spp1 expression (although not significant for either Mstn or Spp1). Differential expression patterns between WT and KO CX3CR1 mice is less clear than between the two different time points (6h and 24h). However, KO mice appear to have increased expression of neutrophil chemotactic Pbp. Interestingly, a number of genes involved in T cell function appear to have different expression patterns between the two genotypes. Ccl19 expression appears to be downregulated in the left ventricles of WT mice at 6h and 24h post reperfusion. In contrast, Cxcl10 and Ccl17 have elevated expression (although not significant) in these mice at 6h and 24h post I/R respectively. While CX3CR1 KO mice appear to have reduced expression of CD40lg and Il-15 in their left ventricles 24h post reperfusion, as well as a non-significant increase in Ccl5 levels at 6h I/R.

Gene name	6h		24h		24h vs 6h			
	WT LV vs RV	KO LV vs RV	WT LV vs RV	KO LV vs RV	WT LV	WT RV	KO LV	KO RV
Adipoq							0.323 (p=0.203)	
Bmp4					0.331 (p=0.095)	0.383 (p=0.328)	0.283 (p=0.205)	0.404 (p=0.296)
Bmp7			0.446 (p=0.737)	0.484 (p=0.566)	0.239 (p=0.275)		0.261 (p=0.062)	
Ccl11			0.413 (p=0.617)	0.459 (p=0.401)			0.353 (p=0.365)	
Ccl12					2.441 (p=0.382)		2.077 (p=0.203)	3.061 (p=0.731)
Ccl17			2.339 (p=0.617)		0.341 (p=0.143)	0.217 (p=0.266)		
Ccl19	0.479 (p=0.458)		0.365 (p=0.203)					
Ccl2	5.926 (p=0.333)	9.316 (p=0.526)	2.575 (p=0.678)	2.479 (p=0.552)		2.194 (p=0.837)		4.094 (p=0.541)
Ccl22							4.261 (p=0.205)	5.733 (p=0.486)
Ccl24					4.274 (p=0.126)		5.582 (p=0.039)	2.678 (p=0.494)
Ccl3	16.447 (p=0.139)	6.859 (p=0.526)	3.454 (p=0.371)	2.747 (p=0.007)	0.334 (p=0.275)		0.304 (p=0.33)	
Ccl4	42.327 (p=0.139)	14.066 (p=0.732)	3.102 (p=0.617)	2.393 (p=0.307)	0.175 (p=0.226)	2.362 (p=0.837)	0.196 (p=0.367)	
Ccl5		2.011 (p=0.656)			0.495 (p=0.526)			
Ccl7	2.204 (p=0.453)	3.426 (p=0.526)	3.022 (p=0.617)		3.213 (p=0.275)	2.119 (p=0.686)		3.48 (p=0.337)
Cd40lg				2.395 (p=0.401)				
Csf1								2.184 (p=0.285)
Csf2	33.970 (p=0.333)	59.545 (p=0.619)			0.016 (p=0.143)		0.023 (p=0.203)	
Csf3	6.006 (p=0.333)	2.218 (p=0.771)			0.028 (p=0.143)	0.071 (p=0.328)	0.06 (p=0.203)	
Ctf1			0.353 (p=0.617)	0.295 (p=0.007)			0.409 (p=0.33)	
Cxcl1	9.9 (p=0.139)	15.715 (p=0.545)			0.138 (p=0.218)		0.093 (p=0.203)	
Cxcl10	5.064 (p=0.641)							
Cxcl11					0.379 (p=0.690)			
Cxcl16					2.003 (p=0.143)			2.211 (p=0.139)
Cxcl3	22.706 (p=0.410)	38.577 (p=0.526)	6.901 (p=0.203)	2.862 (p=0.054)	0.37 (p=0.263)		0.441 (p=0.678)	5.77 (p=0.794)
Cxcl5	5.662 (p=0.333)	12.382 (p=0.639)	2.156 (p=0.737)		0.318 (p=0.275)		0.224 (p=0.109)	
Gusb					2.117 (p=0.083)			
Fasl							0.389 (p=0.48)	

Il-10	2.571 (p=0.543)						0.415 (p=0.444)	0.464 (p=0.731)
Il-11			4.146 (p=0.866)	4.658 (p=0.566)			4.189 (p=0.631)	
Il-12a					0.458 (p=0.275)		0.248 (p=0.334)	
Il-15				0.418 (p=0.054)				
Il-18					2.367 (p=0.275)	2.495 (p=0.308)	3.625 (p=0.205)	3.945 (p=0.285)
Il-1a	15.661 (p=0.4)		3.766 (p=0.617)	3.752 (p=0.206)		3.626 (p=0.854)		
Il-1b	17.305 (p=0.333)	18.763 (p=0.580)	3.745 (p=0.678)	2.765 (p=0.366)	0.391 (p=0.625)		0.337 (p=0.393)	
Il-1rn	4.549 (p=0.190)	8.19 (p=0.526)	3.948 (p=0.634)	3.035 (p=0.307)				3.117 (p=0.493)
Il-6	20.406 (p=0.139)	29.2 (p=0.722)	2.705 (p=0.903)	2.075 (p=0.566)	0.075 (p=0.158)		0.081 (p=0.203)	
Il-7		0.368 (p=0.526)					3.131 (p=0.205)	
Lif	7.453 (p=0.333)	10.702 (p=0.722)			0.228 (p=0.422)		0.228 (p=0.359)	
Lta	2.929 (p=0.909)	2.371 (p=0.526)						
Ltb	6.877 (p=0.669)	6.946 (p=0.526)	2.119 (p=0.719)	2.628 (p=0.671)		5.783 (p=0.837)	2.287 (p=0.691)	6.022 (p=0.37)
Mstn	0.209 (p=0.139)	0.176 (p=0.526)	0.269 (p=0.617)	0.202 (p=0.007)	0.318 (p=0.312)	0.248 (p=0.075)	0.337 (p=0.336)	0.297 (p=0.016)
Osm	13.154 (p=0.146)	8.554 (p=0.526)		2.011 (p=0.552)	0.158 (p=0.275)		0.153 (p=0.238)	
Pf4	0.306 (p=0.674)				4.75 (p=0.126)		3.42 (p=0.129)	3.785 (p=0.37)
Ppbp		2.960 (p=0.526)		2.012 (p=0.007)	2.751 (p=0.095)			2.056 (p=0.694)
Rplp2		0.474 (p=0.626)						
Spp1	4.042 (p=0.333)	5.478 (p=0.526)	4.497 (p=0.880)	3.951 (p=0.366)	23.181 (p=0.158)	21.017 (p=0.358)	25.932 (p=0.129)	36.026 (p=0.016)
Tfrc					0.348 (p=0.269)	0.302 (p=0.308)	0.44 (p=0.384)	0.276 (p=0.285)
Tgfb2	2.348 (p=0.186)					2.724 (p=0.575)		2.414 (p=0.336)
Thpo			0.421 (p=0.371)					
Tnf	3.768 (p=0.897)	4.274 (p=0.526)				3.154 (p=0.624)		3.694 (p=0.136)
Tnfsf11	0.293 (p=0.4)	0.303 (p=0.798)				0.473 (p=0.837)		0.263 (p=0.246)
Tnfrsf11b	6.980 (p=0.424)	10.529 (p=0.526)		2.647 (p=0.671)		3.727 (p=0.837)		4.484 (p=0.396)
Tnfsf10					4.077 (p=0.263)	4.54 (p=0.119)		6.32 (p=0.285)
Zfp91		2.170 (p=0.526)			3.251 (p=0.275)	3.155 (p=0.328)	2.343 (p=0.367)	3.233 (p=0.285)

Table 3.1 Differential Gene Expression. Genes with two-fold upregulation (red) and downregulation (green) with p<1 are shown. Bold indicates p ≤0.05.

3.2.3 T cell margination

Having assessed the overall immune response to I/R injury following MI, this project continued with the investigation of the role of T cells in this response. As the CX3CL1/CX3CR1 axis has a well-known role in cell adhesion, and this is a key event occurring in the early I/R response, the first step in studying the role of T cells in acute MI I/R was to investigate T cell adhesion 2h post reperfusion. Pilot data for this project (not shown) suggested that CD3⁺ T cells have increased adhesion to blood vessels in the 2h I/R left ventricle. This led to a wider study of T cells that appear to have adhered to blood vessel walls in the CX3CR1-EGFP mouse line. This was done using IF staining as shown on Figure 3.17a. In contrast to the small pilot data, very few CD3⁺ cells could be identified on the vessel lumens. In fact, once normalised to the vein length, the average number of T cells is less than 0.01 cells per μm (Figure 3.17b). Further to this, no significant difference in T cell adhesion is observed between the genotypes or between the remote and infarcted area of each genotype.

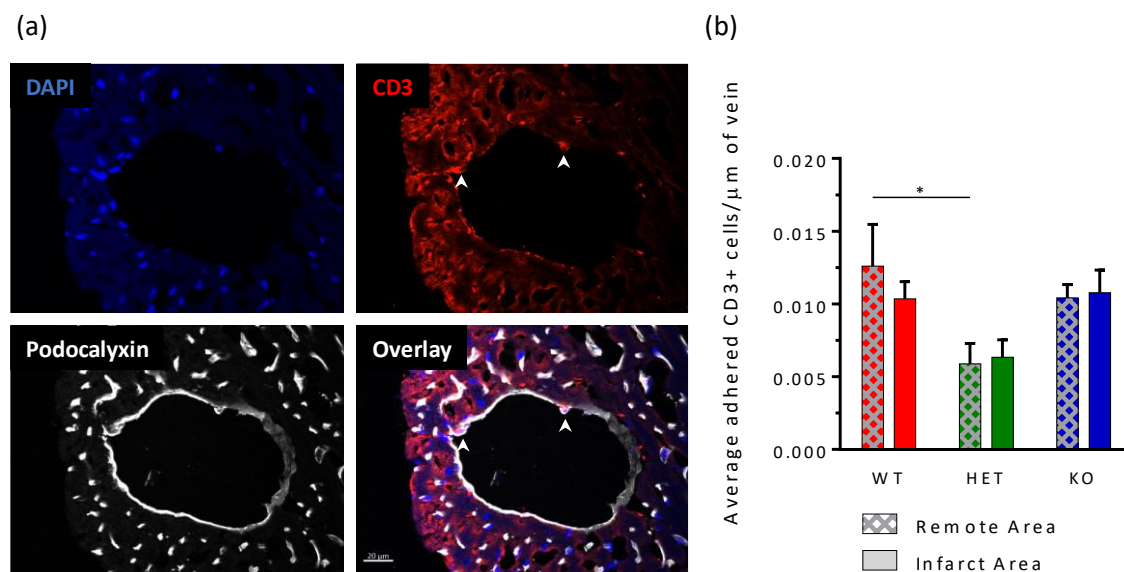


Figure 3.17 T cell adhesion to coronary blood vessels 2h after reperfusion. (a) Representative image of IF staining. Arrowheads show T cells (CD3) adhering to blood vessels (podocalyxin). Scale bar = 20 μm . (b) Average CD3⁺ cell adherence in WT, HET and KO mice, normalised to vessel length. Error bars = SEM; n = 4-6/group. Two-Way ANOVA with Tukey's multiple comparisons test. * $p \leq 0.05$.

3.2.4 T cell infiltration in the myocardium following I/R

As with the CD45⁺ immune cell infiltrate in section 3.2.2, infiltration of T cells in the I/R myocardium was studied using multiple complementary methods.

3.2.4.1 Epifluorescence microscopy

Firstly, CD3⁺ T cell infiltration was investigated across naïve, 2h sham and I/R, and 24h sham and I/R hearts within each genotype (Figure 3.18). Similar to CD45⁺ cell data on Figure 3.7, left ventricles following 24h reperfusion had significantly higher density of T cell infiltration ($p \leq 0.0001$) than any of the other groups. This was true for WT, HET and KO mice. This was followed by analysis of the difference of CD3⁺ cell infiltrate between the three genotypes, where in addition to CD3, EGFP expression was measured using an anti-GFP antibody (Figure 3.19a). The results on Figure 3.19b show that naïve hearts show low level of T cell infiltration with no difference among WT, HET and KO mice. However, following 2h reperfusion (Figure 3.19c), WT mice had nearly 60% more CD3⁺ cells in their infarct area following I/R than HET animals ($p \leq 0.001$), as well as KO mice in the sham group ($p \leq 0.05$). Although not significant, a decrease in the infiltrate in the infarct area of KO mice was also measured. Interestingly, 24h after reperfusion only T cell numbers in the HET mice had recovered to WT levels (Figure 3.19d), with KO mice having over two-fold decrease compared to both WT ($p \leq 0.01$) and HET ($p \leq 0.001$) mice. This is in contrast to CD45 data where both HET and KO immune cell infiltrate had recovered to WT levels. In addition to this, the number of EGFP⁺ T cells infiltrating the left ventricle was also investigated (Figure 3.20). Similar to the total EGFP⁺ cell infiltration (shown on Figure 3.9), HET mice showed an over 90% increase ($p \leq 0.0001$) in the number of EGFP⁺ CD3⁺ cells in their left ventricles following 24h I/R (Figure 3.20a). In contrast, KO mice did not have a significant accumulation of EGFP⁺ T cells, although they showed a trend of increased infiltration both at 2h and 24h post reperfusion (Figure 3.20b). Investigating the difference in EGFP⁺ CD3⁺ cell recruitment between HET and KO mice shows no significant difference between the two genotypes either in naïve hearts (Figure 3.21a) or 2h post reperfusion (Figure 3.21b). However, after 24h I/R, HET mice had nearly 70% increase ($p \leq 0.01$) in left ventricular infiltration of EGFP expressing T cells (Figure 3.21c).

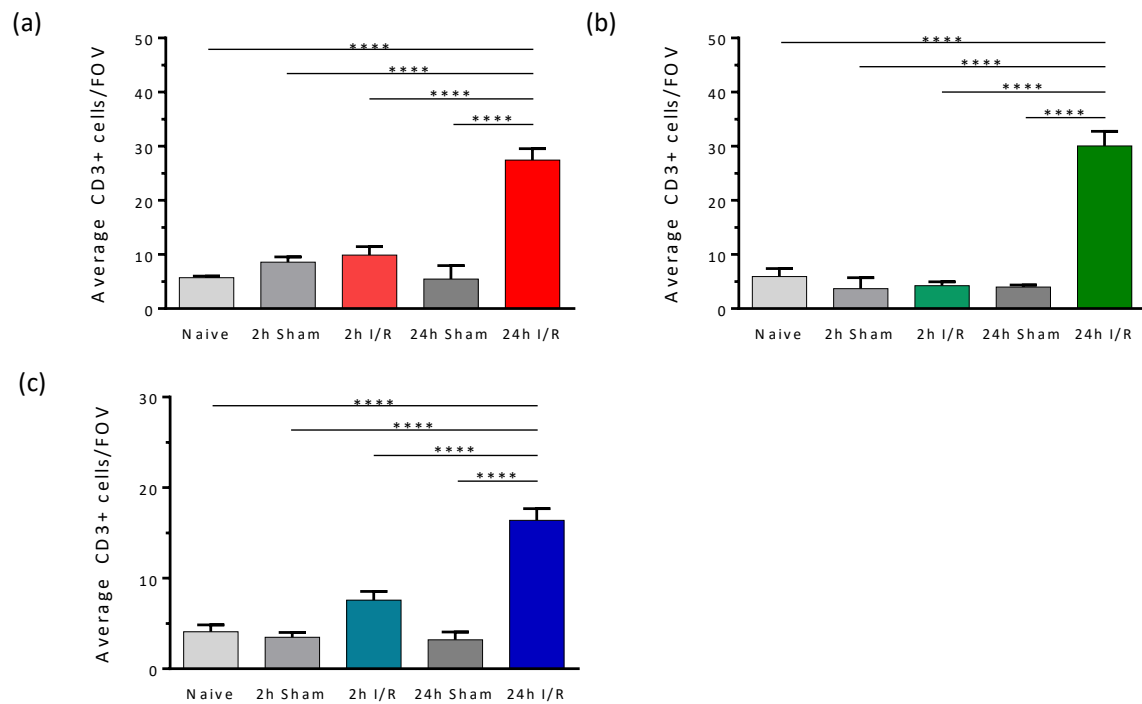


Figure 3.18 T cells accumulate in the infarct area after 24h reperfusion. Average number of CD3⁺ cells infiltrating the left ventricles of (a) WT, (b) HET and (c) KO mice. Error bars = SEM; n=3-6/group; One-way ANOVA with Tukey's multiple comparisons test. **** $p \leq 0.0001$.

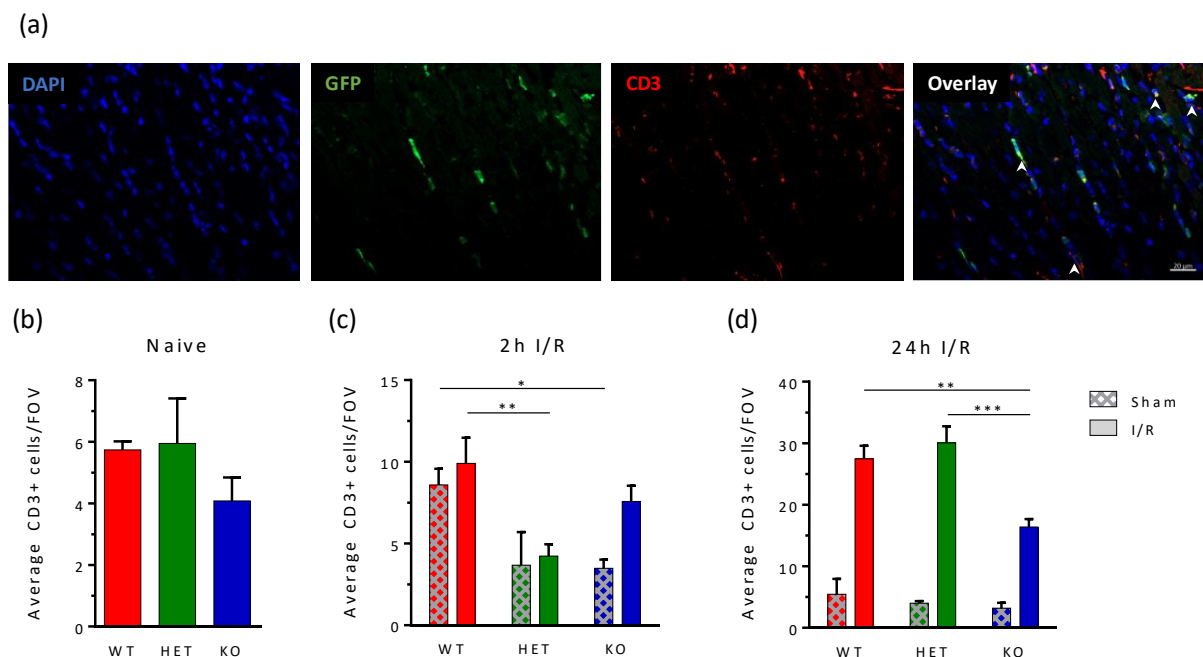


Figure 3.19 Loss of CX3CR1 expression leads to reduced T cell recruitment to the injured heart. (a) Representative image of EGFP⁺ CD3⁺ IF staining. Arrowheads show EGFP⁺ CD3⁺ cells. Scale bar = 20 μ m. Density of T cell recruitment in (b) naïve, (c) 2h and (d) 24h post surgery hearts. Error bars = SEM; n = 3-6/group. One-way (Naïve) and Two-Way (2h and 24h) ANOVA with Tukey's multiple comparisons test. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

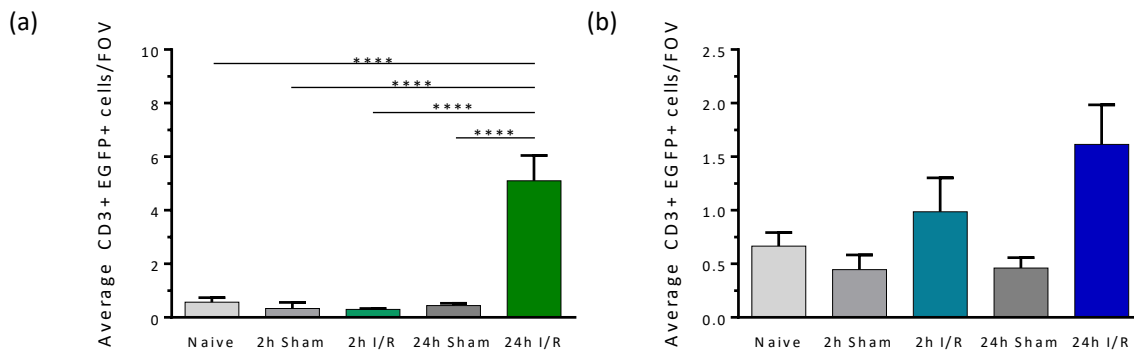


Figure 3.20 EGFP⁺ T cell infiltration in the infarct area following myocardial reperfusion. Average number of EGFP⁺ CD3⁺ cells accumulating in the left ventricles of (a) HET and (b) KO CX3CR1-EGFP mice. Error bars = SEM; n=3-6/group; One-way ANOVA with Tukey's multiple comparisons test. **** $p \leq 0.0001$.

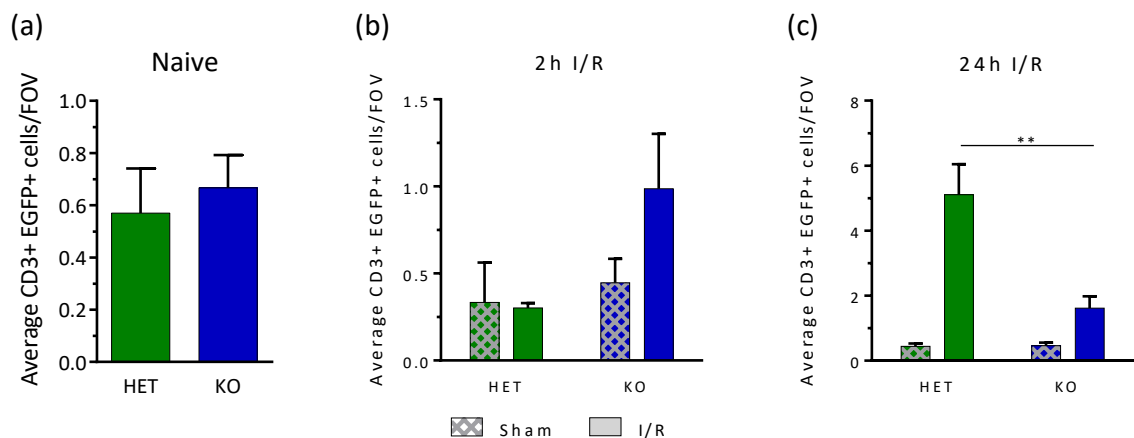


Figure 3.21 Loss of CX3CR1 expression leads to reduced EGFP⁺ CD3⁺ cell recruitment to the injured heart 24h post reperfusion. Density of EGFP⁺ T cell recruitment in (a) naïve, (b) 2h and (c) 24h post surgery hearts. Error bars = SEM; n = 3-6/group. Unpaired two-tailed t-test (Naive) or two-Way ANOVA with Sidak's multiple comparisons test (2h and 24h). ** $p \leq 0.01$.

While this project focuses on the early I/R response and therefore most of the work looks into the role of T cells at 24h reperfusion, it is reported that T cell numbers in the MI myocardium peak at 3 days post infarct (Yan, Anzai et al. 2013b). Therefore, T cell infiltration in WT and KO mice was also assessed for this time point (Figure 3.22). While the results show a trend of reduction for CD3⁺ cell infiltration in the infarcted left ventricle in KO mice, this difference was not significant. However, analysis of the T cell infiltrate at each I/R time point within a genotype (Figure 3.23) shows that 3 days after I/R KO mice have significantly higher T cell infiltration at 2h (an increase of 75%) or 24h (an increase of 50%) post reperfusion. However, 3 day WT mice only showed significantly increased T cell infiltration (80%) when compared to 2h I/R mice.

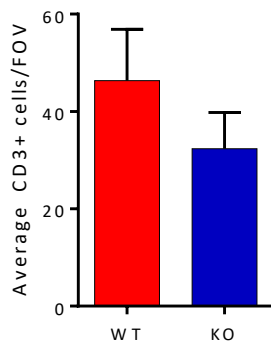


Figure 3.22 Loss of CX3CR1 expression does not lead to significant change in T cell infiltration to the infarct area 3 days post reperfusion. Error bars = SEM; n=4. Unpaired two-tailed t-test.

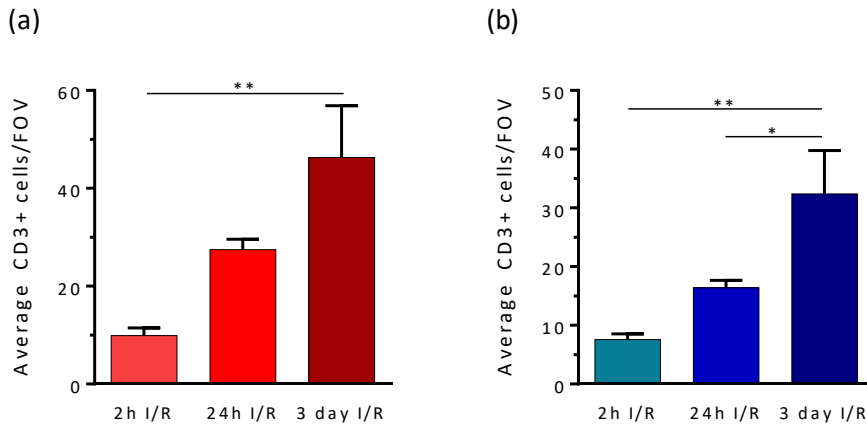


Figure 3.23 T cell accumulate in the injured myocardium after 2h, 24h and 3 days reperfusion. Increasing numbers of CD3⁺ cells infiltrate the injured myocardium in (a) WT and (b) KO mice. Error bars = SEM; n=4-6. One-way ANOVA with Tukey's multiple comparisons test. * $p \leq 0.05$; ** $p \leq 0.01$.

3.2.4.2 Cardiac digest

T cell infiltration 24h after reperfusion was also investigated using flow cytometry analysis of cardiac digested tissue. Since IF studies have technical limitations to the number of markers that can be studied per sample, flow cytometry analysis allows for deeper investigation of the T cell subsets. As with CD45 cardiac digest analysis in section 3.2.2.2, right ventricle data was used as control instead of sham animals. IF analysis on Figure 3.24 shows that both 24h sham left ventricle and 24h I/R right ventricle data were equally good controls compared to the infarcted left ventricle area of 24h I/R hearts. T cell flow cytometry analysis was performed using the gating strategy on Figure 3.25. Additional T cell markers, to identify naïve, effector and memory populations, were not used because of the low number of events detected in the CD4 and CD8 T cell gates.

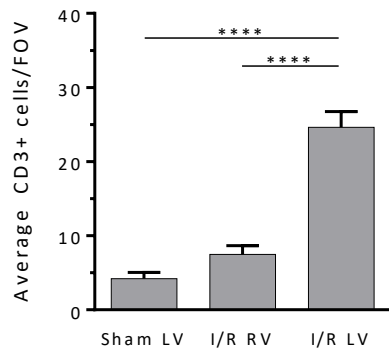


Figure 3.24 IF data of CD3 infiltration in the left ventricle of sham and right ventricle of I/R mice are both equally controls for I/R left ventricle. Pooled data for all CX3CR1-EGFP genotypes. Error bars = SEM; n=9-12/group. One-way ANOVA with Tukey's multiple comparisons test. **** p ≤ 0.0001.

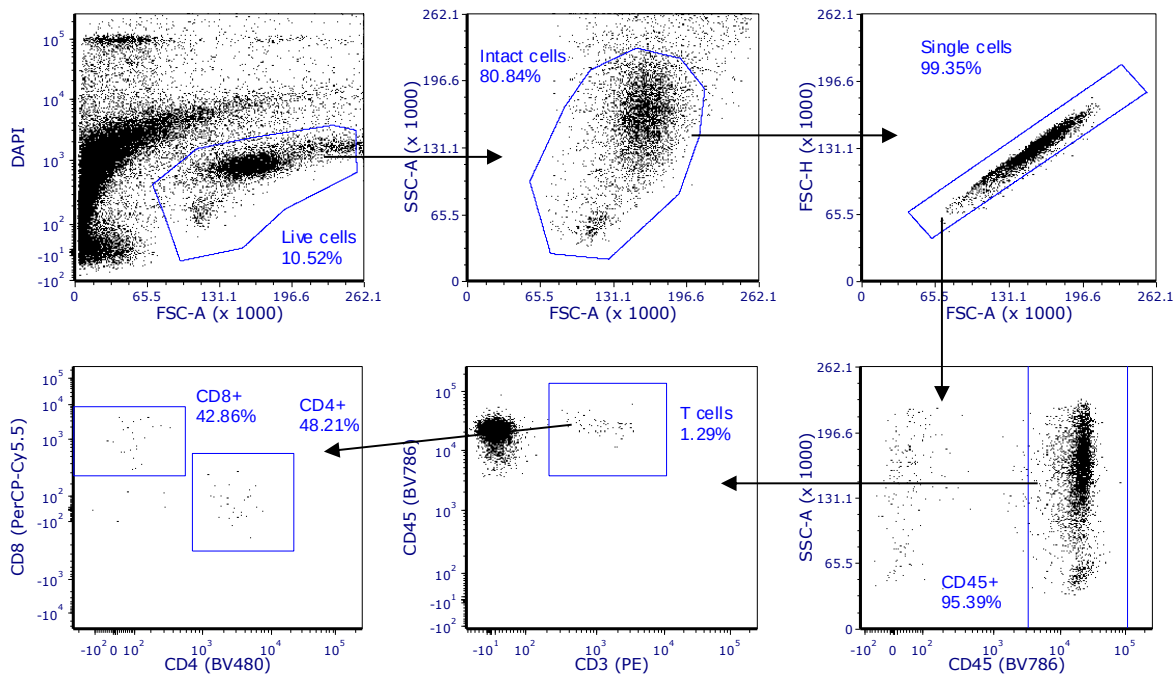


Figure 3.25 Flow cytometry gating strategy to identify T cell populations in digested cardiac tissue. Representative dot plots of a left ventricle sample 24h after reperfusion.

Overall, no difference in the T cell infiltrate was detected among the genotypes (Figure 3.26). Less than 0.05% of the leukocytes per mg tissue infiltrating the infarcted area are CD3 expressing T cells. The low total CD3⁺ T cells naturally resulted in even lower number of CD4 and CD8 gated cells (under 0.025%/mg), whose levels also remained consistent between WT, HET and KO mice. Similar to the CD45⁺ digest data above, the percentage of T cells in the remote right ventricle was higher than their left ventricle counterpart, with a significant 4-6 fold increase for HET and KO mice.

The level of CX3CR1 and EGFP expression was also measured for these T cell populations, as was shown for total leukocytes on Figures 3.12c and d. The results on Figure 3.27a show that there is no differential infiltration of CX3CR1 expressing cells in the left ventricles of WT and HET mice, with around 0.1%/mg of T cells being CX3CR1⁺. On the other hand, Figure 3.27b

shows that KO T cells from both CD4 and CD8 subtypes show a trend of reduction compared to their HET counterpart, although the difference was not significant. The percentage of EGFP expressing HET cells was around two-fold higher than that of CX3CR1⁺ ones. Again, this is believed to be because EGFP is measured without any antibody binding and includes any intracellular signal, while CX3CR1 is detected only with an antibody.

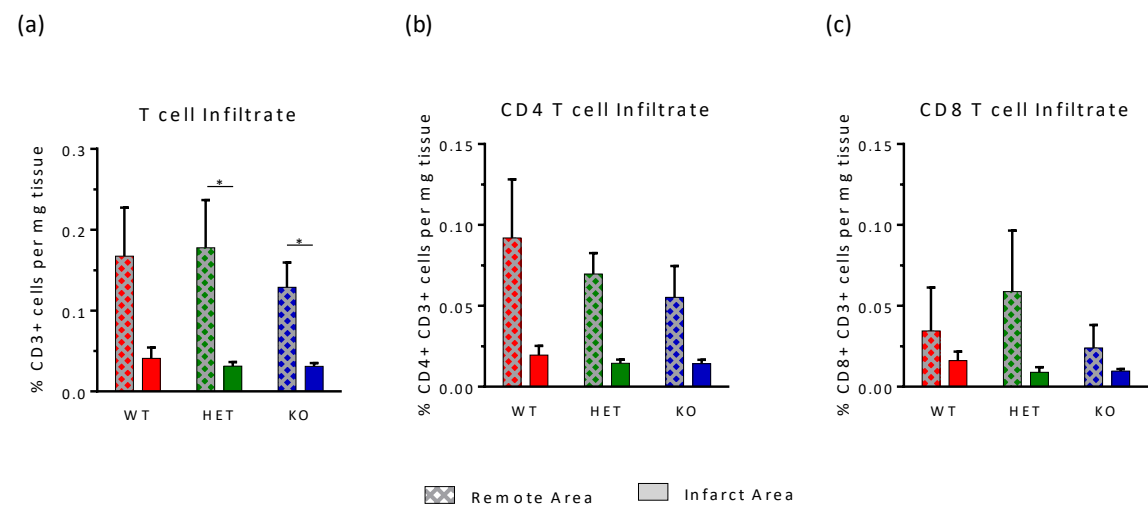


Figure 3.26 Flow cytometry analysis of digested cardiac tissue 24h post reperfusion in WT, HET and KO CX3CR1 mice. Cardiac infiltration of (a) T cell, (b) CD4 T cells and (c) CD8 T cells presented as percentage of total leukocytes per mg of tissue. Two-way ANOVA with Tukey's multiple comparisons test. Infarct area – left ventricle; remote area – right ventricle. Error bars = SEM, n=3-6/group.

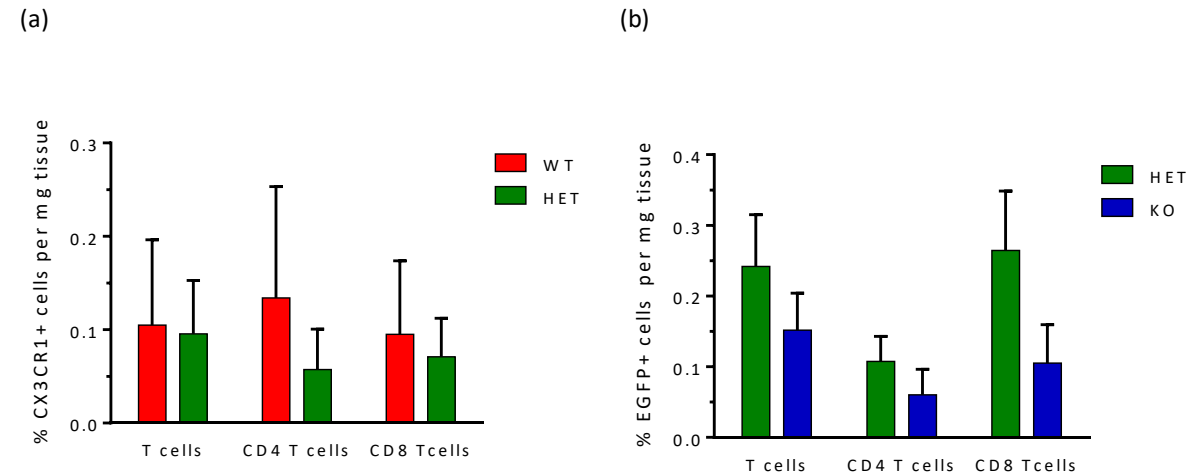


Figure 3.27 Flow cytometry analysis of CX3CR1/EGFP T cell expression after 24h reperfusion. Left ventricular infiltration of T cell populations expressing (a) CX3CR1 and (b) EGFP per mg of tissue. Two-way ANOVA with Sidak's multiple comparisons test. Error bars = SEM, n=3-6/group.

3.2.5 Cardiac function following I/R

Finally, heart function of CX3CR1 WT, HET and KO mice was investigated using cardiac magnetic resonance imaging (MRI). The results on Figure 3.28 show the change in heart function between 1 week and 6 weeks following post MI reperfusion. While loss of CX3CR1 causes changes in the immune cell infiltrate in the acute MI I/R response, this did not lead to significant worse deterioration in cardiac function. No significant difference between the genotypes was measured for end-diastolic volume, end-systolic volume, ejection fraction or left ventricular mass. However, both HET and KO mice showed a clear trend towards adverse left ventricular remodelling and reduced ejection fraction.

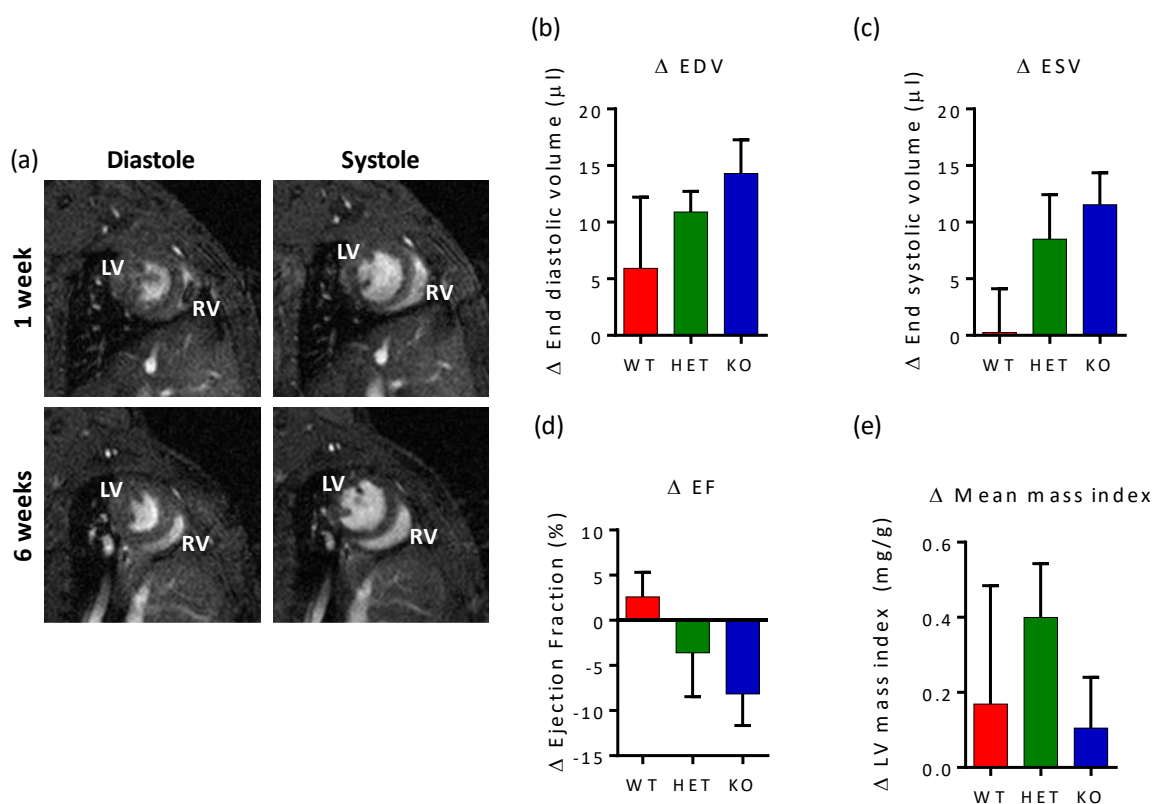


Figure 3.28 MRI analysis assessing cardiac function in CX3CR1 WT, HET and KO mice. Loss of CX3CR1 does not lead to significantly worse deterioration in cardiac function between 1 and 6 weeks after reperfusion, although there is a trend towards increased adverse remodelling. (a) End-diastolic and end-systolic frames in the short axis orientation of a mid-ventricular infarcted heart. LV = left ventricle, RV = right ventricle. Change between 1 and 6 weeks of (b) end diastolic volume, (c) end systolic volume, (d) ejection fraction, (e) mean mass normalised to pre-operation mouse weight. Error bars = SEM, n = 8-10/group. One-way ANOVA with Tukey's multiple comparisons test. Dr Simon Tual-Chalot and Dr Rachael Redgrave performed the MRI. Data was analysed both by Dr Simon Tual-Chalot/Dr Rachael Redgrave and myself.

3.3 Discussion

The work described here sheds light on the effect of CX3CR1 knockout on myocardial I/R injury and the involved inflammatory response. This has been achieved using a CX3CR1-EGFP mouse line which serves both as a gene knockout model, as well as a reported line. The first aim of this chapter was to establish a baseline T cell phenotype. This was required as the initial work by Jung et al. describing this line reports no EGFP expression in T cells (Jung, Aliberti et al. 2000). This is in contrast to more recent evidence that T cells do express CX3CR1 and can even be used as a marker distinguishing certain memory and effector T cell populations (Gerlach, Moseman et al. 2016, Jameson, Masopust 2018, Yamauchi, Hoki et al. 2020), as well as previous work from this group showing depletion of circulating CX3CR1⁺ T cells post MI (Boag, Das et al. 2015). Furthermore, Jung et al. only looked at EGFP expression in total CD3⁺ cells (Jung, Aliberti et al. 2000), while subsequent studies have shown that T cell subtypes have varying levels of CX3CR1 expression, with effector populations, especially Th1 and Tc1, having the highest (Boag, Das et al. 2015, Umehara, Bloom et al. 2004, Imai, Hieshima et al. 1997, Nishimura, Umehara et al. 2002, Fraticelli, Sironi et al. 2001). Indeed, the results described in Section 3.2.1 confirm that CX3CR1 and/or EGFP are expressed in WT, HET and KO CX3CR1-EGFP mice, with a considerably higher proportion in effector and memory T cells. Moreover, level of CX3CR1/EGFP expression was highest in memory and effector CD4 and CD8 populations in blood, spleen and lymph nodes (both peripheral and cardiac). Interestingly, the results show increased number of CX3CR1⁺ CD8 cells and EGFP⁺ memory CD4 cells in the peripheral lymph nodes of HET mice. CX3CR1/EGFP expressing T cells in heterozygous mice may have a phenotype more similar to the CX3CR1^{int} cells described by Gerlach et al. (Gerlach, Moseman et al. 2016), as they do have only 50% detectable CX3CR1 mRNA (Figure 3.1b). In addition to this, these CX3CR1^{int} T cells are known to populate the lymph nodes. However, the present study is not able to distinguish CX3CR1⁻, CX3CR1^{int} and CX3CR1^{high} cells as CD27 was not included in the flow cytometry panel.

The next step of this study was to analyse the inflammatory response to acute myocardial I/R in the CX3CR1-EGFP mouse line. This aim was approached using several different complementary methodologies in order to study the role of CX3CR1 in MI I/R in more detail. Initially, results showed that HET mice had a significantly increased area infiltrated by CD45⁺ cells 24h post reperfusion compared to WT mice (Figure 3.6b). This inflamed area is used as a guide to infarct size as studies have shown that excessive inflammation is associated with poor

cardiac function post MI (Westman, Lipinski et al. 2016). Preclinical models show that expression of pro-inflammatory cytokines, such as IL-6, TNF α and IL-1 β , in the MI myocardium are associated with increased end-diastolic diameter of the left ventricle (Koh, Akira et al. 1998). Ventricular dilation and EDV have also been shown to be affected by the IL-1 receptor pathway (Bujak, Dobaczewski et al. 2008, Antonio, Salloum Fadi N. et al. 2008). However, although larger infarct size correlates with adverse LV remodelling and worse cardiac function, this is not always the case. As Westman et al. showed, 15% of patients with small infarct size have adverse LV remodelling while 60% of those with large MI do not (Westman, Lipinski et al. 2016). This is in part due to the complex inflammatory response taking place in the infarcted left ventricle. Importantly, the data described in this chapter supports this. While CX3CR1-EGFP HET mice have increased “infarct size” (area of leukocyte infiltration) compared to WT controls (Figure 3.6b), they do not have significantly worse cardiac function. Moreover, compared to HET mice, KO animals have a trend of smaller area of CD45⁺ infiltration and more adverse LV remodelling.

Analysis of the density of leukocyte infiltration in the left ventricle of naïve hearts is in line with the baseline T cell phenotyping data, in that there is no significant difference between the three CX3CR1-EGFP genotypes. However, the inflammatory response post MI does show a difference. The results suggest that knockout of CX3CR1 causes a delay in leukocyte recruitment to the site of injury. Both HET and KO mice show a significant reduction in CD45⁺ cell accumulation 2h post infarct compared to WT but their levels return to normal within 24h. Moreover, partial CX3CR1 knockout also causes a delay in the recruitment of T cells in HET mice. Similar to the CD45⁺ infiltration, these mice have reduced T cell accumulation 2h post reperfusion that return to WT levels by 24h. Furthermore, there appears to be a change in the cell composition of the 24h inflammatory infiltrate. HET mice have increased recruitment of total EGFP expressing cells to the injured myocardium that is not observed in KO mice, despite both having comparable leukocyte infiltration. While not only leukocytes express CX3CR1/EGFP, this data clearly shows that CX3CR1-dependent cell recruitment does occur in the post MI I/R. Interestingly, KO mice do not have an accumulation of total EGFP⁺ cells 24h post reperfusion. This suggests that while partial CX3CR1 knockout in HET mice causes delayed immune response immediately post MI, which returns to normal by 24h, this is not the case in KO mice. Complete CX3CR1 knockout does not allow a normal response in the first 24h of post MI I/R. Instead, KO mice have increased recruitment of CX3CR1⁻/EGFP⁻ leukocytes. Most

likely these are neutrophils, as they are the most numerous early responding leukocytes (Swirski, Nahrendorf 2013, Nahrendorf, Swirski et al. 2007, Yan, Anzai et al. 2013b). However, the switch in monocyte/macrophage phenotype from classical/M1 to CX3CR1⁺ non-classical/M2 is important for successful myocardial repair (Swirski, Nahrendorf 2013, Nahrendorf, Swirski et al. 2007), so KO mice appear to have reduced cardioprotective infiltrate. Moreover, complete absence of CX3CR1 expression disrupts CX3CR1-dependent recruitment of T cells. KO mice have not only reduced total T cell infiltration in the post MI myocardium 24h after reperfusion (Figure 3.19), but they also show reduced infiltration of EGFP⁺ CD3⁺ cells (Figure 3.21). In contrast, partial knockout in HET mice does not affect the total T cell number recruited to the MI heart at 24h I/R. As HET animals show a significant increase in the accumulation of EGFP⁺ T cells compared with KO mice at this time point, this suggests that specific recruitment of CX3CR1 expressing T cells is dependent on CX3CR1-CX3CL1 interaction. Analysis of the T cell response at the peak of their infiltration, described by Yan et al. to be at 3 days in myocardial I/R models (Yan, Anzai et al. 2013a), supports results of a delay in CX3CR1-mediated cell recruitment. As shown above (Figures 3.22 and 3.23), unlike WT mice, KO mice have significantly increased accumulation of T cells between 24h and 3 day I/R. And while KO animals maintained lower CD3⁺ cell infiltration at 3 days compared to WT controls, this difference was not shown to be significant in the current study.

Interestingly, KO mice show an increased number of total EGFP⁺ cells in naïve hearts compared to HET animals. Although this difference was not observed for EGFP⁺ CD3⁺ cells or in the baseline T cell phenotyping, these results suggest that complete germline CX3CR1 knockout induces a compensatory mechanism. These EGFP⁺ cells in the uninjured myocardium may include monocytes, tissue resident macrophages and vascular smooth muscle cells (Rowinska, Koepfel et al. 2017). Although CX3CL1 and CX3CR1 are each other's only known ligand and receptor, this pathway is involved in multiple different inflammatory signalling pathways (Zhuang, Ou et al. 2019). The CX3CL1/CX3CR1 axis is activated by pleiotropic pro-inflammatory cytokines such as IFN γ , TNF α , IL-1 β and IL-6. Moreover, key inflammatory pathways such as JAK-STAT, MAPK, NF κ B and TLR show crosstalk with fractalkine and its receptor (Zhuang, Ou et al. 2019). Fractalkine can even induce its own expression in an autocrine fashion via these signalling pathways (Chandrasekar, Mummidi et al. 2003). Furthermore, these pathways are involved in signalling of multiple cytokines and chemokines, many of which have overlapping and even redundant function (Ozaki, Leonard 2002). Indeed, the evolutionary purpose of this

is believed to be that it provides a compensatory mechanism for cytokines which have a critical role for survival or whose inappropriate activation would not be pathogenic (Kelso 1994). Therefore, interpreting data from germline knockout mice must take into account the fact that the function of the KO gene may be compensated by another gene in the network (Mattison, Nie et al. 2013). More specifically for the CX3CL1/CX3CR1 axis, Gregg et al. noted the lack of consistency in findings using *Cx3cr1*^{-/-} mice and proposed that disruption in signalling of the CX3CL1/CX3CR1 axis can have an impact on the balance of other inflammatory mediators (Gregg, Lumeng et al. 2014). Further to this, they suggest that the effect of this is specific to the local tissue environment and disease state (Gregg, Lumeng et al. 2014). In a model of neural inflammation where CX3CR1 has a neuroprotective role, Mattison et al. propose that in order to compensate for the loss of CX3CR1, downstream signalling is dysregulated in *CX3CR1*^{-/-} mice, effectively reducing the response to pro-inflammatory stimulation (Mattison, Nie et al. 2013). In addition to this, Poupel et al. describe contradicting results of monocyte recruitment in atherosclerosis between pharmacological inhibition of CX3CR1 and studies in *CX3CR1*^{-/-} mice. Again, this is contributed to compensatory mechanisms arising during development in mice with genetically knocked down genes (Lucie, Alexandre et al. 2013). This challenge of compensatory mechanisms developing as a result of germline gene deletion is getting increasing recognition, as at least 15% of knockout mouse lines do not show phenotypic differences from WT controls (Fredholm, Chen et al. 2005, Barbaric, Miller et al. 2007).

Given this cytokine redundancy and compensatory mechanisms discussed above, it is not surprising that CX3CR1 KO mice did not show significantly different inflammatory gene expression in the infarcted myocardium. However, the inflammatory gene expression pilot study described in Section 3.2.2.3 does show interesting expression patterns (despite not being statistically significant). The injured left myocardium of both WT and KO mice appears to have some expected changes in mRNA levels compared to remote right ventricle controls, as shown on Table 3.1.

Left ventricles of both WT and KO mice at 6h and 24h I/R appear to have non-significant increase in expression of Il-1a, Il-1b and Il-1rn. The pro-inflammatory IL-1 pathway has been described to play an important role in the early myocardial I/R response and inhibiting it has shown improved cardiac function in rodent models (Zhu, Jinzhou, Huang et al. 2019). IL-1a is an important sterile inflammation danger signal released by necrotic cardiomyocytes and

blocking its signalling has been shown to reduce post MI cardiac inflammation (Lugrin, Parapanov et al. 2015). Similarly, Il-1b expression in the infarcted myocardium is upregulated and modulating its function with an IL-1b antibody leads to improved left ventricular remodelling and cardiac function in a rat myocardial I/R model (Frangogiannis 2015, Harouki, Nicol et al. 2017). On the other hand, Il-1rn encodes IL-1ra (receptor antagonist) which inhibits the function of IL-1a and IL-1b by competing for binding to their receptors, and therefore has cardioprotective function in myocardial I/R models (Ken, Bari et al. 2001). Its increase in the 24h MI left ventricles has been reported for a permanent mouse MI model (Ruparelia, Digby et al. 2013). Similarly, left ventricles of WT and KO mice appear to have increased expression of Lta and Ltb, although this was not statistically significant. These genes encode TNF family members lymphotoxin a and b respectively, and are expressed by activated T and B cells (Lau, Zhang et al. 2013). Lta has been shown to upregulate expression of adhesion molecules (Suna, Sakata et al. 2008), play a role in atherosclerosis (Suna, Sakata et al. 2008), as well as have elevated expression in murine models of permanent MI (Zhang, Yanqing, Zhao et al. 2013) and I/R injury (Lau, Zhang et al. 2013). Another pro-inflammatory factor, osteoprotegerin, is a soluble receptor for RANKL and TRAIL (members of the tumour necrosis factor receptor superfamily) and is encoded for by Tnfrsf11b. It is expressed in many different tissues including the cardiovascular system and its elevated expression is not only associated with increased risk in development of cardiovascular disease but also with worse outcome for ST elevation MI (Shetelig, Limalanathan et al. 2017, Pedersen, Pedersen et al. 2012). Therefore, the results shown here, suggesting upregulation of Tnfrsf11b expression in left ventricles 6h post infarct, are in accordance with existing literature.

Interestingly, left ventricles of both WT and KO mice also appear to have upregulated expression of several cardioprotective genes. Mice after 24h reperfusion had non-significant increase in expression of Il-11 in their left ventricles, which has been shown to have a cardioprotective effect in mouse and rat I/R models (Kimura, Maeda et al. 2007, Tamura, Kohno et al. 2018). Furthermore, expression of Lif appears to be upregulated in the left ventricles of 6h I/R mice. The protein encoded by this gene, Leukemia Inhibitory Factor, has been described to have cardioprotective function in early MI (Yunzeng, Hiroyuki et al. 2003, Zouein, Kurdi et al. 2013). Moreover, both WT and KO mice appear to have upregulated Oncostatin M (Osm) expression in their left ventricles after 6h and 24h I/R. This is in accordance with previous studies describing upregulation of cardioprotective Osm in murine

MI (Hu, Zhang et al. 2017, Sun, Li et al. 2015) and canine myocardial I/R (Gwechenberger, Moertl et al. 2004) models. Another cardioprotective factor Osteopontin-1, encoded by *Spp1*, is upregulated in the left ventricles of WT and KO mice at 6h and 24h I/R. Osteopontin-1 is a matricellular protein and cytokine which is produced by macrophages, smooth muscle cells, endothelial cells, as well as cardiomyocytes and cardiac fibroblasts (Duerr, Mesenholl et al. 2014). It is reported to have upregulated expression in a porcine heart I/R model (Kossmehl, Schönberger et al. 2005), as well as in canine myocardial I/R where its levels are elevated from 5h post reperfusion (Frangogiannis, Youker et al. 1998). The left and right ventricles of both WT and KO CX3CR1 mice show upregulation in *Spp1* expression at 24h compared to their 6h counterparts. This is consistent with the reports by Frangogiannis et al. that the peak of osteopontin expression is at 24h post reperfusion (Frangogiannis, Youker et al. 1998). Interestingly, Tregs have been reported to modulate the pathogenic recruitment and function of CD4 and CD8 T cells, as well as monocytes/macrophages via upregulation of Osteopontin (Weirather, Hofmann et al. 2014).

The results on Table 3.1 show that myostatin (*Mstn*) expression is significantly downregulated in the left ventricle of KO mice 24h post reperfusion, as well as in the KO 24h right ventricle compared to 6h. In addition to this, its expression appears to be downregulated across all groups (in left ventricles of WT and KO mice compared right ventricles and in 24h compared to 6h I/R), although this did not reach statistical significance. Myostatin is a potent negative regulator of muscle growth (Silljé, de Boer 2010). While its effects on skeletal muscle development and hypertrophy are well studied in *Mstn* knockout models, its role on cardiac muscle is more subtle and even controversial (Hanna, Frangogiannis 2019). The decrease in *Mstn* expression described here, is in contrast to the majority of studies focusing on the myocardium which report a rapid increase post injury. However, these studies focus on permanent MI models (Castillero, Akashi et al. 2015, Lim, McMahon et al. 2018) or heart failure in both clinical studies (George, Bish et al. 2010, Gruson, Ahn et al. 2011, Lenk, Erbs et al. 2012) and rodent models (Shyu, Lu et al. 2006). These reports are counterintuitive, as one would expect that cardiac injury would promote cardiac hypertrophy in the saved/remote myocardium instead of upregulation of its inhibitors. However, the rationale behind these studies lies in that myostatin upregulation is a counterbalance to muscle hypertrophy, preventing excessive cell growth. In addition to this, while myostatin is well known to cause muscle hypertrophy, it has also been reported that it does not lead to greater muscle force

generation (Amthor, Macharia et al. 2007). Moreover, the contrasting results described here could be due to cardiac I/R injury, which these studies did not focus on. In fact, similar to the results described here, Pavo et al. recently reported that Mstn expression is downregulated both in the ischaemic and remote myocardium in a model of repetitive I/R injury (Pavo, Lukovic et al. 2017).

More importantly, in addition to these expected MI changes, some genes appear to be differentially expressed only in WT or KO mice, potentially suggesting different inflammatory response in the absence of CX3CR1. Interestingly, only WT left ventricles appear to have downregulation of Ccl19 expression at 6h and 24h post reperfusion. CCL19 is the receptor for CCR7, expressed on T cells, and has been reported to rise in the circulation following coronary intervention (Hofmann, Frantz 2016). Furthermore, clinical work from our group has shown that CCR7⁺ CD4⁺ T cells are selectively lost in the coronary circulation post reperfusion (Boag, Das et al. 2015). In addition to this, WT mice appear to have an increased expression of Ccl17 at 24h post infarct. This chemokine is secreted by anti-inflammatory M2 macrophages and is chemotactic to Th2 cells (Peet, Ivetic et al. 2019, Xu, J., Xiong et al. 2019). Another chemokine involved in recruitment of pro-inflammatory T cells, such as Th1 and cytotoxic T cells (CTLs), is CXCL10. Its levels are known to be elevated in cardiovascular diseases, as is the pro-inflammatory polarisation of T cell populations (Altara, Mallat et al. 2016). Furthermore, the role of CXCL10 in MI pathogenesis is supported as a result of its expression in atherosclerotic lesions (Mach, Sauty et al. 1999, Cheng, Tempel et al. 2007) and the increased infiltration of Th1 and CTLs (Rohm, Atiskova et al. 2015). In addition to this, studies in mouse and canine models show that CXCL10 levels are upregulated in the early response to myocardial I/R injury (Frangogiannis, Mendoza et al. 2001, Marcin, Marcin et al. 2009). In fact, Bujak et al. reported that mice have elevated expression of CXCL10 after 6h reperfusion, selective for infarcted area (Marcin, Marcin et al. 2009). This is in accordance with the data on Table 3.1 suggesting that WT CX3CR1 mice have upregulated CXCL10 expression in the left ventricle area at the early I/R time point.

On the other hand, KO mice appear to have upregulated expression of Ccl5 in their infarcted area 6h post reperfusion. CCL5, or RANTES (regulated on activated normal T cell expressed and secreted) is secreted by a number of different cell types including activated T cells, macrophages, endothelial and smooth muscle cells. Its role is in recruitment of granulocytes, monocytes, T cells, NK cells and DCs via binding to its receptors CCR1, CCR3 and CCR5

(Braunersreuther, Pellieux et al. 2010). CCL5 has been described to have a critical role in macrophage and neutrophil recruitment in atherosclerosis and is subsequently implicated in MI (Montecucco, Braunersreuther et al. 2011). Circulating and cardiac levels of CCL5 have been shown to be upregulated following myocardial ischaemia and its inhibition has a cardioprotective effect (Braunersreuther, Pellieux et al. 2010, Montecucco, Braunersreuther et al. 2011, Vajen, Koenen et al. 2018). Furthermore, blocking CCL5 interaction with CXCR4 leads to reduced I/R inflammation, due to lower neutrophil and monocyte/macrophage infiltration (Vajen, Koenen et al. 2018). Therefore, the elevated Ccl5 expression in the left ventricles of KO mice 6h post reperfusion, suggests that absence of CX3CR1 may lead to increased recruitment of neutrophils and monocytes to the injured myocardium in the early I/R response. Another gene showing differential expression only in KO mice is Pbp. It encodes pro-platelet basic protein or CXCL7 which is expressed in megakaryocytes and in monocyte stimulated by stromal cells (de Couto, Liu et al. 2015, Pillai, Iwata et al. 2006). It has a role in neutrophil chemotaxis (Brown, Sepuru et al. 2017), as well as blood coagulation, and has been reported to be increased in coronary heart disease (Maneerat, Prasongsukarn et al. 2017) and myocardial infarction (Xu, S., Jiang et al. 2019, Cheow, Cheng et al. 2016). Interestingly, only left ventricles of KO mice at both 6h and 24h appear to have upregulated expression of CXCL7, suggesting an increased recruitment of neutrophils to the infarct area of mice lacking CX3CR1. These mice also showed a decrease in expression of cardioprotective IL-15, although this was not statistically significant. This is a pleiotropic cytokine that can affect multiple pathways including cell survival, differentiation and proliferation. It has been reported to be cardioprotective for hypoxic cardiomyocytes, and its administration in a permanent MI mouse model improves cardiac function (Ameri, Bayardorj et al. 2020). Despite this, expression of IL-15 has been reported to be downregulated in permanent MI models 24h post infarction (Iyer, de Castro Brás et al. 2016) as well as 2 days post infarction (Wang, Y., Huang et al. 2018, Zhang, Shu, Liu et al. 2017). Similarly, the results shown here suggest that CX3CR1 KO mice have a decreased IL-15 expression in the infarct area of hearts 24h post reperfusion. Finally, CD40lg encodes CD40 ligand (CD40L) which binds to its receptor CD40, expressed on lymphocytes, monocytes/macrophages, platelets and endothelial cells. Activation of the CD40/CD40L axis induces cellular adhesion molecules and pro-inflammatory cytokine production (Mami, Thorsten et al. 2005). Furthermore, it has been reported that both CD40L and CD40 expression is upregulated following cerebral ischaemia reperfusion and that signalling via this pathway aggravates brain infarction (Mami, Thorsten et al. 2005). Interestingly, CX3CR1 KO mice

appear to show increased expression of CD40lg in their left ventricles 24h post reperfusion. This may be suggestive that a compensatory upregulation of adhesion molecules, other than CX3CR1, is occurring in mice lacking any CX3CR1.

This gene expression study does hint towards a potentially different inflammatory response in KO mice. Although the changes in gene expression in this study are not statistically significant, in contrast to 24h WT samples, KO left ventricles do not appear to show increased Th2 chemotaxis via M2 macrophage/non-classical monocytes secreted CCL17. M2 macrophages and non-classical monocytes have high CX3CR1 expression (Burgess, Wicks et al. 2019, Geissmann, Jung et al. 2003) and are essential for cardiac repair (Andreadou, Cabrera-Fuentes et al. 2019). Therefore, complete absence of the receptor is likely to have a significant impact on their recruitment and accumulation in the I/R heart, explaining the lack of upregulated CCL17 expression in KO LVs. Indeed, models of pulmonary hypoxia have shown that knockout of CX3CR1 leads to disrupted M1:M2 macrophage polarisation (Amsellem, Abid et al. 2017). Moreover, CX3CR1 KO mice appear to show enhanced neutrophil chemotaxis via CCL5 and CXCL7 to their left ventricles compared to the remote right ventricle area. A pattern not observed in WT mice. Together, this data suggests a more potent and prolonged pro-inflammatory response and a reduction in recruitment of reparative cells in CX3CR1 KO mice. This observation is supported by MRI results, showing worse deterioration of cardiac function between 1 week and 6 weeks post reperfusion.

Overall, the results described in this chapter show that while reduced or absent expression of CX3CR1 slows down leukocyte and T cell recruitment, myocardial I/R injury stimulates the activation of compensatory chemotactic/cell adhesion mechanisms. The present study has not investigated the late I/R response (3-7 days I/R) but the absence of a significant reduction in T cell infiltration between WT and KO mice at 3 days I/R (Figure 3.22) is suggestive that leukocyte infiltration in KO mice may return to WT levels after >3 days I/R. However, this appears to only be regarding the overall number of infiltrating immune cells, not their phenotype. Although EGFP⁺ cell recruitment at 3 days I/R was not investigated, the results described here clearly show that complete knockout of CX3CR1 changes the composition of the inflammatory infiltrate in the acute MI response (2-24h I/R). As the results on Figures 3.19 and 3.22 show that complete absence of CX3CR1 leads to reduced infiltration of CX3CR1/EGFP⁺ T cells, while the number of total leukocytes remains consistent between WT and KO mice (Figure 3.8). However, more detailed analysis of the differential infiltrate was not possible in this study.

This was the result of the technical limitations of IF microscopy and the number of parameters that can be investigated, as well as the limited number of cells that can be isolated from cardiac tissue digest. The latter did not allow for a more expansive T cell flow cytometry panel to be used. Despite this, cardiac digest data does support IF studies in that there is a reduction in EGFP⁺ T cells infiltrating the left ventricular myocardium 24h post reperfusion between HET and KO mice (Figure 3.27). More importantly, the results on Figure 3.26 show that while there is no detectable difference in total T cells infiltration between the genotypes, there is trend of reduction in CD8 T cells with decreasing CX3CR1 expression. Indeed, baseline T cell phenotyping of this mouse line shows that circulating CD8 T cells have higher CX3CR1/EGFP expression than CD4 cells (Figures 3.4 and 3.5). Moreover, circulating effector CD8 T cells have the highest proportion of CX3CR1/EGFP expression (40-60% of total CD8 T cells). Therefore, these circulating CX3CR1⁺ T cells would be the ones to be rapidly recruited to the injured myocardium and are likely the ones to be “missing” from the inflammatory infiltrate in KO mice. However, the low numbers of isolated T cells did not allow for deeper T cell phenotyping in post MI cardiac tissue in order to investigate naïve, memory and effector T cell populations. Crucially, analysis of total CD3⁺ cell infiltrate may mask vast reduction in the small subset of CX3CR1⁺ CD8 effector T cells.

Furthermore, no investigation of T cell function or changes in Th/Tc polarisation was performed in the CX3CR1-EGFP mouse line. This may provide important insight as CX3CR1⁺ T cells are likely to be highly cytotoxic and pro-inflammatory such as Th1 or Th17 (Kobayashi, Okamoto et al. 2007, Lee, M., Lee et al. 2018). Although Th2 CX3CR1⁺ T cells have also been described (Mionnet, Buatois et al. 2010). Cytotoxic T cells are associated with elevated IFN γ and TNF α (Caza, Landas 2015, Mittrücker, Visekruna et al. 2014). Two pro-inflammatory cytokines that are known to be elevated in the early pro-inflammatory phase of MI (Westman, Lipinski et al. 2016) and can upregulate CX3CL1 expression (Lee, M., Lee et al. 2018). And while this project is based on the hypothesis that blocking CX3CL1/CX3CR1 mediated T cell recruitment is cardioprotective, the MRI data presented here is in contrast to this expectation. Indeed, HET and KO mice appear to have worse cardiac function than WT CX3CR1 mice. However, the model described in this chapter is investigating the effect of a global CX3CR1 knockout, not specific to T cells. While interaction between T cells and monocytes has been described in MI models showing delayed monocyte transition into reparative phenotype subsequent to CD4 T cell deficiency (Hofmann, Beyersdorf et al. 2012), CX3CR1⁺ monocytes

far outnumber CX3CR1⁺ T cells. Therefore, it may be hypothesised that the potentially cardioprotective effect of limiting cytotoxic T cell recruitment is lost by the reduced recruitment and development of M2 macrophages and non-classical monocytes. However, in order to successfully study the specific role of CX3CR1⁺ T cells in myocardial I/R injury, a T cell selective KO model is required.

In summary, the work described in this chapter shows that CX3CR1 knockout causes a delay and phenotypic change in the inflammatory infiltrate in the post MI myocardium. Absence of fractalkine receptor also appears to lead to more deteriorating left ventricular remodelling, although this was not statistically significant. However, there are multiple challenges with using a global knockout mouse model, especially when investigating the role of a rare cell population such as CX3CR1⁺ T cells. Moreover, the response to I/R injury is very complex, involving crosstalk of multiple cell types (and subtypes) and signalling pathways. Fractalkine itself has cell type- and tissue-specific roles that are often contrasting (Lee, M., Lee et al. 2018). Therefore, more detailed and specific study of the immune cell response is required. These two challenges are the focus of the rest of this PhD thesis.

Chapter 4. Deep immune profiling of myocardial I/R injury using mass cytometry

4.1 Introduction

In the previous chapter I described the need to use both immunofluorescence (IF) microscopy and flow cytometry to study the immune cell infiltrate in the infarcted heart. The use of either techniques has its advantages, as well as limitations. Flow cytometry provides a detailed picture of the overall inflammatory cell phenotypes. IF microscopy is limited to only four parameters (in our available system) providing limited information on cell phenotype but giving important spatial information about inflammatory infiltrate. In this chapter I will describe the use of a recently developed tool, called imaging mass cytometry (IMC) (Giesen, Wang et al. 2014), which allows for both high-dimensional, single cell analysis, as well as spatial information.

Mass cytometry is a novel alternative method to fluorescence flow cytometry, where a suspension of single cells is labelled with isotopically pure metal-labelled antibodies, rather than fluorescently conjugated ones. Around 40 different metal isotopes can be detected and quantified simultaneously using inductively coupled plasma mass spectrometer (ICP-MS), avoiding the challenges of spectral overlap in standard fluorescent flow cytometry. The technique is described in detail in several publications (Tanner, Baranov et al. 2013, Olsen, Leipold et al. 2019) and is depicted in Figure 4.1. In short, cells stained with metal-labelled antibodies are aerosolised through a nebuliser and carried in a flowing argon stream into a high-temperature plasma (ICP) torch. As the metal-tagged cells travel through the $\sim 7000^{\circ}\text{K}$ plasma, they are vaporised, atomised and ionised. The resulting positively charged ion cloud passes through a series of vacuum cones where it cools down and focuses into a beam of ions. The ion beam then travels through an electrostatic quadrupole ion deflector which filters out neutral particles. Lower mass ions (under 100Da), originating from organic molecules, are further filtered out using a radio frequency quadrupole. This leaves a filtered ion cloud, consisting only of ions of analytical interest, which are passed through a time-of-flight (TOF) chamber. There the ions are accelerated by voltage pulses and leave the TOF chamber according to mass (from low to high mass as lighter ions travel faster and reach the detector first). Upon reaching the detector, the ions trigger a series of electron amplifications, leading to an electron pulse that can be recorded and digitised as single cell readouts.

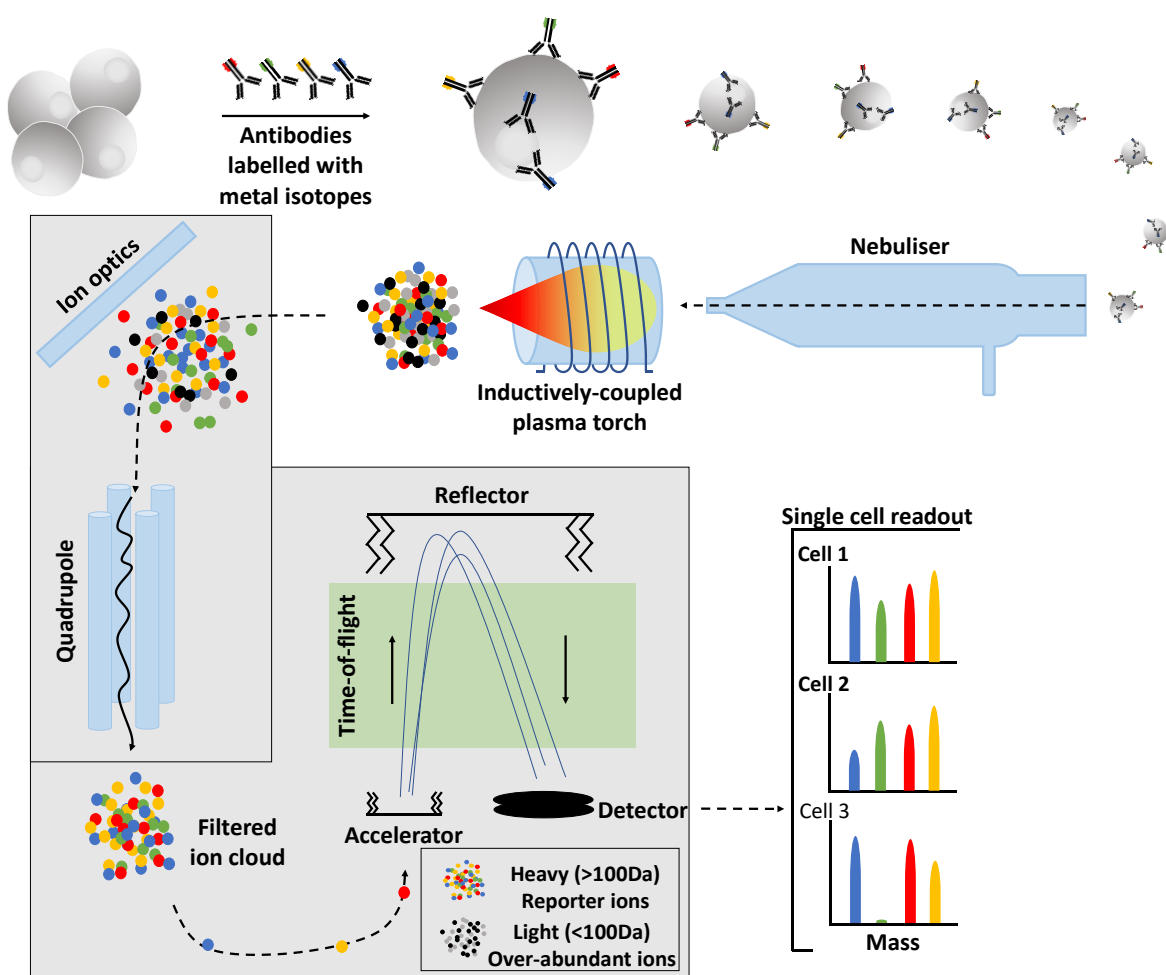


Figure 4.1 Suspension mass cytometry (CyTOF) workflow. Cells are stained with metal isotope-conjugated antibodies and run through the mass cytometer. The cells are aerosolised in the nebuliser and carried by an argon stream into an inductively-coupled plasma torch which vaporises, atomises and ionises the conjugated cells. Large positively charged ions are selected by electrostatic and radio frequency quadrupoles before passing through a time of flight (TOF) chamber. Electron pulses are then detected and digitised as a single cell readout.

This results in high-dimensional single cell data that currently cannot be matched by the most advanced fluorescence flow cytometers. However, suspension mass cytometry, like fluorescence flow cytometry, does not provide any spatial information as the tissue has to be dissociated either mechanically or enzymatically. The need for this gap of high-dimensional single cell spatial data to be filled has led to the development of IMC. First described by Giesen et al in 2014, the technique presents a fusion between CyTOF mass cytometry and immunohistochemistry (Giesen, Wang et al. 2014). Tissue sections immobilised on slides are stained with metal-conjugated antibodies, followed by a series of 1µm UV laser ablation shots at a frequency of 100-200Hz (Figure 4.2). The stained tissue is vaporised on each laser shot and the particles are carried via an argon/helium flow into the plasma of the mass cytometer. The abundance of metal isotopes in each 1µm of tissue is then simultaneously measured as

described above using the CyTOF mass cytometer. A region of interest (ROI) from the tissue is scanned spot-by-spot by the laser beam, ultimately producing a map of the intensity of all target markers across the ROI. The images produced have a resolution comparable to x20 microscope magnification but with a much higher number of parameters due to the use of isotopically labelled probes. Furthermore, IMC eliminates common IF challenges like tissue autofluorescence and limited number of target markers due to spectral overlap.

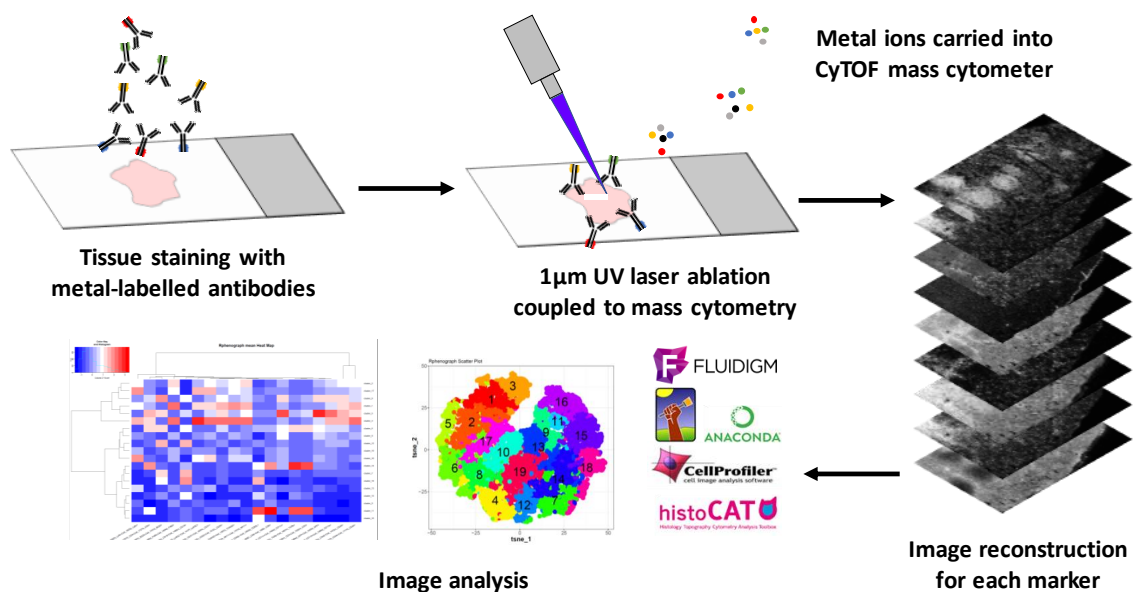


Figure 4.2 Imaging Mass Cytometry (IMC) Workflow. Tissue sections are stained with metal isotope-conjugated antibodies and series of 1µm UV laser shots ablate a selected region of interest. The laser ionises the stained tissue and the ion cloud is carried into a CyTOF chamber. The abundance of each isotope is measured for each 1µm of tissue, producing an image showing the intensity of each marker. Single cell data is then extracted following image processing using an IMC analysis pipeline.

It is important to note that there are certain challenges with the analysis of mass cytometry data, shared with other emerging high-dimensional -omics data. Conventional fluorescence flow cytometry data is usually analysed using “manual gating” as described in the previous chapters. However, gating on two-dimensional scatter plots has certain limitations including subjectivity and difficulty in identifying unknown populations (because gating is based on pre-existing knowledge of marker expression patterns). The use of biaxial plots is an even greater challenge for high-dimensional data such as mass cytometry due to the large number of measured parameters per cell. Therefore, partially or fully automated analysis methods have to be used for the multidimensional structure of these data sets to be visualised and reliably and reproducibly analysed. Clustering analysis is a commonly used method of computer-assisted gating which can be either supervised or unsupervised, depending on whether or not

the operator inputs external variables to train a model which can then predict the features of new samples (Weber, Robinson 2016). Ultimately, these specialised algorithms group together (cluster) cells with similar marker expression which can then be analysed individually or across multiple samples. As this is an un-biased, data-driven, multi-dimensional analysis approach, in contrast to the manual gating of “known knowns”, it is better suited to detect and more confidently identify previously unknown or rare cell populations.

This makes this technique very valuable and useful for this project as it can be used to investigate in detail the immune response following myocardial ischaemia reperfusion. CyTOF and IMC allow for deep immunophenotyping of I/R inflammation – identifying multiple major immune cell populations with a broad panel of markers (described below) while retaining the spatial information (currently only possible with microscopy-based techniques) which is critical for advancing our understanding of myocardial infarction. IMC is still a novel methodology with existing studies primarily focusing on human samples, in part due to the high number of historic formalin-fixed-paraffin-embedded (FFPE) patient samples. However, while studies on human tissue are more clinically relevant, animals studies are invaluable by allowing studies of gene deletion and time course studies. At the time of writing this thesis, only a few research groups have published studies on mouse tissue analysis using IMC (Japp, Hoffmann et al. 2017, Cole, Park et al. 2018, Widjaja, Singh et al. 2019, David, Rezende et al. 2016), which are key in advancing scientific research by performing experiments that would be unethical in human clinical trials. Given the limited published application of this method on mouse research and fewer available validated IMC mouse antibodies, it is essential that such IMC analysis protocols and pipelines are carefully developed and validated. Therefore, this part of my PhD research project was to: (1) successfully establish this novel method to confidently detect a range of different immune cell types within the mouse spleen, and (2) to characterise which immune cell populations are present during the acute I/R response in a surgical mouse model of heart injury.

4.2 Results

4.2.1 Antibody validation

The mass cytometry antibody panel, shown on Table 4.1, was designed to have minimal cross-talk between markers that are expressed on the same cells and antibodies were metal-conjugated in-house. Therefore, the antibodies had to be validated by testing them on beads and splenocytes in suspension to confirm successful conjugation to the correct metals, as well as to show that antibody specificity has not been affected by the metal conjugation process.

Marker Name	Metal	Population
Gr-1 (Ly6G/C)	141Pr	Granulocytes/Macrophages
CD11c	142Nd	DCs/some NKs
CX3CR1 (GFP)	145Nd	Macrophages/Monocytes
F4/80	146Nd	Macrophages/DCs
CD45	147Sm	Pan-Leukocyte
CD11b	148Nd	Monocytes/Granulocytes/Macrophages
CD19	149Sm	B cells
CD25	151Eu	Tregs
CD3	152Sm	T cells
TCRgd	159Tb	gd T cells
CD62L	160Gd	Naïve T cells
Ly6C	162Dy	Monocytes
CD31	165Ho	Endothelial cells
CD8	168Er	T cells
TCRb	169Tm	ab T cells
NK1.1 (CD161)	170Er	NK/NKT cells
CD44	171Yb	All leukocytes
CD4	172Yb	T cells
Pan-Actin	175Lu	Structural
B220 (CD45R)	176Lu	B cells

Table 4.1 Mass Cytometry Antibody Panel.

4.2.1.1 Antibody conjugation

The antibody conjugation method, described in section 2.9.2, results in a 100µg of each antibody being conjugated to a specific lanthanide metal tag. This is confirmed using suspension mass cytometry, where each antibody-metal complex is bound to beads via the Fc region of the antibody and then run through the suspension mass cytometer. A signal would be detected only if there is antibody present to bind to the beads and if there is a metal tag successfully conjugated to the antibody. The results on Figure 4.3 are an example of three antibodies (CD11b, CD62L and NK1.1) being tested in a single sample. The figure shows that bead-antibody-metal complexes are detected for 148Nd-CD11b, 160Gd-CD62L and 170Yb-

CD161 (NK1.1), confirming successful antibody conjugation. This conjugation validation step was performed for each antibody vial that was used prior to any further experiments.

The three metal isotopes on Figure 4.3 show a low level of cross-talk, represented by the three bead-antibody-metal populations with low, medium and high signal intensity for ^{148}Nd , ^{160}Gd and ^{170}Yb . This is not unexpected as these metal isotopes are known to have $\geq 0.5\%$ cross-talk into/from other metal probes due to probe purity and oxides (information provided by probe manufacturer Fluidigm). However, this low channel cross-talk would not affect cell population identification in subsequent analysis as a result of the careful antibody panel design described in section 2.9.1. Namely, these metal isotopes are conjugated to antibodies targeting CD11b, CD62L and CD161 (NK1.1), which are not co-expressed in any of the cell types of interest. Furthermore, the populations that these markers identify (myeloid cells, naïve T cell and NK cells) will be identified by the presence and/or absence of additional markers.

4.2.1.2 Suspension cells

Following successful conjugation, antibody binding was validated on suspension cells. Mouse splenocytes were used for this as the spleen contains all major immune cell populations that are targeted by the panel and because they can be isolated without the use of any enzymatic digestion which could affect epitope expression. Similar to flow cytometry data, the results on Figure 4.4a show the manual gating strategy used to identify all cell populations. In addition to the manual gating, the suspension mass cytometry data is also analysed using a less manual clustering method, as described in section 2.9.3.2. The results in Figure 4.4b and c show that the clustering algorithm is successfully identifying the major lymphocyte populations, as well as some of the myeloid cells, such as macrophages, DCs, neutrophils and $\text{Ly6C}^{\text{high}}$ monocytes. However, myeloid cells present an additional challenge in clustering and correctly identifying each population on a heatmap as most of them express several of these markers to a various degree, in contrast to bimodal expression patterns of most lymphocyte markers. While Ly6C is used to distinguish $\text{Ly6C}^{\text{high}}$ classical and Ly6C^{low} non-classical mouse monocytes, it is also expressed on macrophages, populations of memory T cells and endothelial cells (Jutila, Kroese et al. 1988, Cerwenka, Carter et al. 1998). Furthermore, CD11b is a widely expressed marker, including on the surface of neutrophils, monocytes, and DCs (Springer 1994, Coxon, Rieu et al. 1996, Christensen, Andreasen et al. 2001). F4/80 is a commonly used marker to identify macrophages but its expression has also been shown for a population of DCs, as well as eosinophils (Ruedl, Rieser et al. 1996, Dos Anjos Cassado 2017). Similarly, CD11c is a marker

for DCs but its expression has also been reported for monocytes, macrophages and neutrophils (Springer 1994, Helft, Böttcher et al. 2015).

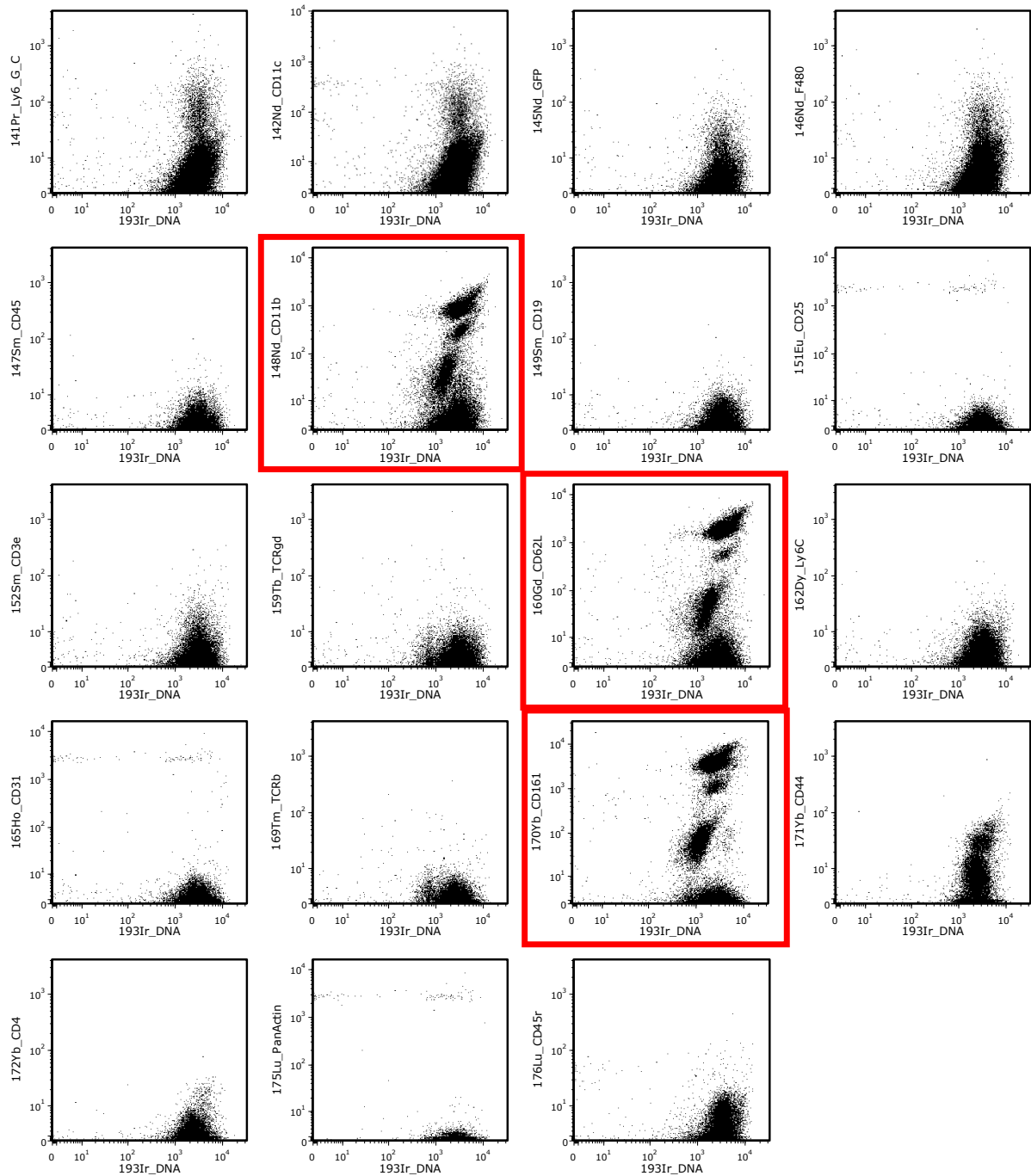
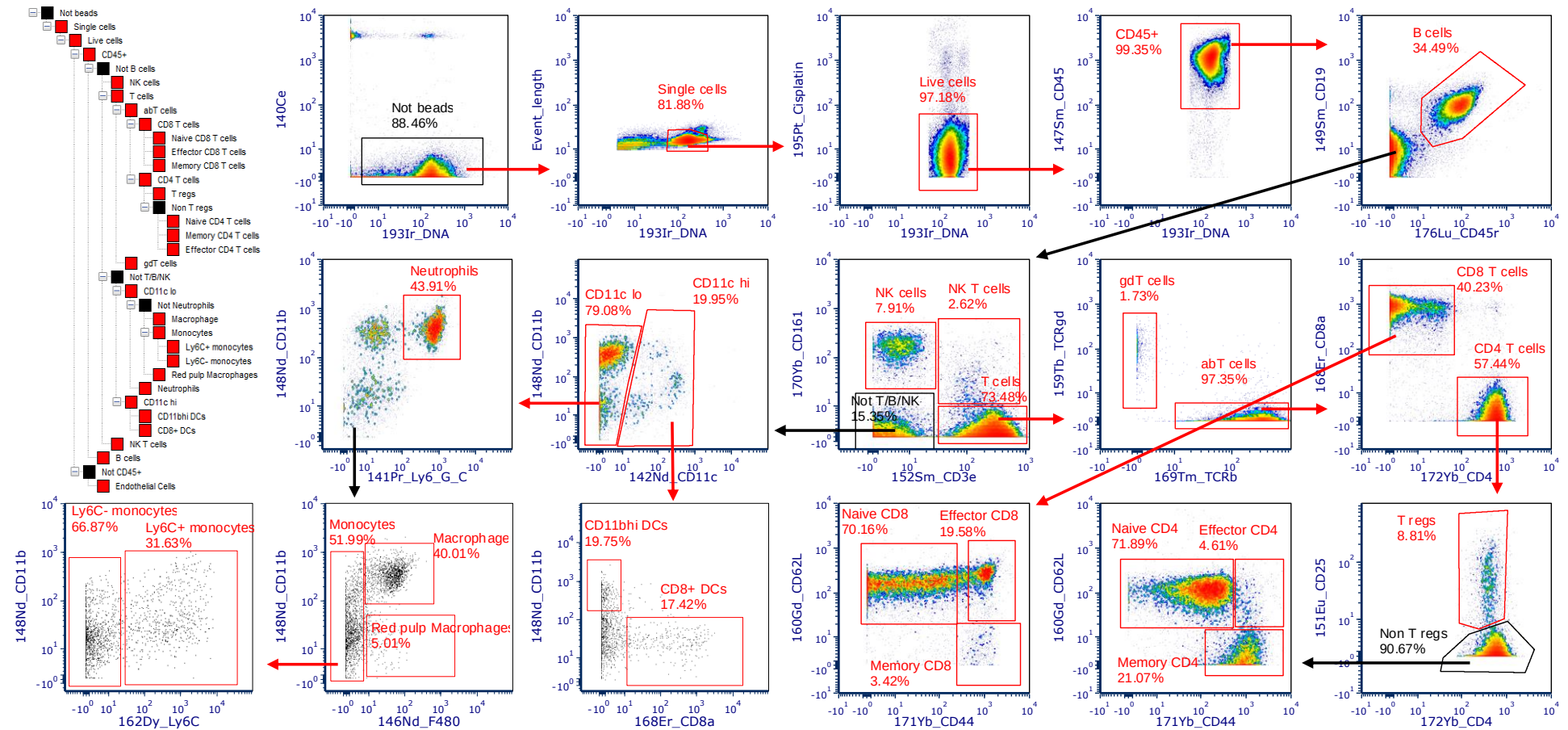


Figure 4.3 Validation of antibody conjugation. The metal-tag conjugation for three antibodies was tested on beads using suspension mass cytometry. Signals for 148Nd CD11b, 160Gd CD62L and 170Yb CD161 (NK1.1) are detected in the corresponding channels (highlighted).

(a)



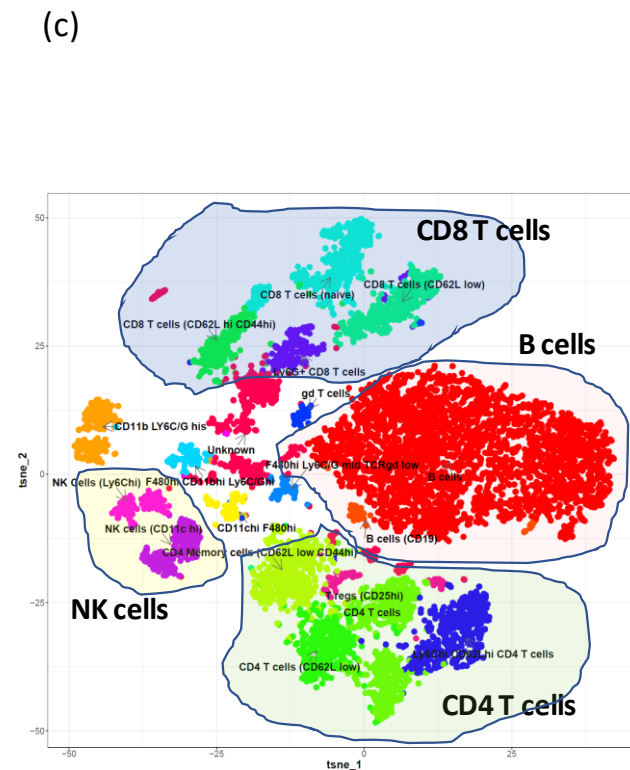
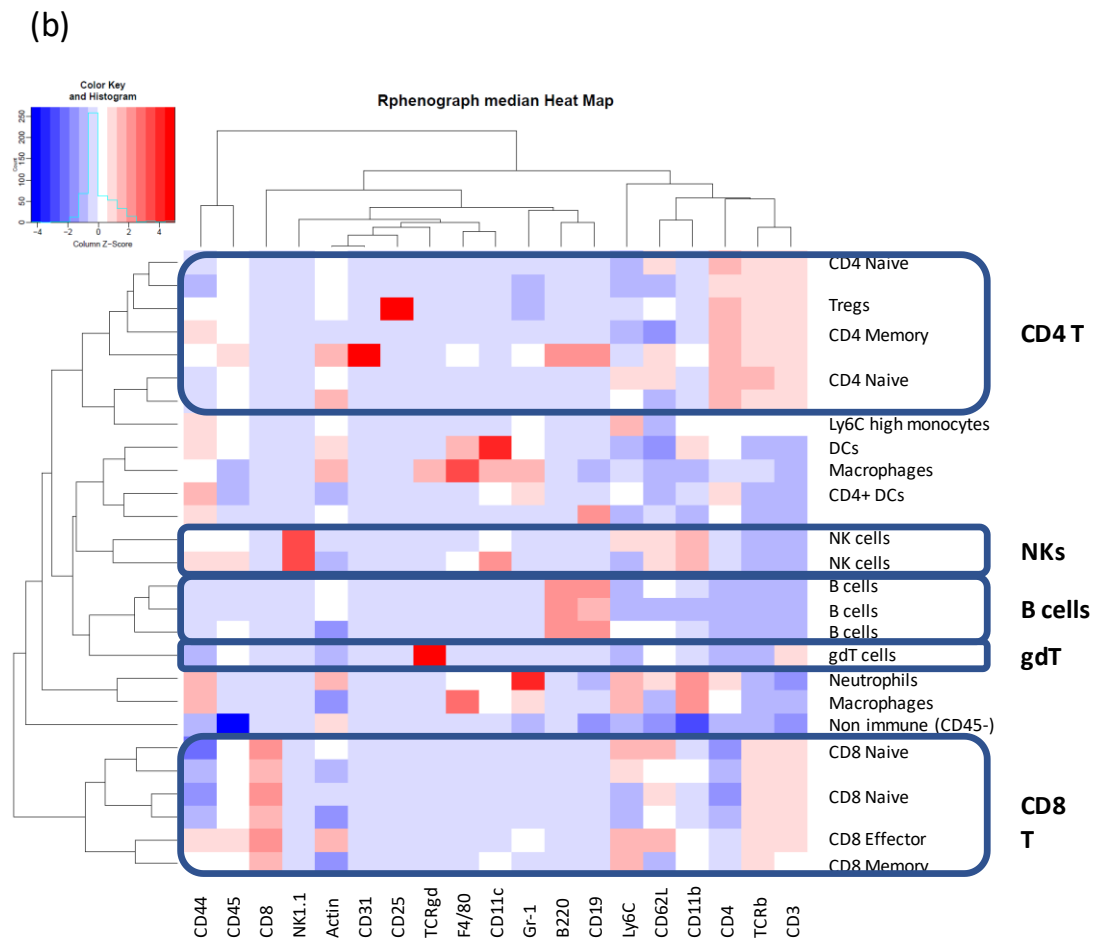


Figure 4.4 Analysis of suspension mass cytometry mouse splenocyte data. (a) Manual gating strategy for suspension mass cytometry. Cells were stained for surface markers and the populations in the gating hierarchy (top left) were identified using manual gating. Arrows show which populations each gate originates from. Red arrows/gates show gates that originate from the specified population. Black arrows/gates show gates that exclude a population, i.e. Not Gate. (b) Heatmap showing Phenograph clustering. Cell populations are manually identified based on the expression pattern. (c) t-SNE visualisation of the cell clusters, identified by the Phenograph algorithm.

The last step of the validation pipeline is to assess the effect of the fixation, used for the tissue samples (4% formaldehyde for 10min at room temperature), that can affect the protein epitope and thereby reduce antibody binding. To do this, dissociated spleen cells from the same mouse were either stained and then fixed (normal suspension cell staining protocol) or fixed and then stained (cryo preserved tissue section staining protocol). Antibody binding to eight of the 18 cell surface markers was significantly reduced following fixation. As shown on Figure 4.5 mean marker levels were significantly reduced for CD45, CD19, CD25, CD62L, CD31, CD8, TCRb and CD4 when staining was performed after formaldehyde fixation. The effect on some of the epitopes was also observed in the percentage of each population. As shown on Figure 4.6, T cells were primarily affected as the overall percentage of TCRb⁺, CD4⁺, CD8⁺ T cells was reduced following fixation. Furthermore, there was significant reduction in the CD4/CD8 subpopulations (naïve, effector memory), as well as an 80% drop in the percentage of detected Tregs. In addition to the drop in T cells, the CD8 antibody epitope appeared to be affected by the formaldehyde as the percentage of CD8⁺ Dendritic Cells was significantly changed when the cells were fixed prior to antibody staining. The results also show there is no effect on the percentage of total leukocytes detected (CD45⁺ cells) and a 10% increase in the number of B cells was observed. However, by comparing the gating dotplots for both methods (Figure 4.7), it is evident that the main effect of 4% formaldehyde fixation before staining is on binding of CD25 and CD62L antibodies. While there is no change in the Treg population resolution, the number of events in the CD25⁺ gate is more than halved due to the fixation. CD62L gating is even more challenging as fixation makes it virtually impossible to identify and gate on the CD62L low population (memory CD4/CD8 T cells) because it is merged with the CD62L⁺ CD44⁺ effector CD4/CD8 T cell population (Figure 4.8). Therefore, we would expect that cells expressing CD25 and CD62L will be underrepresented in the IMC data due to the tissue fixation. This is a limitation of the staining method that cannot be completely avoided but which can be attempted to be reduced by using higher concentrations for these two antibodies in IMC staining to make sure all available epitopes are saturated. Further tests on suspension cells using the increased CD25 and CD62L antibody concentrations were not performed as it was decided to test them on spleen sections using IMC.

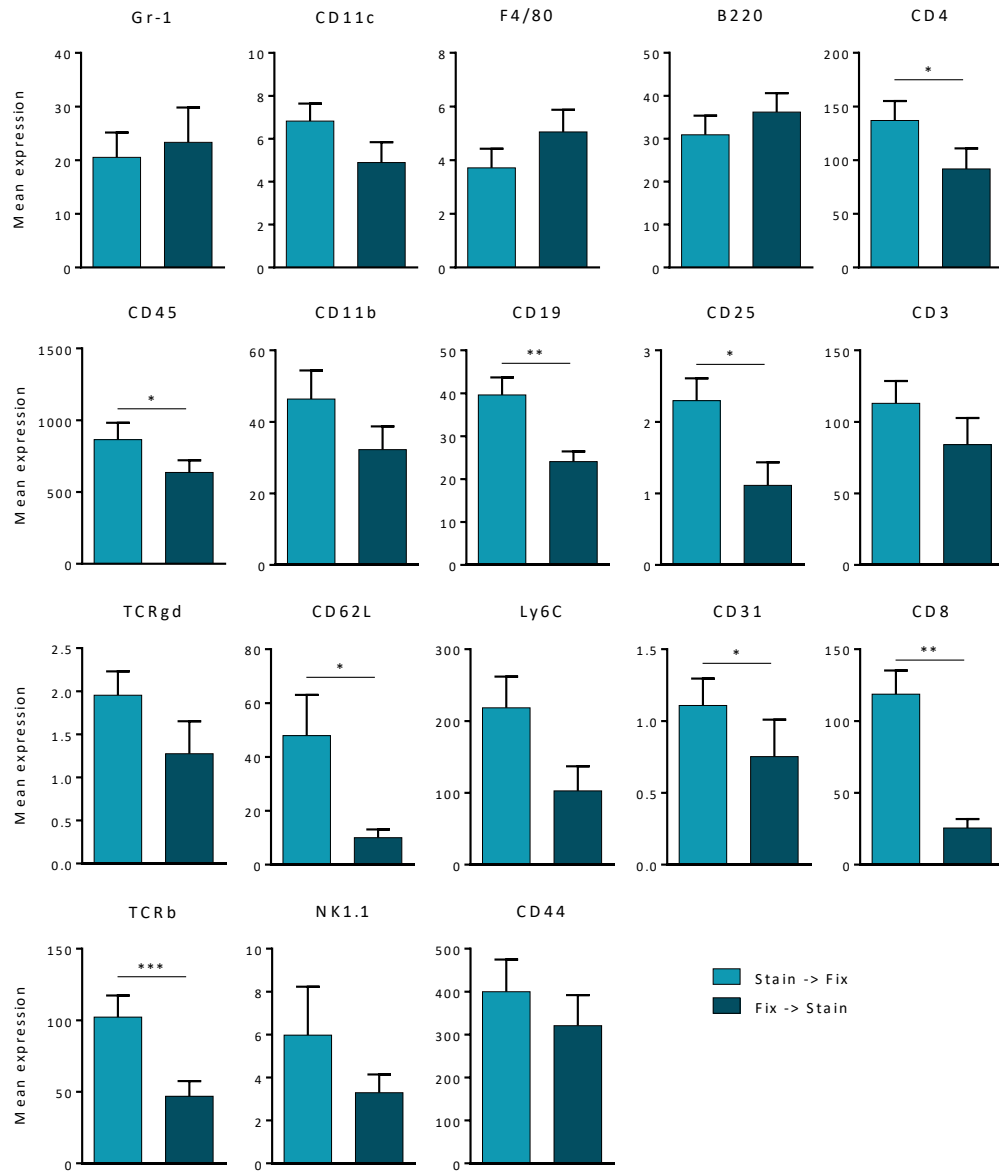


Figure 4.5 Mean surface marker expression. The level of each marker was measured for the total Live Cells gate when cells from the same spleen were stained either before or after fixation with 4% formaldehyde for 30 minutes at room temperature. Error bars = SEM; n=5; Paired t-test; * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

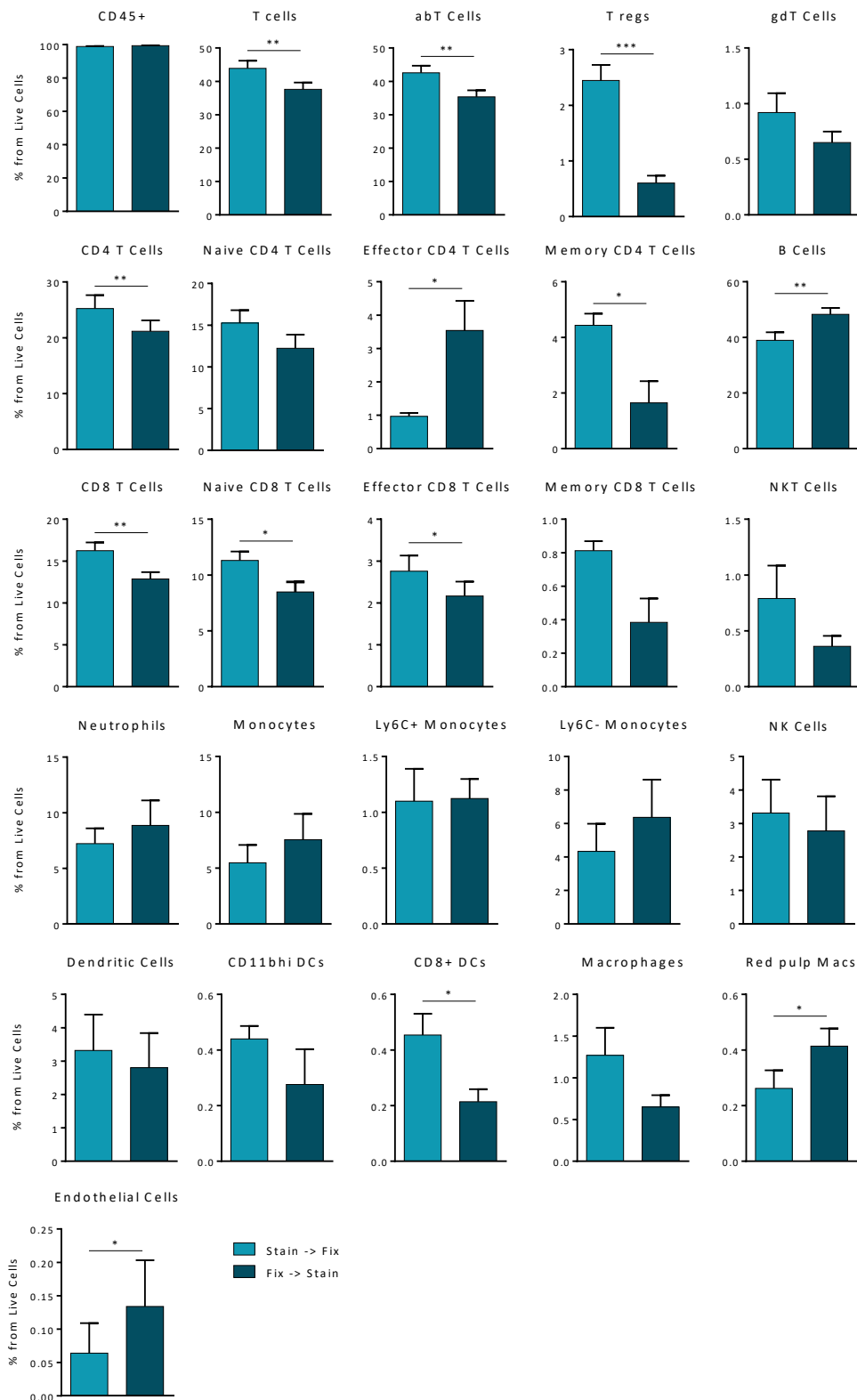


Figure 4.6 Population distribution of mouse splenocytes. The percentage of each population from total Live Cells was measured for cells from the same spleen that were stained either before or after fixation with 4% formaldehyde for 30 minutes at room temperature. Error bars = SEM; n=5; Paired t-test; * = $p \leq 0.05$, ** = $p \leq 0.01$.

(b)

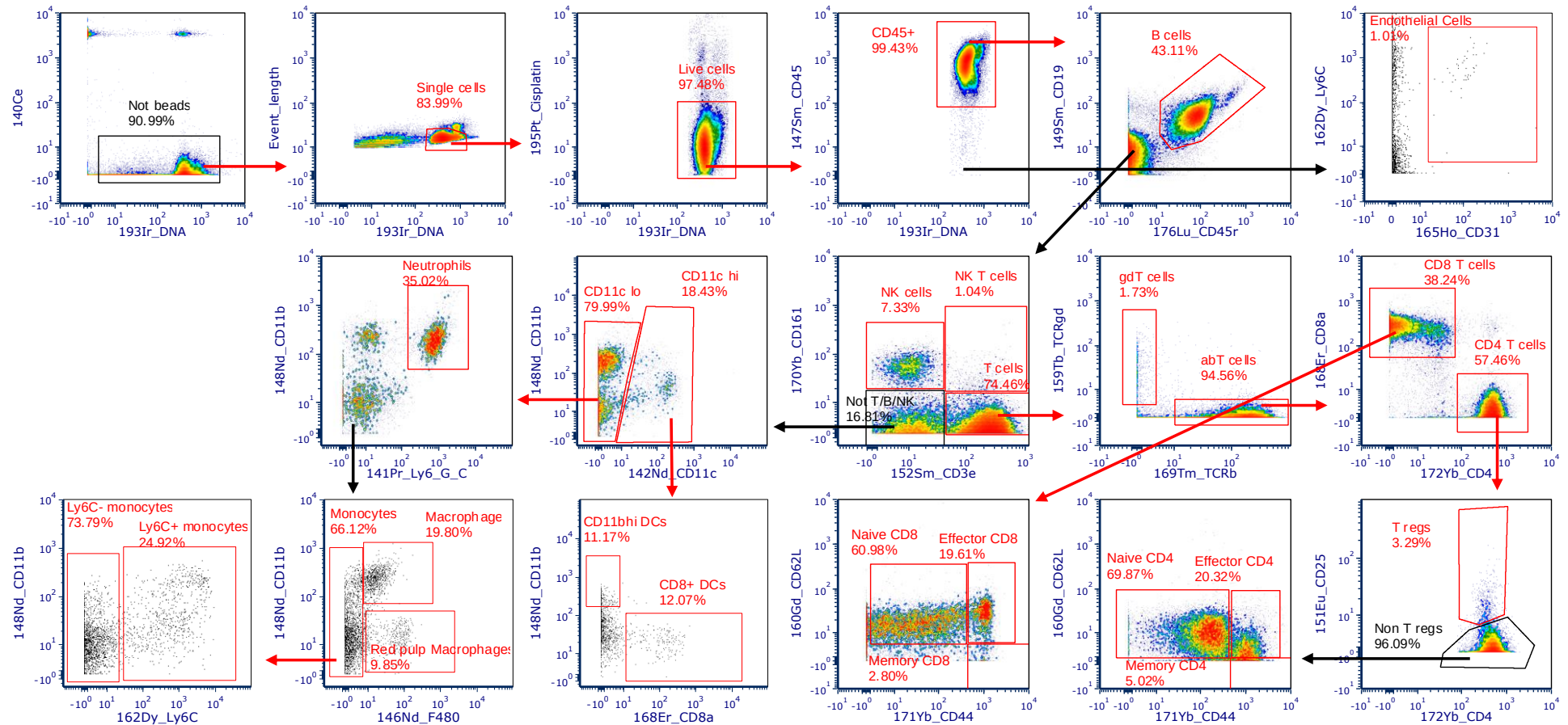


Figure 4.7 Manual gating plots for different staining protocols. Representative examples for (a) cells were stained and then fixed and (b) cells were fixed and then stained. Arrows show which populations each gate originates from. Red arrows/gates show gates that originate from the specified population. Black arrows/gates show gates that exclude a population, i.e. Not Gate.

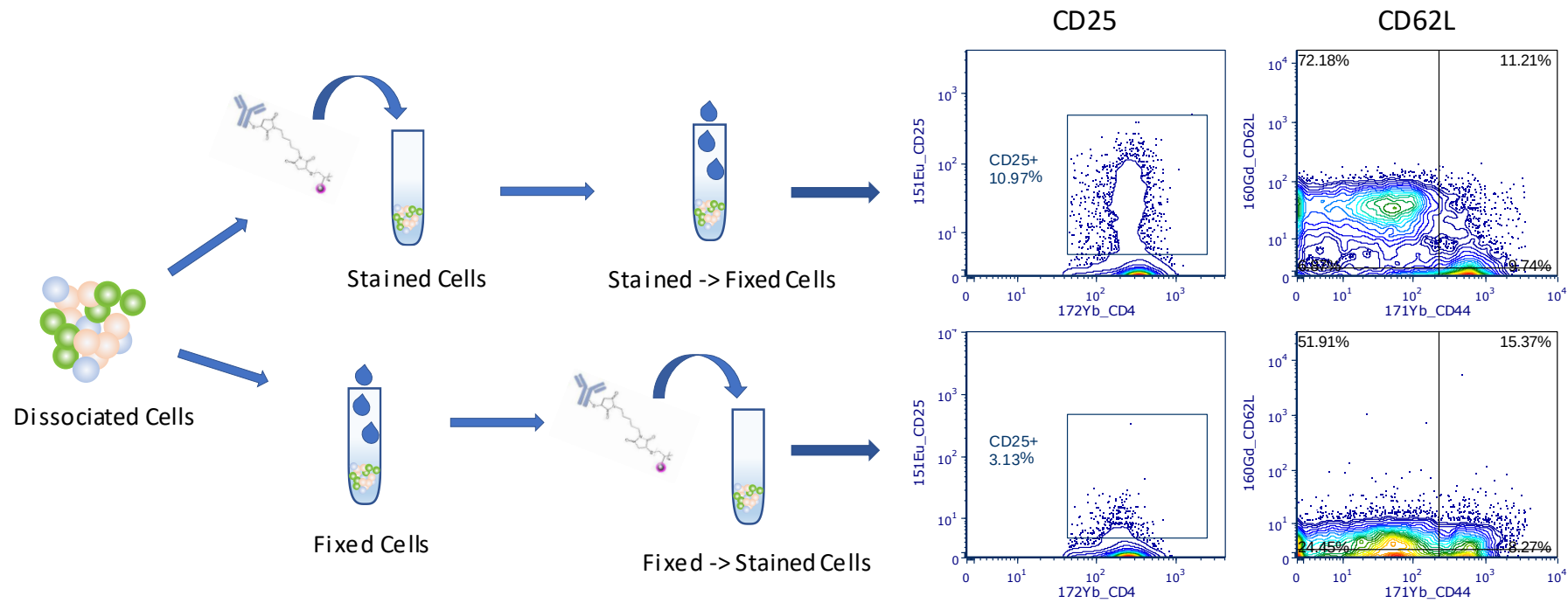


Figure 4.8 Effect of formaldehyde fixation on binding of CD25 and CD62L antibodies. Mouse splenocytes were either stained and then fixed with 1% formaldehyde or fixed prior to antibody staining. Cells were fixed for 30min at room temperature.

4.2.2 IMC method development

Following the successful panel design and antibody validation described above, the IMC method in section 2.9.4 was established and optimised in fixed frozen mouse spleen tissue. This tissue was chosen for the method development as it provided several advantages. Firstly, all major immune cell populations, targeted in our panel, are present in high numbers. Secondly, it allows for direct comparison to be made with the suspension data in section 4.2.1.2, confirming that populations have been correctly identified. Lastly, mouse splenic architecture is very well described, with distribution of known cell populations in the clearly defined red and white pulp (Lewis, Williams et al. 2019). This allows for any non-specific antibody binding to be easily identified if biologically impossible staining is observed, e.g. presence of B cells in the red pulp.

4.2.2.1 Tissue processing

Initially, the same tissue processing method was used for the spleens as described for the hearts in section 2.3.1. However, this method resulted in poor DNA staining in spleen both using standard immunofluorescence microscopy, as well as IMC. As shown on Figure 4.9a and b, there is clear, bright DNA staining on the periphery of the spleen, which becomes dimmer and more diffused further into the tissue. This is especially observed in the lymphocyte follicles (encircled on the image) where the cell density is highest. This issue is limited to the spleen, as no such staining pattern was observed in the less densely cellular heart tissue (Figure 4.9c and d). Therefore, several different tissue processing methods were tested on parts of the same spleen sample (Figure 4.10). The results show that fixing the tissue with either 1% Formaldehyde or 10% Formalin overnight at 4°C, respectively, prior to 30% sucrose immersion and cryo embedding, results in patchy DNA staining at x20 magnification (IMC equivalent). However, no pre-fixing of the tissue gives bright DNA staining throughout the spleen, including clearly defined lymphocyte follicles. The same staining pattern was observed using IMC. The iridium staining on Figure 4.11 shows that no pre-fixing gives the clearest DNA staining with clearly identifiable white pulp follicles. Subsequently, this method of no-prefixing, 30% sucrose immersion overnight, followed by cryo embedding on dry ice was chosen for future spleen tissue processing.

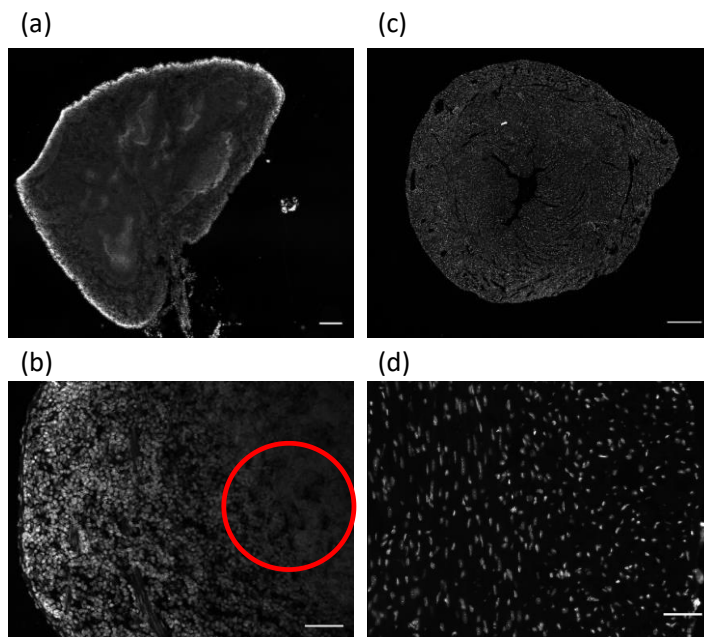


Figure 4.9 DNA staining of heart and spleen sections. Sections were processed using the original heart processing method (1% Formaldehyde overnight at 4°C, 30% sucrose overnight, cryo embedding over dry ice). 10µm sections were stained with DAPI and imaged using fluorescence microscopy. (a) x5 spleen, 200µm scale bar; (b) x20 spleen, 50µm scale bar; (c) x5 heart, 500µm scale bar and (d) x20 heart images, 50µm scale bar.

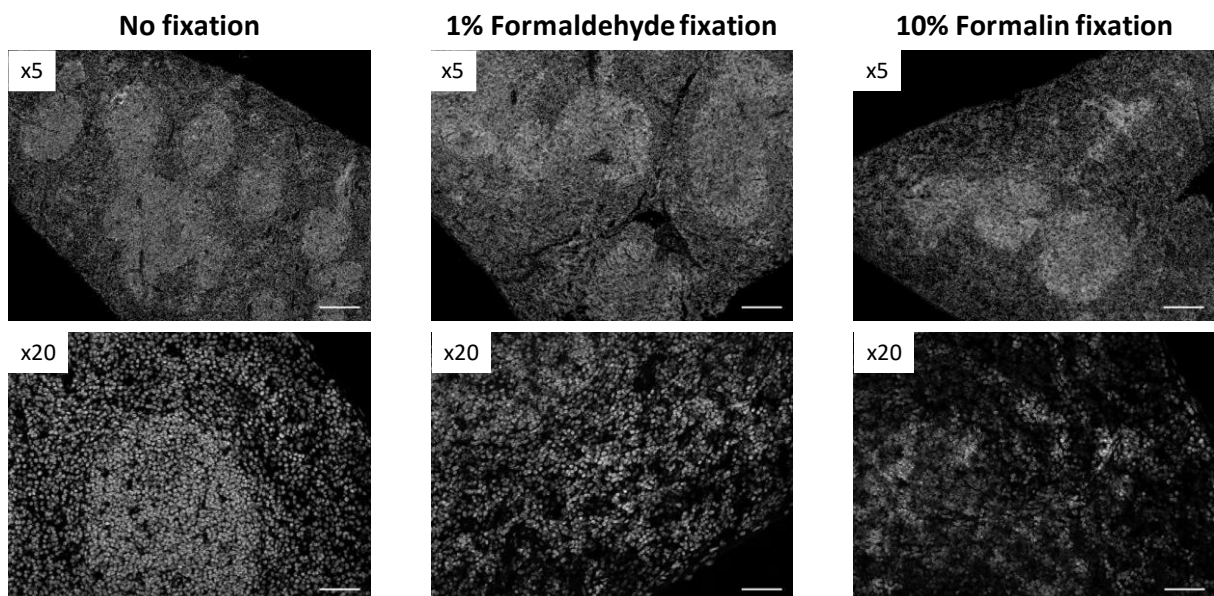


Figure 4.10 DNA staining of spleen processed using different methods. Spleen samples were processed using three different overnight tissue processing methods before cryo embedding. 10µm sections were stained with DAPI and imaged using fluorescence microscopy. Scale bars are 200µm for x5 images 50µm for x20 images.

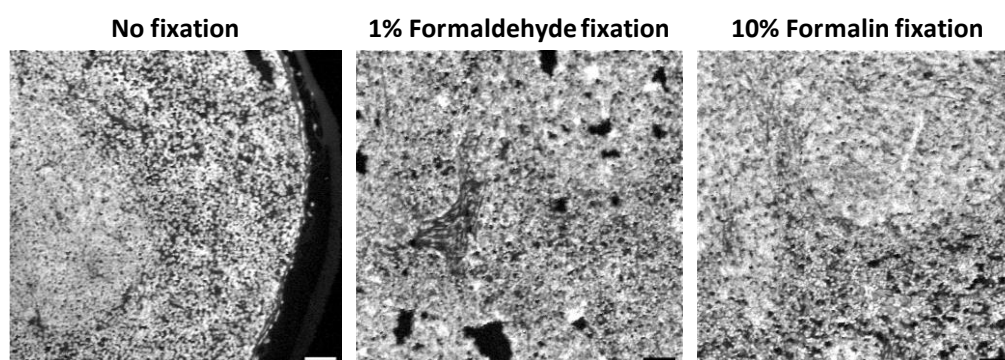
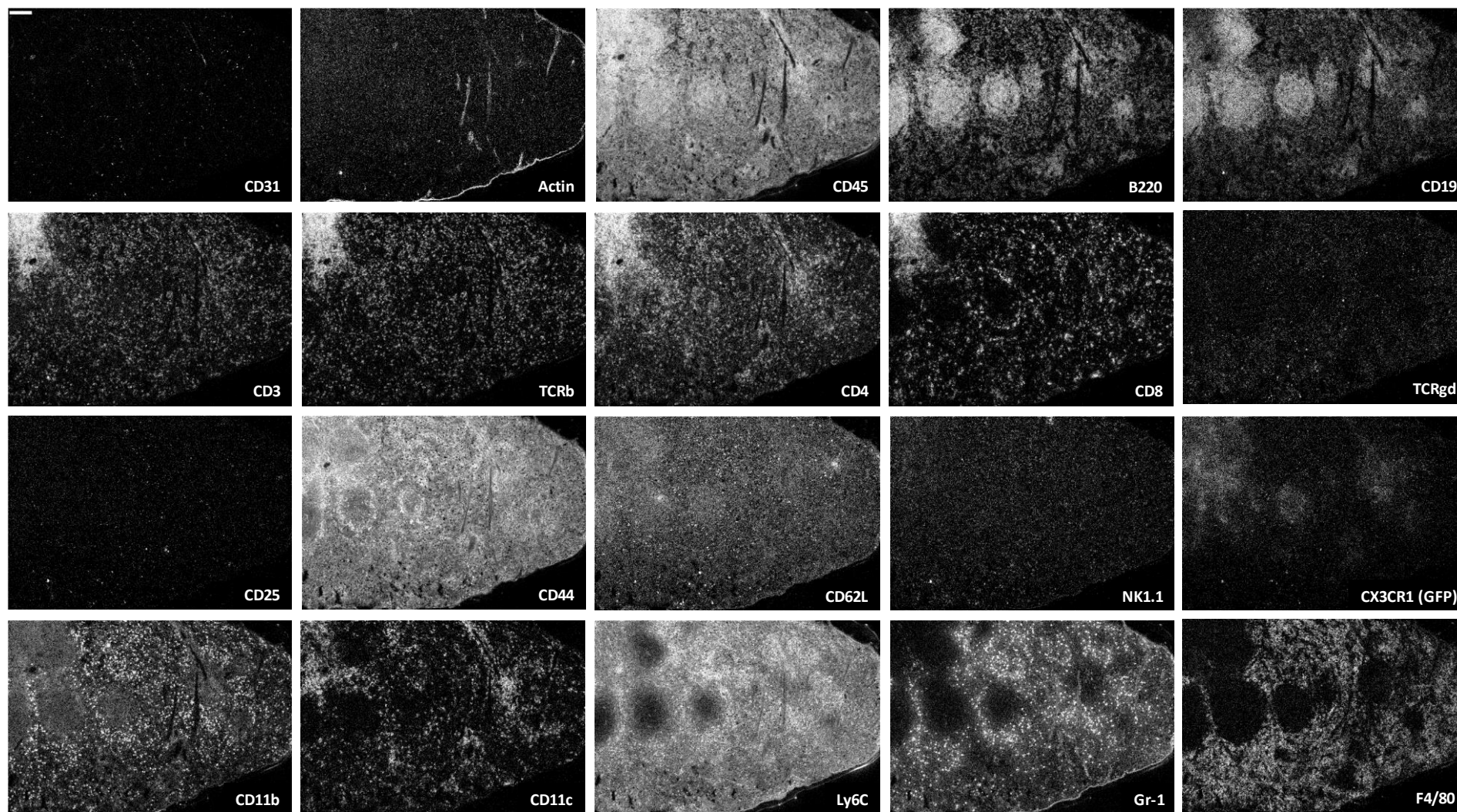


Figure 4.11 DNA staining of spleen processed using different methods. Spleen samples were processed using three different overnight tissue processing methods before cryo embedding. 10µm sections were stained with Iridium and data was acquired using imaging mass cytometry. Scale bars = 50µm.

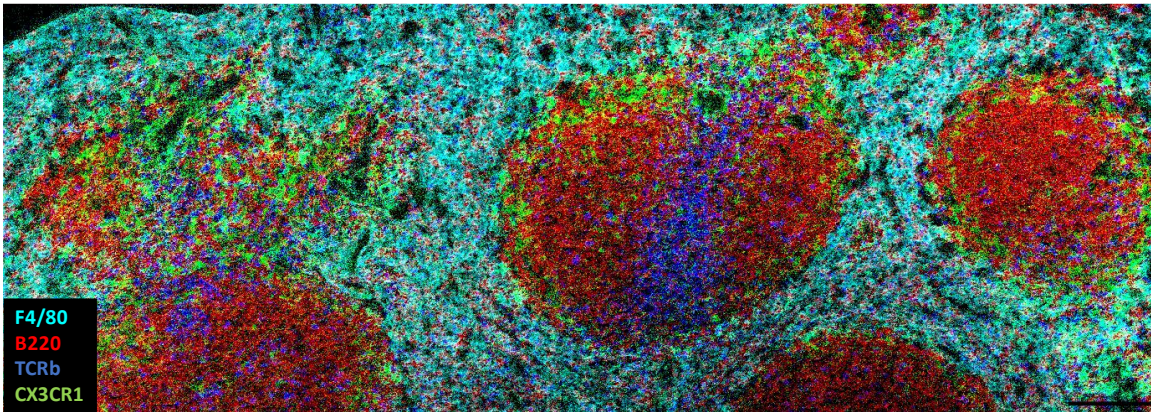
4.2.2.2 Data analysis pipeline to single cells

Following the thorough antibody validation and optimisation of the tissue processing method, described above, all antibodies were successfully detected on normal CX3CR1-EGFP heterozygous mouse spleen (Figure 4.12). As seen on the figure, all antibodies produce a clear signal in the “correct” spleen area. B cell markers B220 and CD19 strongly stain the B cell follicles which are surrounded by T cells (CD3⁺, TCRb⁺, CD4⁺, CD8⁺). The red pulp is stained for macrophages (F4/80⁺), neutrophils (Gr-1⁺), dendritic cells (CD11c⁺), as well as common myeloid markers CD11b and Ly6C. As expected, there is not as strong CD25, NK1.1 and TCRgd staining because these cell populations (Tregs, NK cells and gdT cells, respectively) are much rarer. The whole section is stained for CD45 and CD44, which are expressed by all leukocytes. CD31 staining did not show clear endothelial staining and therefore the amount of this antibody was doubled from (0.57µl/test to 1.14µl/test) in future experiments to improve subsequent staining.

(a)



(b)



(c)

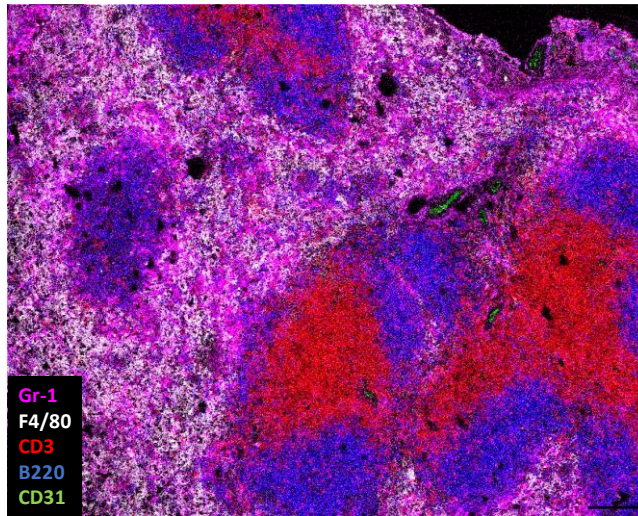


Figure 4.12 Imaging mass cytometry of mouse spleen. The full antibody panel was used to stain 10 μ m spleen sections from a CX3CR1-EGFP heterozygous mouse. (a) Grey-scale images of one marker per image; (b) and (c) false colour images of several markers per image; scale bar = 100 μ m.

The output of IMC data is a stack of grey-scale images with per-pixel measurements, each one showing the abundance of one protein (as shown on Figure 4.12a). The challenge with such high content data is to extract single-cell information by segmenting these images. The method used here is described by Sommer et al. called Ilastik (C. Sommer, C. Straehle et al. 2011). This is a semi-supervised, automated, pixel classification machine learning technique. The classifier is trained to recognise background, nuclei and membranes (based on selected markers), producing uncertainty and probability maps shown on Figure 4.13, which guide the training. Following this, the images are processed through a CellProfiler pipeline which creates a segmentation mask (Figure 4.13d). This segmented cell data is then used for further downstream analysis using histoCAT, allowing visualisation, clustering classification and cell

interaction analysis. Like for the suspension data, these cell clusters are then manually identified based on the expression pattern presented in the heatmap.

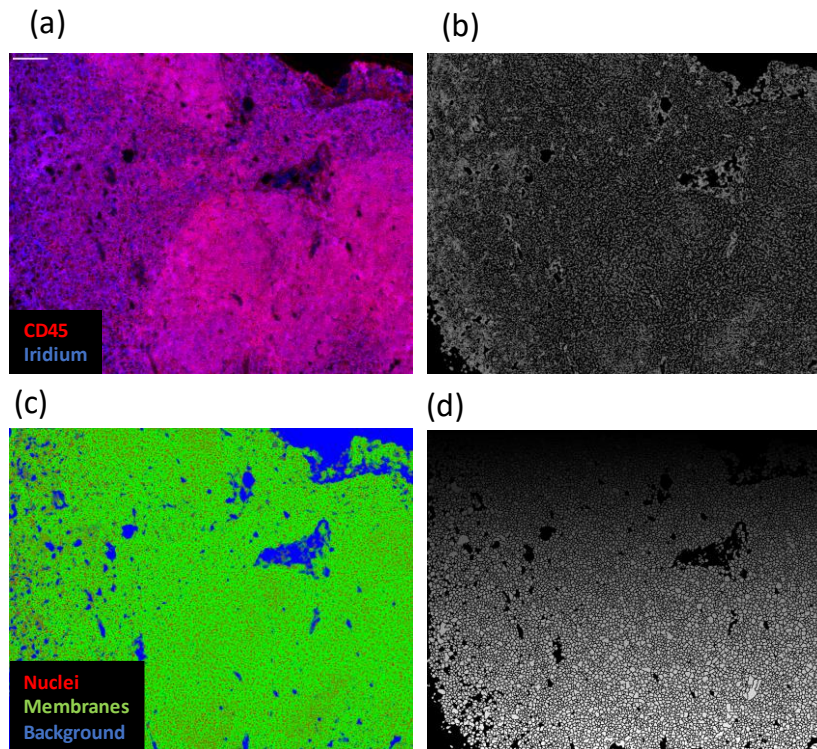


Figure 4.13 Image processing using the Ilastik pipeline. (a) False colour IMC image showing CD45 and Iridium staining. (b) Uncertainty map showing which pixels the classifier benefits the most if they are labelled/trained. A well trained classifier has low uncertainties within a class region (nuclei) and high within a class border (nuclei-membrane border). (c) Probability map of each class which is used for image segmentation. (d) Image mask showing single cell segmentation, used for downstream analysis. Scale bar = 100µm.

The analysis on Figure 4.14, using CD45, CD3, CD8, CD44, CD4 and B220 as markers for cell segmentation, results in the identification of a single T cell cluster that is double positive for CD4 and CD8 (green box). This is not biologically possible in a normal spleen sample, and the suspension data confirms this is not an artefact from the staining method/antibody binding. Therefore, this suggests this is due to incomplete segmentation of the images which was subsequently addressed by reducing the section thickness or ablation depth.

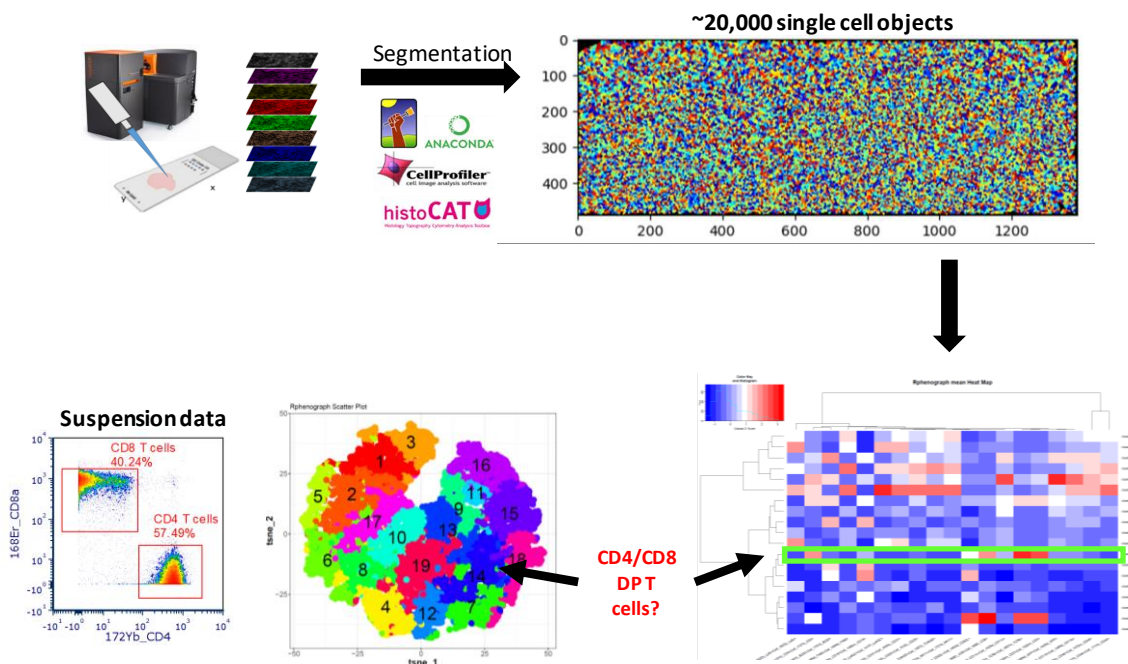
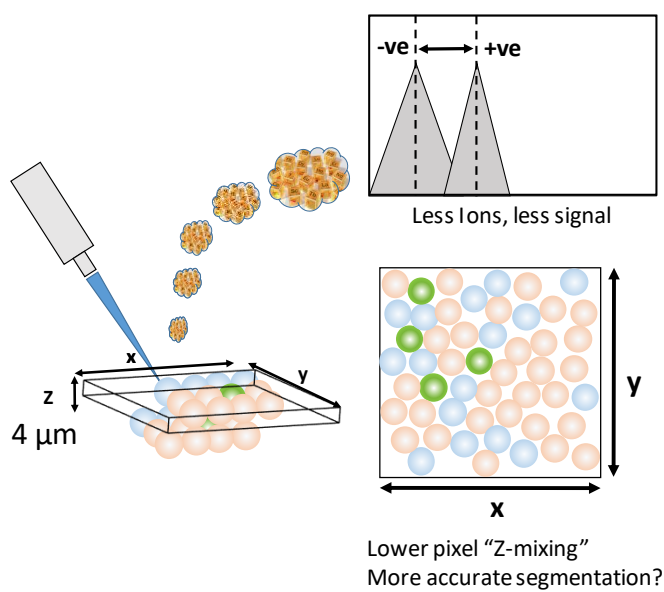
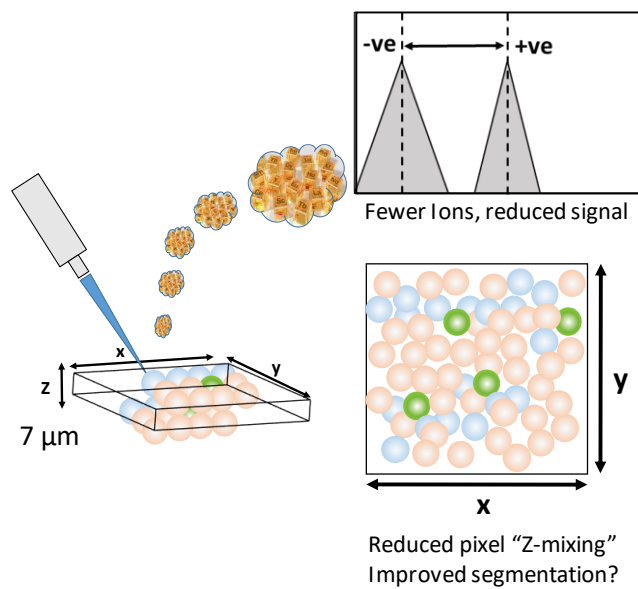
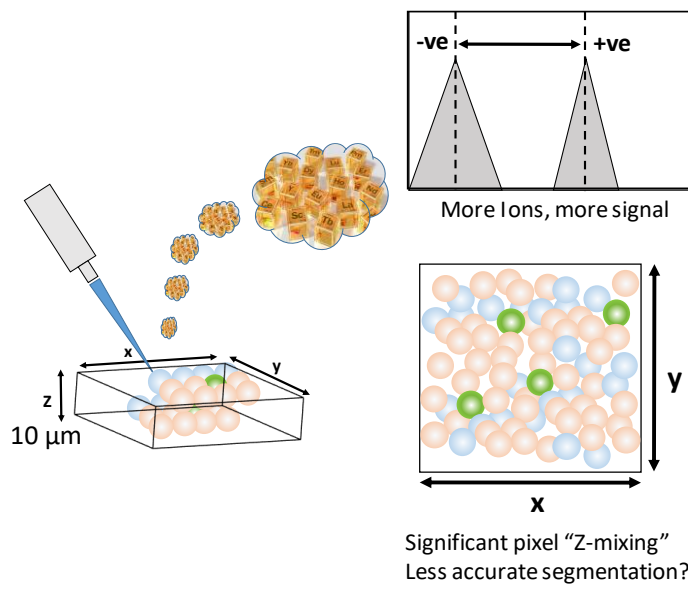


Figure 4.14 IMC data analysis pipeline to single cells. A CD4/CD8 double positive (DP) cluster is identified on the Phenograph clustering heatmap (#14 on the t-SNE plot). The suspension data dotplot confirms this is not an artefact due to antibody binding/staining method.

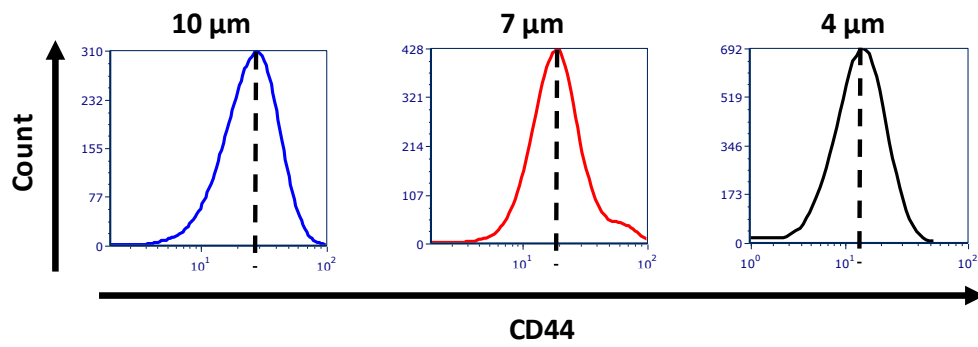
4.2.2.3 Section thickness/ablation depth

Ablating a 10 μ m section results in a stronger signal because more ions are detected but this comes with a significant pixel “Z-mixing” where there is more than one cell on a single Z plane. This leads to challenges with cell segmentation and clustering as biologically impossible markers are detected in the same pixel (e.g. CD4⁺ CD8⁺ T cells). Reducing the section thickness or the ablation depth would result in fewer ions being released and detected resulting in reduced “Z-mixing” and therefore more accurate segmentation. This was investigated by using spleen cryo sections at 10 μ m, 7 μ m and 4 μ m (Figure 4.15a). The results on Figure 4.15b confirm that reducing the section thickness/ablation depth does reduce the signal strength as shown for CD44. Furthermore, it appears that the 4 μ m section in Figure 4.15c has reduced overlap between CD4 and CD8 as observed by the reduced “cyan” T cell area. Downstream single cell analysis of these data on Figure 4.16a show a single T cell cluster for the 10 μ m sections. However, for 7 μ m and 4 μ m sections (Figure 4.16b and c), two T cell clusters can be identified. One of these clusters has higher levels of CD4 expression than CD8, suggesting that the clustering algorithm is starting to pull out some of the CD4⁺ T cells from the total T cell cluster. Although, this indicates that reducing the section thickness/ablation depth is improving the segmentation, pixel “Z-mixing” is not sufficiently reduced to successfully identify some cell populations.

(a)



(b)



(c)

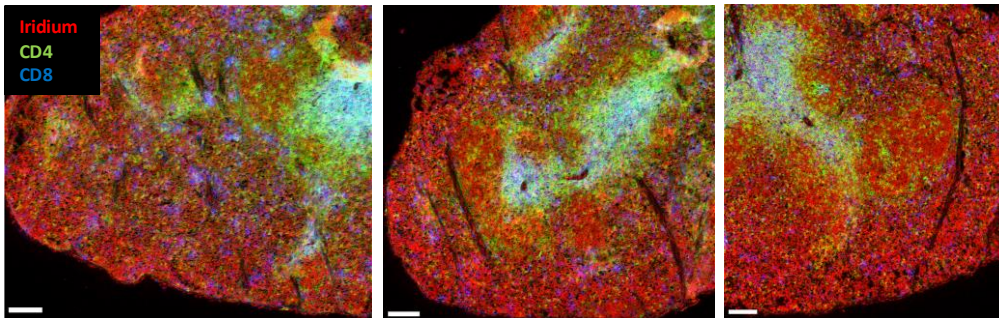
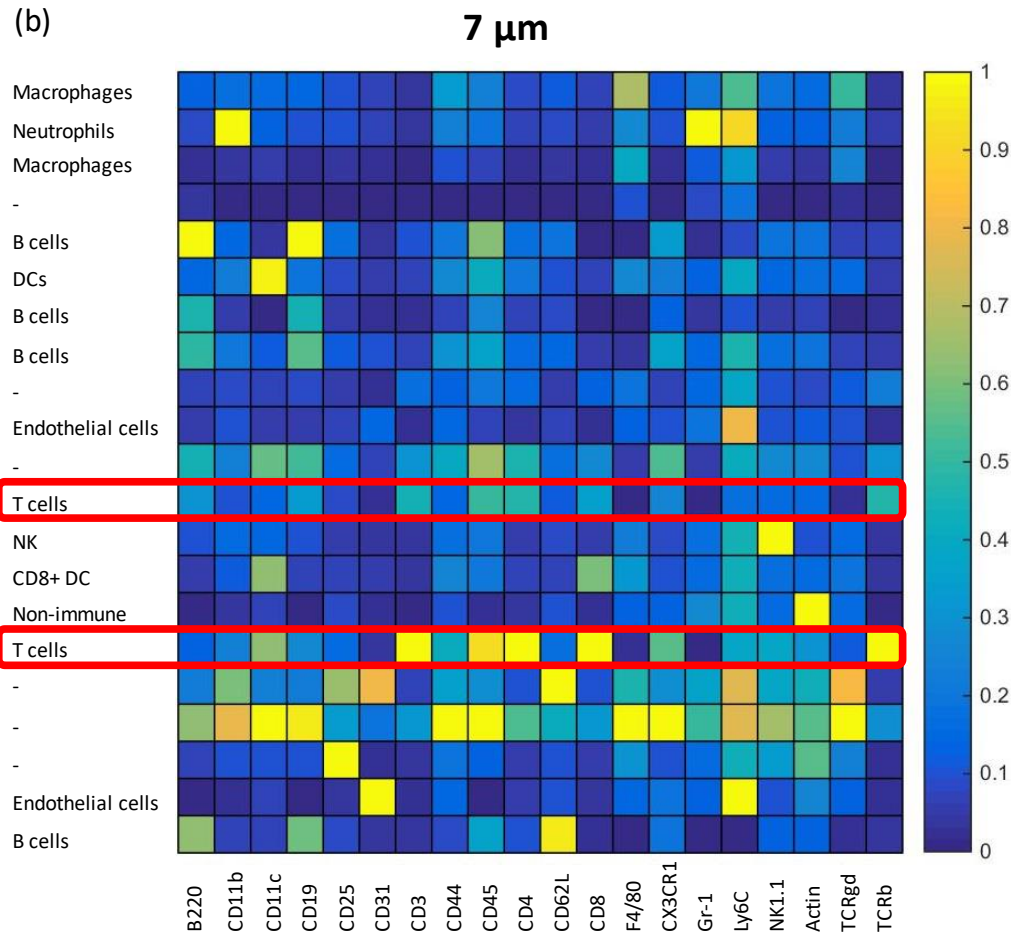
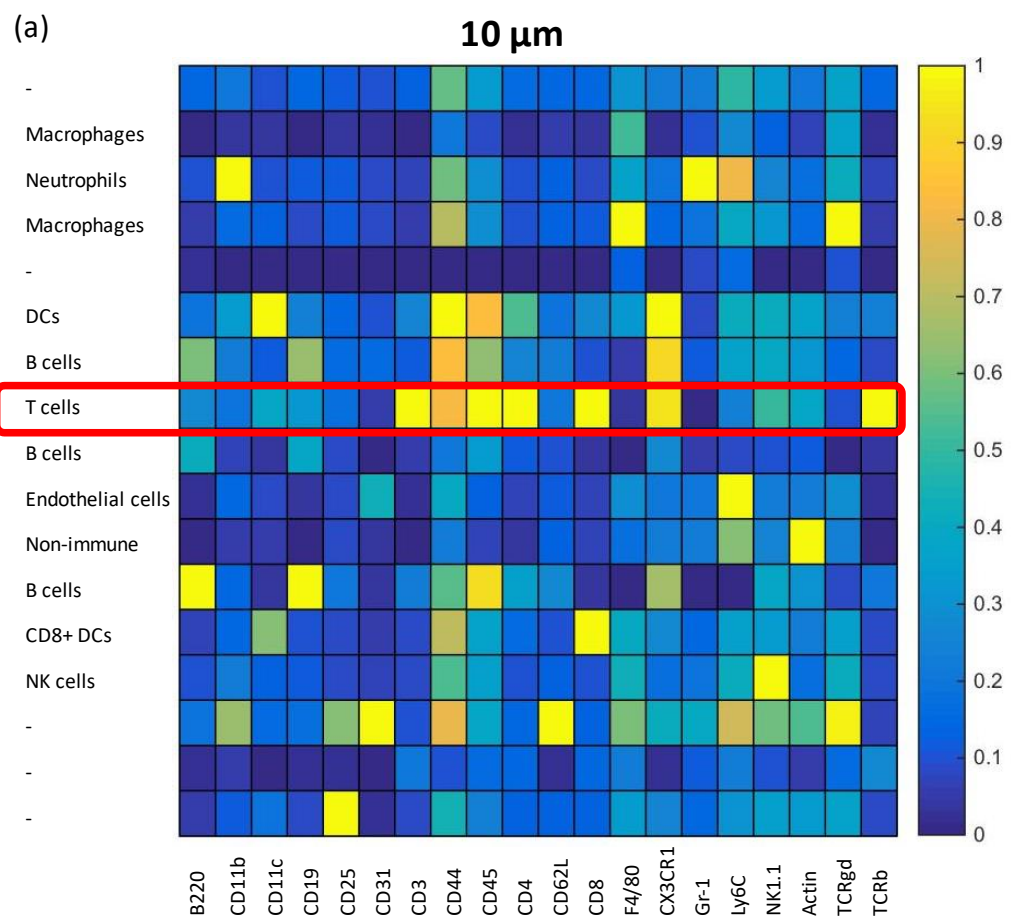


Figure 4.15 Section thickness/ablation depth in IMC. (a) Relationship between section thickness/ablation depth, signal strength and pixel Z-mixing. (b) Reducing section thickness/ablation depth, reduces CD44 signal strength. (c) Thinner sections have reduced CD4/CD8 overlap (cyan). Scale bars = 100 μm .



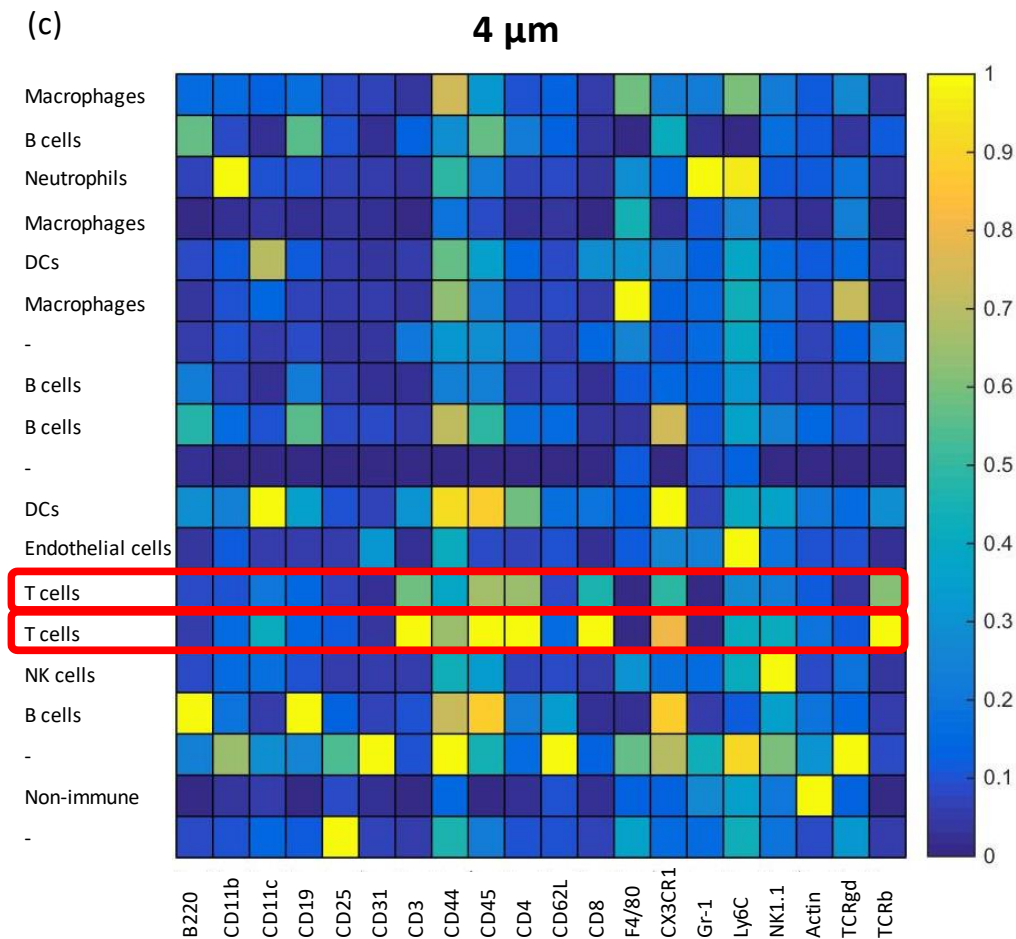


Figure 4.16 Reduced section thickness/ablation depth improves T cell cluster identification. Heatmaps show Phenograph clustering of (a) 10 μm , (b) 7 μm and (c) 4 μm sections. T cell clusters are encircled in red.

Lastly, it was attempted to improve the segmentation by improving the image resolution. The Hyperion was set up on 0.5 μm^2 step mode, rather than the normal 1 μm^2 , by making overlapping shots (Figure 4.17). While this ablation mode is not the standard operating procedure for the Hyperion system and it greatly increases the image acquisition time, it does result in higher image resolution. However, the heatmap on Figure 4.17 show that this did not improve T cell clustering. As with the 4 μm section ablated using the 1 μm step mode, this resulted in two T cell clusters being identified, one with higher CD4 expression than CD8 but still double positive. As there was no noticeable improvement in identifying single positive CD4 and CD8 T cells, it was decided that the 0.5 μm^2 step mode will not be used in further experiments as it is four times slower to acquire images than the standard 1 μm^2 step mode.

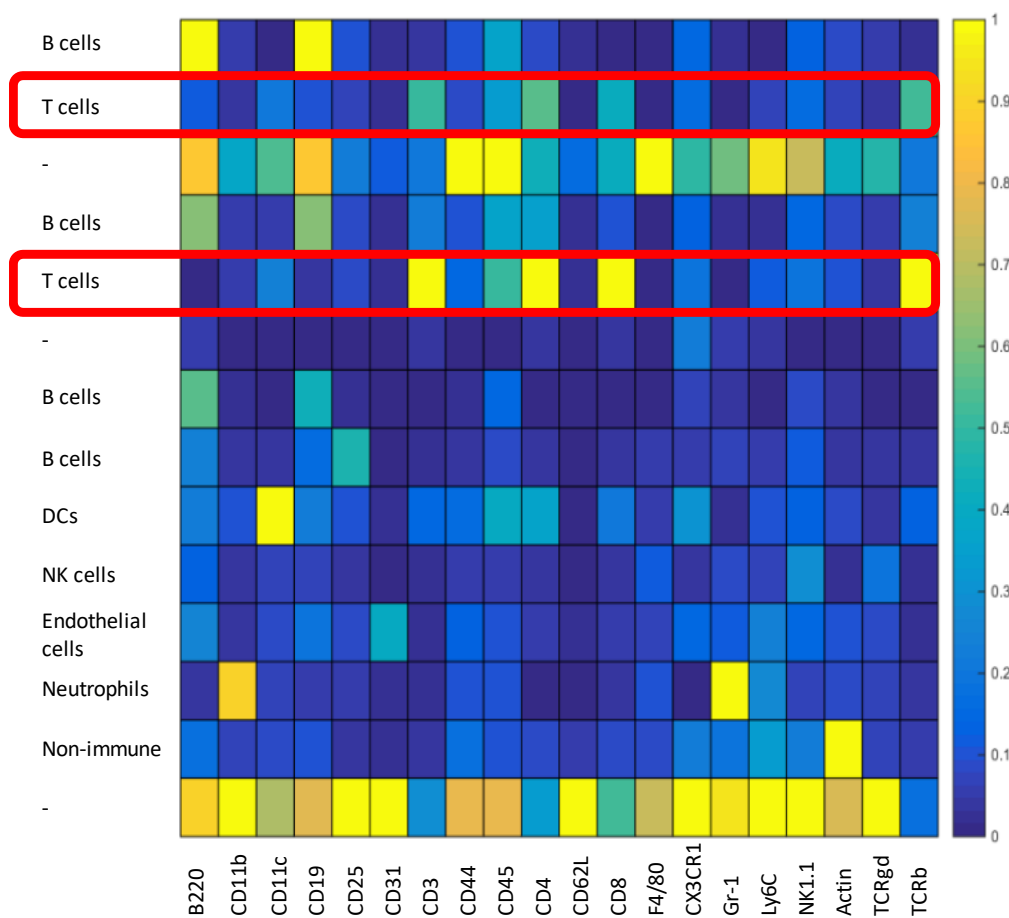
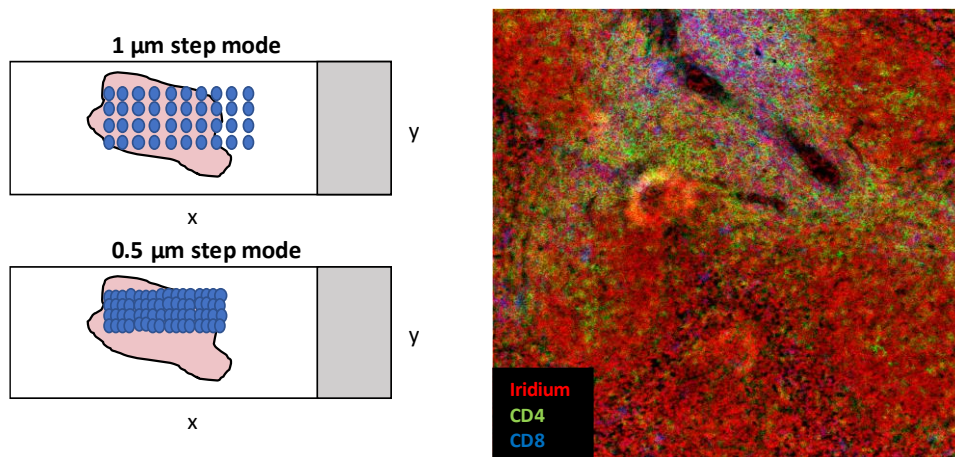


Figure 4.17 Smaller step sizes improve resolution but not segmentation. Using 0.5 μm laser step sizes on a 4 μm section, instead of the normal 1 μm , improves images resolution but does not improve CD4/CD8 single positive T cell cluster identification.

4.2.3 Deep profiling of immune cell infiltration 24h post MI in CX3CR1-EGFP mice

The original aim of this chapter to characterise which immune cells infiltrate the I/R myocardium in a mouse MI surgery model. While mass cytometry machine unavailability did

not allow for the entire planned tissue bank to be analysed, the results below describe the pilot study of a single CX3CR1-EGFP knockout (KO) heart.

Results in the previous chapter showed dramatic accumulation of leukocytes in the left ventricles of CX3CR1-EGFP KO mice 24h post reperfusion, subsequent to MI. IMC analysis of sister sections of the same heart confirms that the myocardium is infiltrated with multiple immune cell populations. Figure 4.18 shows that the infarct area (mid-left ventricle) is populated by various CD45⁺ cells, the most prominent of which are Gr-1⁺ neutrophils, CD11b⁺ myeloid cells, CD3⁺ and CD4⁺ cells. Additionally, prominent CD44 and Ly6C staining is observed across the whole section. CD44 is a widely expressed marker across all leukocyte populations and Ly6C can also identify endothelial cells. Analysis of 24h I/R IMC heart data shows that automated Phenograph clustering allows the identification of several major immune cell populations (Figure 4.19). The analysis identified two clusters of Gr-1⁺ neutrophils, two clusters of endothelial cells, expressing CD31 and Ly6C in the absence of CD45, CD11b⁺ myeloid cells (GFP⁺ and GFP⁻) and a cluster of F4/80⁺ macrophages. For the lymphocyte lineage, Figure 4.19 shows that two clusters of T cells are identified as TCRb⁺ CD3⁺ cells, although they both appear to have similar CD4/CD8 expression which indicates that single positive CD4 or CD8 T cell identification was not possible. Cluster 4 is also likely to represent a population of CD25⁺ CD3⁺ T cells but they cannot be confidently identified as such due to the lack of TCRb or CD4 expression. In addition to this, two populations of CD19⁺ B220⁺ B cells are identified, as well as the two rarest populations – gdT cells and NK cells. Furthermore, two clusters of immune and non-immune cells are identified. The former cannot be more accurately allocated to any specific immune cell type due to being positive for conflicting markers such as Gr-1 (neutrophils), CD25 (Tregs), CD19 (B cells), CD11b (myeloid cells), CD11c (DCs). However, the high CD45 expression clearly indicates that this cluster is composed of immune cells and indeed this is one of the largest clusters, second to neutrophils (Table 4.2). The results on Table 4.2 show that a large proportion of the cells identified by the Phenograph clustering are endothelial and non-immune cells, most likely cardiomyocytes and fibroblasts. Interestingly, the inflammatory infiltrate in this CX3CR1-EGFP KO mouse appears to have comparable numbers of T and B cells. However, T cell numbers would greatly increase if the aforementioned Cluster 4 is included.

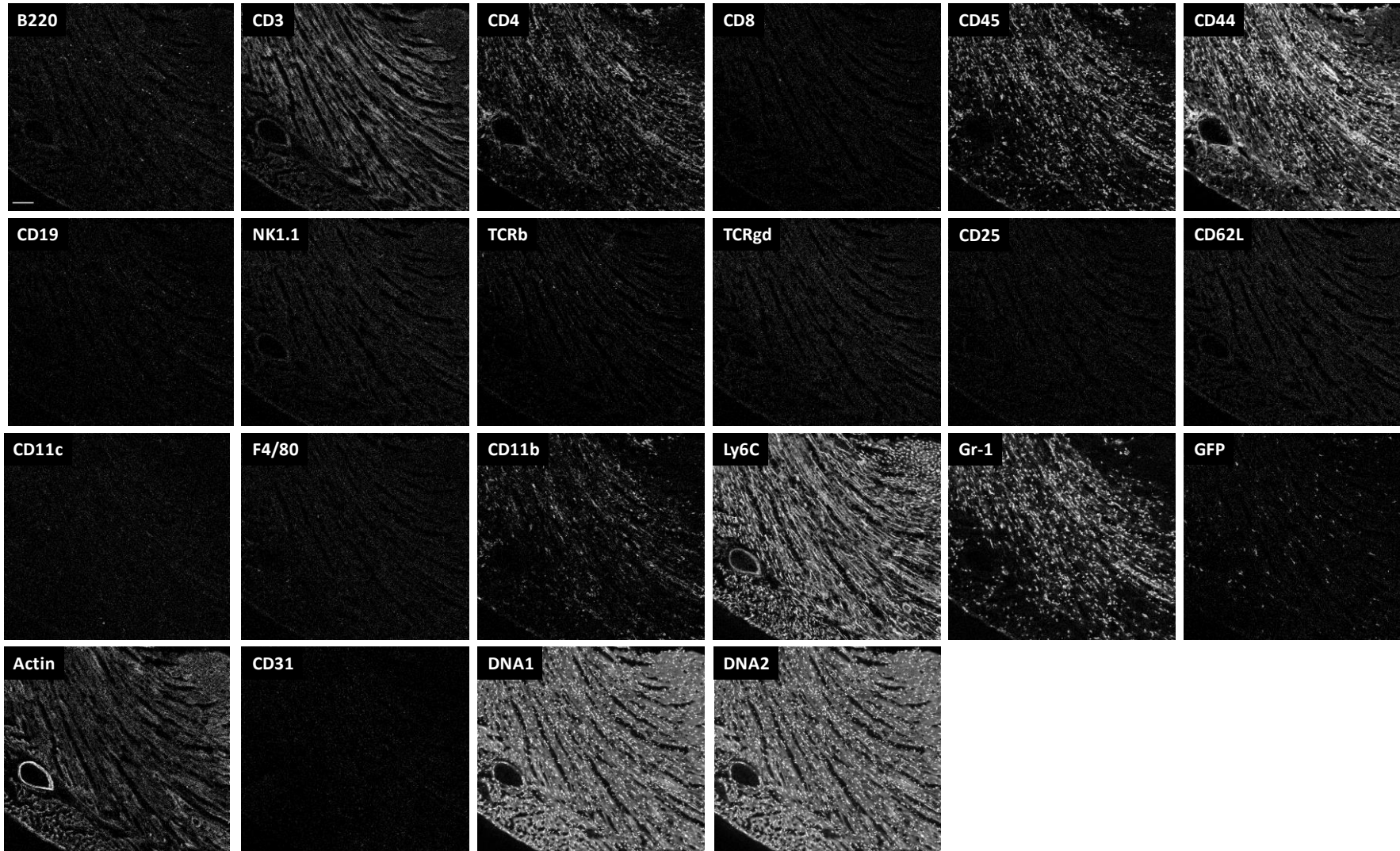


Figure 4.18 Imaging mass cytometry of mouse heart 24h post reperfusion. The full antibody panel was used to stain 10µm heart section in the infarct region of a CX3CR1-EGFP knockout mouse. Representative grey-scale images of one marker per image; scale bar = 100µm.

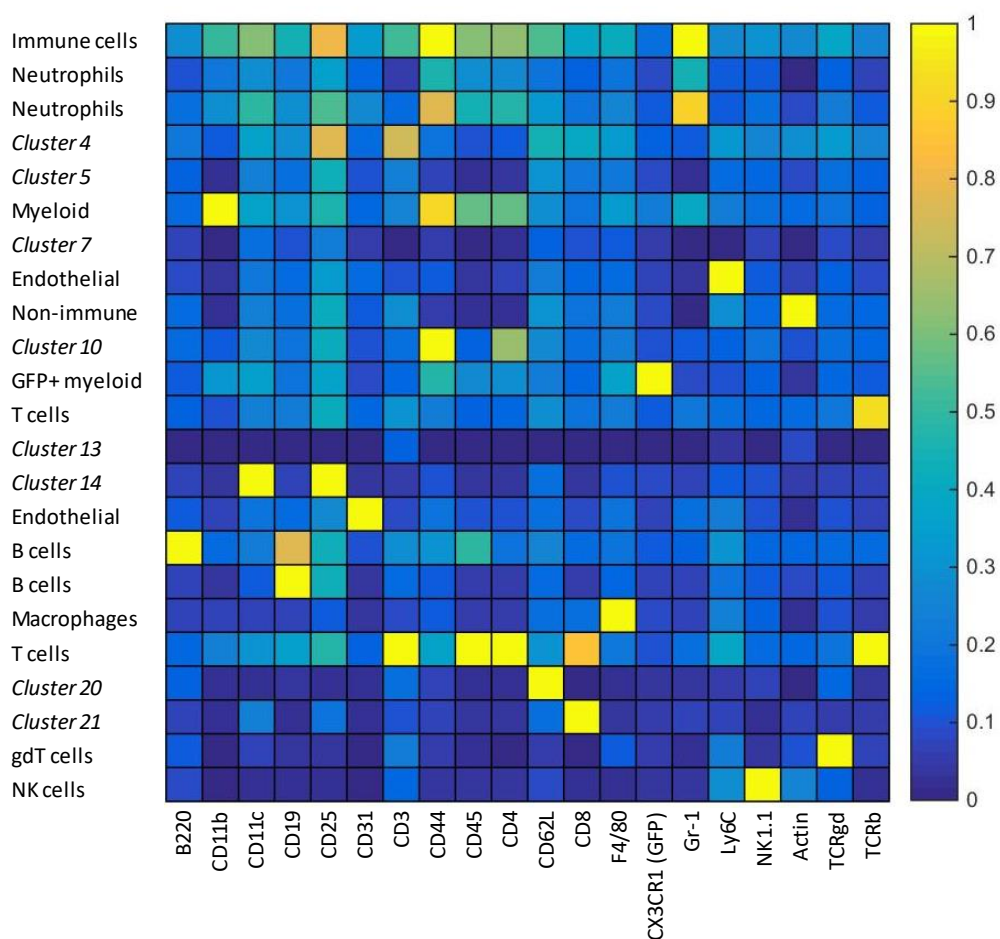


Figure 4.19 Immune cell populations infiltrate the I/R myocardium 24h post MI. Heatmap shows Phenograph clustering of cells in the left ventricle of a CX3CR1-EGFP knockout mouse.

Cell population	Number of cells
Neutrophils	6968
Immune cells	4601
Endothelial cells	2451
Myeloid cells	2371
Non-immune	1802
GFP+ myeloid cells	786
T cells	682
B cells	655
Macrophages	258
gdT cells	87
NK cells	72

Table 4.2 Cell populations in the 24h I/R myocardium identified following Phenograph cluster analysis

Finally, one of the major benefits of IMC over high-content flow or mass cytometry analysis of digested tissue is that the spatial information of different cell types is not lost. IMC analysis allows for the Phenograph clusters on Figure 4.19 to be mapped on each IMC image, as shown on Figure 4.20. As expected the large cluster of immune cells is localised to the infarct area of the left ventricle, while non-immune CD45⁻ cells are preferentially located in the periphery (Figure 4.20a). The two neutrophil clusters are also localised largely to the infarct area, although some neutrophil infiltration is seen across the whole ventricle (Figure 4.20b). On the other hand, the clusters of myeloid cells, GFP⁺ myeloid cells and macrophages are more dispersed throughout the ventricle, indicating that 24h post reperfusion they are migrating from the vasculature to the infarct area (Figure 4.20c). As seen on Figure 4.20d cells from the two T and B cells clusters are less numerous than granulocytes and myeloid cells, but they also appear to be preferentially localised to the infarct left ventricular area.

Ultimately, these results show that the IMC method developed as part of this PhD is successful in providing deeper immunophenotyping of the post MI immune response in murine hearts. This method can be used both to gain better understanding of which cell types infiltrate the infarct area, as well as where they locate which would provide crucial insight into immune cell interactions.

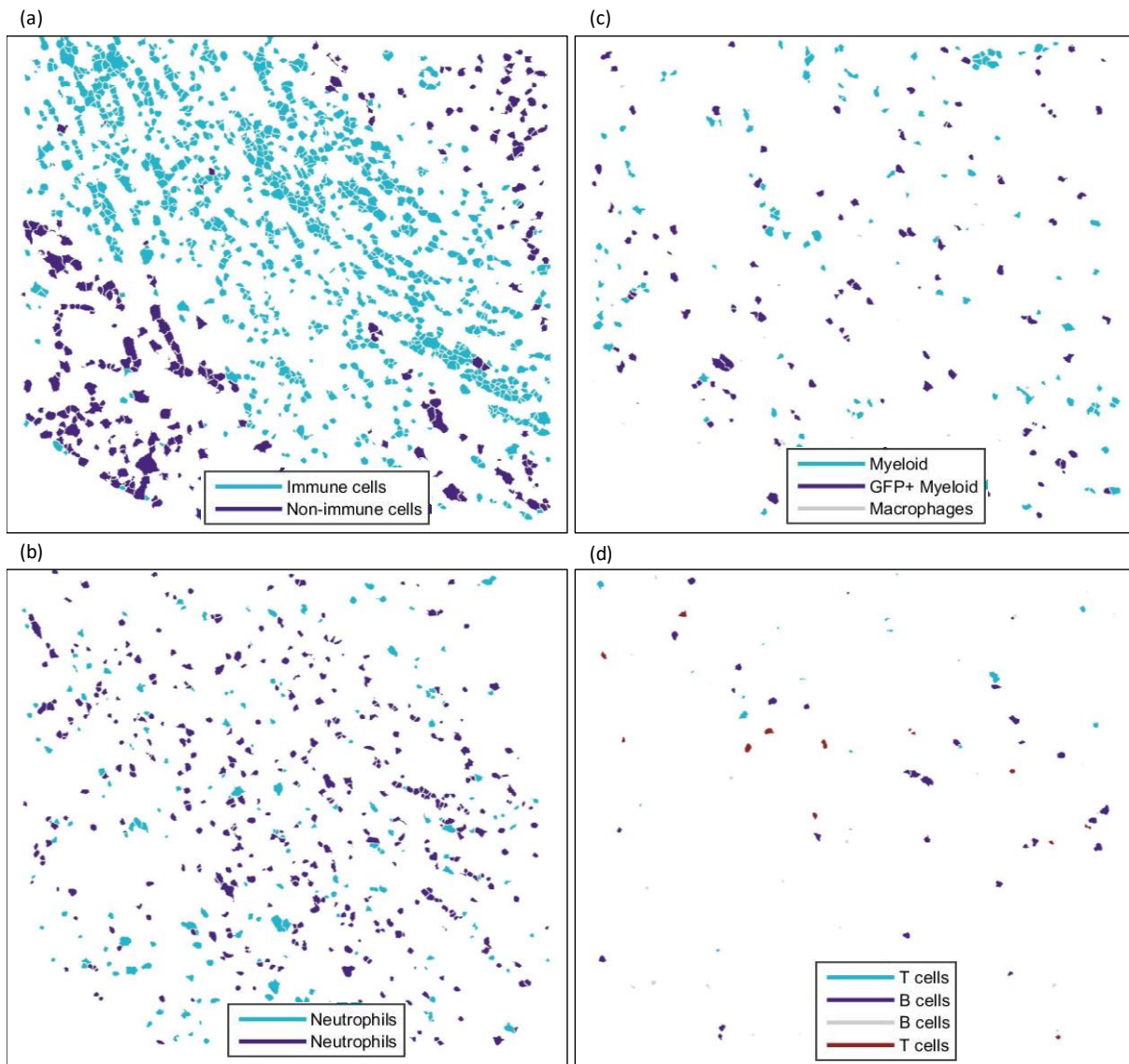


Figure 4.20 Immune cell clusters locate to the infarct region of the left ventricle. Phenograph clusters are mapped to a representative 24h I/R heart ROI, showing (a) immune and non-immune cell (clusters 1 and 9), (b) neutrophil (clusters 2 and 3), (c) myeloid and macrophage (clusters 6 and 18), (d) T and B cell clusters (clusters 12, 16, 17 and 19). All images are at the same magnification.

4.3 Discussion

Mass cytometry is one of the first techniques to enable multi-parameter single cell analysis at an unprecedented level of detail. Utilising inductively coupled plasma mass spectrometry and rare earth metal tagged antibodies, this methodology allows for simultaneous and rapid detection of up to 40 parameters per cell. Imaging mass cytometry is a novel addition to CyTOF mass cytometry, which allows for this high-dimensional analysis to be applied on tissue sections, giving invaluable spatial information at subcellular resolution. This chapter describes the successful establishment and optimisation of this technology to study in detail immune cells in the mouse spleen. Subsequently, this work served as the ground truth for the investigation of inflammatory infiltrate to the heart following 24 hour ischaemia/reperfusion (I/R) injury in this PhD project.

To date, mass cytometry research has primarily been focused on human samples with very few research groups currently working on mouse tissue (Japp, Hoffmann et al. 2017, Cole, Park et al. 2018, Widjaja, Singh et al. 2019, David, Rezende et al. 2016). Therefore, as this is still a novel area, the methodology that is presented here was carefully designed, optimised and validated on mouse spleen cells and tissue sections before further application to myocardial I/R studies. However, despite this novelty, sample preparation for IMC is identical to that for the immunofluorescence (IF) staining, described in the previous chapter. Therefore, this allowed for faster implementation of the method as the heart tissue was already available and minor changes had to be made to the existing tissue staining protocol. Furthermore, sample preparation and manual gating analysis of suspension mass cytometry is similar to the already well established fluorescence flow cytometry pipeline, allowing for an easier implementation of the technique.

The main challenge with the suspension mass cytometry data, described above, is the semi-automated clustering analysis, due to the expression of multiple shared markers between haematopoietic cells. Cluster identification for certain populations can be challenging as transitioning between cell types can be a continuum rather than clear distinction between them. This is particularly the case for cells of the myeloid branch of the immune system which express a continuum of common markers (CD11b) and only one cell type specific marker included in our panel (F4/80, CD11c). In contrast, the bimodal expression of lymphocyte markers such as CD3 vs B220 or CD4 vs CD8, does not present this challenge. This lack of clearly defined populations creates a challenge for both the clustering algorithm and the correct

cluster identification. Despite this, the results above show that the suspension mass cytometry pipeline has been successfully set up and all expected cell populations can be identified in mouse spleen tissue. Furthermore, the population distribution is in accordance with existing reports for mouse splenocytes. While different cell isolation methods would be better suited for isolating red pulp derived cells such as DCs and macrophages, the results described in this chapter showing approximately 25% CD4 T cells, 16% CD8 T cells, 38% B cells, 2.5% Tregs, 3% NK cells, 3% DCs, 1% macrophages, 7% neutrophils and 5% monocytes in mouse spleen are in agreement with published reports (Hensel, Khattar et al. 2019, Chen, Z., Huang et al. 2017, Goltsev, Samusik et al. 2018). This confirms that the developed panel and mass cytometry methodology can successfully identify the expected immune cell populations in the mouse spleen.

Although the mass cytometry antibody panel was carefully designed to use antibody clones that have been reported to work well in fixed frozen mouse tissue, the nature of immunohistochemistry (and therefore IMC) staining requires fixation of the tissue section prior to staining. The use of any fixative, prior to antibody staining, is well known to cause alterations in epitopes which can result in signal loss. Therefore, the effect on our antibody panel from the 4% formaldehyde fixation that we use was assessed using suspension splenocytes and CyTOF. Formaldehyde exposure causes covalent cross-linking of antigens, leading to antibody/epitope binding interference and reduction or complete loss of signal (Maes, Davidson et al. 2007, Olsen, Leipold et al. 2019). The results described in section 4.3.1.2 are consistent with previous publications showing overall reduction in mean marker levels in samples that were fixed prior to staining (Maes, Davidson et al. 2007, McCarthy, Macey et al. 1994, Pizzolo, Melamed 1996). Furthermore, an increase in the percentage of B cells and endothelial cells was observed in samples stained post fixation. This difference could be due to the increased permeability of cells exposed to formaldehyde (Hoffman, Frey et al. 2015, Maes, Davidson et al. 2007) and therefore represent staining of “intracellular” antigens. These challenges of tissue staining cannot be avoided. However, using suspension mass cytometry on the same tissue type prior to IMC serves as a guide for the IMC pipeline. Based on the suspension cell results, it can be easily established which antibodies would work in a given IMC staining method. Overall, this saves time and resources in establishing IMC experiments as the slow and laborious single antibody testing on tissue sections can be reduced or even avoided.

While suspension based technologies have traditionally been used to advance high dimensional single cell research, they require tissues to be disaggregated for analysis and therefore destroying key spatial information. Recently a number of technologies have been developed to make multi-parameter measurements of fixed frozen or FFPE tissue section, with matching dimensionality to that of fluorescence or mass flow cytometry. Imaging mass cytometry is one of these techniques and while it preserves spatial information, it also raises additional analysis challenges. In order to accurately identify cellular phenotypes within the tissue, the images must first be segmented to give single cell information. This requires machine learning tools, generally employing the nuclear images as the “seed”, and has been explored for high content microscopy images for a number of years (Kan 2017, Belevich, Joensuu et al. 2016, F. Xing, Y. Xie et al. 2018). Segmentation is a key part of the IMC analysis pipeline as failure to segment single cells is equivalent to including doublets and cell aggregates in the analysis, leading to mis-identification of cell types. In contrast to suspension cytometry, where whole isolated cells are analysed, cells in tissue sections often overlap, are packed together and have only part of their membrane available for analysis. These difficulties are shared with multiplex microscopy, however, the fact that IMC is not optical in nature presents unique challenges. IMC relies on precise laser shots that ablate $1\mu\text{m}^2$ tissue in its entirety. Therefore, all cells in different Z-planes will be merged, creating a challenge for both image segmentation and subsequent downstream analysis.

The work in this chapter, addresses this issue by investigating the success of the segmentation and population clustering in sections at different thickness. We have found that $4\mu\text{m}$ is the optimal thickness (ablation depth) for a spleen section, producing enough ions to resolve each marker and improving population identification. However, our data shows that despite reducing the section thickness/ablation depth, some cell populations such as CD4+ or CD8+ single positive T cells cannot be clearly identified. Although logic would suggest that increasing the spatial resolution of the images by using smaller laser size would improve the segmentation and therefore better define T cell clusters, the data presented here did not confirm this. This suggests that this is a limitation due to the resolution of IMC and/or that it is due to the high cell density in the mouse spleen. The latter is of particular importance as our observation of poor cluster identification is in cell types within white pulp follicles which has very high cell density. Furthermore, the majority of published T cell imaging work uses a very limited number of markers, compared to our extensive T cell panel. T cells are often

identified as either CD3⁺/CD4⁺, CD3⁺/CD8⁺ or simply CD4⁺ or CD8⁺ (Safeukui, Gomez et al. 2015, Wang, Z., Shufesky et al. 2009, Wang, X., Xu et al. 2011, Liu, X., Li et al. 2014, Kim, M., McConnell et al. 2006). While multiplex CD3/CD4/CD8 T cells immunohistochemistry is well established, this is often not followed by image segmentation and clustering analysis (Yarilin, Xu et al. 2015, Feng, Jensen et al. 2016). Other highly multiplexed single cell imaging techniques, similar to IMC, do exist. Histo-Cytometry combines multiplexed antibody staining with tiled high resolution confocal microscopy and quantitative analysis that can identify CD4⁺ and CD8⁺ single positive T cells (Gerner, Kastenmuller et al. 2012). While CODEX allows for deep immune phenotyping of mouse splenocytes by utilising oligonucleotide-conjugated antibodies and fluorescence microscopy fitted with an automated fluidic setup (Goltsev, Samusik et al. 2018). However, both of these methods use high resolution microscopy which can focus on a single focal plane, avoiding a key challenge of IMC – ablation of multiple cells across the tissue depth and subsequent z-pixel mixing. Moreover, the challenge of cell density in IMC analysis is evident by the fact that the majority of published work involves cells lines (Bouzekri, Esch et al. 2019, Giesen, Wang et al. 2014), cancerous tissue (Carvajal-Hausdorf, Patsenker et al. 2019, Giesen, Wang et al. 2014) or other non-dense tissue such as skin (Guo, Zhang et al. 2019) and intestines (Bassan, Willis et al. 2019, Cao, Chang et al. 2019). Durand et al recently described lymphocyte work in human tonsil sections (Durand, Walter et al. 2019). However, they limited their IMC image segmentation and analysis to total B cell follicles and T cell zone, rather than single cells, further suggesting the difficulties of IMC single cell segmentation in lymphoid tissue.

Our work reveals certain limitations of using IMC to study highly cell-dense tissue which cannot be overcome using the current technology. However, we shed light on the importance of using multiple parameters to identify cell populations, the correct image segmentation and cell phenotype identification. It is imperative to be aware that depending on the tissue, section thickness and cell type, published IMC data may not necessarily represent truly single cell results. As new high-dimensional image analysis technologies emerge, analytical tools must also continue to be developed. This is an area of intensive research, with novel software and analysis pipelines being developed. ImaCyte is one such tool, that was recently described to provide interactive visual analysis workflow for cell phenotype identification and microenvironment exploration that could improve future IMC analysis (Somarakis, Van Unen et al. 2019). However, these were not be further investigated in this work as the focus of my

project was to establish and optimise an imaging mass cytometry pipeline that can be confidently used to study the I/R heart. This has been successfully achieved as the image segmentation challenges described above were not expected to affect heart sections which have visibly lower cell density.

However, initial results from IMC analysis on heart tissue 24h after post MI reperfusion, did not result in successful identification of CD4 and CD8 single positive T cells. Despite the lower cell density, compared to spleen sections, heart sections at 10µm were analysed. Therefore, this simply may be due to the higher tissue depth which is twice the average diameter of a lymphocyte. It may also be as a result of overall lower numbers of CD4 and CD8 single positive T cells compared to the larger cell clusters such as neutrophils, and the pooled immune and non-immune cell clusters. Furthermore, similar to clustering of splenocytes, it was not possible to identify naïve, effector and memory T cells based on the expression of CD62L and CD44. While both antibodies resulted in staining in the heart, the gradual changes in the expression of these markers between the different T cell populations does not allow for separate Phenograph clusters to be formed using the current analysis pipeline. This is also the reason behind identifying only a pooled myeloid cell cluster instead of being able to confidently identify myeloid cell subsets such as DCs, classical and non-classical monocytes. However, mass cytometry is a rapidly developing research area with multiple groups developing novel analysis methodologies, which may allow better population clustering in the future.

Moreover, these results highlight a major challenge with any tissue section imaging technique, IF or IMC. Analysis is performed on a tissue section and depending on where each cell is cut through, a T cell may not be positive for both CD3 and TCRb simply because this particular section of the cell membrane does not contain a CD3 as well as a TCRb molecule. IMC provides greater validity of the data because cell populations can be identified by more than one marker, which is increasingly important for immunological studies. However, it does create an additional challenge in that a cluster of cells may express markers that would usually lead to the identification a cell population, but because they do not express another lineage-specific marker, the investigator cannot confidently label this population. Indeed, this is the case for Cluster 4 on Figure 4.19. While this cluster is highly positive for CD3, which would have immediately classed this cluster as T cells in IF analysis, the lack of TCRb or TCRgd prevent this in IMC analysis. However, this could also be due antigen engagement of the TCR which is

known to cause TCR internalisation (Alcover, Alarcón 2000, Martínez-Martin, Fernández-Arenas et al. 2011). Antigen binding to the TCR could also cause steric hindrance and prevent anti-TCRb antibody binding leading to lower TCRb staining. This is more likely to occur in an inflammatory response such as I/R injury, compared to the unchallenged spleen environment described above.

Ultimately, the work in this chapter describes the successful development of an IMC method to perform deep immunophenotyping of myocardial inflammatory infiltrate in response to myocardial I/R injury. The current system did not allow the identification of all intended cell populations, such as naïve, effector and memory T cells. However, the IMC panel can be further modified in order to gain additional insight into the specific T cell subtypes involved. Intracellular staining markers including IFN γ , IL-4, IL-17 and IL-10 can be included in the panel to identify various Th and Tc subtypes. Importantly, this panel can be easily modified to fit the specific research purpose by utilising the spleen optimisation steps described above. Although it was not possible to achieve the final goal of this chapter and use this method to fully characterise the inflammatory infiltrate during the acute I/R response, here I have described the successful development of a cutting-edge technology. The method that was developed would not only help elucidate the role multiple immune cells in MI, but it can also be utilised in the investigation of different tissues and pathologies. Importantly, adopting this method more widely could reduce the need of certain mouse experiments such as adoptively transferring various populations of cells into different mouse lines. Deeper immunophenotyping would allow for more information to be gained from a single experiment, especially when functional markers are included in the panel. The IMC heart data presented here is based on 10 times larger area and 5 times more markers than the corresponding IF analysis on the same heart in Chapter 3. This not only reduces the manual time to gain far more information but also allows for additional experiments to be performed on the remaining heart tissue such as gene expression and proteomics. Therefore, this work is in support of the 3Rs (Replacement, Reduction and Refinement) principles for performing animal research.

Chapter 5. Effect of T cell phenotypes on inflammation following myocardial I/R

5.1 Introduction

The results in Chapter 3 confirm that the CX3CL1-CX3CR1 axis plays a role in myocardial I/R injury. While knockout of CX3CR1 results in poorer cardiac function in the weeks post MI, its effect on the acute inflammatory response is less clear. Absence of CX3CR1 causes a change in the phenotype of the inflammatory response, with a significant reduction in T cell numbers 24h post reperfusion. However, there are multiple different T cell subpopulations with very different functions which are often tissue/pathology-specific. These various effector T cell populations were not investigated in the work presented in Chapter 3, therefore significantly limiting our ability to understand the exact role that CX3CR1⁺ T cells play in myocardial I/R injury. Moreover, the complex crosstalk between different immune cells, as well as the overall innate and adaptive immunity, represents a further challenge in immunological research. While the work in Chapter 4 describing the successful development of a novel method of deep immunophenotyping may prove crucial in understanding this crosstalk, the CX3CR1-EGFP mouse model would not help elucidate the exact role of CX3CR1⁺ T cells in myocardial I/R. There are three main reasons behind this, revealed by the results in Chapter 3. Firstly, the CX3CR1-EGFP mouse line that was used is a global knockout of CX3CR1. Therefore, any CX3CR1⁺ T cell specific response would be masked by the absence of the more numerous CX3CR1⁺ monocytes and macrophages, which are crucial for myocardium repair. Addressing this challenge would require additional studies using T cell-specific mouse models. Secondly, germline knockout mice, like the CX3CR1-EGFP line used in Chapter 3, most likely have compensatory mechanisms that have developed in the absence of the functioning protein encoded by this gene. While studying this compensatory mechanism was not the focus of Chapter 3, its existence is expected to have an effect on leukocyte chemotaxis which may affect the inflammatory response to myocardial I/R injury. Thirdly, while the work in Chapter 3 confirmed that CX3CR1⁺ T cells can be identified in various tissues of young immunologically unchallenged mice, their numbers are very low. As CX3CR1 expression on T cells is highest in cytotoxic effector and memory populations, the mice used in Chapter 3, with predominantly naive T cell phenotype, are not a suitable model to study the role these T cells. Therefore, the work described in this fifth chapter is focused on identifying and establishing a more clinically relevant mouse model of myocardial infarction, that is better suited to investigate the specific role of CX3CR1⁺ T cells.

First of all, this chapter begins by investigating the specific role of lymphocytes in myocardial I/R injury. This has been done using mice deficient in recombination-activating gene (RAG) 2 (RAG2). Together with RAG1 mice, these are considered the gold standard mouse model to study adaptive immunity. Rag1 and Rag2 are required for T cell receptor (TCR) and B cell receptor (BCR)/immunoglobulin (Ig) rearrangement (Rios Francisco J., Montezano Augusto C. et al. 2020). The ability of antibodies and TCRs/BCRs to recognise a remarkable number of antigens is due to the somatic DNA rearrangements of these genes. This process, known as V(D)J recombination, involves RAG1/2 proteins joining germline variable (V), diversity (D) and joining (J) gene segments, creating a unique combination for each naïve B and T cell. RAG1/2 synergistically confer V(D)J rearrangement in the Ig heavy (κ) and light (λ) chain, as well as the TCR α , β , γ and δ chain loci (Mombaerts, Iacomini et al. 1992, Shinkai, Rathbun et al. 1992). Therefore, RAG1 and RAG2 are essential for B and T cell development and maturation. Knockout (KO) lines for each of the two genes were first described nearly two decades ago by Mombaerts et al. (Mombaerts, Iacomini et al. 1992) and Shinkai et al. (Shinkai, Rathbun et al. 1992) respectively, who described a severe combined immune deficient (SCID) phenotype with complete lack of mature B and T cells. While RAG2 is only known to be expressed in lymphocytes, RAG1 has also been detected in the murine central nervous system (CNS) (Mombaerts, Iacomini et al. 1992). However, RAG1 KO did not lead to any CNS phenotype so the two lines are considered to have identical phenotypes, lacking any mature lymphocytes (Mombaerts, Iacomini et al. 1992).

These mice have been used to study the effect of lymphocytes in cardiovascular diseases, including atherosclerosis (Wu, He et al. 2018) and hypertension (Guzik, Hoch et al. 2007). More importantly, RAG KO mice were used in the seminal study by Yang et al. who showed that T cells play a detrimental role in myocardial I/R injury. Additionally, they confirmed that it is IFN γ secreting CD4 T cells that have a deleterious effect by performing adoptive transfer of CD4 T cells in these RAG KO mice (Yang, Day et al. 2006). As these mice have proven essential in studying the role of T cells in MI, the work in this chapter aims to build upon these findings to further elucidate the adaptive immune response to myocardial I/R.

This chapter then reveals some important differences in the dynamics of circulating immune cells between MI patients and the commonly used mouse myocardial I/R model, before focusing on exploring alternative mouse models. Models that are more representative of the

clinical MI presentation and subsequently better suited to investigate the specific role of CX3CR1⁺ T cells are described in the second half of this chapter.

The first consideration when designing an improved mouse model of human disease is the effect of ageing. The process of inflammaging and the subsequent challenges of using young mice to study human immunopathologies is rapidly gaining more recognition (Sadighi Akha 2018, Mestas, Hughes 2004, Bjornson-Hooper, Fragiadakis et al. 2019). It is well known that changes are induced in the immune system as a result of increasing age, especially in the adaptive arm of the immune system. And since the average age of patients at first MI is around 65 years for men and 72 years for women (Mozaffarian, Benjamin et al. 2015), inflammaging with its baseline low-grade inflammation is an important model to investigate (Montecino-Rodriguez, Berent-Maoz et al. 2013, Sadighi Akha 2018).

The second consideration is subsequent to the increased longevity in humans compared to mice and consists of an infection model. During their lifetime, humans are continuously exposed to multiple pathogens, including chronic latent infections. This leads to a drastic difference in the immune cell repertoire, especially the adaptive immune system and most relevant for this project – T cells (Mestas, Hughes 2004, Bjornson-Hooper, Fragiadakis et al. 2019). In contrast, laboratory inbred mice, kept in specific pathogen-free (SPF) environment, rarely encounter pathogens in their short lifetime (Tao, Reese 2017). As a result, the final part of this project is to investigate a mouse model of a viral infection using murine leukaemia virus (MuLV).

Therefore, the specific aims of this chapter are to (1) investigate the effect of no lymphocytes on mouse myocardial I/R injury, (2) assess the dynamics of circulating leukocytes and their CX3CR1 expression, and explore CX3CR1⁺ T cells in a mouse model of (3) ageing and (4) MuLV infection.

5.2 Results

5.2.1 Effect of no T cells

In order to investigate the role of no T cells on myocardial I/R injury, a RAG2 KO mouse line was used. While this line has impaired lymphocyte development and is devoid of both mature B and T cells, it is a widely used model to study specific T cell responses by adoptive transfer of selected T cell populations. Indeed, one of the original aims of this chapter was to investigate the role of CX3CR1⁺ T cells by transferring CX3CR1/EGFP⁺ T cells isolated from the CX3CR1-EGFP mouse line. However, it was not possible to do this by the end of this PhD project due to construction work in the mouse housing unit, resulting in disrupted mouse colony breeding and reduced availability of procedure rooms to perform MI surgeries.

5.2.2.1 Baseline phenotyping of RAG2 KO mouse line

The first step in studying the myocardial I/R response in the RAG2 KO mouse line was to establish the baseline T cell phenotype. This was achieved using flow cytometry analysis of lymphoid tissues, the gating strategy of which is shown on Figure 5.1a and Figure 5.1b for WT and RAG2 KO mice respectively. The proportion of total T cells, CD4 and CD8 T cells in spleen, lymph nodes and blood is shown on Figure 5.1c. As expected, RAG2 KO mice show dramatic loss in T cells across all tissues. Loss of T cells is most prominent in the lymph nodes where there is 95% ($p \leq 0.0001$) reduction in the percentage of total T cells. Loss of CD3⁺ cells in the spleen and blood is also evident with 60% ($p \leq 0.001$) and 82% ($p \leq 0.01$) decrease respectively. While no CD4 or CD8 single positive T cells were detected in the spleen or blood of RAG2 KO mice, lymph node samples had around 27% CD4⁺ CD3⁺ cells. However, it must be noted that the lymph nodes of RAG2 KO mice had only 3.5% of total cells being CD3⁺. Therefore, the 27% CD4⁺ CD3⁺ cells is the result of a very few events falling in the CD4 gate. In addition to this, as shown on Figure 5.1b, RAG2 KO mice do have a number of CD4/CD8 double positive T cells. These represent immature T cells that are not normally found in peripheral lymphoid tissues.

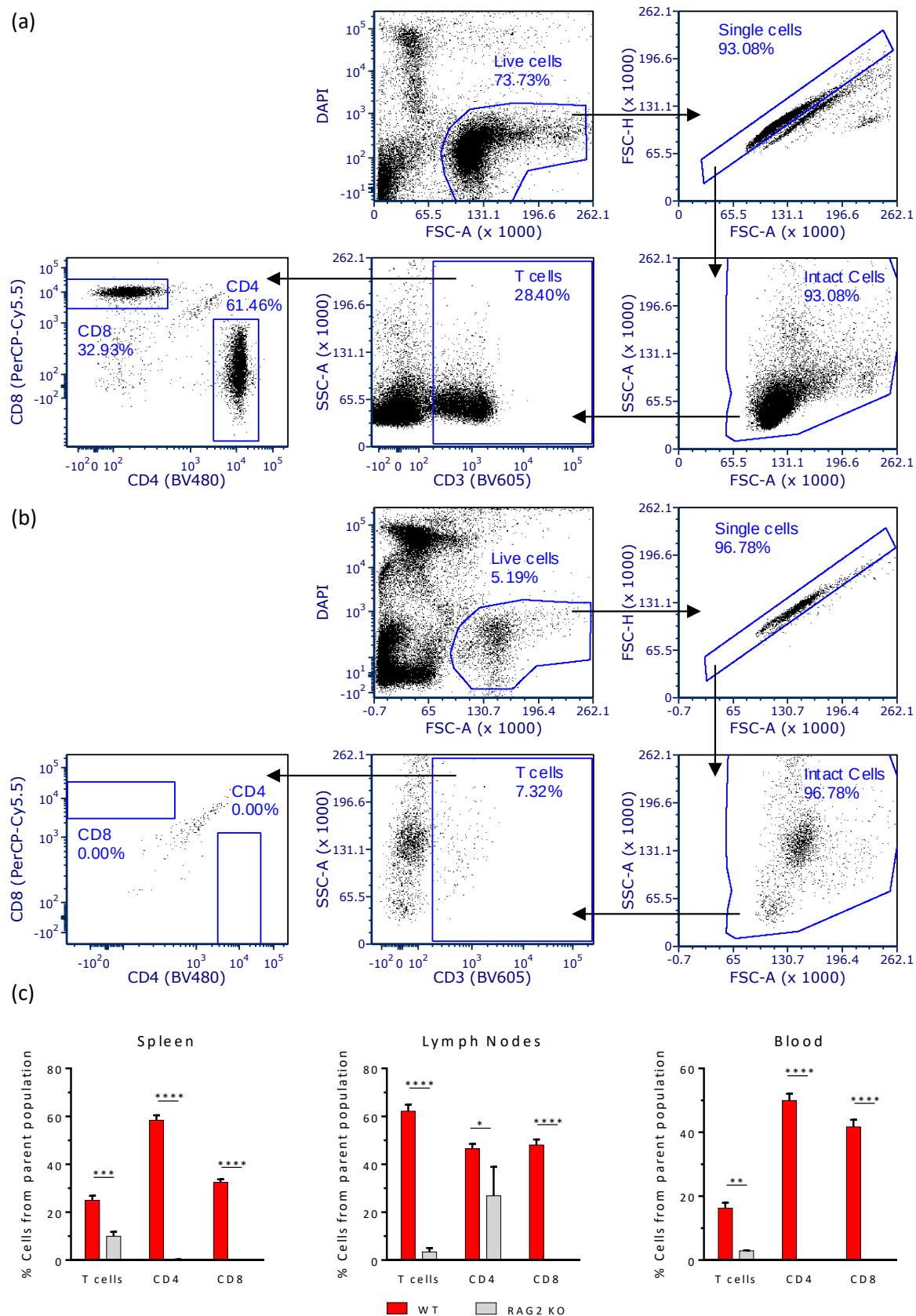


Figure 5.1 Baseline T cell phenotyping of RAG2 KO mice by flow cytometry. Gating strategy for (a) WT and (b) RAG2 KO mice. (c) Proportion of T cells in spleen, lymph nodes (popliteal and subiliac) and blood. Error bars = SEM; n = 3-9; Two-way ANOVA with Sidak's multiple comparisons test; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

5.2.2.2 Myocardial inflammation at 24h I/R

After having confirmed that RAG2 KO mice do not have mature T cells in the major peripheral lymphoid tissues, the inflammatory response to myocardial I/R was investigated. This was done at 24h post reperfusion as this is the peak of the pro-inflammatory response and the results in Chapter 3 show dramatic accumulation of CD45⁺ cells in the infarcted area. As shown on Figure 5.2a, RAG2 KO mice also showed such leukocyte infiltration to the left ventricular wall. Furthermore, loss of autofluorescence in the red channel, used as a surrogate marker for infarct size, coincided with CD45⁺ cell infiltration. However, the results on Figure 5.2b show that no significant difference was measured between the area of leukocyte infiltration (infarct area) in WT and RAG2 KO mice 24h after reperfusion ($p = 0.6991$). Interestingly, analysis of the density of CD45⁺ cell infiltrate showed that the lack of lymphocytes in RAG2 KO mice results in 35% increase in leukocyte density (Figure 5.3). While this difference from WT mice was not shown to be statistically significant ($p = 0.067$), it does show a clear trend towards more leukocytes, most likely neutrophils and monocytes, infiltrating the myocardium in the acute MI response of RAG2 KO animals.

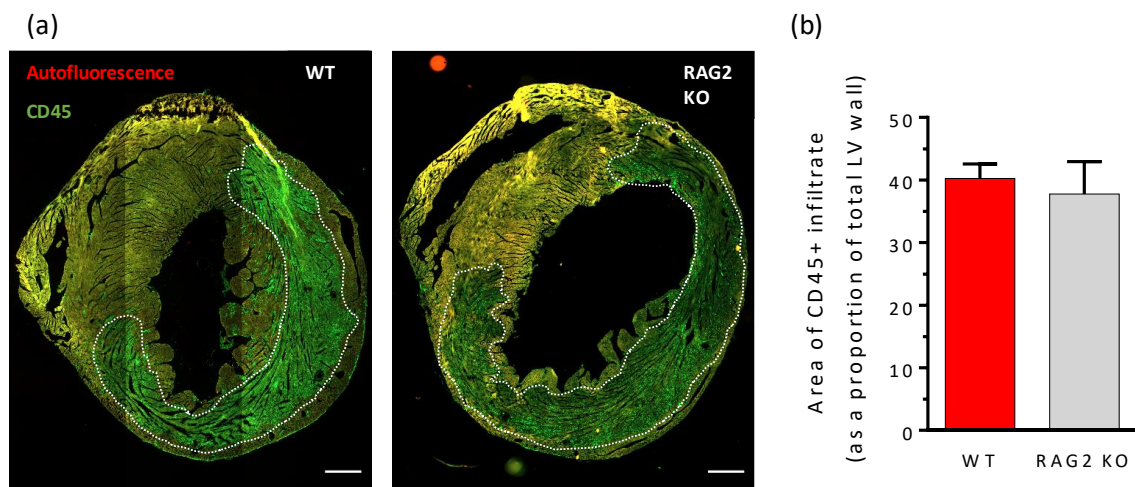
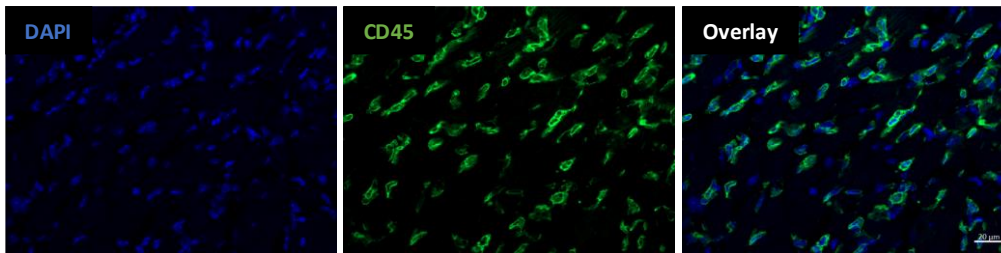


Figure 5.2 Area of leukocyte infiltration in the injured myocardium following 24 hours reperfusion. (a) Representative image of immune cells (green) infiltrating the heart tissue over the infarcted area, indicated by the loss of autofluorescence (red) that is typical of viable myocardial tissue (dotted line). Scale bar = 500 μ m; x5 magnification. Lines on WT image are due to microscope bulb misalignment that did not affect image analysis. (b) Area of CD45⁺ cell infiltration of the left ventricles WT and RAG2 KO mice. Error bars = SEM; $n = 6-8$ /group; Unpaired two-tailed t-test. Work done with Thomas Hosfield – under my supervision he sectioned RAG2 KO tissue and performed CD45 imaging and analysis.

(a)



(b)

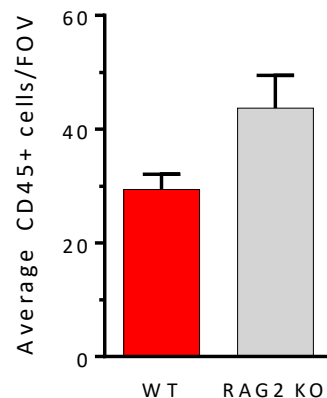


Figure 5.3 Density of leukocyte infiltrate in the absence of lymphocytes. (a) Representative image of CD45 IF staining. Scale bar = 20μm; x40 magnification. (b) Density of cell infiltration of the left ventricles WT and RAG2 KO mice. Error bars = SEM; n = 6-8/group; Unpaired two-tailed t-test. Work done with Thomas Hosfield – under my supervision he sectioned RAG2 KO tissue and performed CD45 imaging and analysis.

5.2.2.3 Adoptive transfer of CX3CR1-EGFP cells

Ultimately the aim of using the RAG2 KO mouse line for this PhD project was so that the effect of adoptively transferred T cells (with and without functional CX3CR1 expression) on the I/R response can be studied. However, as mentioned above, it was not possible to achieve this in time for the completion of the project because of disruption in the mouse housing unit. However, the results of a pilot study investigating the implementation of this technique in our research group are described below.

RAG2 KO mice were transfected with mononuclear splenocytes, isolated from CX3CR1-EGFP heterozygous and knockout donors. Circulating T cell and EGFP⁺ cell reconstitution was measured at Day 2 and Day 22 post transfer using flow cytometry, as shown on Figure 5.4a and b respectively. Additionally, splenic T cells were analysed at the terminal Day 22 time point using the same flow cytometry assay. The results on Figure 5.5a show that cell transfer was successful, with circulating EGFP⁺ cells being detected as early as 2 days after transfer and

having a 4-fold increase by Day 22 ($p \leq 0.05$). Successful transfer of T cells was also measured, both in the circulation (Figure 5.5b) and the spleens (Figure 5.5c). While the proportion of circulating T cells was increasing from Day 2 to Day 22, it failed to reach WT levels. Although the ~10% difference between WT control and RAG2 KO 22 days post transfer was not statistically significant. Importantly, the results on Figure 5.5b show that CD4 and CD8 T cells were also detected at Day 2 and Day 22 post transfer, showing dramatic increase from baseline RAG2 KO mice. The proportion of CD4 T cells in the circulation two days after adoptive transfer is comparable to WT controls 60% and 50% of T cells for RAG2 KO and WT respectively. But by Day 22 only 35% of T cells are CD4⁺, a significant decrease from both WT and Day 2 levels ($p \leq 0.0001$). In contrast, circulating T cell expressing the CD8 co-receptor show a 14% increase from Day 2 to Day 22 post transfer. Their level is comparable to that of WT mice, showing no significant difference. The results for T cell populations in the spleen 22 days post transfer show a similar pattern (Figure 5.5c). While total T cell levels do not reach the 25% of CD3⁺ live splenocytes of WT controls, there is a clear trend of increase compared to baseline RAG2 KO mice. Similarly, CD4 T cells at 22 days post transfer remain significantly lower than WT (40% reduction, $p \leq 0.0001$) but the percentage of CD8 T cells returns to WT levels (28% and 32% for Day 22 and WT controls respectively).

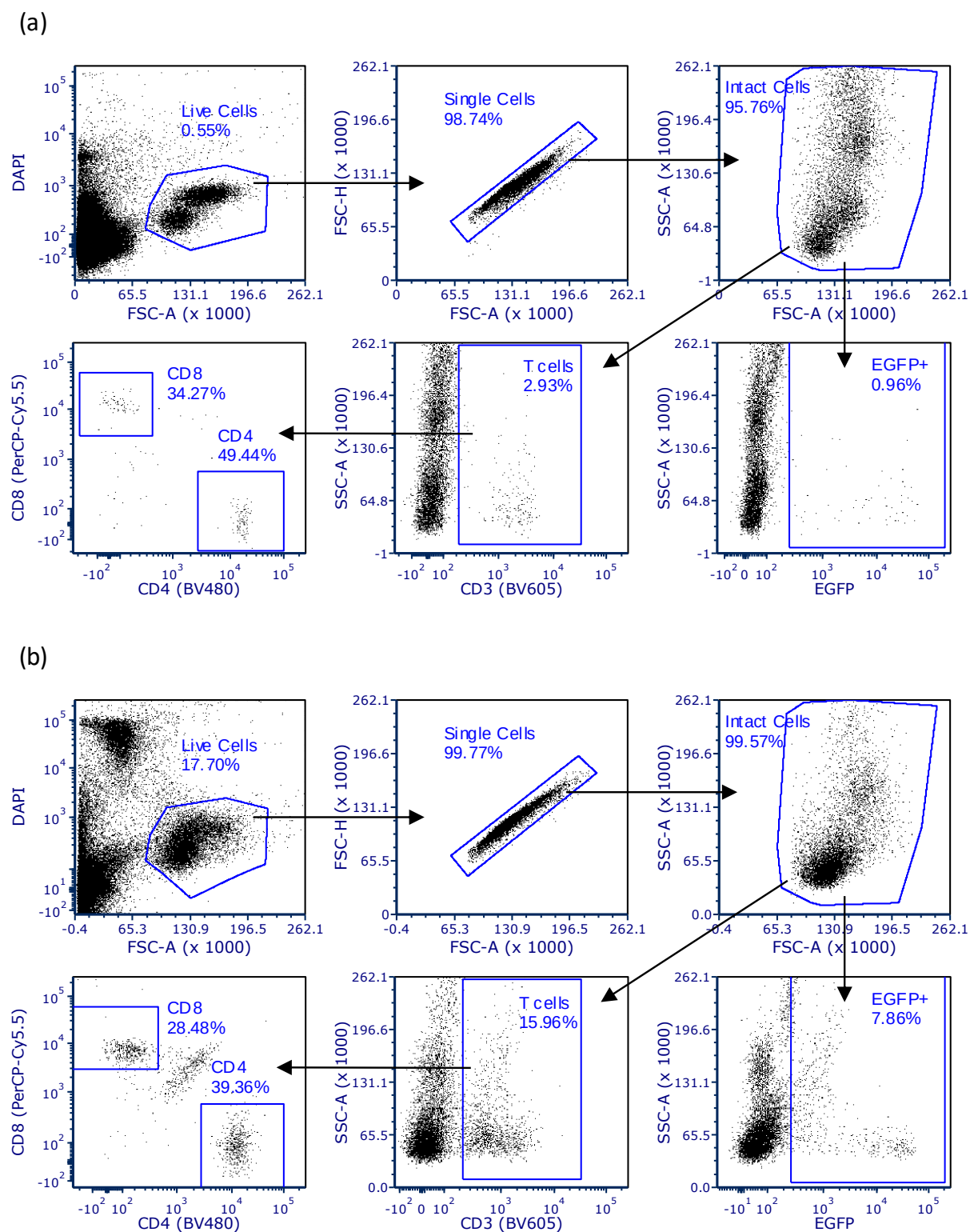


Figure 5.4 Confirmation of successful adoptive transfer using flow cytometry. Gating strategy for blood samples analysed at (a) Day 2 and (b) Day 22 post transfer of 3.3×10^7 cells/mouse.

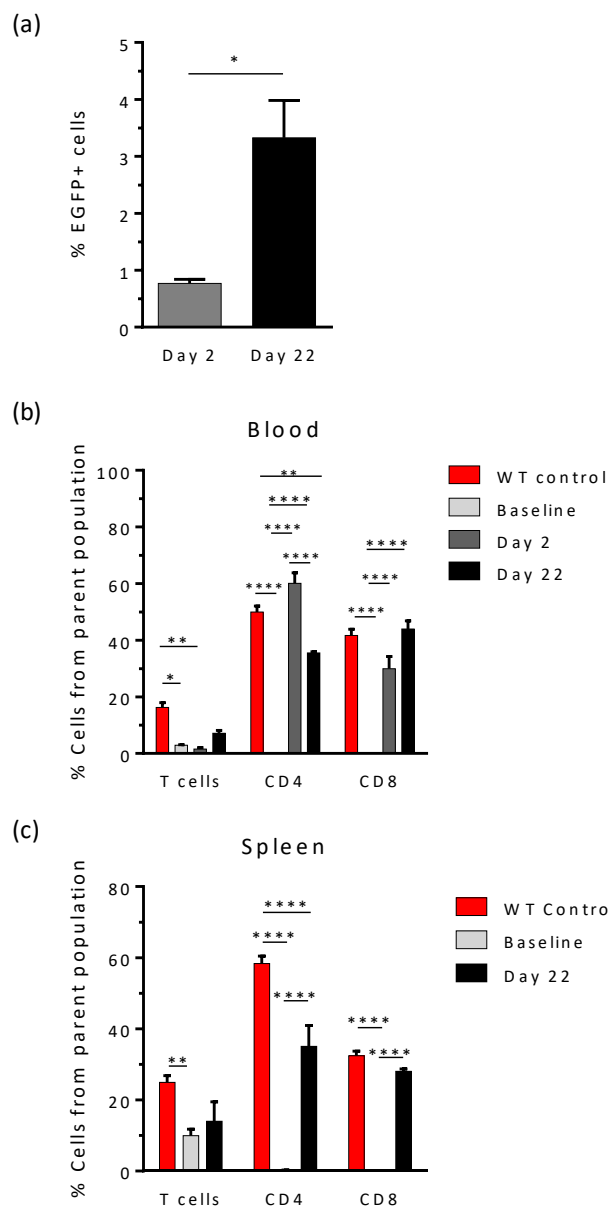


Figure 5.5 Adoptive transfer of CX3CR1-EGFP mononuclear cells into RAG2 KO mice. (a) Percentage of circulating EGFP⁺ cells (as proportion of total Intact cells) at Day 2 and Day 22 post transfer. Error bars = SEM; n = 3; unpaired two-tailed t-test; * $p \leq 0.05$. T cell populations in (b) blood and (c) spleen of WT control mice, baseline RAG2 KO mice, RAG2 KO mice at Day 2 and Day 22 post adoptive transfer. Error bars = SEM; n = 3-9. Two-way ANOVA with Tukey's multiple comparisons test; * $p \leq 0.05$; ** $p \leq 0.01$; **** $p \leq 0.0001$.

5.2.2 Dynamics of circulating CX3CR1⁺ T cells following cardiac I/R

Work from our group has shown that STEMI patients undergoing PPCI have transient depletion of circulating effector CD4 and CD8 T cells, most prominent at 90 minutes post reperfusion. This depletion is associated with CX3CR1 expression, and Boag et al have shown that T lymphocytes appear to be retained in the injured myocardium (Boag, Das et al. 2015). This study was key in driving the hypothesis of this PhD project as it is the only one to date showing a key role in myocardial I/R injury for CX3CR1⁺ T cells. Therefore, the work in this section

explores whether our current myocardial I/R mouse model is representative of the clinical environment that Boag et al. described. To achieve this, the absolute number of circulating immune cells and their CX3CR1 expression was measured in C57BL/6 mice before MI and 2h post reperfusion (Figure 5.6). In addition to performing this analysis on mice using our standard “Open MI” model, the temporal dynamics of circulating leukocytes were also analysed in mice which infarction was induced using a “Closed MI” model (described in Chapter 2 Section 2.2.1). In this latter model, mice underwent a surgery to place the LAD suture, without tying it, 7 days before inducing MI. This was done in order to assess the effect of systemic inflammation due to the open chest surgery of the original Open MI model, which does not occur in clinical MI patients.

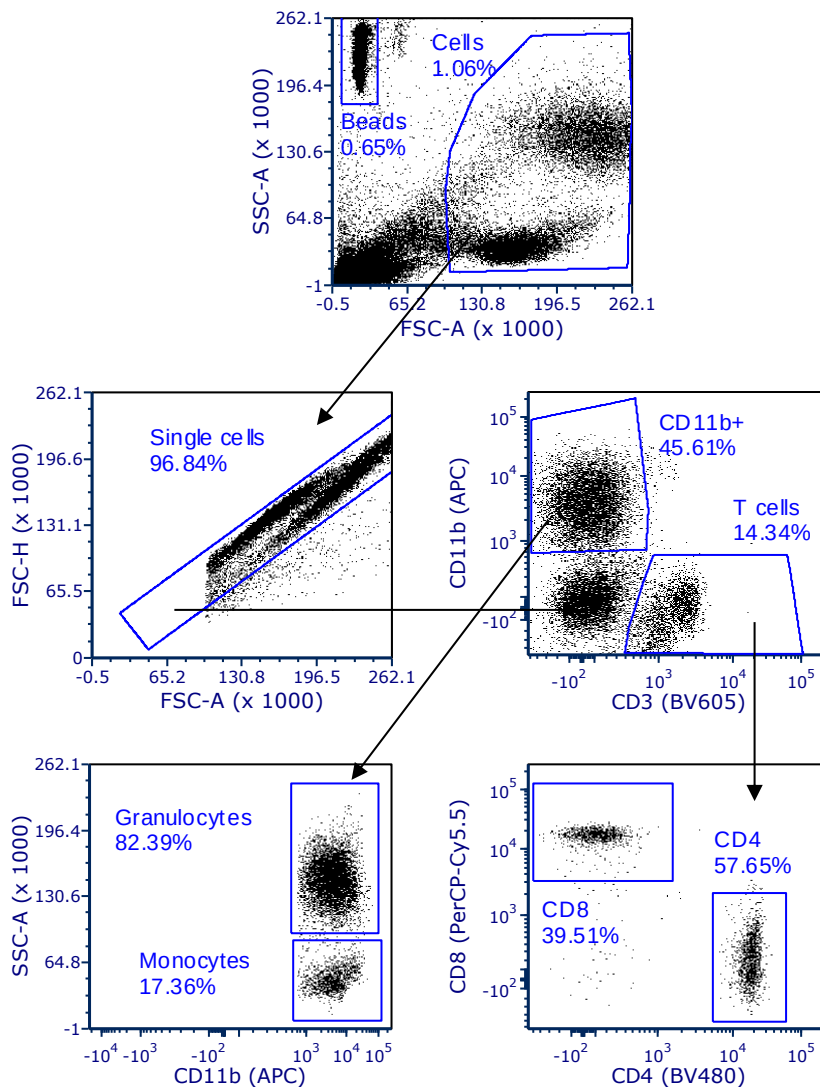


Figure 5.6 Gating strategy for absolute cell count analysis of blood samples of mice at 2h I/R

In contrast to STEMI patients, the results for mice undergoing Open MI (Figure 5.7) show that no depletion in the number of total, CD4 or CD8 T cells is detected 2h post reperfusion. While

there is a trend of reduction from baseline across all these populations, this was also observed for mice undergoing Sham surgery. There was also no significant difference in the dynamics of circulating granulocytes, monocytes or CX3CR1⁺ monocytes. Both Sham and I/R surgery result in a small increase in granulocyte numbers and decrease in monocytes and CX3CR1⁺ monocytes. However, myocardial I/R induced a 70% decrease ($p \leq 0.01$) in the number of CX3CR1⁺ CD4 T cells. As well as a trend of reduction, although not significant, for both CD8 T cells and total T lymphocytes (40% and 30% reduction respectively). This response was specific to mice in the I/R group, as no such pattern was observed in sham animals. However, the significant CX3CR1⁺ cell decrease in I/R mice appears to be due to high baseline reading, as there was no significant difference between sham or I/R mice 2h after surgery. In fact, the number of CX3CR1⁺ T cells and CD4 T cells at baseline was significantly different between mice in the sham and I/R group ($p \leq 0.05$ and $p \leq 0.001$ for total T cells and CD4 T cells respectively). The baseline number of CX3CR1⁺ CD8 T cells in the blood of mice in the I/R group also showed a 2-fold increase than sham mice, although this was not significant. This significant baseline variability is surprising as baseline sampling was performed 7 days prior to any surgery. Moreover, at this point these mice had not yet been assigned to each group (sham or I/R) so baseline sampling and analysis was blinded.

Figure 5.8 shows that mice in the Closed MI model group also have significant baseline variability in the number of total T cells, CD4 T cells and CD8 T cells ($p \leq 0.01$). Moreover, baseline CX3CR1⁺ CD4 and CD8 T cell numbers were 2-fold higher in the sham surgery group than their I/R counterpart ($p \leq 0.05$). This is in contrast to the results for Open MI mode shown on Figure 5.7. Subsequently, this baseline variability leads to a significant reduction in the number of all T cell populations between sham baseline and 2h post sham surgery ($p \leq 0.05$ – $p \leq 0.01$). Interestingly, mice undergoing closed model MI show an 85% increase in the number of circulating granulocytes 2h post surgery compared to sham animals ($p \leq 0.05$). Furthermore, closed model surgery (both sham and I/R) induced around 50% depletion of circulating monocytes and CX3CR1⁺ monocytes 2h after surgery ($p \leq 0.01$). Although the latter was not shown to be statistically significant for sham mice.

Ultimately, the results described in this section, using either MI model, fail to recapitulate the significant lymphopenia described by Boag et al. (Boag, Das et al. 2015). This is suggestive that the current MI mouse model may not be well suited to investigate the role of CX3CR1⁺ T cells in myocardial I/R injury.

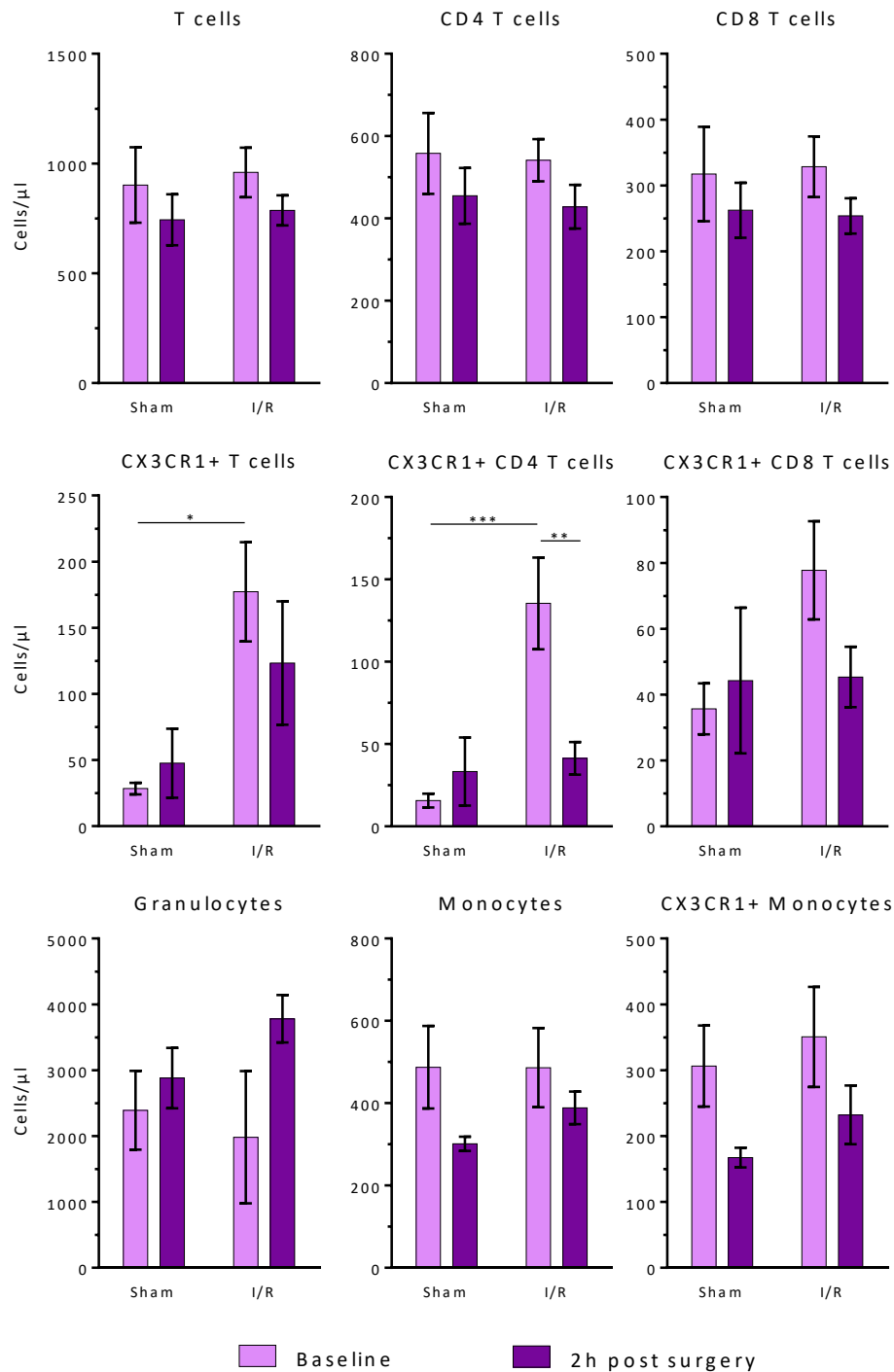


Figure 5.7 Dynamics of circulating immune cells following open model MI surgery and 2h reperfusion. Error bars = SEM; n = 6-8. Two-way repeated measures ANOVA with Sidak's multiple comparisons test; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

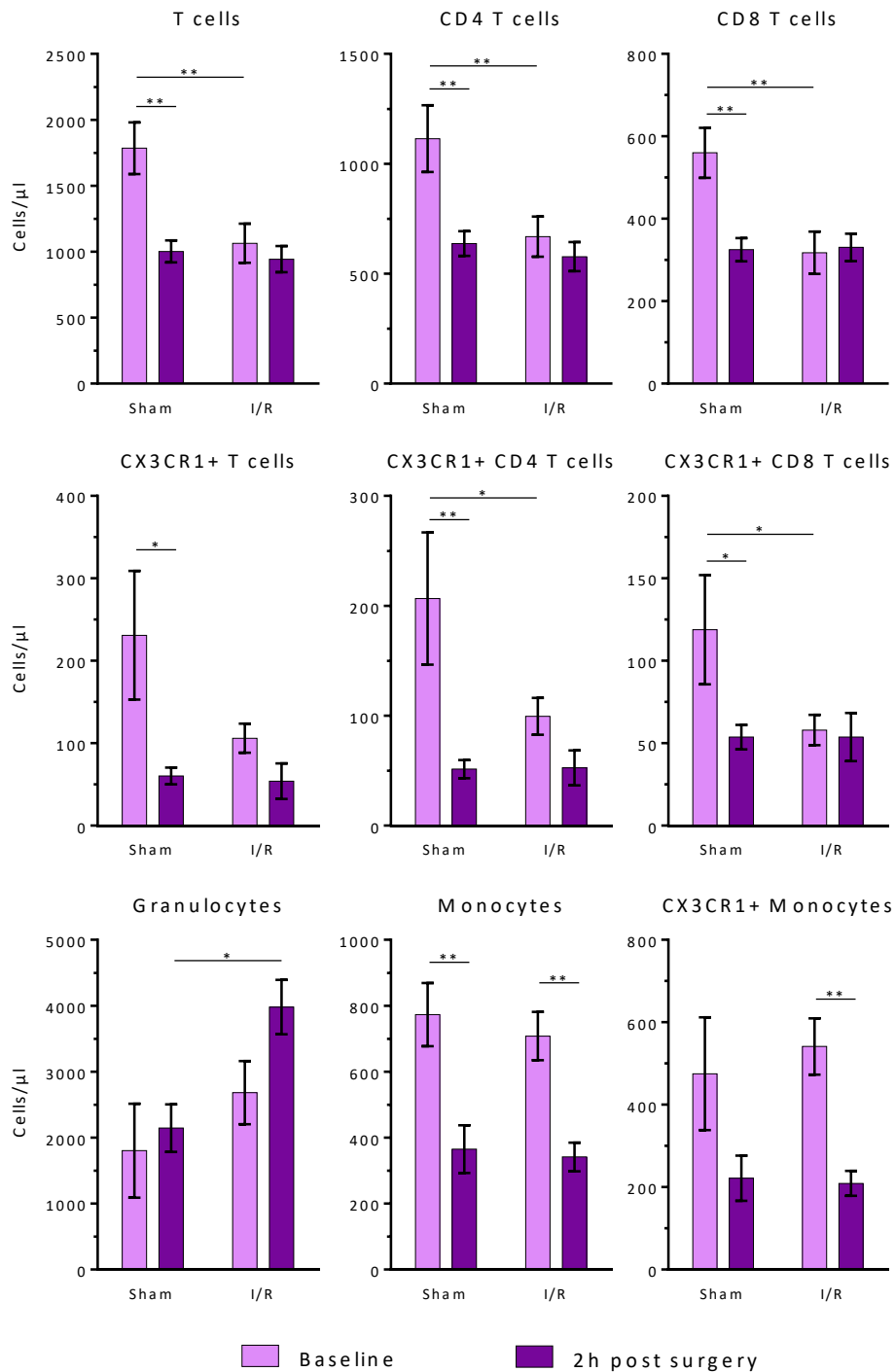


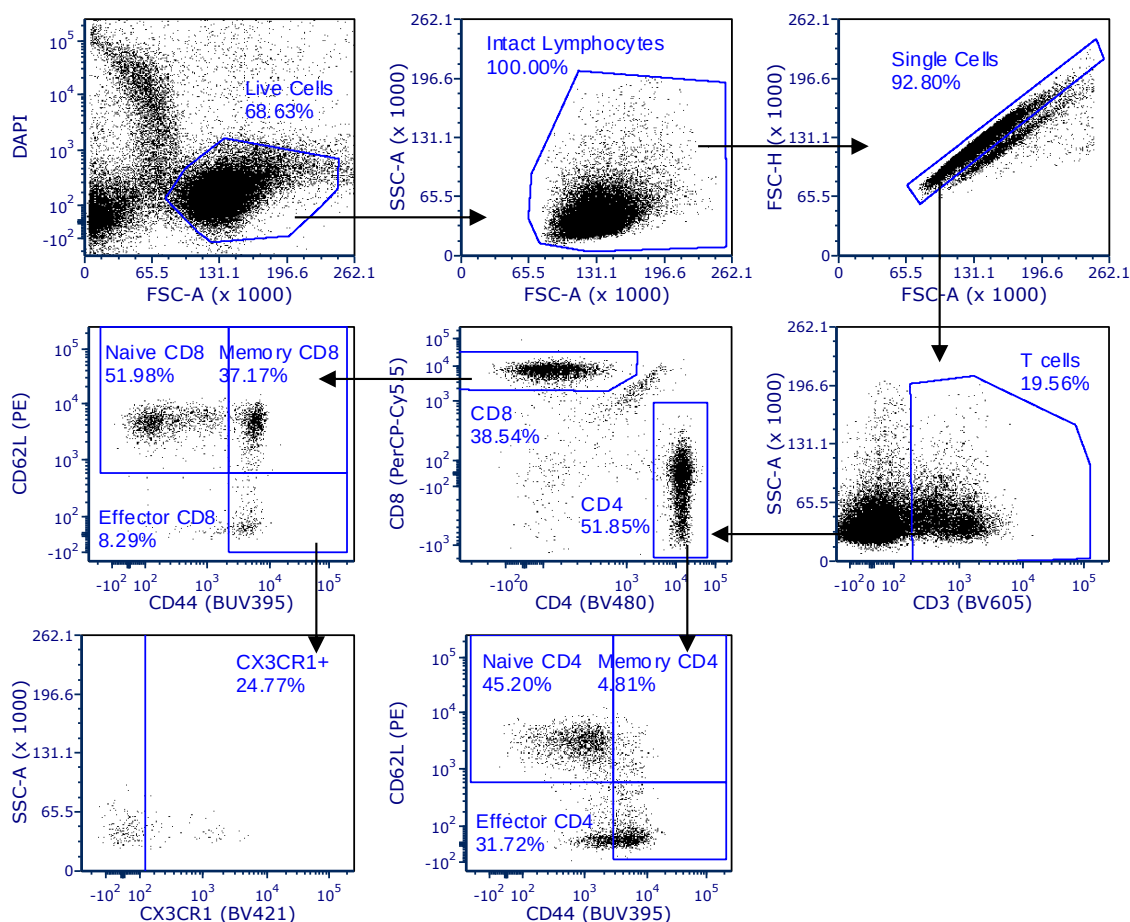
Figure 5.8 Dynamics of circulating immune cells following closed model MI surgery and 2h reperfusion. Error bars = SEM; n = 7-8. Two-way repeated measures ANOVA with Sidak's multiple comparisons test; * p ≤ 0.05; ** p ≤ 0.01.

5.2.3 CX3CR1+ T cells in aged mice

The results from Section 5.2.2 led to the need to explore alternative mouse models which are more representative of clinical MI. One of the primary risk factors for development of cardiovascular disease is age (Dhingra, Vasan 2012). Increased age is also associated with

higher proportion of effector and memory T cells (which have higher % CX3CR1 expression) due to more naïve T cells having encountered their cognate antigen (Sadighi Akha 2018, Tao, Reese 2017). Therefore, CX3CR1 expression in splenic T cells from old mice (100 weeks or 23 months) was investigated by flow cytometry (Figure 5.9a). The results on Figure 5.9b show that there is no significant difference between the proportion of total T cells, CD4 or CD8 T cells, although there appears to be small reduction in the CD4:CD8 ratio. Importantly, old mice showed a highly significant reduction in naïve CD4 (60% reduction, $p \leq 0.0001$) and CD8 T (50% reduction, $p \leq 0.0001$) cells with corresponding increase in effector CD4 (3-fold increase, $p \leq 0.0001$) and memory CD8 (4-fold increase, $p \leq 0.0001$) T cells. An 8-fold increase in effector CD8 T cells was also detected but this was not statistically significant. Furthermore, T cells and CD8 T cells showed a significant increase in the percentage CX3CR1⁺ cells with a 4-fold ($p \leq 0.01$) and 14-fold ($p \leq 0.05$) increase respectively. While there was no change in the proportion of cells with CX3CR1 expression in effector populations, old animals had increased percentage of CX3CR1⁺ CD4 and CD8 T cells. CD4 memory cells showed a 4-fold increase in CX3CR1⁺ cells ($p \leq 0.0001$) while memory CD8 had a 6-fold increase that was not statistically significant.

(a)



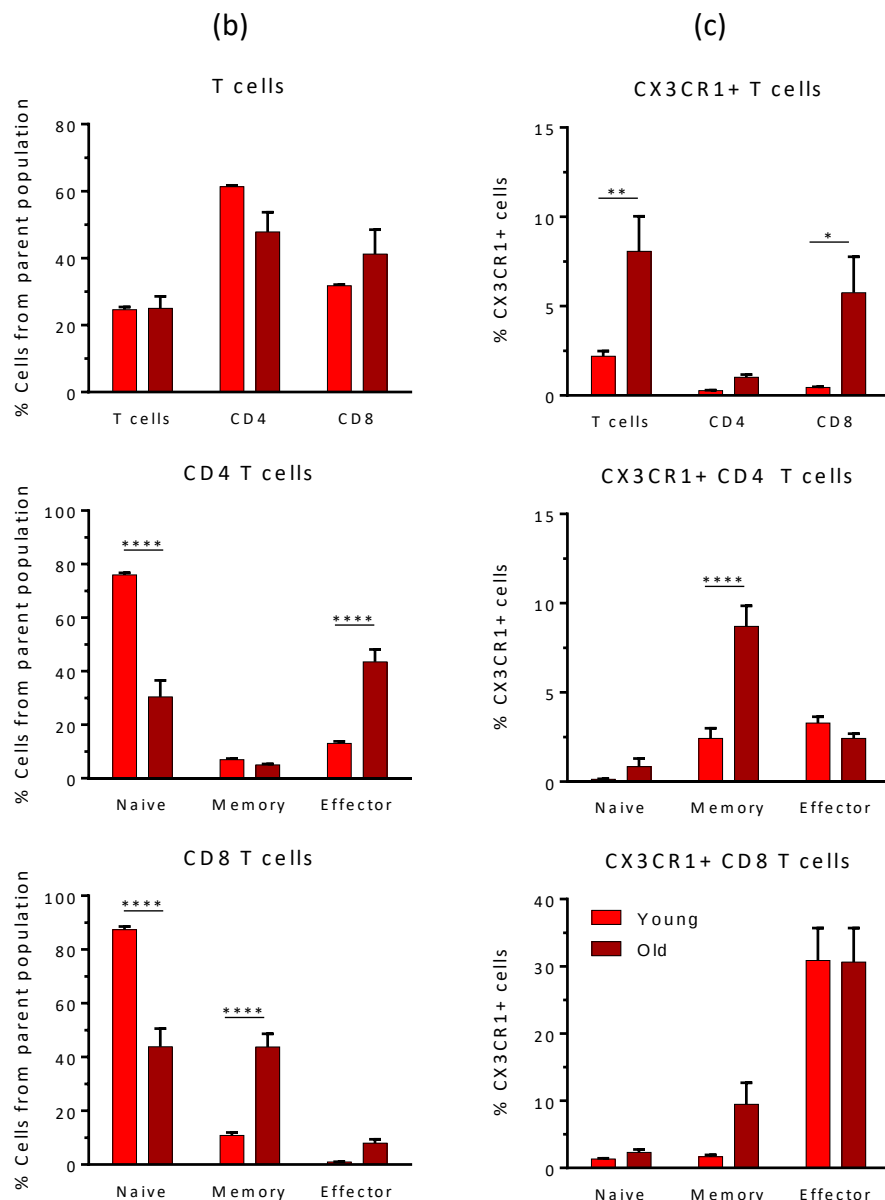


Figure 5.9 Baseline phenotyping of splenic T cells in aged mice. (a) Flow cytometry gating strategy. Proportion of (b) major T cell populations and their (c) CX3CR1 expression. Young mice = 3 months old. Old mice – 23 months old. Error bars = SEM; n = 6-7; Two-way ANOVA with Sidak's multiple comparisons test; * $p \leq 0.05$; ** $p \leq 0.01$; **** $p \leq 0.0001$.

5.2.4 MuLV infection causes increase in CX3CR1+ T cells

Virus infections are known to cause increase in CX3CR1⁺ T cells (Mudd, Panigrahi et al. 2016) and given that MI patients have been exposed to multiple pathogens during their lifetime, the final aim of this PhD project was to investigate CX3CR1 expression in T cells in a mouse infection model. T cell phenotyping was performed on mice following infection with murine leukaemia virus (MuLV) using flow cytometry (Figure 5.10). Spleen and blood T cells were investigated in mice 5 weeks after infection. Figure 5.11 shows that while MuLV infection does not alter total T cell levels, it does cause an increase in the CD4:CD8 ratio, both in the spleen

and blood. Infected mice have around 50% increase in proportion of CD4 T cells and around 85% decrease in CD8 T cells in the spleen and blood ($p \leq 0.0001$ and $p \leq 0.001$ respectively). Splenic T cells also show a dramatic reduction in naïve CD4 and CD8 T cells, with a drop of 95% ($p \leq 0.0001$) and 85% ($p \leq 0.0001$) respectively. This is matched by significant increase of effector cells, with a 4-fold increase in effector CD4 ($p \leq 0.0001$) and over 7-fold increase ($p \leq 0.0001$) in effector CD8 cells. While splenic memory CD8 T cells do not show significant upregulation, their CD4 counterpart show a 2-fold increase ($p \leq 0.05$). CD4 and CD8 subpopulations show a similar pattern in the blood of MuLV infected mice. Although less drastic, depletion of naïve CD4 (70% reduction ($p \leq 0.0001$)) and CD8 (55% reduction ($p \leq 0.0001$)) T cells is evident. Furthermore, memory CD4 and CD8 cells in MuLV mice have 13-fold ($p \leq 0.01$) and 2-fold ($p \leq 0.05$) increase respectively, as well as 5-fold increase in effector CD4 ($p \leq 0.001$) and significant 10-fold increase in effector CD8 T cells ($p \leq 0.05$).

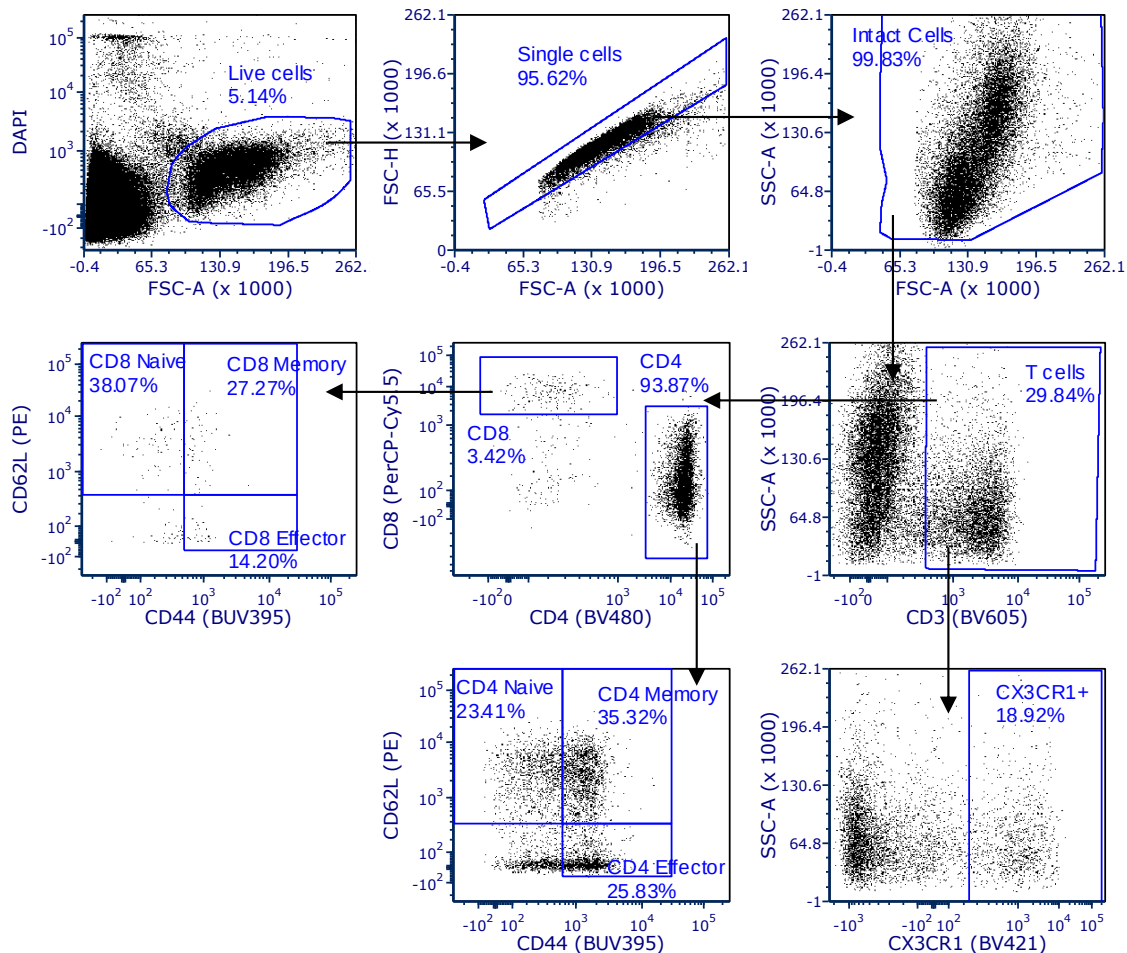


Figure 5.10 Flow cytometry gating strategy for baseline phenotyping of circulating T cells in mice infected with MuLV.

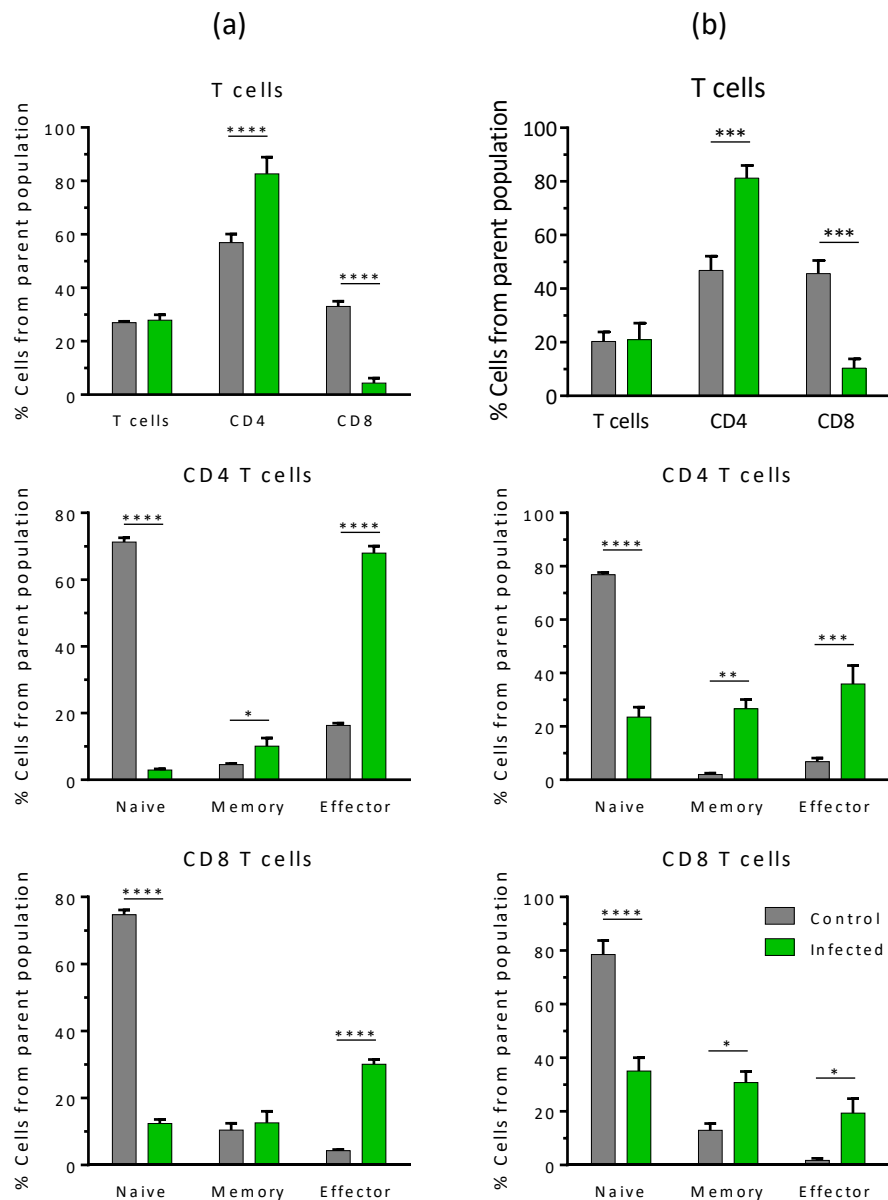


Figure 5.11 Baseline T cell phenotyping of mice infected with MuLV. Viral infection causes T cell maturation in (a) spleen and (b) blood. Error bars = SEM; n = 3-4; Two-way ANOVA with Sidak's multiple comparisons test; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

MuLV infected mice also show a dramatic increase in CX3CR1 expressing T cells across all populations, as shown on Figure 5.12. Both blood and spleen derived T cells have elevated CX3CR1 expression although this is not significant for CD4 and total T cells in the spleen. However, these populations in the blood show a 10-fold increase ($p \leq 0.0010$). But CD8 T cells in both lymphoid tissues show a dramatic increase in CX3CR1 expression, with circulating CD8 cells having a 17-fold increase ($p \leq 0.0001$) post MuLV infection and splenic CD8 T cells showing a 33-fold increase ($p \leq 0.0001$). Furthermore, naïve CD4 T cells in the spleen and blood have dramatic increase in CX3CR1 expression (100-fold, $p \leq 0.0001$, and 40-fold, $p \leq 0.05$ respectively). While all CD4 effector and memory population show increased percentage of

CX3CR1⁺ cells, this was only significant for splenic memory CD4 T cells (8-fold increase, $p \leq 0.0001$). Similarly, effector CD8 cells in the spleen and blood showed 3-fold ($p \leq 0.001$) and 2-fold ($p \leq 0.01$) increase in CX3CR1 expression respectively. Memory CD8 T cells had 22-fold increase in the blood ($p \leq 0.001$) and a 4-fold increase in the spleen ($p \leq 0.0001$). On the other hand, only splenic naïve CD8 T cells showed statistically significant 2-fold increase ($p \leq 0.05$).

Overall, these results show that mice have a dramatic increase in the proportion of T cells expressing CX3CR1 5 weeks following MuLV infection.

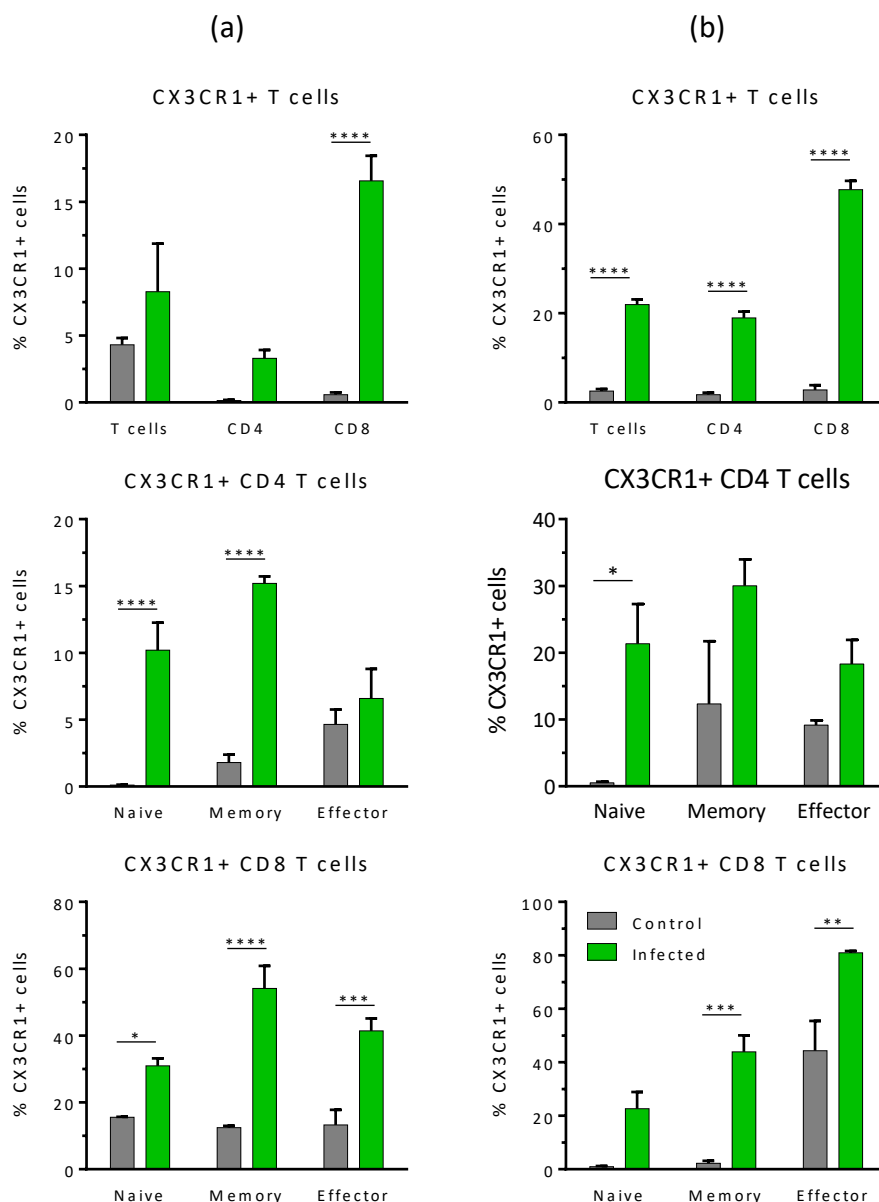


Figure 5.12 CX3CR1 expression in T cells of mice infected with MuLV. Viral infection causes an increase in CX3CR1⁺ T cells in (a) spleen and (b) blood. Error bars = SEM; n = 3-4; Two-way ANOVA with Sidak's multiple comparisons test; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

5.3 Discussion

The results in Chapter 3 highlighted the challenges of studying the specific role of CX3CR1⁺ T cells in myocardial infarction by utilising a widely used mouse model of young immunologically unchallenged animals with a global gene knockout. Therefore, the final part of this PhD project, presented in this chapter, focused on addressing these challenges and investigated the effect of different T cell phenotypes on the inflammatory response to myocardial ischaemia/reperfusion. The results described above show that complete lack of lymphocytes causes a strong innate immune cell infiltrate in the post MI myocardium. And while it was not possible to study the specific role of CX3CR1⁺ T cells in time for this PhD project due to disruption in the mouse housing unit, this chapter presents novel results the implication of which are discussed below. Furthermore, the work in this chapter provides an important example of the difficulties behind studying the inflammatory response in humans using mouse models. Immunology is a rapidly developing area of science and our increasing understanding of its complexity requires careful consideration in order to identify the optimal mouse model. This chapter describes two such models, ageing and infection, which are key immunogenic processes that are tremendously underappreciated in mouse research.

The first step of investigating the effect of no lymphocytes on the myocardial I/R response was to confirm that no mature T cells are detected in the lymphoid tissue of RAG2 KO mice. Baseline T cell phenotyping of this line using flow cytometry analysis of T cell markers in the blood, spleen and lymph nodes confirms dramatic loss of these cells. However, it should be noted that some immature CD3⁺ T cells were detected that were double positive for CD4 and CD8. This is not unexpected as RAG1 has been shown to mediate V(D)J recombination in the absence of RAG2 (Carmona, Fugmann et al. 2016). While RAG1 and RAG2 KO mice have been shown to have identical phenotypes, the two proteins have distinct roles in V(D)J recombination (Akamatsu, Oettinger 1998). RAG1 recognises the recombination signal sequence on the DNA, mediates binding to it and recruits RAG2 to form the RAG1/2 complex. While RAG2 binding directly to the DNA in the absence of RAG1 has not been reported (Akamatsu, Oettinger 1998). On the other hand, RAG2 identifies transcriptionally active chromatin by interacting with histone H3 in which Lys4 is trimethylated (H3K4me³), ultimately enhancing binding between the DNA and the RAG1/2 complex (Carmona, Fugmann et al. 2016). Importantly, RAG1 carries the catalytic domain of the complex with RAG2 having no known enzymatic function (Carmona, Fugmann et al. 2016). Therefore, it is not unexpected

that a low level of CD3⁺ events is detected in the lymphoid tissues of RAG2 KO mice. Despite this, the results on Figure 5.1 clearly, show that RAG2 KO mice have dramatic reduction in the number of CD3⁺ cells in blood, spleen and lymph nodes, and complete lack of mature single positive CD4 or CD8 T cells.

Afterwards, the effect of RAG2 KO on the inflammatory response to MI 24h post reperfusion was assessed. The data presented here shows that while lack of lymphocytes does not alter the area of leukocyte infiltrate, it does lead to a 35% increase in its density (albeit not significant). The lack of differential area of CD45⁺ cell infiltration between WT and RAG2 KO mice is unexpected, as this is used as a surrogate marker of infarct size. This is in contrast to the seminal study by Yang et al. who describe a decrease in the infarct area of RAG1 KO mice following myocardial I/R (Yang, Day et al. 2006). Moreover, Yang et al. show that this protective effect in RAG1 KO mice is due to the absence of CD4 T cells (Yang, Day et al. 2006). A finding later confirmed by Mathes et al. who showed reduced infarct size in MHC class II KO mice, deficient in CD4 T cells (Mathes, Weirather et al. 2016). However, the discrepancy between the results may be due to the different methods used. While the results here use CD45⁺ cell infiltration and loss of autofluorescence as a guide to infarct size, both Yang et al. and Mathes et al. used triphenyl tetrazolium chloride (TTC), together with Phthalo Blue or Evans Blue staining respectively, which allowed them to normalise the infarct size to the area of myocardium at risk (INF/AAR). Indeed, Mathes et al. only describe a significant reduction in infarct size of MHC class II KO mice when presented as INF/AAR. In addition to the different method used to evaluate infarct size, the myocardial I/R model used in these two research groups differs from the one used in ours. This PhD project exclusively uses a model of 60 minutes ischaemia, while Yang et al. use 40-45 minute coronary occlusion and Mathes et al. use 30 minute ischaemia. It is unclear what effect this difference in ischaemic time could have (though it is likely to increase necrotic myocardium), but it does suggest caution must be taken when comparing these studies.

More interestingly, the results on Figure 5.3 shows that RAG2 KO mice show increased density in the infarct area 24h after reperfusion. While this difference did not reach statistical significance, the increasing trend is clear. This suggests that similar to CX3CR1 KO in Chapter 3, lack of mature lymphocytes results in some form of compensatory inflammatory response. As RAG2 KO mice are devoid of adaptive immunity, this indicates that a more prominent innate immune cell repertoire is recruited following MI. Most likely it consists of neutrophils

as they are one of the most numerous cell type infiltrating the MI myocardium at this time point (Yan, Anzai et al. 2013a). Within hours of MI, they localise to the injured myocardium, peaking between one and three days post MI (Ong, Hernández-Reséndiz et al. 2018). Their function is to scavenge cell debris and phagocytose dead or dying cells (Swirski, Nahrendorf 2018). They do this by releasing granules containing proteolytic enzymes such as matrix metalloproteinases (MMPs), generation of reactive oxygen species, as well as pro-inflammatory cytokines such as IL-1, IL-6 and TNF (Jordan, Zhao et al. 1999, Swirski, Nahrendorf 2018, Ong, Hernández-Reséndiz et al. 2018). The latter include factors that are chemotactic to monocytes and result in further amplification of the inflammatory response (Ong, Hernández-Reséndiz et al. 2018). Moreover, high neutrophil count in the circulation of STEMI patients undergoing PPCI is an independent predictor of adverse cardiac remodelling and is directly related to larger infarct size (Chia, Nagurney et al. 2009, Guasti, Dentali et al. 2011). Therefore, neutrophils are generally considered detrimental in the context of myocardial I/R. Despite this, they are essential for cardiac healing as evidenced by worse cardiac function in a neutrophil depletion permanent MI mouse model (Horckmans, Ring et al. 2016). In addition to this, more recent evidence shows that neutrophils can influence macrophage polarisation by promoting their differentiation into the reparative M2 phenotype (Horckmans, Ring et al. 2016). Furthermore, Ma et al. have suggested that neutrophils themselves can be polarised following MI (Ma, Yabluchanskiy et al. 2016). They report that neutrophils harvested at day one following MI are highly pro-inflammatory, while those harvested at days 5-7 post MI had an anti-inflammatory phenotype (Ma, Yabluchanskiy et al. 2016). Similar to macrophages, the two populations of neutrophils have been termed N1 and N2 for pro- and anti-inflammatory respectively (Ma, Yabluchanskiy et al. 2016). Therefore, expansion of the neutrophil infiltrate in the left myocardium 24h post MI may provide a cardioprotective function by rapidly clearing dead cells and debris, as well as promote the polarisation of N2 and M2 cells. The latter of which are crucial for the reparative phase of myocardial I/R injury. Indeed, this is supported by unpublished data from our group showing increased density of Ly6G⁺ neutrophils in the hearts of RAG2 KO mice 24h post reperfusion, as well as improved cardiac function at 6 weeks reperfusion.

However, as discussed above, T cells have been shown to be directly responsible for causing myocardial damage as measured by increase in infarct size and more detrimental cardiac function. So far studies have failed to elucidate the exact mechanisms by which T cells confer

their detrimental function. This presents a significant challenge due to the complex interplay between different immune cells, as well as the multiple T cell subtypes with remarkably different functions. Indeed, neutrophil clearance required for the reparative phase of myocardial I/R may be due to the action of T cells. This may be induced directly by T cell secretion of anti-inflammatory cytokines which attenuate neutrophil recruitment and function, or indirectly by T cells releasing factors chemotactic for M2 macrophages and non-classical monocytes that in turn modify the neutrophil response.

While CD4 T cells are more widely investigated in the context of MI, studies on the role of CD8 T cells in myocardial infarction are limited. A permanent MI rat model described that CD8 T cells isolated from these rats exert potent MHC restricted cytotoxicity against cardiomyocytes (Varda-Bloom, Leor et al. 2000). In addition to this, two clinical studies identify two distinct CD8 T cell subpopulations that are increased in the circulation and contribute to myocardial injury post MI (Tae Yu, Youn et al. 2015, Zhang, Lili, Wang et al. 2018). They report that levels of CD57⁺ and CD28⁺ CD8 T cells are correlated with worse cardiac function and survival (Tae Yu, Youn et al. 2015, Zhang, Lili, Wang et al. 2018). Expression of the co-stimulatory receptor CD28 is a marker of cytotoxic CD8 T cells and the level of CD28⁺ CD8 T cells correlates with patients that present with larger infarcts and continue to develop more deteriorating cardiac function in the 12 months following infarction (Zhang, Lili, Wang et al. 2018). More recently, Ilatovskaya et al. implicated CD8 T cells as negative regulators of myocardial inflammation (Ilatovskaya, Pitts et al. 2019). Using a permanent MI mouse model, they showed that CD8 T cells deficiency results in improved cardiac function and increased survival 7 days after infarct. However, these mice also had higher number of neutrophils and macrophages as early as 3 days post infarct. Unexpectedly, Ilatovskaya et al. showed that lack of CD8 T cells results in severe phenotype of cardiac rupture in mice post MI. They suggest that the reason behind this is the increased pro-inflammatory innate immune cell infiltrate that results in poor scar formation. While the increase in innate immune cell infiltration is in accordance with the data presented in this chapter, showing increased total leukocytes in mice lacking any mature lymphocytes, the poor scar formation is not. Unpublished data from our group shows that RAG2 KO mice have smaller scar size 6 weeks after reperfusion. This discrepancy between the findings suggests that B cells or CD4 T cells are responsible for detrimental cardiac remodelling in the late myocardial I/R response. Th1 CD4 T cells are a likely candidate as their numbers are

increased in CD8 deficient mice, a trend not observed for Th2 cells (Ilatovskaya, Pitts et al. 2019).

Indeed, the role of CD4 T cells on MI and myocardial I/R has been more widely studied than that of CD8 T cells. Hoffman et al. report that CD4 T cells have a cardioprotective role in experimental MI mice (Hofmann, Beyersdorf et al. 2012). Using a permanent MI model, they show that IFN γ expressing CD4 T cells infiltrate the infarcted myocardium and confirm that TCR-dependent activation is required for wound healing and remodelling. These cells are likely of the Th1 CD4 subtype and the authors suggest that they are activated by cardiac autoantigen recognition (Hofmann, Beyersdorf et al. 2012). In their study, CD4 KO mice showed detrimental cardiac remodelling 56 days after infarct, while mice with disrupted MHC class II function (MHC II KO and OT-II mice) had greater mortality due to myocardial rupture (Hofmann, Beyersdorf et al. 2012). Moreover, they describe increased leukocyte and pro-inflammatory monocyte infiltration in the absence of CD4 T cells 7 days after MI (Hofmann, Beyersdorf et al. 2012). Despite the different time point, this is in accordance with the results described in this chapter, showing increased CD45⁺ cell density in RAG2 KO mice 24h post reperfusion.

Further to the role of Th1 CD4 T cells, a number of research groups report conflicting evidence for the role of immunomodulatory Tregs in response to MI with both detrimental and healing function being described. Weirather et al. show evidence that Foxp3⁺ CD4 Tregs are important for myocardial healing after MI (Weirather, Hofmann et al. 2014). Using a Treg depletion model (either genetic or therapeutically using anti-CD25 antibody), they reported reduced survival after permanent MI. This phenotype could be rescued by the administration of superagonistic anti-CD28 antibody that results in Treg population expansion. They observed this effect 7 days after permanent MI and reported that absence of Tregs results in proinflammatory M1 macrophage polarisation (Weirather, Hofmann et al. 2014). Interestingly, the same group later discovered that Foxp3⁺ CD4 Tregs are detrimental to myocardial healing in ischaemia/reperfusion injury (Mathes, Weirather et al. 2016). As described above, they used a MHC class II KO mice in a model of 30 minute ischaemia. However, similar to the I/R model used throughout this PhD project, Mathes et al. focused on the early T cell role by investigating the response in the first 24 hours. Unexpectedly, they found that Tregs actually contribute to the early I/R injury and they appear to do this in an antigen-independent manner outside lymphoid organs (Mathes, Weirather et al. 2016).

Foxp3⁺ Tregs are known to activate in response to pro-inflammatory stimuli, including TNF α , in an antigen-independent manner, resulting in upregulate expression of the lymphocyte activation marker CD69 (Bremser, Brack et al. 2015). Although Mathes et al. did not investigate the mechanism by which Tregs contribute to myocardial I/R injury (Mathes, Weirather et al. 2016), this is likely due to promoting a pro-inflammatory environment. Tregs have been reported to secrete IFN γ in a Th1 cytokine environment (Koenecke, Lee et al. 2012, Daniel, Wang et al. 2014) which can in turn promote M1 polarisation (Wang, F., Zhang et al. 2018, Lu, Li et al. 2019). The latter was reported earlier in the permanent MI work by Weirather et al. (Weirather, Hofmann et al. 2014). Furthermore, the seminal study by Yang et al. showing that IFN γ producing CD4 T cells contribute to myocardial I/R (Yang, Day et al. 2006), is in support of this proposed deleterious IFN γ secreting CD4 Tregs.

Although B cells are classically recognised for their role in antibody-mediated immunity, recent studies point to a key modulatory function of B cells in both the adaptive immune response, as well as innate immunity (Adamo, Rocha-Resende et al. 2020a). As with all immune cell types, B lymphocytes can be divided into two lineages with each one of them having several subtypes. The two main lineages consist of B1 B cells which produce natural antibodies and are mostly produced in prenatal life, and B2 conventional B cells which develop postnatally and represent the vast majority of B cells in adults (Adamo, Rocha-Resende et al. 2020a). In mice, B1 cells are distinguished by their CD11b expression which is absent in B2 lymphocytes (Adamo, Rocha-Resende et al. 2020a). This is important to note as the study of circulating immune cells 2h post I/R described in Section 5.2.2 may include some B1 cells in the CD11b⁺ monocyte gate. B cells play an important role in presenting antigens to T cells via MHC class II-restricted interactions (Adamo, Rocha-Resende et al. 2020a). This is more important in mice than humans, as mouse monocytes have been reported to be poor antigen presenting cells (APCs) to CD4 T cells due to their low MHC class II levels (Bjornson-Hooper, Fragiadakis et al. 2019). In addition to the APC role of B cells, they are also important immunoregulators by the secretion of various cytokines such as IFN γ , IL-6 and IL-17 which can polarise T cell subtypes, as well as monocyte chemoattractant CCL7 (Adamo, Rocha-Resende et al. 2020a). Furthermore, regulatory B cells (Bregs) have been identified. Similar to Tregs, they exert their immunosuppressive function by secretion of IL-10 and TGF β (Rosser, Mauri 2015). However, they can also further enhance immunosuppression by promoting Tregs (Adamo, Rocha-Resende et al. 2020a). Although this project focuses on the role of T cells in

myocardial I/R injury, RAG2 KO mice lack mature B cells as well as T cells. B cells are also one of the most prevalent immune cell populations in the naïve mouse heart, with both B1 and B2 subtypes being detected (Bönner, Borg et al. 2012, Adamo, Staloch et al. 2018, Adamo, Rocha-Resende et al. 2020b). Further to this, Adamo et al. reported a two-fold increase in the number of B cells in the myocardium of mice 4 days after post MI reperfusion (Adamo, Staloch et al. 2018). Moreover, Yan et al. report that T and B cells share similar temporal dynamics in the I/R myocardium, peaking at day 3 post reperfusion (Yan, Anzai et al. 2013a). But while they do not report significant increase in T cell numbers at one day after I/R, they do show that B cell numbers have a statistically significant increase even at the early time point. This suggests that the lack of B cells in RAG2 KO mice may be exerting a stronger effect on the change in leukocyte density 24h post I/R (Figure 5.3). Interestingly, Alamo et al. suggest that B1 and B2 cells in the myocardium have different roles in the acute I/R response. CD11b⁺ B1 cells responds with chemokine and TLR signalling, while the far more numerous CD11b⁻ B2 cells respond in an antigen-dependent BCR activation (Adamo, Staloch et al. 2018). However, as antigen-dependent response usually takes 7 days, it is likely that any B cell effect observed in the acute 24h I/R time point is due to B1 lymphocytes. Indeed, Zhang et al. reported that B1 cells produce IgM natural antibodies that activate the complement cascade (Zhang, Ming, Austen et al. 2004). Using a RAG1 KO model of intestinal I/R injury, they then confirmed that these natural antibodies restore pathogenic injury within 3 hours of reperfusion (Zhang, Ming, Austen et al. 2004). Importantly, seminal work by Zouggari et al. confirms that B cells play a detrimental role in MI (Zouggari, Ait-Oufella et al. 2013). Using a permanent MI model depleted of B cells by an anti-CD20 antibody, they show reduced infarct size and improved cardiac function 14 days post infarct. Furthermore, they suggest that this deleterious effect of B cells is mediated by CCL7 production which induces mobilisation of classical Ly6C⁺ monocytes (Zouggari, Ait-Oufella et al. 2013). B cells play a clear role in the inflammatory response to MI and this is becoming increasingly recognised and studied using mouse models. However, unlike naïve mouse hearts that have more B cells than T cells (Bönner, Borg et al. 2012), normal human hearts appear to have similar numbers of B cells, CD4 and CD8 T cells (Noutsias, Pauschinger et al. 2002). In addition to this, patients with dilated cardiomyopathy present with increased number of T cells, while there is no change in B cell numbers (Noutsias, Pauschinger et al. 2002, Adamo, Rocha-Resende et al. 2020b). Therefore, this suggests that T cells may play a bigger role in human cardiomyopathies compared to the one in mouse models.

This difference between the inflammatory response in mice and humans is reaffirmed by the analysis of leukocytes dynamics immediately after reperfusion described in this chapter. The results in Section 5.2.2 fail to reproduce the clinical presentation of STEMI patients undergoing PPCI, as described by Boag et al. (Boag, Das et al. 2015). Marked lymphopenia by 2h post reperfusion could not be reproduced by using either the standard open MI mouse model or by using a closed chest LAD occlusion. The latter avoids activating a systemic inflammatory response due to the open chest surgery required for MI induction in mice. This inability to reproduce human I/R response could be due to several important differences between mice and humans. The normal cardiac output of mice, normalised to weight, is around nine times higher than that of humans (Doevendans, Daemen et al. 1998). Therefore, it is possible that the lymphopenia in response to acute myocardial I/R simply occurs earlier than the 90 minute time point observed in humans (Boag, Das et al. 2015). This is supported by findings by Yang et al. who report significant reduction in the number of lymphocytes, as well as neutrophils, 60 minutes following reperfusion (Yang, Day et al. 2006). Another key difference between the immune system of mice and human is the balance between lymphocytes and neutrophils. Human blood is neutrophil rich with around 50-70% neutrophils and 30-50% lymphocytes (Mestas, Hughes 2004). In contrast, mouse blood is lymphocyte rich with 75-90% lymphocytes and only 10-25% neutrophils (Mestas, Hughes 2004, Doeing, Borowicz et al. 2003). But despite this difference in the proportion of innate and adaptive cells, mice have been shown to have a more neutrophil rich response to delayed type hypersensitivity (DTH) than humans who have 3-5 times more circulating neutrophils (Mestas, Hughes 2004). While DTH has a completely different pathophysiology than myocardial I/R injury, this is an interesting example displaying the difference between mouse and human immunology.

This challenge of replicating the T cells response in STEMI patient, as well as the low number of CX3CR1⁺ T cells in the CX3CR1-EGFP mouse model in Chapter 3, led to the investigation of alternative mouse models that better recapitulate the phenotype of an average MI patient. Expression of CX3CR1 on T cells is primarily in memory and effector populations (Section 3.2.1). Memory and effector T cell populations increase with advanced age and following infections with various pathogens (Kumar, Connors et al. 2018). This is the result of more naïve cells activating upon encountering their cognate antigen and subsequently differentiating into effector and memory cells. While healthy mice at around 3 months of age are a widely used model for MI, their immune system, and therefore I/R inflammatory response, is considerably

different from that of a typical ~65 year old MI patient with underlying co-morbidities and a lifetime of infections. Therefore, the T cell phenotype was explored in mouse models of ageing and infection with MuLV. And as expected, both old and young infected mice showed a significant change in the proportion of naïve to effector/memory T cells. CD4 and CD8 T cells in both models showed a T cell phenotype more akin to that of MI patients. Furthermore, both models showed a significant increase in the proportion of CX3CR1 expressing T cells. Although it was not possible to investigate the response of these models to myocardial I/R during this PhD project, the importance of using such models in future research is described below.

Immunosenescence is the term used to describe the process of ageing that causes multiple changes in the components of the immune system, disrupting its ability to maintain homeostasis, prevent new infections and respond to tissue injury (Nikolich-Zugich 2018, Sadighi Akha 2018). This process is characterised by multiple facets including increased low-grade inflammation due to increase in soluble mediators such as IL-1 β , TNF and IL-6 (inflammaging), disruption of self-tolerance and impaired wound healing (Nikolich-Zugich 2018, Sadighi Akha 2018). All of these can lead to a more deleterious inflammatory response post MI in aged patients, highlighting the importance of investigating I/R in aged mice. One of the most profound effects of ageing in both mice and humans is a shift towards myeloid response and a decreased capacity to produce lymphoid cells (Montecino-Rodriguez, Berent-Maoz et al. 2013). In humans, this is in part due to reduced thymic output of naïve T cells although human naïve T cells have a remarkably long life span of 5-10 years compared to 6-10 weeks for mice (Kumar, Connors et al. 2018). But while the naïve T cell pool in mice is maintained by seeding of new naïve T cells, the one in adult humans is maintained by homeostatic proliferation (Sadighi Akha 2018). This results in reduced repertoire of naïve T cell clones and more importantly in the context of MI, inequality in the clonal size which is more prominent for CD8 T cells (Sadighi Akha 2018). Therefore, with increased age, certain clones of T cells are becoming more enriched, which would be of particular importance when those clones are autoreactive. Recent evidence by Tang et al. confirm that clonally expanded T cells with cardiac tissue specificity, contribute to the ischaemic heart failure (IHF) (Tang, T., Zhu et al. 2019). Moreover, they showed that these T cells display memory and effector phenotype and are predominantly Th1 CD4 T cells and CD8 T cells. These cells are highly cytotoxic, expressing high levels of IFN γ , granzyme B and perforin (Tang, T., Zhu et al. 2019). Importantly, their study is based on IHF patients with an average age of 57 years old, 14% of

which suffered previous STEMI (Tang, T., Zhu et al. 2019). While these findings are in line with evidence that CD8 CTLs and CD4 Th1 cells contribute to myocardial I/R injury, they further highlights the effect that ageing has on T cell repertoire and the significantly different immune response that can be induced in a young or old model.

Age related reduction in the phagocytic ability of macrophages has also been reported, both in mice and humans (Nikolich-Žugich 2018). In addition to this, DC have been shown to have decreased antigen uptake which can result in reduced T cells stimulation (Nikolich-Žugich 2018). In contrast, autoantibody production is more likely with increasing age and subsequently autoreactive T cells may receive co-stimulation by B cells (Weyand, Goronzy 2016). Van der Borght, et al. propose α -myosin as a potential autoantigen in response to the post MI sterile inflammation (Van der Borght, Scott et al. 2017). In a permanent mouse model of MI, they show that dendritic cells present cardiac α -myosin to CD4 T cells, inducing their differentiation into IFN γ and IL-17 secreting Th1 and Th17 effector cells (Van der Borght, Scott et al. 2017). Interestingly, studies on the role of ageing in organs transplantation shows that organs from older donors are more prone to I/R injury (Sadighi Akha 2018, Colvin, Smith et al. 2017). This is attributed to inflammaging and its increased baseline inflammation, with DC co-stimulation, IFN γ producing CD8 T cells and memory T cells secreting IL-17 playing a role (Sadighi Akha 2018, Colvin, Smith et al. 2017). Overall, these studies show that in addition to T cells being directly affected by immunosenescence, ageing can cause an indirect effect by modifying the function of other immune cells that T cell interact with.

With increased ageing, both CD4 and CD8 T cells can become senescent and develop a highly secretory phenotype known as senescence-associated secretory phenotype (SASP) (Sadighi Akha 2018). Combined with the age-associated decline in TCR signalling (Li, Yu et al. 2012), this suggests that T cells in older patients may have a stronger antigen-independent cytokine-driven response to myocardial I/R than young mice (Sadighi Akha 2018). Although age-induced changes in circulating factors are still not well understood, given the complex interplay between different leukocyte populations, it is likely that senescent T cells, secreting pleiotropic pro-inflammatory cytokines, exacerbate myocardial I/R injury. This is supported by recent studies investigating T cells with a senescent phenotype. Absence of CD28 is considered a marker of senescence and CD28⁻ CD4 T cells are found in atherosclerotic plaques of patients with fatal MI (Giovanna, Goronzy Jörg J. et al. 2000, Weyand, Goronzy 2016). These cells secrete IFN γ and are highly cytotoxic towards endothelial cells (Giovanna, Kopecky Stephen L.

et al. 1999, Weyand, Goronzy 2016). CD57 is another marker of senescence and in humans, it has been reported that a population of CD57 expressing CD8 T cells plays a pathogenic role in acute MI (Tae Yu, Youn et al. 2015). These cells have an activated (HLA-DR⁺, PD-1⁺, FasL⁺), senescent (CD28⁺, IL-7Rα⁺, CD45RO⁺) and pro-inflammatory (IFNγ⁺, TNFα⁺, granzyme a/b⁺, perforin⁺) phenotype. Tae Yu et al. report that the frequency of CD57⁺ CD8 T cells in the circulation of MI patients is associated with poor short-term survival, 6 months after MI (Tae Yu, Youn et al. 2015). The authors propose that this population of CD8 T cells contributes to MI injury by promoting a pro-inflammatory cytotoxic environment. Importantly, they show that CD57⁺ CD8 T cells express increased levels of CX3CR1 compared to CD57⁻ CD8 cells (Tae Yu, Youn et al. 2015), which is in support of the hypothesis of this PhD project. Furthermore, while this project did not investigate the levels CX3CL1 in response to myocardial I/R, a recent study by Dookun et al. shows that the level of fractalkine in the left ventricles of 3-4 month old mice is significantly elevated 7 days post transient MI (Dookun, Walaszczyk et al. 2020). Moreover, the authors showed that this increase can be reversed by administering senolytic therapy, confirming a role for senescence in modulating the CX3CL1/CX3CR1 axis.

In addition to ageing alone, the immune system in MI patients is profoundly affected by the multiple infections encountered throughout their lifetime and in particular life-long chronic infections. Indeed, the lack of bystander infections in the majority of mouse models is considered a major contributor to the limited reproducibility of preclinical mouse research (Justice, Dhillon 2016, Tao, Reese 2017). A study by Beura et al. investigated the immunological differences between research mice bred in SPF facilities and wild or pet shop mice (Beura, Hamilton et al. 2016). They showed that SPF mice had low levels of memory T cells, which was in contrast to mice exposed to pathogens which showed a T cell phenotype more akin to that of adult humans (Beura, Hamilton et al. 2016, Tao, Reese 2017). Interestingly, they showed that laboratory mice had peripheral blood mononuclear cell (PBMC) signature similar to that of neonatal humans, with high number of naïve lymphocytes. Whereas, PBMCs of wild and pet shop mice was more similar to that of adult humans, especially in terms of interferon related pathways (Beura, Hamilton et al. 2016). Importantly, they confirmed that the immature immune system of SPF mice is not due to age alone by cohousing them with pet shop mice. This resulted in increased CD8 T cell effector response to a *de novo* viral infection in SPF mice cohoused with pet shop mice (Beura, Hamilton et al.

2016). Ultimately, this shows that irrespective of age, bystander infections induce immune system maturation, leading to a more adult human-like immunity.

T cells have been shown to be particularly affected as a result of co-infections and changes in the overall immune environment (Tao, Reese 2017). Studies have shown that chronic infections by viral or parasitic pathogens modify the responses of unrelated CD8 memory T cells (Stelekati, Shin et al. 2014, Tao, Reese 2017). Murine cytomegalovirus (MCMV) is a persistent beta-herpesvirus that is commonly found in wild mice but is not found in SPF laboratory mice (Tao, Reese 2017). Mice with latent MCMV infections have decrease in their naïve T cells with corresponding increase in effector memory CD8 T cells (Cicin-Sain, Brien et al. 2012, Mekker, Tchang et al. 2012, Tao, Reese 2017). CD8 response in these mice share similarities with immunosenescent animals (Cicin-Sain, Brien et al. 2012, Mekker, Tchang et al. 2012, Tao, Reese 2017). Their CD8 T cell response to viral infections is impaired and they have been reported to have increased incidence of autoimmune diseases including myocarditis with detectable anti-myosin autoantibodies (Tao, Reese 2017, Fairweather, Lawson et al. 1998, O'Donoghue, Lawson et al. 1990).

CMV infections in humans are also associated with an elevated immune risk and even decreased life expectancy (Cicin-Sain, Brien et al. 2012). MCMV infected mice have similar presentation to that of adult humans with chronic human CMV (HCMV) infections, who also have pronounced changes in T cell populations and impaired naïve T cell response (Cicin-Sain, Brien et al. 2012, Nikolich-Žugich 2018). CMV seropositivity is associated with accumulation of CD28⁻ CD8 T cells (Cicin-Sain, Brien et al. 2012) that are immunosenescent and have been proposed to be a predictor of mortality in humans over the age of 65 although the mechanisms behind this is not well understood (Strindhall, Nilsson et al. 2007, Montecino-Rodriguez, Berent-Maoz et al. 2013). Importantly, while ageing causes a relative shift from naïve to memory T cell response, only CMV infection causes an absolute increase in the number of memory T cells (Wertheimer, Bennett et al. 2014, Nikolich-Žugich 2018). This is the result of clonal expansion of T cells with specificity for various HCMV antigens and is known as “memory inflation” (Souquette, Frere et al. 2017, Nikolich-Žugich 2018). More recently, a study by Pera et al. showed that CMV, not ageing, induces expansion of pro-atherogenic CD28⁻ CD4 T cells which leads to increased risk of cardiovascular disease (Pera, Caserta et al. 2018). Ultimately, given that approximately 50% of 6-47 year old and up to 90% of 75-80 year old

individuals are CMV seropositive (Souquette, Frere et al. 2017), studying myocardial I/R response in an infection mouse model is crucial for translational research.

The infection model used in this project, murine leukaemia virus (MuLV), is gammaretrovirus that causes splenomegaly and lymphadenopathy 4-6 weeks post infection (Morse, H. C., 3rd, Chattopadhyay et al. 1992, Chattopadhyay, Morse et al. 1989, Hartley, Fredrickson et al. 1989). Unlike CMV, MuLV does not cause a latent chronic infection but instead results in a murine acquired immunodeficiency syndrome (AIDS) phenotype (Cook, Green et al. 2003). However, this occurs after 8-9 weeks post infection (Morse, H. C., Yetter et al. 1989) and the T cell phenotyping described in Section 5.2.4 is at 5 weeks post infection. This is the acute phase of the infection during which instead of mice being immunocompromised, they present with a transient splenomegaly and an MuLV specific CD8 T cell response (Francois, Peng et al. 2013). While the results on Figure 5.11 show that MuLV infection causes an increase in the CD4:CD8 ratio, which is not typical for the MI response, it does provide clear evidence that infected mice have changes in the distribution of naïve, memory and effector T cells. As the proportion of effector, and to a lesser extent memory, CD4 and CD8 T cells is significantly increased, this indicates that acute phase MuLV infection may be a useful model to study myocardial injury. Furthermore, this model would be suitable for studies of the role of CX3CR1 expressing T cells as the proportion of CX3CR1⁺ cells is increased among all T cell populations, and most importantly memory and effector cells (as shown on Figure 5.12).

While an MCMV mouse model is likely to be a more accurate representation of the typical MI patient, the work presented here is from a pilot study using an MuLV model that was available as part of a collaboration. MuLV causes murine AIDS and cytotoxic IFN γ secreting CX3CR1⁺ CD8 T cells have been shown to be increased in patients with HIV infection (Mudd, Panigrahi et al. 2016), especially in those with CMV co-infection (Morris, Chen et al. 2020). Ultimately, the results described above do achieve the aim of this chapter of establishing an infection mouse model with significantly higher CX3CR1 expression in the CD4 and CD8 T cell pool. Combined with the increase in effector and memory T cells in the aging mouse model, as well as corresponding upregulation in CX3CR1 expression, the work described in this chapter describes a more human-like model to study MI. Importantly, the findings described in this chapter present detailed phenotyping of CX3CR1 expression in T cell populations of old and infected mice. At the time of writing, this is the first report to describe these novel findings and no other group has reported on CX3CR1 expression in T cell subpopulations in ageing and

MuLV infection models. Furthermore, reports by Dookun et al. of CX3CL1 modulation as part of the SASP phenotype (Dookun, Walaszczyk et al. 2020), and recent evidence of immunosenescent CD57⁺ CD28⁻ CX3CR1⁺ CD8 T cells in individuals with chronic HIV/CMV co-infection (Morris, Chen et al. 2020), are crucial in supporting the findings of this chapter that a more optimal mouse model is required in order to study the role of CX3CR1⁺ T cells in myocardial I/R injury.

Chapter 6. General Discussion and Conclusions

6.1 Summary of major findings

First of all, this PhD project is the first body of work to provide a detailed analysis of different T cell populations across multiple lymphoid tissues in three different mouse models – CX3CR1-EGFP mice (WT, HET and KO), C57BL/6 23 month old mice, C57BL/6 mice 5 weeks post MuLV infection. Furthermore, this report describes CX3CR1 expression on T cells in these mouse models, as well as in normal WT C57BL/6 mice. CX3CR1⁺ T cells in humans have received more attention from the scientific community in recent years, but no other group has done such a detailed analysis under different conditions. Here, I report that CX3CR1 is expressed primarily in effector CD4 and CD8 T cells in the spleens, blood and lymph nodes of mice, with the highest level being in the circulation (40-60% of effector CD8 T cells). Furthermore, I show that both ageing and infection cause accumulation of CX3CR1⁺ T cells.

Secondly, this project investigates the inflammatory response to myocardial I/R injury in WT, HET and KO CX3CR1-EGFP mice, especially focused on the role of T cells. Here I show that absence of CX3CR1 leads to a delay in leukocyte recruitment to the infarcted myocardium in the first two hours post reperfusion which returns to WT levels by 24h I/R. However, while total CD45⁺ cell infiltration returns to normal 24h after MI, complete absence of CX3CR1 results in a change in the composition of this inflammatory infiltrate. These mice have reduced number of CX3CR1/EGFP⁺ cells in the infarcted myocardium, as well as fewer T cells. Importantly, reduced T cell recruitment to the 24h I/R heart is CX3CR1-dependent, as complete knockout of the receptor results in significantly lower number of CX3CR1/EGFP⁺ T cells compared to HET animals. Gene expression analysis during this acute MI response could not elucidate the mechanisms behind this delayed and altered inflammatory response. Despite this, the results in Chapter 3 point to a compensatory mechanism being activated in mice with germline CX3CR1 disruption, with CX3CR1 KO mice having enhanced neutrophil recruitment and lower M2 chemotaxis. Furthermore, despite not reaching statistical significance, CX3CR1-EGFP HET and KO mice show a trend of more deteriorating cardiac function and remodelling between 1 and 6 weeks post MI. However, the worsening cardiac function is likely not due to the absence of CX3CR1⁺ T cells but to disruption in CX3CR1⁺ non-classical monocytes which are crucial for myocardial healing after MI.

Thirdly, Chapter 4 of this PhD project describes the detailed development of a cutting-edge method utilising mass cytometry to study inflammation in the mouse. Mass cytometry, and more specifically imaging mass cytometry, is a relatively novel methodology which has so far

had limited application to mouse research. During my PhD project, I established a method whereby mass cytometry (suspension and imaging) of mouse splenocytes is used as a guide to rapidly develop and optimise an immunological panel. This can then be applied to investigate inflammatory responses in non-lymphoid tissues which have limited resident immune cells. Unfortunately, it was not possible to use this novel method to further elucidate the immune cell populations which are present during the acute I/R response in the surgical mouse model of heart injury.

Fourthly, this PhD project reveals that lack of lymphocytes in RAG2 KO mice causes denser inflammatory infiltrate in myocardium 24h after reperfusion. While this model has previously been reported to have improved cardiac function post MI, this thesis shows that in the absence of B and T cells, MI induces an enhanced leukocyte (primarily neutrophil) response. Disruption of the mouse housing unit in the final months of my PhD project, prevented the study of the specific role of CX3CR1⁺ T cells in this model by adoptively transferring them into RAG2 KO recipients. Despite this, I have shown results from the pilot study, confirming that CX3CR1-EGFP cells are successfully transferred into RAG2 KO mice.

Finally, this PhD project has revealed important differences in the response of CX3CR1⁺ T cells between mice and humans. In Chapter 5, I report that 2h after reperfusion MI induces significant depletion of CX3CR1⁺ CD4 T cells. However, the mouse model failed to reproduce the marked lymphopenia and selective depletion of CX3CR1⁺ cytotoxic CD8 T cells. This was attributed to fundamental differences between the species and more importantly, the fact that mouse MI models are not an accurate representation of MI patients. Humans presenting with MI are of an average of around 65 years old and have existing co-morbidities. Moreover, a significant proportion of them have chronic viral infections. All of this results in dramatic changes in the immune system before MI even occurs. My work in Chapter 5 highlights this by investigating the baseline T cell phenotypes, and their CX3CR1⁺ expression, in mouse models of ageing and viral infection. Both of these models have a marked accumulation in effector and memory CD4 and CD8 T cells, as well as significant increase in the proportion of CX3CR1⁺ T cells across CD4 and CD8 T cell populations.

6.2 Implications

One of major implications of the research I performed during my PhD project is the detailed T cell phenotyping summarised in the previous section. Having a detailed baseline phenotype

of T cell populations in different mouse models would contribute to the research of other groups. Indeed, the T cell immunophenotyping panel that I developed has already contributed to two publications (Richardson, Sage et al. 2018, Martin-Ruiz, Hoffmann et al. 2020). Furthermore, at the time of writing, no other published report exists, describing CX3CR1 expression on T cell subpopulations in multiple mouse models. Therefore, the detailed analysis I performed in CX3CR1-EGFP, young and old C57BL/6 23 or MuLV infected mice, is a major novel finding that would have implications on all future CX3CR1⁺ T cell research.

Similarly, the IMC method that I developed is a significant technical achievement that has the potential to impact future mouse research. The work in Chapter 4 describes how suspension mass cytometry of easily isolated splenocytes, can be used as a guide to rapidly and confidently optimise an IMC panel. Successfully employing IMC to a wide number of projects, would allow the study of multiple immune cells simultaneously, while preserving crucial spatial information. My work points to certain technical challenges of IMC, such as reduced single cell resolution as a result of tissue depth and cell density, that have been largely overlooked by IMC researchers as they focus on the study of cancerous cell lines or less dense tissues. Despite this, future bioinformatics advancements may optimise IMC analysis and the challenge of distinguishing CD4/CD8 single positive T cell I describe may prove to be a useful model.

Importantly, the work described in Chapter 3 reveals that MI response in CX3CR1-EGFP heterozygous mice differs from that in wild type or knockout animals. While there are no major differences in the baseline T cell phenotype of these mice, the results described here clearly show that upon challenge, the inflammatory response in HET animals is different from that in their WT or KO counterparts. This finding is of particular importance as CX3CR1-EGFP HET mice are often used as a normal “WT” control due to the convenience of the EGFP reporter. However, the results in Chapter 3 are in agreement with a recent study by Gyoneva et al. which shows that CX3CR1-EGFP HET animals exhibit functional haploinsufficiency using microglial transcriptomics (Gyoneva, Hosur et al. 2019). This is not unexpected given that this project confirms that HET animals have only 50% of CX3CR1 mRNA compared to WT animals (Figure 3.1b). As a result, all three genotypes (WT, HET and KO) were used for analysis of CD45⁺ and CD3⁺ cell infiltration post MI, confirming the findings by Gyoneva et al. that CX3CR1-EGFP HET mice have an intermediate WT-KO response (Gyoneva, Hosur et al. 2019). Interestingly, the authors of this study also point to a premature ageing phenotype in CX3CR1-EGFP KO mice

(Gyoneva, Hosur et al. 2019). Although their study was based on CX3CR1 expression in the CNS, this finding may provide additional insight into why CX3CR1-EGFP HET and KO mice appeared to have more deteriorating heart function 6 weeks post infarct. While this effect is likely to be due to disrupted non-classical monocytes and M2 macrophage response (which are the predominant CX3CR1⁺ leukocyte population), it is well known that ageing alone results in worse cardiac function. Therefore, the more deleterious cardiac remodelling in CX3CR1-EGFP HET and KO mice may be in part due to premature ageing of these mice. While this phenotype is not supported by the baseline analysis of T cell populations in Chapter 3, investigating ageing in this mouse line was not the aim of this project. Therefore, the T cell panel did not include any senescence markers such as CD28 or CD57, with CD28⁻ and CD57⁺ being a hallmark of immunoageing. Furthermore, T cell function in these mice was not assessed in comparison to that of old mice.

The discussion in Chapter 5 highlights some of the differences between mouse and human immunology and the need to use more clinically relevant mouse models. While most of the existing research in mouse MI and I/R injury uses young, healthy SPF mice, the work in this PhD project points to the requirement of mice with increased infection exposure in order to have enhanced correlation to human inflammation. The seminal study by Yang et al. confirming a role for IFN γ expressing CD4 T cells in a RAG1 KO MI model used CD4 T cells from 8-14 week old donor mice (Yang, Day et al. 2006). While they did not report a role for CD8 T cell in this model, this may be due to the immature T cell pool of these mice. The work I describe in Chapter 5, adds to the existing literature that both ageing and infection cause a shift towards a more mature T cell pool that is more akin to that of MI patients. Therefore, this PhD project reaffirms the need for better mouse models in translational research.

6.3 Challenges and limitations

Mice are the most widely used model organism and they are essential for scientific research. Despite this, they are inherently imperfect models of human disease, especially when investigating the complex immune system. Most preclinical models of MI use healthy young animals which is in contrast to the typical MI ~65 year old patient with underlying conditions such as atherosclerosis and hypertension. Indeed, the lack of pre-existing atherosclerosis may be a significant limitation of the work that was done during this PhD project, as recent evidence by Mudd et al. suggest (Mudd, Panigrahi et al. 2016). CX3CR1⁺ CD8 T cells have been

shown to have upregulated expression of protease-activated receptor 1 (PAR-1), a thrombin-activated receptor, in HIV patients receiving antiretroviral therapy (ART) (Mudd, Panigrahi et al. 2016). The authors showed that these patients have overall increase in PAR-1 levels and that thrombin can directly enhance IFN γ secretion by CD8 T cells. The authors propose that CX3CR1⁺ CD8 T cells can be directly activated by blood clotting factors, recruited to the endothelium via CX3CR1, and result in IFN γ secretion (Mudd, Panigrahi et al. 2016). This is a key finding that supports a role of IFN γ secreting cytotoxic CD8 T cells that express CX3CR1 are recruited to the myocardium following MI reperfusion. Importantly, this effect would be near impossible to detect in the existing mouse MI and especially I/R models. These mice do not suffer an infarct due to a blood clot occluding a coronary artery. Therefore, the population of CX3CR1⁺ CD8 T cells identified in HIV patients by Mudd et al. would not become activated in these mice (Mudd, Panigrahi et al. 2016), whereas it would in an MI patient. Furthermore, a typical atherosclerotic individual may already have enhanced CX3CR1⁺ CD8 T cells activation, mediated by small blood clots and vascular occlusions that are not located in a coronary artery. This may result in a more rapid and cytotoxic CX3CR1⁺ CD8 T cell response at the time of MI. Ultimately, this suggests that an atherosclerosis mouse model may be required to study the true effects of this population in myocardial I/R injury.

In addition to atherosclerosis, chronic infections such as CMV contribute to the risk of cardiovascular disease. While in Chapter 5 I discuss the importance of using a mouse model with a mature immune system, this PhD did not investigate myocardial I/R in these conditions. This limitation of particular importance for the role of CX3CR1⁺ cells as Tang and Chen et al. recently reported a link between gut microbiota and cardiac repair (Tang Tony W.H., Chen Hung-Chih et al. 2019). Using antibiotic treatment of mice prior to permanent MI, they showed that mice with depleted gut microbiota have increased mortality which is in part attributed to reduced infiltration of CX3CR1⁺ monocytes in the myocardium which are required for tissue repair (Tang Tony W.H., Chen Hung-Chih et al. 2019). Although SPF mice used in this project do not have similarly depleted gut microbiota, they do lack exposure to multiple pathogens as seen in wild or pet shop mice. This would inevitably result in abnormal gut microbiota that is in contrast to humans.

Furthermore, the CX3CR1-EGFP mouse model that was used for this project has a germline mutation in the CX3CR1 gene. As discussed in Chapter 3, these mice probably have a compensatory mechanism activated, which is another inherent limitation to all research using

germline mutations. Moreover, this line does not have T cell-specific CX3CR1 knockout, which likely results in masking of the effect of CX3CR1⁺ T cell loss due to the more widespread expression of CX3CR1 in monocytes. In particular, the reparative function of non-classical monocytes which highly express CX3CR1 may be preferentially affected, preventing the detection of the potentially beneficial depletion of CX3CR1⁺ T cells. Similarly, the effect of increased density in leukocyte infiltration in the RAG2 KO model is not due to specific absence of T cells. These mice lack both mature B and T cells. Originally, one of the aims for the final chapter in this thesis was to investigate the specific role of CX3CR1⁺ T cells in the RAG2 KO model using adoptive transfer of CX3CR1⁺ and CX3CR1⁻ populations. However, this was not possible due to disruption of the mouse colonies and surgery rooms in the final months of my PhD.

This PhD project does not shed light on whether or not T cell response to myocardial I/R injury is antigen dependent or independent. A clear role for autoreactive B and T cells is strong evidence of the involvement of antigen dependent activation (Van der Borgh, Scott et al. 2017, Rieckmann, Delgobo et al. 2019). On the other hand, given the short time frame of acute I/R response 2-24h post MI, it is likely that an antigen-independent response plays a bigger role. In the context of CX3CR1 expressing T cells, studies by Mudd et al. and Morris et al. also show conflicting response (Mudd, Panigrahi et al. 2016, Morris, Chen et al. 2020). The former study describes that IFN γ production by CX3CR1⁺ CD8 T cells is induced by TCR-mediated response to thrombin (Mudd, Panigrahi et al. 2016). Whereas Morris et al. show that CX3CR1⁺ CD57⁺ CD8 T cells are promoted by IL-15 whereas direct TCR stimulations induces their apoptosis (Morris, Chen et al. 2020). Most likely the overall T cell response to MI is a mixture of both antigen dependent and independent activation, and the importance of each changes with the progressing stages of the I/R response. However, further research is needed to elucidate the exact role each one plays in the acute response immediately post reperfusion. It is important to highlight that differences between SPF mice and chronically infected aged humans may play a bigger role for the early response as humans would have higher proportion of rapidly responding effector and memory T cells, which are CX3CR1⁺ rich populations. In addition to this, in contrast to humans, mouse endothelial cells do not express MHC class II so they do not present antigens to CD4 T cells (Mestas, Hughes 2004). This suggests that any local vascular activation of CD4 T cells that occurs in humans would be reduced in mice as antigen presentation would only occur via non-endothelial cells. However, the work described in this

thesis did not focus on investigating T cell function either in the CX3CR1-EGFP line or in the more clinically relevant ageing and infection model.

Finally, the complexity of the immune system presents a challenge to all immunological research, evidence for which are the multiple conflicting findings. In the context of myocardial I/R, Bönner et al. describe a 50 minute transient LAD occlusion model that shows around 2-fold increase in the number of CD4 and CD8 T cells infiltrating the myocardium 3 days post reperfusion (Bönner, Borg et al. 2012). In contrast, Zouggari et al. show that T cell numbers in the heart increase 24h post MI and then rapidly decline by day 3 (Zouggari, Ait-Oufella et al. 2013). Furthermore, Zouggari et al. showed that B cell depletion using anti-CD20 antibody does not affect T cell recruitment to the myocardium at 3 days post I/R (Zouggari, Ait-Oufella et al. 2013). However, by 5 days post MI, T cell numbers are reduced by around 1/3rd although this did not reach statistical significance (Zouggari, Ait-Oufella et al. 2013). These contradicting results point to the complex, rapidly changing inflammatory response that involves constant and diverse crosstalk between various immune cell populations and multiple immunomodulating factors. This challenge can be addressed using the IMC method developed as part of this project. Novel mass cytometry technology enables the simultaneous study of all major immune cell populations on a single tissue section, allowing the acquisition of high-content spatial data that can elucidate the complex interplay between different immune cell populations in the post MI response.

6.4 Future directions

Future directions for this project would initially focus on the work that was not possible to be completed in time because of mass cytometry machine unavailability. Therefore, I would firstly aim to complete the detailed phenotyping of different immune cell populations that infiltrate the I/R myocardium in mice with surgically induced MI. Afterwards, I would focus on investigating the specific role of CX3CR1 expressing T cells in myocardial I/R injury by performing adoptive transfer of CX3CR1⁺ and CX3CR1⁻ T cells. However, unlike the original aim of Chapter 5 to use RAG2 KO recipient mice, a more clinically relevant model of infection would be utilised. Using MuLV resistant recipient mice that receive T cells from donor mice 5 weeks post MuLV infection, would allow the study the response of a more human-like immune system. Similar to the work in Chapter 3, I would focus on studying the acute inflammatory response at 2h and 24h reperfusion, as well as cardiac function at 1 and 6 weeks post infarct.

Future work should also investigate the effect of atherosclerosis on CX3CR1⁺ CD8 T cell activation, as well as general immunosenescence in aged mice.

Furthermore, following the results of two recent studies, I would include investigation of inflammatory infiltrate at later time points post reperfusion in the work following this project. Ilatovskaya et al. showed that mice with permanent MI have over 2-fold increase in plasma Cx3cl1 expression 7 days after infarct (Ilatovskaya, Pitts et al. 2019). This is similar to the results by Dookun et al. showing increased CX3CL1 in the left ventricles of mice 7 days post transient MI (Dookun, Walaszczyk et al. 2020). This evidence does indicate that by 7 days after infarction, the CX3CL1/CX3CR1 axis is activated, and suggests that perhaps the 24h acute response that has been the focus of this PhD is too early for major changes in CX3CL1 or CX3CR1 to be detected. Furthermore, it is important to note the different myocardial infarction models used by Dookun et al. and Ilatovskaya et al. – transient and permanent MI respectively (Dookun, Walaszczyk et al. 2020, Ilatovskaya, Pitts et al. 2019). A key difference is the temporal dynamics of the immune cells, as described by Yan et al. who show that leukocytes react more rapidly to I/R injury compared to permanent MI (Yan, Anzai et al. 2013a). In their study, the authors show that in a permanent MI model most leukocyte populations peak at day 7 post MI, while they do this at day 3 in response to I/R (Yan, Anzai et al. 2013a). Therefore, as the acute 24h time point investigated here is below the peak of I/R inflammatory infiltrate, CX3CL1/CX3CR1-mediated changes may not be as prominent, suggesting that investigating the inflammatory infiltrate at 3, 5 and 7 days post reperfusion may be useful.

Finally, Ilatovskaya et al. showed that WT T cell infiltrate 3 days post infarct is primarily due to CD8 T cells (Ilatovskaya, Pitts et al. 2019). This indicates that CD8 T cells are the early responders to myocardial I/R injury. Furthermore, the same study shows that there is no change in macrophage phenotype in the absence of CD8 T cells. Therefore, CD4 T cells are the T cell populations that primarily affects polarisation of monocytes into either M1 or M2 phenotype. Instead, CD8 T cells are shown to have a role in regulating the removal of necrotic myocytes via IFN γ -mediated neutrophil activation (Ilatovskaya, Pitts et al. 2019). These findings highlight that different T cell populations can have dramatically different roles in response to myocardial I/R injury. This indicates that in order to better understand myocardial inflammation, and to develop effective therapeutics, future research needs to focus on investigating the specific roles of various Th and Tc subtypes, as well as elucidate ways to

selectively modulate them. Therefore, it is imperative that future work involves the study of specific T cell subpopulations and their function. I would approach this using IMC analysis that includes hallmark markers of Th/Tc subtypes, such as IFN γ , IL-4, IL-10 and IL-17, as well as immunosenescence markers such as CD28 and CD57.

6.5 Conclusions

The work in this PhD project aimed to investigate the role of fractalkine and CX3CR1 expressing T cells in myocardial I/R injury. The results described in this thesis support the hypothesis that the CX3CL1-CX3CR1 axis contributes to immune cell infiltration to the injured myocardium. Investigation of CX3CR1 knockout mice shows that absence of CX3CL1-CX3CR1 interaction results in delayed and altered inflammatory infiltrate 24h post reperfusion. Furthermore, recruitment of CX3CR1⁺ T cells is affected in mice devoid of functioning CX3CR1. Although analysis of cardiac function in these mice did not show a significant change, both CX3CR1-EGFP HET and KO mice showed a trend of more deleterious cardiac function and remodelling. Additionally, this project investigated the effect on post reperfusion inflammation in the absence of mature lymphocytes. RAG2 KO mice, which have no T or B cells, showed a trend of increased density of leukocytes infiltrating the infarcted myocardium. While this project did not investigate the specific role of CX3CR1⁺ T cells in myocardial I/R injury, it was successful at developing a novel method of high-content IMC analysis of mouse tissue, as well as identify two mouse models that are more representative of the inflammatory system of MI patients.

In conclusion, the findings of this PhD project confirm that CX3CR1⁺ T cells are recruited to the injured myocardium following MI. The work described in this thesis also shows the lack of either CX3CR1 or mature lymphocytes results in altered inflammatory infiltrate to the I/R myocardium. While this work does not reveal the mechanism of adverse cardiac remodelling as a result of CX3CR1 knockout or the T cell-specific effect on leukocyte infiltration, the results suggest that CX3CR1 expressing T cells, which increase with ageing and in response to infection, contribute to myocardial I/R injury. Although additional work is required to elucidate the mechanisms behind it, this project supports the hypothesis that selectively disrupting the CX3CL1-CX3CR1 axis holds therapeutic potential.

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