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A Characterisation of Mononuclear Phagocyte Dynamics in the Healthy and Regenerating Zebrafish Heart

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School of Physiology, Pharmacology and Neuroscience

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17th March 2022

A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of Doctor of Philosophy in the Faculty of Life Sciences.

Word count: 43,786
Abstract

The inability of mammals to regenerate damaged tissue following myocardial infarction results in extensive scarring and dysfunction of the heart. The adult zebrafish undergoes a similar scarring response, but is subsequently able to regenerate fully functional cardiac muscle and concomitantly resolve scar tissue. Mononuclear phagocytes (MNPs), comprising monocytes, macrophages and dendritic cells, play key roles in orchestrating both of these responses. In mammals, tissue resident macrophages have been shown to exert a beneficial influence on healing, yet specific subpopulations of pro-inflammatory, recruited macrophages have been attributed deleterious roles in mediating adverse scarring. How zebrafish MNP populations differ to promote a regenerative outcome is not well understood, largely due to the paucity of cell-specific markers that can segregate populations. In an attempt to better delineate MNPs, the co-expression of classical macrophage markers, mpeg1.1 and csf1ra, was investigated in adult zebrafish hearts. Surprisingly, this identified a discrete population of mpeg1.1+csf1ra- B cells and NKL cells, but also identified a transient population of injury-responsive csf1ra+mpeg1.1- monocytes, indicating that mpeg1.1 expression can stratify populations of monocytes and macrophages. Establishment of an *ex vivo* imaging platform also enabled the live imaging of cardiac macrophage populations, and, alongside detailed three-dimensional imaging, revealed new insights into their motility, migration and cellular interactions. Analysis of csf1ra mutant fish also revealed a dramatic deficiency of both resident, and injury-associated macrophages deep within the myocardium, highlighting the requirement of csf1ra for normal cardiac MNP migration. This deficiency also appeared to alter scar dynamics following cardiac injury. Similarly, the absence of *il1b* signalling, which is mediated by MNP populations and enhances recruitment of pro-inflammatory leukocytes, also appeared to affect collagen I resolution. Collectively, this has expanded our knowledge of the interplay between cardiac MNP dynamics and scarring, and has built a foundation that will facilitate the future study of these populations.
Author Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University’s Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate’s own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: .....B R Moyse.................................................  DATE:...17th March 2022......
Covid Mitigation Statement

I started my PhD in the Richardson lab in September 2018 with the initial deadline of September 2021 to submit my thesis. Therefore, when the Covid-19 pandemic started in March 2020 I was 18 months into my research project when lockdown and compulsory working from home commenced on the 17th March for over 3 months. Although this was an opportunity to write up my existing data and plan the future direction of the project, it significantly disrupted the execution of experiments during an uncertain time.

During the initial lockdown period, and for several months after, only essential fish breeding for the maintenance of lines was allowed due to the limited animal husbandry staffing and access to the aquarium. This prevented the breeding of fish required for experiments, that must be at least 6 months old for many of the planned studies, and also delayed the use of existing fish, which was not optimal for study design. The fish were also put on a reduced feeding routine which had an unknown impact on the development, health and consistency of lines raised during this period.

Maximum room occupancies that were imposed for over a year following the return to work also significantly restricted access to the lab and equipment. Within our lab group, we commonly needed to work from home on alternating weeks to accommodate for everyone’s access needs to the lab. The availability of technical facilities, particularly microscope access, was also highly limited, minimising the time available to perform experiments. This significantly prolonged the time required to perform experiments and required additional planning to ensure that access to all the required equipment was available to conduct an experiment, which would not have been required under normal circumstances.

Although I was lucky to be granted a 6-month extension and have achieved many of the aims of the project, disruptions caused by the pandemic have extended beyond this time period and undoubtedly affected the progression and productivity of this project.
Acknowledgements

First and foremost, I would like to thank Dr Rebecca Richardson for her excellent supervision over the past four years, for always being supportive and enthusiastic, and always having time for me. I would also like to thank the rest of the Richardson lab, particularly Dr Rebecca Ryan, Laura Bevan and Aaron Scott for making it a fun and enjoyable experience, in addition to all the helpful and plentiful advice. Thanks also to the Martin, Hammond and Weavers labs for making a lovely scientific community that was always willing to help and generate ideas.

For technical support, I would like to thank the Wolfson Bioimaging Facility, particularly Dr Stephen Cross for his excellent imaging analysis expertise, and Dr Dominic Alibhai for his time and support with establishing the *ex vivo* imaging system. Furthermore, thank you to Dr Andrew Herman, Lorena Sueiros Ballesteros and Helen Rice for their help with numerous flow cytometry experiments and the Histology Services Unit for always being happy to help.

I would also like to thank the directors, administrative staff and peers of the Dynamic Molecular Cell Biology PhD programme.

Finally, I would like to thank my friends and family for their support, and always being willing to hear about my fish.
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<th>Description</th>
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<tbody>
<tr>
<td>AFOG</td>
<td>Acid Fuschin Orange G</td>
</tr>
<tr>
<td>AGM</td>
<td>Aorta-gonads-mesonephros</td>
</tr>
<tr>
<td>ALM</td>
<td>Anterior lateral mesoderm</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>BDM</td>
<td>2,3-Butanedione monoxime</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ce3D</td>
<td>Clearing enhanced 3D</td>
</tr>
<tr>
<td>CHT</td>
<td>Caudal hematopoietic tissue</td>
</tr>
<tr>
<td>CSF-1</td>
<td>Colony stimulating factor 1</td>
</tr>
<tr>
<td>csf1ra</td>
<td>colony stimulating factor 1 receptor alpha</td>
</tr>
<tr>
<td>csf1rb</td>
<td>colony stimulating factor 1 receptor beta</td>
</tr>
<tr>
<td>cTM</td>
<td>Cardiac tissue macrophage</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>dc</td>
<td>Days cultured</td>
</tr>
<tr>
<td>dpi</td>
<td>Days post (cryo)injury</td>
</tr>
<tr>
<td>dpmi</td>
<td>Days post myocardial infarction</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EdU</td>
<td>5-Ethynyl-2'-deoxyuridine</td>
</tr>
<tr>
<td>EMP</td>
<td>Erythromyeloid progenitor</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>hpf</td>
<td>Hours post fertilisation</td>
</tr>
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</table>
hpi  Hours post injury
HSC  Hematopoietic stem cell
HSPC  Hematopoietic stem progenitor cells
Il10/IL-10  Interleukin 10
Il1a/IL-1α  Interleukin 1, alpha
Il1b/IL-1β  Interleukin 1, beta
Il1r1/IL-1RI  Interleukin 1 receptor type 1
Log2FC  Log2 fold change
M-CSF  Macrophage colony stimulating factor
MDM  Monocyte-derived macrophage
mfap4  Microfibril Associated Protein 4
MI  Myocardial infarction
mmd  Monocyte to macrophage differentiation-associated
MMP  Matrix metalloproteinase
MNP  Mononuclear phagocyte
mpeg1.1  Macrophage expressed 1, tandem duplicate 1
mpeg1.2  Macrophage expressed 1, tandem duplicate 2
NK  Natural killer
NKL  Natural killer-like
O/N  Overnight
P  Postnatal day
PBI  Posterior blood island
PBS  Phosphate buffered saline
PBST  Phosphate buffer saline/triton
PH3  Phospho-Histone H3
ROS  Reactive oxygen species
rpm  Revolutions per minute
RT  Room temperature

XVI
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>Tg</td>
<td>Transgenic</td>
</tr>
<tr>
<td><em>tnfa</em></td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>U/ml</td>
<td>Units per millilitre</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>ZFIN</td>
<td>Zebrafish Information Network</td>
</tr>
</tbody>
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1 Introduction

1.1 Heart failure

1.1.1 Health burden of heart failure

Heart failure is characterised by the inability to sustain adequate circulation due to weakened pump function of the ventricle (Cahill et al., 2017, Murphy et al., 2020). This is caused by the loss of functional cardiomyocytes and scarring of the cardiac tissue which can occur as a result of hypertension, valvular heart disease or various cardiomyopathies. However, this predominantly arises following myocardial infarction (MI) which results in extensive cell death within the ventricular myocardium (Laflamme and Murry, 2011). Although advancements in medical interventions following MI have significantly improved the survival rate of MI to 70%, the resulting irreversible tissue damage causes lifelong morbidity and heart failure and currently affects over 650,000 people in the UK (British Heart Foundation, 2022). As 2.3 million people in the UK suffer from coronary heart disease, predisposing patients to MI due to the narrowing of coronary arteries, MI and heart failure impose massive clinical and financial burdens in the UK alone (British Heart Foundation, 2022).

Heart failure occurs because humans have a very limited ability to regenerate cardiac tissue and the infarcted region becomes infiltrated with dysfunctional scar tissue (Tzahor and Poss, 2017). This diminishes normal pumping mechanics and results in further structural adaptations and neurohormonal activation, such as vasoconstriction and fluid retention, to preserve blood circulation (Laflamme and Murry, 2011, Murphy et al., 2020). This causes further deterioration of the cardiovascular system and leads to inevitable disease progression. The consequent scarring also disrupts electrophysiology of the heart predisposing patients to recurrent cardiac arrest and sudden cardiac death (Uygur and Lee, 2016, Talman and Ruskoaho, 2016, Prabhu and Frangogiannis, 2016).
1.1.2 Current treatments for heart failure

Despite extensive research, we are yet to understand how to reverse the damage caused by MI. Routine medical interventions for heart failure are therefore largely limited to minimising side effects caused by reduced cardiac output and prolonging preservation of the remaining cardiac function (Choi et al., 2019, Murphy et al., 2020). This commonly involves pharmacological modulation of neurohormonal activation, such as the management of blood pressure, and the use of implantable devices to compensate for cardiac dysfunction (Murphy et al., 2020). However, currently the only mainstay of fully restoring cardiac function in patients with severe heart failure is through heart transplantation, which is not a viable option for many (Choi et al., 2019).

Therapeutics to regenerate the injured muscle and reverse or prevent remodelling of the cardiac architecture would be the gold-standard in heart failure treatment. To this end, multiple approaches to replenish the lost cardiomyocytes have been investigated. Cell therapy techniques whereby cells are introduced to the infarct site in the hope of restoring cardiomyocytes or contractile muscle have also been extensively trialled (Hashimoto et al., 2018, Lin and Pu, 2014). This has included the injection or engraftment of skeletal myoblasts, embryonic or induced pluripotent stem cell-derived cardiomyocytes, cardiac progenitor cells and bone-marrow derived cells (Tzahor and Poss, 2017, Cahill et al., 2017, Ahuja et al., 2007). Although some of these approaches have demonstrated the engraftment of functional muscle and minor improvements in cardiac function, there remain many complications. The low engraftment rate requires excessive numbers of cells to be cultured and injected, which is costly and practically challenging, and allogeneic cell therapies require the use of long-term immunosuppressants to prevent rejection (Tzahor and Poss, 2017, Hashimoto et al., 2018). Furthermore, successful engraftment of new muscle does not guarantee restoration of function; commonly, the electrophysiology of exogenous cells does not synchronise with endogenous cardiomyocytes and results in harmful arrhythmias (Tzahor and Poss, 2017, Cahill et al., 2017). Interestingly, there is also evidence to suggest that the benefits elicited by such cell therapies are due to pro-regenerative responses stimulated by the introduction of foreign tissue, rather than the engrafted cells themselves (Vagnozzi et al., 2020).
Strategies to enhance the proliferative capacity of endogenous tissue have also been sought. This has included promoting the intrinsic capabilities of cardiomyocytes and sparse populations of cardiac progenitor cells to proliferate by manipulating cell cycle regulators and promoting pro-mitotic pathways and paracrine signals (Tzahor and Poss, 2017, Ahuja et al., 2007, Laflamme and Murry, 2011). Attempts to reprogramme resident fibroblasts into cardiomyocytes in situ by alteration of transcription factors have also shown some promise (Chen et al., 2017, Tzahor and Poss, 2017, Hashimoto et al., 2018). However, these approaches have yet to be tested in clinical trials.

Thus, there are many obstacles that must be overcome before these strategies have potential as routine and cost-effective treatments. These approaches also fail to address the issue of obstructive scarring, which may even be inhibitory to the success of the above therapies (Hashimoto et al., 2018). Consequently, there is increasingly an appreciation that promoting a pro-regenerative environment by the manipulation of other cell types within the infarct site may be essential to foster the restoration of cardiomyocytes (Cahill et al., 2017), possibly in combination with the strategies outlined above.
1.2 **Cellular responses to ischaemic damage following MI**

Following a MI, a rapid series of cellular processes is initiated to preserve cardiac function and instigate repair of the tissue. This can be broadly categorised into three phases: the inflammatory phase, proliferation and remodelling, and maturation (Figure 1.1) (Frangogiannis, 2014, Talman and Ruskoaho, 2016).

1.2.1 **Inflammatory phase**

Cell death within the infarct site is caused by the initial ischaemic insult, and reperfusion of the tissue following clearance of the coronary blockage can further exacerbate tissue damage through the release of reactive oxidative species (ROS) (Cowled and Fitridge, 2011). Necrotic cells release damage associated molecular patterns (DAMPs) which, in addition to ROS, trigger the release of pro-inflammatory cytokines and lipid mediators from resident cells (Epelman et al., 2015, Frangogiannis, 2014). This includes tumour necrosis factor (TNF), interleukin 1β (IL-1β) and interleukin 6 (IL-6) which induce the upregulation of chemokines and adhesion molecules, leading to the extravasation of leukocytes from the circulation (Frangogiannis, 2014, Dewald et al., 2004). Neutrophils rapidly infiltrate the tissue, shortly followed by pro-inflammatory monocytes which differentiate into monocyte-derived macrophages (MDMs) (Frangogiannis, 2014, Talman and Ruskoaho, 2016, Epelman et al., 2015). Neutrophils, monocytes and MDMs act to phagocytose cellular and extracellular matrix (ECM) debris and contribute to the secretion of pro-inflammatory mediators and chemokines to recruit additional leukocytes (Frangogiannis, 2014, Swirski and Nahrendorf, 2018).

1.2.2 **Proliferation and remodelling**

Resolution of the inflammatory phase is required for the induction of tissue repair processes and requires the coordinated response of many cell types and the surrounding matrix (Frangogiannis, 2012). Neutrophils undergo apoptosis and are cleared by macrophages, usually within the first week post-MI (Frangogiannis, 2012). This phagocytosis of apoptotic neutrophils and dead cells triggers a transition to the release of anti-inflammatory mediators by macrophages, such as IL-10 and TGF-β (Frangogiannis, 2012, Soehnlein and Lindbom, 2010). T regulatory cells also secrete these inhibitory molecules which further promotes an anti-inflammatory macrophage phenotype, and downmodulates the expression of chemokines and endothelial-leukocyte adhesion molecules preventing further immune cell
infiltration (Frangogiannis, 2012). This is also augmented by the release of pro-resolving mediators, such as lipoxins and resolvins (Frangogiannis, 2012, Pinto et al., 2014).

The loss of cardiomyocytes leads to weakness within the ventricle. To combat this, interstitial fibroblasts, which are prevalent within cardiac tissue, proliferate and migrate to the infarct site where they differentiate into myofibroblasts and deposit a matrix to stabilise the tissue, which is largely comprised of collagen III fibrils (Cahill et al., 2017, Talman and Ruskoaho, 2016, Frangogiannis, 2014). Myofibroblasts also secrete other ECM proteins such as fibronectin and matricellular proteins required for healing responses (Talman and Ruskoaho, 2016). Macrophages also secrete growth factors which support angiogenesis and endothelial cell proliferation causing revascularisation of the tissue (Bujak and Frangogiannis, 2009, Lai et al., 2019).

1.2.3 Maturation and adverse remodelling

Although some cardiomyocytes at the border zone proliferate to replace cardiomyocytes, this occurs at a very low level and is insufficient to regenerate the tissue (detailed further in section 1.5.1) and the establishment of a chronic scar occurs to restore tissue mass and prevent rupture of the ventricular wall (Talman and Ruskoaho, 2016, Tzahor and Poss, 2017). Therefore, once the infarct has become infiltrated with an immature collagenous scar, myofibroblast number subsides, and the persisting myofibroblasts switch to production of type I collagen, which becomes cross-linked, forming a mature scar (Talman and Ruskoaho, 2016).

However, fibrotic tissue is dysfunctional and the resulting mechanical stress and release of pro-fibrotic mediators cause further deposition of collagen by fibroblasts and myofibroblasts in surrounding tissue (Talman and Ruskoaho, 2016, Bujak and Frangogiannis, 2009). Surviving cardiomyocytes also become hypertrophied to compensate for the loss of cardiomyocytes and show increased contractility, further contributing to disease progression and dysfunction (Gao et al., 2021, Cahill et al., 2017).
Figure 1.1. Phases of tissue repair following MI-induced damage. An initial sterile inflammatory response is initiated by cell death and ROS-mediated damage. Pro-inflammatory phagocytes are recruited to the infarct site to clear debris and secrete pro-inflammatory mediators. Following resolution of the inflammatory response, myofibroblasts proliferate, differentiate and infiltrate the infarct site to secrete an immature collagenous scar which remodels the infarct site. Myofibroblast number subsides, and the collagenous matrix matures to form a chronic scar with limited cardiomyocyte renewal contributing to repair of the tissue. Reactive scarring also occurs in tissue distal to the infarct site causing further adverse remodelling (Frangogiannis, 2014, Talman and Ruskoaho, 2016, Lai et al., 2017).
1.3 **Cardiac regeneration**

Fibrotic repair of cardiac muscle, and many other tissue types, is the default response to injury in postnatal mammals, and much of the characterisation of this process has been performed using models of MI in rodents (Cochain *et al.*, 2012, Senyo *et al.*, 2013, Simões *et al.*, 2020). However, several other vertebrate species, such as zebrafish, newts and axolotls, can efficiently regenerate and resolve scar tissue following injury to completely restore structure and functionality of many organs, including the heart (Tzahor and Poss, 2017, Cahill *et al.*, 2017, Poss *et al.*, 2002). Foetal and neonatal rodents also possess a transient ability to regenerate cardiac muscle (Porrello *et al.*, 2011, Porrello *et al.*, 2013, Haubner *et al.*, 2012) and, strikingly, human infants also demonstrate an enhanced ability to restore functional cardiac tissue with limited adverse scarring following MI, surgical interventions and diphtheria infection (Haubner *et al.*, 2016, Saker *et al.*, 1997, Vivien *et al.*, 2016). This indicates that mammalian cardiac tissue has an intrinsic capacity to regenerate and poses the exciting potential to reinstate this ability. Extensive studies have therefore been performed in regenerative organisms to identify the roadblocks to regeneration in postnatal mammals, however, we are still yet to establish the multitude of key signalling pathways and cell populations that could facilitate this favourable regenerative outcome versus fibrotic repair (Kikuchi and Poss, 2012).
1.4  **Studying cardiac regeneration in adult zebrafish**

1.4.1  **Zebrafish as a model organism**

Zebrafish (*Danio rerio*) are small vertebrates of the teleost fish family and have become increasingly popular as a model organism for *in vivo* studies, in particular within the tissue regeneration field (Lieschke and Currie, 2007, Gemberling *et al.*, 2013). This is due in part to the presence of homologs to 70% of the human genome and 84% of human disease genes, despite whole genome duplication resulting in the potential for multiple orthologs to mammalian genes (Howe *et al.*, 2013). Genetic mapping has also allowed relatively simple genetic modification of the zebrafish genome and has facilitated the interrogation of many gene functions through the creation of numerous mutants and transgenic reporter lines, which is also assisted by their high fecundity and relative ease to maintain large colonies (Lieschke and Currie, 2007). This, combined with short external development time and translucency during embryonic and larval stages, has enabled *in vivo* imaging and tracing of cellular processes which is not possible in many mammalian models.

1.4.2  **Studying heart regeneration in zebrafish**

Following cardiac injury, zebrafish fully regenerate their myocardium within 60-180 days (Poss *et al.*, 2002, Chablais *et al.*, 2011). Comparably to mammals, this occurs by dedifferentiation and proliferation of mature cardiomyocytes adjacent to the injury site (Jopling *et al.*, 2010, Kikuchi *et al.*, 2010), but occurs much more efficiently and is accompanied by less extensive scar deposition which is fully resolved following cardiomyocyte renewal (Talman and Ruskoaho, 2016, Poss *et al.*, 2002, Kikuchi, 2015). Regenerative capacity is also maintained throughout the lifespan of the zebrafish, therefore elucidating how this occurs in an adult organism may be advantageous over neonatal models, in which developmental processes are still occurring.

The similar cellular composition and anatomy of zebrafish hearts to that of mammals also allows for comparisons to be drawn (Figure 1.2) (González-Rosa *et al.*, 2017). Somewhat similar to mammalian hearts, zebrafish hearts are also four chambered, but consist of a single ventricle and atrium and two additional chambers, the sinus venosus and bulbus arteriosus, which deliver and receive blood from the atrium and ventricle, respectively (Hu *et al.*, 2000).
The wall of the mammalian and zebrafish heart is also similar, consisting of epicardium, trabecular and compact myocardium and epicardium (Jensen et al., 2013, Lien et al., 2012, Pieperhoff et al., 2014).

Figure 1.2. Structural similarities of the adult mammalian and zebrafish heart. Mammals have a four chambered heart comprised of two ventricles and two atria, whereas zebrafish hearts have a single ventricle and atrium with additional inflow/outflow chambers (sinus venosus and bulbus arteriosus, respectively) (Hu et al., 2000, Jensen et al., 2013). Cardiac muscle is comprised of dense compact myocardium which overlays trabeculated myocardium that protrudes into the lumen of the chamber and is lined by a thin endocardial layer. This composition is maintained in the atria, however zebrafish atria have a much thinner myocardial layer and resembles a sack-like structure (Hu et al., 2000). The exterior of the heart is covered by epicardium and pericardium which are separated by a fluid-filled region called the pericardial space (Andrés-Delgado and Mercader, 2016). Cardiomyocytes are shown as mononucleated, representing the nucleation of zebrafish cardiomyocytes, but mammalian cardiomyocytes are commonly multinucleated (Vivien et al., 2016).
1.4.3 Cardiac injury models

Models of MI in mice commonly involve permanent or transient ligation, via suturing, of the left anterior descending coronary artery to prevent blood flow to a region of the ventricle (Gao et al., 2010, Lindsey et al., 2018). Due to the variable position and size of the coronary artery, modelling MI on the zebrafish ventricle by coronary ligation is challenging (Vivien et al., 2016, Harrison et al., 2015). Alternative methods to induce cardiac damage have therefore been developed, each of which result in slightly different patterns of repair and regeneration, in order to exploit zebrafish as a model of cardiac regeneration (González-Rosa et al., 2017).

1.4.3.1 Resection

The earliest studies of regeneration in zebrafish involved surgical removal of up to 20% of the apical region of the ventricle (Poss et al., 2002). The resulting regenerative response is characterised by low levels of scarring and contraction of the wound site, resulting in full regeneration of the ventricle within one to two months (Poss et al., 2002, Chablais et al., 2011). However, this resection model does not recapitulate the highly necrotic environment of a MI which is important for conditioning the inflammatory response and may contribute to the minimal scarring observed (Chablais et al., 2011, González-Rosa et al., 2017).

1.4.3.2 Systemic cardiac injury

An inducible technique resulting in the ablation of approximately 60% of cardiomyocytes has also been established, more closely recapitulating widespread cardiac cell death induced by cardiomyopathies and end stage heart failure (Wang et al., 2011). This provokes extensive cardiomyocyte proliferation to recover the myocardium within 30 days post-ablation in the absence of scarring (Wang et al., 2011), likely because cardiomyocyte death is not localised and non-myocyte populations are not damaged (González-Rosa et al., 2017). Exposing adult fish to hypoxia and reoxygenation has also been utilised and has been shown to induce cardiac regeneration, but this inevitably has other systemic effects (Parente et al., 2013).

1.4.3.2.1 Cryoinjury

To more accurately mimic the localised and mass cell death induced by MI, cryoinjury of the ventricle was developed as an injury model and is now commonly used. Following thoracotomy, a liquid nitrogen-cooled probe is placed on the ventricle to induce a consistent region of cell death within ~20% of the ventricle area (Chablais et al., 2011, González-Rosa et al., 2017).
This induces extensive collagen deposition within the injury site but this is replaced by functional muscle, although this occurs more slowly than in other models, occurring 60-180 days post injury (dpi) depending on injury severity (Chablais et al., 2011, González-Rosa et al., 2011, Hein et al., 2015). Cryoinjury of the zebrafish heart therefore provides an excellent opportunity to understand the mechanism of transient scarring alongside cardiomyocyte regeneration.

### 1.4.4 Involvement of myocytes, non-myocytes and ECM in zebrafish heart regeneration

Characterisation of the cellular response to cardiac injury has shown that zebrafish undergo highly similar inflammatory and remodelling phases to mammalian models of MI (section 1.2) (González-Rosa et al., 2017, Lai et al., 2019). A fibrin layer immediately seals the injury site and there is successive infiltration of pro-inflammatory and anti-inflammatory leukocytes and deposition of a collagenous scar by myofibroblasts (Chablais and Jaźwińska, 2012, Bevan et al., 2020, Xu et al., 2018). Yet, robust proliferation of existing cardiomyocytes and concomitant scar removal proceed to regenerate the tissue (Poss et al., 2002).

The reactivation of specific cardiomyocyte populations within and surrounding the injury site is essential for regeneration in the zebrafish (Kikuchi et al., 2010, Jopling et al., 2010), but this regenerative process has been shown to be dependent on the actions of many non-myocyte cell types (Ryan et al., 2020, González-Rosa et al., 2017). Cytokines derived from the endocardium and leukocytes are required to instruct cardiomyocyte proliferation (González-Rosa et al., 2017), in addition to the requirement for revascularization and innervation of the tissue, which is also essential for scar resolution (Marín-Juez et al., 2016, Harrison et al., 2015, Mahmoud et al., 2015). Regeneration and invasion of the injury site by the epicardium is also an important source of paracrine signals, ECM and cells with transdifferentiation potential to support regeneration (Wang et al., 2015, González-Rosa et al., 2017). The deposition of scar tissue (~4 dpi in cryoinjury models) is also beneficial to heart regeneration in the zebrafish, promoting proliferation and likely provides essential structural support, which is not required in other low pressure tissue regeneration models (Chablais and Jaźwińska, 2012, Palatinus et al., 2010, Chen et al., 2016). However, reversal of this scar deposition is also essential (~14 dpi) and the mechanism by which this occurs remains somewhat elusive, although collagenase
activity by matrix metalloproteinases (MMPs) and regulated TGFβ signalling has been shown to contribute (Gamba et al., 2017, Chablais and Jaźwińska, 2012).

Immune cells also play an essential role in orchestrating many of these processes (Lai et al., 2019, Ryan et al., 2020, Gao et al., 2021). As described in section 1.2, MNPs play important roles in conditioning an appropriate inflammatory environment to foster tissue repair and will be explored further in section 1.6. However, T regulatory lymphocytes also contribute to this and provide trophic factors that are essential for normal proliferation and scar resolution (Hui et al., 2017). Likewise, robust T and B lymphocyte responses have also been associated with a favourable regenerative outcome (Lai et al., 2017). Treatment with immunosuppressants has been shown to impede cardiac regeneration due to a blunted inflammatory response (Huang et al., 2013, de Preux Charles et al., 2016), collectively demonstrating the importance of immune cells in mediating regenerative programs.

Figure 1.3. Phases of tissue regeneration following cardiac cryoinjury in the adult zebrafish. Cardiac regeneration undergoes similar phases of inflammation and remodelling to mammalian models (Figure 1.1). However, the zebrafish subsequently undergoes efficient cardiomyocyte proliferation and scar removal to achieve full restoration of cardiac muscle (Chablais and Jaźwińska, 2012, González-Rosa et al., 2017).
1.5 **Factors affecting the regenerative capacity of the heart**

The extent to which a tissue can regenerate is highly tissue dependent in postnatal mammals, with the skeletal muscle and liver being highly regenerative, whilst other tissues such as skin, showing more modest levels of regeneration (Laflamme and Murry, 2011, González-Rosa et al., 2017). However, cardiac tissue is one of the most resistant tissues to regeneration, which is likely due to many factors (Tzahor and Poss, 2017).

1.5.1 **Cardiomyocyte proliferation**

The inability of the postnatal mammalian heart to regenerate is hampered by the low proliferative capacity of cardiomyocytes (Vivien et al., 2016, Senyo et al., 2014). In mammals, homeostatic and injury-associated cardiomyocyte renewal occurs by dedifferentiation and proliferation of healthy cardiomyocytes (Porrello et al., 2013, Senyo et al., 2013). Cardiac progenitor populations can also give rise to cardiomyocytes, but it is thought that these cells have minimal contributions to cardiomyocyte renewal, although some disputed evidence suggests these progenitors are important for replenishment following injury (Ellison et al., 2013, Hsieh et al., 2007). Nevertheless, homeostatic cycling of cardiomyocytes is rare, with ~0.3-1% of cardiomyocytes being renewed per year and only increasing to ~3% following MI, with many of these cells also failing to undergo cytokinesis (Tzahor and Poss, 2017, Bergmann et al., 2009, Senyo et al., 2013). This is insufficient to replace extensive regions of injured tissue following a severe MI, in which it is estimated that 25% of cardiomyocytes, corresponding to approximately 1 billion cells in humans, can be lost (Vivien et al., 2016, Laflamme and Murry, 2011).

Low proliferation rate is partly attributed to cellular changes that occur during differentiation into mature cardiomyocytes in mammals. This involves increased ploidy and nucleation of the cells, with many mammalian cardiomyocytes being polyploid and multinucleated, and an increased complexity of myofilament organisation which must be disassembled during re-entry into the cell cycle (Vivien et al., 2016, Jopling et al., 2010, Kikuchi et al., 2010). This contrasts with the cardiomyocytes of regenerative organisms, including zebrafish and neonatal mammals, which are commonly mononucleated and have a simpler myofilament/sarcomere structure (Kikuchi, 2015). This is compounded by the observation that the loss of regenerative ability in the neonatal mouse, around postnatal day (P) 7,
coincides with the period in which the majority of cardiomyocytes fully differentiate. This involves a transition of cardiomyocytes to become diploid and binuclear, and cardiac growth continues by hypertrophy and not proliferation (Tzahor and Poss, 2017).

Furthermore, oxygen availability and consequent cell metabolism has also been associated with proliferative capacity. The transition to postnatal life in mammals is associated with a switch from hypoxia to normoxia, shifting metabolism from glycolysis to oxidative phosphorylation, which produces ROS and is proposed to induce cell-cycle arrest in cardiomyocytes (Vivien et al., 2016, Tzahor and Poss, 2017, Cahill et al., 2017, Puente et al., 2014). Furthermore, the aquatic environment of zebrafish is innately hypoxic, and has been associated with their enhanced cardiomyocyte proliferation (Vivien et al., 2016).

1.5.2 Tissue mechanics

The high ventricular pressure required to sustain the circulation of mammals has also been shown to be inhibitory to cardiomyocyte proliferation (Vivien et al., 2016, Canseco et al., 2015). This pressure is also further intensified in the infarcted heart following the infiltration of stiff matrix proteins and redistribution of loading due to dysfunctional tissue (Tzahor and Poss, 2017, Yahalom-Ronen et al., 2015).

1.5.3 Scarring

Removal of adverse scar tissue is as critical as the replenishment of cardiomyocytes to achieve regeneration of a functional myocardium and, until recently, has been somewhat overlooked in the cardiac regeneration field. The mechanism behind the maintenance of the chronically scarred mammalian heart is unknown. It is possible that persistent myofibroblasts actively replenish the scar and/or the mature scar may be resistant to degradation, or signals directing scar removal are absent (Talman and Ruskoaho, 2016). Further investigation into dynamics of chronic scarring will be essential to reverse the adverse remodelling that is intrinsic to the pathology of the failing heart.

1.5.4 Immune system

It was originally posited that increased complexity of an organism’s immune system was inversely associated with regenerative capacity (Vivien et al., 2016). Nevertheless, as it has been established that regenerative vertebrates, such as the zebrafish, have many analogous
innate and adaptive immune cell populations to that of non-regenerative mammals which respond similarly to tissue injury, this hypothesis is now largely disregarded (Herbomel et al., 1999, Wittamer et al., 2011, Dee et al., 2016).

Rather, it is becoming increasingly evident that subtle differences in dynamics of immune cell populations and inflammatory profile of a wound impacts the relative success of both reparative and regenerative responses (Godwin et al., 2017, Frangogiannis, 2012, de Lemos et al., 2007, Frangogiannis, 2014). Regulation of the attending immune cells and the inflammatory response also appears to be intimately linked with the establishment of an appropriate scarring response. Studies in multiple models of tissue injury have shown that the initial, acute pro-inflammatory phase is essential to program the subsequent repair/regeneration (Huang et al., 2013, de Preux Charles et al., 2016, Han et al., 2015). However, prolonged inflammatory responses are associated with less mature scar tissue and can lead to myocardial rupture (Frangogiannis, 2014).
1.6 Mononuclear phagocytes in tissue repair and regeneration

As outlined in sections 1.2 and 1.4.4, mononuclear phagocytes (MNPs), which comprise monocytes, macrophages, and dendritic cells (DCs), have multifaceted and essential roles in mediating the cascade of responses following tissue damage (Zlatanova et al., 2016, Gao et al., 2021).

1.6.1 Inflammatory profile

Macrophages are capable of secreting both pro- and anti-inflammatory mediators to regulate the inflammatory environment and this inflammatory profile or activation state is commonly used to classify and attribute roles to populations of macrophages during tissue repair (Figure 1.4) (Lee, 2019).

Pro-inflammatory macrophages present at the initial stages of the injury response have been historically referred to as classically activated M1 macrophages. This M1 phenotype is induced by exposure to IFNγ and TNFα and is characterised by the secretion of factors such as ROS and IL-1β, TNFα and MMPs, which recruits additional inflammatory cells, and is accompanied by phagocytic activity to clear necrotic tissue and pathogens (Krzyszczyk et al., 2018, Das et al., 2015). Subsequently, macrophage populations attending the injury site transition to an anti-inflammatory/pro-reparative M2 phenotype which is induced by IL-4 and IL-10 (Lee, 2019). M2 macrophages promote initiation of tissue repair and regeneration by the secretion of factors that promote proliferation, angiogenesis and the differentiation of ECM-secreting myofibroblasts (Krzyszczyk et al., 2018, Das et al., 2015).

The accumulation of macrophages and transition of phenotype is observed in human, murine and zebrafish hearts following cardiac insult (Yan et al., 2013, van der Laan et al., 2014, Bevan et al., 2020). In both murine and zebrafish hearts, this expansion occurs within the first week post-injury, largely representing the recruitment of monocytes to the injury site during the inflammatory phase (Yan et al., 2013, Bevan et al., 2020). Within the murine heart, the majority of monocytes and macrophages express an M1-like profile at 1-3 days post MI (dpmi) whereas M2-like cells are abundant from 5-7 dpmi (Nahrendorf et al., 2007, Yan et al., 2013). Similarly, pro-inflammatory MNPs have been observed in the first 3 days following cardiac injury in the
adult zebrafish ventricle, but transition to a anti-inflammatory phenotype in subsequent days (Simões et al., 2020, Xu et al., 2019, Bevan et al., 2020).

However, this M1/M2 classification oversimplifies the vast phenotypic heterogeneity displayed by wound macrophages (Das et al., 2015, Epelman et al., 2015). This is demonstrated by expression profiling of infarct-associated macrophages in the murine heart. At 1 dpmi, macrophages show high upregulation of Il1b, Il6 and Ccr2 (M1 genes), whereas at 7-14 dpmi, macrophages express high levels of Il10 and Cx3cr1 (M2 genes) (Yan et al., 2013). Nevertheless, when stratifying macrophages by their M1/M2 phenotype (which can be determined by CD206 expression in mice), conventional M1 markers Tnfa and Il6 were not differentially expressed between populations (Yan et al., 2013). This likely demonstrates that there are sub-populations within these M1/M2 populations that have distinct expression profiles, and thus unique functions, which are more complex and cannot be resolved using stratification of conventional M1/M2 markers. Furthermore, macrophage phenotype is highly plastic and responsive to environmental cues (Das et al., 2015). Although it is unestablished whether macrophages switch their polarization state during the duration of wound healing or different subpopulations are recruited during injury, it is likely that the spatiotemporal distribution imparts differences in phenotypic profile to some extent (Das et al., 2015, Frangogiannis, 2014).
Figure 1.4. M1/M2 inflammatory spectrum of monocytes and macrophages. Monocytes and macrophages can be broadly characterised into two phenotypes based on their expression profile and function. M1, classically activated cells are highly phagocytic and secrete pro-inflammatory mediators which are important for immune cell recruitment whereas M2, alternatively activated cells secrete anti-inflammatory mediators and growth factors that support matrix production and angiogenesis. In reality, macrophages likely sit on a spectrum between these two phenotypes expressing certain characteristics of each phenotype (Das et al., 2015, Lee, 2019, Krzyszczyk et al., 2018).
1.6.2 Influences of macrophage inflammatory phenotype on tissue repair and regeneration

Given the extensive and varied functions of macrophages, it is unsurprising that dysregulated macrophage frequency, dynamics or inflammatory profile can have detrimental effects on the successful repair and regeneration of many tissues. Depletion of macrophages has been shown to delay skin wound healing by impairing scar deposition, angiogenesis and proliferation (Mirza et al., 2009), completely abrogates limb regeneration in the salamander (Godwin et al., 2013) and cardiac regeneration in the neonatal mouse (Aurora et al., 2014).

Similar effects are also seen in the adult zebrafish, where reduced macrophage infiltration impairs both fin and cardiac regeneration (Petrie et al., 2014, Xu et al., 2018, Bevan et al., 2020). Curiously, the timing or selectivity of macrophage depletion post-injury results in variable influences on tissue repair/regeneration, reflecting the unique function and importance of the populations at different phases. Genetic ablation of zebrafish macrophages at immediate stages following fin resection stunts regeneration, yet ablation at later stages is less detrimental, indicating that early macrophages are important for establishing the course of wound healing (van Amerongen et al., 2007). However, other studies show that depleting macrophages at later stages is inhibitory to axonal and fin repair, due to a prolonged pro-inflammatory response in the absence of anti-inflammatory macrophages (Tsarouchas et al., 2018, Morales and Allende, 2019). Furthermore, ablation of macrophages at the initial phases post-cardiac cryoinjury compromises scar deposition, whereas late ablation impedes scar regression, demonstrating their roles in regulating both aspects of scar dynamics (Bevan et al., 2020). These differing outcomes have been linked with the opposing M1 and M2 phenotypes that dominate at early and late stages, respectively (Krzyszczyk et al., 2018).

Similarly, clodronate mediated depletion of macrophages within the first week, or selective depletion of M2 macrophages in the murine heart, has also been shown to impair healing due to insufficient collagen synthesis which is required for provide structural support of the tissue (Shiraishi et al., 2016, van Amerongen et al., 2007). However, although macrophages are important to mediate repair of the tissue, they also mediate pathological responses and remodelling in mammals (Lavine et al., 2014, Ismahil et al., 2014, Sager et al., 2016). Macrophages with an M2-like profile have been shown to promote chronic scar formation.
which contributes to the pathology of heart failure and other diseases (Krzyszczyk et al., 2018, Ueha et al., 2012). Interestingly, in mammals, these adverse responses have been linked with adverse inflammatory profiles of subpopulations of monocytes and macrophages from different origins (Lavine et al., 2014, Dick et al., 2019, Panizzi et al., 2010, Nahrendorf et al., 2007).

1.7 **Tissue macrophages**

In addition to being recruited to sites of inflammation via monocyte precursors, extensive populations of macrophages, and smaller populations of DCs, exist within tissues during the steady state (Guilliams et al., 2020). Here, they act as sentinels to instigate responses to infection and injury, but also have diverse functions in development and preservation of tissue homeostasis (Davies et al., 2013). The plasticity of macrophages also allows them to adopt tissue specific functions. For example, cardiac macrophages are important in maintaining electrical conductance of the heart; osteoclasts (bone-associated macrophages) have specialist roles in bone resorption; and microglia (macrophages of the central nervous system) are important for maintaining neural networks (Reynolds and Haniffa, 2015, Hulsmans et al., 2017).

Given the monocyte-derived ontogeny of wound macrophages, it was previously thought that tissue resident macrophages were constantly replenished by circulating monocytes. However, it has become evident that this is not the case in most tissues, with origin being diverse and tissue dependent, and consequently influences macrophage function (Ginhoux and Jung, 2014, Hashimoto et al., 2013, Bajpai et al., 2018).

1.7.1 **Ontogeny of tissue macrophages**

Within vertebrates, there are multiple, conserved waves of haematopoiesis which give rise to MNPs (myelopoiesis) and all other hematopoietic cells; these are initiated during embryonic stages of development and occur in several distinct and transient sites (Figure 1.5) (Gore et al., 2018, McGrath et al., 2015). During the primitive wave, macrophages emerge independently of hematopoietic stem cells (HSC) from a monopotent progenitor and migrate into tissues to seed initial tissue macrophage populations (McGrath et al., 2015, Herbomel et al., 1999). This is succeeded by an interim form of myelopoiesis whereby MNPs derive from a transient erythromyeloid progenitor (EMP), before finally arising from HSCs during definitive
haematopoiesis, which continues as the sole form of monocyte-derived MNP production throughout the lifespan of the organism (Ginhoux and Jung, 2014, Davidson and Zon, 2004, McGrath et al., 2015).

In mice, primitive haematopoiesis occurs in the yolk-sac blood islands, and primitive macrophages emerge at embryonic day (E) 8.5-9.0 to colonise tissues (Figure 1.5). The first macrophages arising from HSC-dependent definitive hematopoiesis develop in the foetal liver at E11.5-12.5, following which they circulate and enter tissues at E13.5-14.5. Terminal myelopoiesis occurs in the bone marrow, however extramedullary hematopoiesis in the spleen can give rise to myeloid cells (McGrath et al., 2015, Soares-da-Silva et al., 2020, Swirski and Nahrendorf, 2018). Primitive myelopoiesis in the zebrafish initiates in the anterior lateral mesoderm (ALM) and the first primitive macrophages emerge directly from a hemangioblast precursor at 20 hours post fertilisation (hpf) (Figure 1.5). The onset of the circulation at 24 hpf shifts myelopoiesis to the posterior blood island (PBI) where macrophages are transiently EMP-derived until definitive hematopoiesis is initiated in the aorta gonads mesonephros (AGM) at 36 hpf. In the AGM, the hemogenic endothelium gives rise to a HSC progenitor. These HSC progenitors migrate transiently to the caudal hematopoietic tissue (72 hpf – 6 dpf) then terminally to the whole kidney marrow (from 96 hpf) and give rise to monocytes and MDMs (Chen and Zon, 2009).

Lineage tracing has begun to uncover the origin of tissue macrophage populations in mice. In the majority of tissues, the primitive, yolk-sac derived macrophages which initially colonise embryonic tissues are replaced by definitive, foetal liver-derived macrophages and subsequently self-renew to continually populate their tissue niche (Ginhoux and Jung, 2014, Hashimoto et al., 2013). Yet, this is not consistent in all tissue types: microglia are solely derived from primitive macrophages; Langerhans cells (specialist DCs of the skin) can originate from yolk-sac or foetal liver monocyte populations, and intestinal macrophages are constantly replenished by circulating monocytes (Ginhoux and Jung, 2014, Epelman et al., 2014, Hashimoto et al., 2013).

Therefore, although definitive haematopoiesis is the primary source of monocytes and MDMs, which are essential for responses to infection and injury in postnatal organisms, there is evidence to suggest that embryonic myelopoiesis is critical to seed tissue macrophage
populations that exist throughout the lifespan of the organism (Hashimoto et al., 2013). Furthermore, these tissue resident populations have been described to have unique roles during homeostasis and during tissue repair and regeneration.

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Zebrafish</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGM</td>
<td>AGM</td>
</tr>
<tr>
<td>Foetal liver</td>
<td>PBI</td>
</tr>
<tr>
<td>Spleen</td>
<td>CHT</td>
</tr>
<tr>
<td>Yolk sac</td>
<td>ALM</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>Whole kidney marrow</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>9</td>
<td>24 (1)</td>
</tr>
<tr>
<td>10</td>
<td>36</td>
</tr>
<tr>
<td>11</td>
<td>48 (2)</td>
</tr>
<tr>
<td>12</td>
<td>60</td>
</tr>
<tr>
<td>13</td>
<td>72 (3)</td>
</tr>
<tr>
<td>14</td>
<td>84</td>
</tr>
<tr>
<td>15</td>
<td>96 (4)</td>
</tr>
<tr>
<td>16</td>
<td>108</td>
</tr>
<tr>
<td>17</td>
<td>120 (5)</td>
</tr>
<tr>
<td>18</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>144 (6)</td>
</tr>
</tbody>
</table>

Figure 1.5. Ontogeny and sites of myelopoiesis in mouse and zebrafish. The location and type of myelopoiesis at different development stages in mice and zebrafish. A colour-coded schematic is also provided to show these sites within the larval zebrafish. Figure adapted from various sources (McGrath et al., 2015, Soares-da-Silva et al., 2020, Davidson and Zon, 2004). Erythromyeloid progenitor (EMP); aorta gonads mesonephros (AGM); anterior lateral mesoderm (ALM); posterior blood island (PBI); caudal hematopoietic tissue (CHT); whole kidney marrow (WKM).
1.7.2 Cardiac MNPs

The murine heart has extensive populations of cardiac tissue macrophages (cTMs) dispersed within the myocardium and surrounding endothelial cells within the steady state (Pinto et al., 2012, Swirski and Nahrendorf, 2018, Epelman et al., 2015). Lineage tracing of murine cTMs has shown that primitive macrophages initially colonise the myocardium, followed by definitive, foetal-liver macrophages. Both populations proliferate to maintain cTMs throughout the lifespan of the mouse, with little input from circulating monocytes (Figure 1.6) (Heidt et al., 2014, Epelman et al., 2014, Pinto et al., 2014). These cTMs have a M2-like profile and function to phagocytose cellular debris, maintain the extensive vasculature and modulate the inflammatory environment (Pinto et al., 2014, Pinto et al., 2012, Leid et al., 2016).

1.7.3 Cardiac MNPs following tissue injury

1.7.3.1 Mice

Analysis of cTMs following MI has shown that these embryonic lineages have distinct responses to tissue injury compared to recruited macrophages, and their dynamics differ between neonatal and postnatal mice. Embryonic cTMs can be distinguished from monocyte-derived cells by the absence of the cell surface receptor, CCR2, in both mice and humans (Epelman et al., 2014, Leid et al., 2016, Bajpai et al., 2018). In neonatal mice, these CCR2-populations of embryonic cTMs expand and mediate tissue regeneration, and there is minimal recruitment of CCR2+ monocytes (Lavine et al., 2014). In contrast, cardiac injury in adult mice induces a profound recruitment of CCR2+ monocytes, which infiltrate the myocardium and differentiate into CCR2+ MDMs and replace embryonic cTMs. However, these CCR2+ MNPs have been attributed harmful pro-fibrotic activity in contrast to embryonic populations (Lavine et al., 2014, Zhang et al., 2020).

The regenerative versus reparative response mediated by these populations has been partly attributed to the opposing inflammatory profile of embryonic cTMs and CCR2+ MNPs. In neonatal and postnatal mice, embryonic cTMs retain a principally M2-like expression profile whereas CCR2+ monocytes show elevated expression of pro-inflammatory markers, expressing high levels of Il-1β, Ccl2 and Il6 (Lavine et al., 2014, Zhang et al., 2020, Bajpai et al., 2018). Similarly, the retention of pro-inflammatory CCR2+ MNPs have been repeatedly shown to drive adverse cardiac remodelling in the mouse (Bajpai et al., 2019, Patel et al., 2018, França...
et al., 2017). Furthermore, attenuation of CCR2+ monocyte recruitment has been associated with reduced adverse remodelling, but the maintenance of essential replacement fibrosis, which is correlated with a reduction in pro-inflammatory cytokines such as IL-1β and TNFα (Dewald et al., 2005). Manipulating CCR2+ monocytes may therefore be a therapeutic strategy to promote cardiac regeneration. However, we are yet to understand exactly how these cells mediate the adverse scarring phenotype in mammals.

1.7.3.2 Zebrafish

Zebrafish are known to have populations of cTMs in the steady state and cardiac MNP populations expand following cardiac injury (Bevan et al., 2020, Lai et al., 2017). However, we are yet to understand whether distinct MNP lineages are responsible for mediating the regenerative response observed. This is because parallel populations have yet to be fully characterised within zebrafish, including those that constitute cTMs and recruited monocyte-derived populations, and prevents their segregation during the analysis of MNPs following injury. Future studies are therefore required to understand the dynamics of these distinct macrophage populations in the zebrafish to determine whether differences in ontogeny and regulation of their inflammatory profile contribute to regeneration.
Figure 1.6. cTMs in neonatal and postnatal mice during homeostasis and following MI. Embryonic cTMs and sparse monocytes comprise the cardiac MNP populations in neonatal mice. Embryonic cTMs expand following MI, with little recruitment of CCR2+ monocytes, and promote regeneration of the myocardium. Embryonic cTMs also seed the majority of cTMs in the postnatal mouse, however, pro-inflammatory CCR2+ monocytes and MDMs displace embryonic cTMs during infarction and contribute to chronic scarring. Subsequently, MDMs become the predominant cTM population (Epelman et al., 2014, Lavine et al., 2014).
1.8  Rationale

In summary, MNPs have been repeatedly reported to be essential for tissue repair and regeneration but have also been shown to mediate adverse scarring that is central to the pathology of heart failure. The recruitment of pro-inflammatory monocytes and displacement of embryonically derived cTMs has been associated with this adverse scarring in mice. Zebrafish are known to recruit pro-inflammatory monocytes during inflammation, but form a transient scar, indicating that they modulate monocyte responses differently to mammals. Furthermore, although zebrafish have populations of cTMs, we do not yet know the ontogeny of these populations and how these cells respond to cardiac injury and elicit pro-regenerative functions. This is, in part, due to the paucity of markers that stratify populations of monocytes and macrophages and lack of in-depth studies on the dynamics of cTMs in the steady state and following cardiac injury within the zebrafish.

This PhD therefore investigates the hypothesis that adult zebrafish modulate MNP populations in response to cardiac injury differently to postnatal mammals to favour a regenerative outcome. This hypothesis was therefore explored by addressing the following aims:

1. Using existing zebrafish and mammalian MNP markers, can MNP subpopulations (namely monocytes and tissue macrophages) be stratified to establish their dynamics and functions in the steady state and following cardiac cryoinjury?
2. How does csf1ra mutation affect cTM populations in the steady state and following injury? Can this help to elucidate the ontogeny of cTMs and injury associated MNPs and the contribution of these cells to scar resolution?
3. Do zebrafish MNPs contribute to il1b signalling following cardiac cryoinjury and how does loss of this pro-inflammatory pathway affect heart regeneration?
2 Materials and Methods

All materials were acquired from Sigma unless otherwise stated.

2.1 Zebrafish maintenance and procedures

2.1.1 Zebrafish husbandry

All zebrafish lines were maintained and procedures performed in accordance with UK Home Office (PPL #P6F93B20F) and local University of Bristol regulations. Zebrafish were maintained in 3 litre tanks on a 14/10 hour light/dark cycle. For breeding of new generations, zebrafish were transferred to Tecniplast 1.7 litre sloping breeding tanks in the evening in a 4:2 female:male ratio and allowed to spawn at the onset of automatic lighting (9 am) the following day. Fertilised eggs were collected, cleaned and kept in Danieus solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5.0 mM HEPES, 0.025 % methylene blue) at 28 °C. At 5 days post fertilisation, larvae were transferred to Tecniplast tanks containing aquarium water and fed and cared for by aquarium facility staff thereon.

Adult zebrafish, aged 6-18 months, were randomly selected for analysis/experimental procedures from mixed-sex tanks housing up to 20 individuals. Animals were euthanised using the Schedule 1 method of immersion in an overdose of MS-222 anaesthetic in aquarium water. Details of the mutant and transgenic lines used are listed in Table 2.1.

2.1.2 Cardiac cryoinjury

Cardiac injuries on adult zebrafish were carried out as described previously by Chablais et al (2011). Animals were anaesthetised via immersion in 0.025% MS-222 in aquarium water and placed ventral side up on a wetted sponge under a dissecting microscope. A ~4 mm incision was made through the skin and the pericardial sac directly above the heart using a microscalpel (World Precision Instruments; 500249) to expose the ventricle using a
microscalpel and forceps. The ventricle was dried using a sterile cotton swab and a liquid nitrogen cooled metal probe was applied to the exposed ventricle for 30 seconds. Aquarium water containing anaesthetic was pipetted onto the ventricle/probe to release the probe from the tissue. The skin incision was gently closed using forceps and the animals were returned to aquarium water to recover. Fish were monitored for normal recovery of breathing rate (typically within 2 minutes) and swimming behaviour (typically within 5 minutes) and allowed to fully recover in a quiet room for ~30 minutes. Once fully recovered injured fish were placed back in the aquarium until the desired timepoint.

2.1.3 Larval tail fin resection

5 dpf larvae were anaesthetised and the tail fin was resected distal to the pigment gap and circulation using a microscalpel. Larval fish were recovered from the anaesthetic in clean Danieus solution at 28 °C. Larvae were then culled with overdose of anaesthetic and the tail region posterior to the cloacae was harvested for RNA analysis.
<table>
<thead>
<tr>
<th>Line</th>
<th>ZFIN ID</th>
<th>Mutation</th>
<th>Protein</th>
<th>Founding lab</th>
</tr>
</thead>
<tbody>
<tr>
<td>TgBAC(<em>csf1ra:GFP</em>)</td>
<td>ZDB-TGCONSTRCT-170322-18</td>
<td>GFP</td>
<td>Cell specific, promoter-driven GFP expression</td>
<td>(Dee et al., 2016)</td>
</tr>
<tr>
<td>Tg(<em>mpeg1.1:mCherry</em>)</td>
<td>ZDB-TGCONSTRCT-120117-2</td>
<td>mCherry</td>
<td>Cell specific, promoter-driven mCherry expression</td>
<td>(Ellett et al., 2011)</td>
</tr>
<tr>
<td>Tg(<em>mfap4:Turquoise</em>)</td>
<td>ZDB-TGCONSTRCT-150424-7</td>
<td>mTurquoise</td>
<td>Cell specific, promoter-driven Turquoise expression</td>
<td>(Walton et al., 2015)</td>
</tr>
<tr>
<td>Tg(<em>mfap4:tdTomato</em>)</td>
<td>ZDB-TGCONSTRCT-160122-3</td>
<td>tdTomato</td>
<td>Cell specific, promoter-driven tdTomato expression</td>
<td>(Walton et al., 2015)</td>
</tr>
<tr>
<td>TgBAC(<em>il1b:EGFP</em>)</td>
<td>ZDB-TGCONSTRCT-190307-7</td>
<td>GFP</td>
<td>Cell specific, promoter-driven GFP expression</td>
<td>(Ogryzko et al., 2019)</td>
</tr>
<tr>
<td>Tg(<em>fli1:EGFP</em>)</td>
<td>ZDB-TGCONSTRCT-070117-94</td>
<td>GFP</td>
<td>Cell specific, promoter-driven GFP expression</td>
<td>(Lawson and Weinstein, 2002)</td>
</tr>
<tr>
<td><em>il1bsh446/sh446</em></td>
<td>ZDB-ALT-190307-9</td>
<td>44 bp deleted in Exon 4</td>
<td>Frameshift/Premature stop</td>
<td>(Ogryzko et al., 2019)</td>
</tr>
<tr>
<td><em>csf1raj4e1/j4e1</em></td>
<td>ZDB-ALT-001205-14</td>
<td>G&gt;A at position 1949</td>
<td>Val&gt;Met at position 614</td>
<td>(Parichy et al., 2000)</td>
</tr>
</tbody>
</table>

Table 2.1. List of transgenic and mutant lines used.
2.2 Genotyping mutant zebrafish lines

Genotyping primer sequences are listed in Table 2.2.

2.2.1 Adult zebrafish

Zebrafish were anaesthetised using a minimal concentration of 0.013\% MS-222 in aquarium water and a small region of the caudal fin was resected using a razor blade. Zebrafish were singly housed for the duration of genotyping. Resected tissue was digested in 100 µl 50 mM NaOH at 95 °C for 20 minutes in a thermocycler (Bio-Rad), cooled to 4 °C and neutralised with 10 µl 1M Tris-HCl (pH 8) and vortexed to dissociate tissue. Debris was pelleted by centrifugation and 2 µl of supernatant was used in a 20 µl PCR reaction to amplify the desired region containing the mutation (10 µl Qiagen Fast Cycling Mastermix, 203745; 1 µl 10µM forward and reverse primer (listed in Table 2.2); 7 µl DNase/RNase free H2O). PCR products were analysed using agarose gel electrophoresis in a 2\% agarose (Fisher Scientific; BP160-100) gel containing SafeView nucleic acid stain (NBS Biologicals; NBS-SV1). Frameshift mutations of il1b\textsuperscript{sh446} fish could be visualised by a band shift. csf1ra\textsuperscript{j4e1/j4e1} were genotyped by visualisation of the pigment defect reported by (Parichy \textit{et al.}, 2000).

2.2.2 Larval zebrafish

Genotyping was performed as described by (Wilkinson \textit{et al.}, 2013). 3 dpf larvae were anaesthetised and tail biopsies were taken distal to the pigment gap and circulation using a microscalpel. Larvae were transferred to individual wells of a 24-well tissue culture plate containing Danieus media and forceps were used to transfer the tissue biopsy to 15 µl 50 mM NaOH and genotyping was performed as described in section 2.2.1.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Oligonucleotide sequence of forward (F) and reverse (R) primers (5’-3’)</th>
<th>Annealing temperature (°C)</th>
<th>PCR product</th>
</tr>
</thead>
<tbody>
<tr>
<td>il1b</td>
<td>F: TGGAGATGTGGACTTCGCAG</td>
<td>57 °C</td>
<td>285 bp/241 bp</td>
</tr>
<tr>
<td></td>
<td>R: AGCTTCTGGTTCATGCAG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2. Oligonucleotide sequences of genotyping primers.
2.3 **Immunostaining, tissue clearing, wholemount and live imaging**

All buffers and solutions are listed in Table 2.3.

2.3.1 **Conventional whole mount imaging**

Tissues were dissected into ice-cold phosphate-buffered saline (ThermoFisher; 18912014; PBS) supplemented with 10 U/ml heparin (Alfa Aesar; A16198) washed three times in PBS then fixed ice-cold Fixation Buffer at 4°C overnight (O/N), or at RT for 2 hours on a rotator. Tissues were washed with at least three changes of PBS and mounted in 1% low gelling temperature agarose in 35 mm glass bottom CELLview culture dishes (Greiner; 627861) for imaging. Imaging of endogenous fluorescence was performed using Leica TCS SP8 AOBS confocal laser scanning microscopes (Lenses used: 10x HC PL APO Dry; 20x HC PL APO CS2 Immersion; 10x HCX APO Water; 25x HC Fluotar).

2.3.2 **In vivo imaging blood circulation**

Protocol was adapted from (Richardson *et al.*, 2016). Anaesthetised zebrafish were positioned on a wetted sponge and the tail was immobilised in 1% low gelling temperature agarose. Within the heated (28 °C) microscope chamber, zebrafish were placed in a reservoir and intubated with flowing (3-4 ml/minute) aquarium water containing 0.016% MS-222. Images were acquired using a Leica SP8 Multiphoton system using a 25x/0.95 HC Fluotar water dipping lens and resonance scanning at 8000 Hz.

2.3.3 **Immunostaining wholemount tissues**

Tissues were prepared and fixed as described in section 2.3.1. Tissues were washed three times for > 30 minutes with 1 ml Ce3D Washing Buffer. Tissues were incubated at 37 °C for 8–24 hours or at RT for 48 hours on shaker in Ce3D Blocking Buffer to permeabilise and block tissues. Tissues were subsequently incubated with antibodies (listed in Table 2.4) diluted in 300 µl Ce3D Blocking Buffer for 3-4 days at 37 °C, 150–220 rpm. Tissues were washed with 1 ml Ce3D Washing Buffer at 37 °C for 8–14 h then O/N at RT. Tissues were incubated with secondary antibodies (listed in Table 2.4) and washed as performed for primary antibodies.
2.3.4 Wholemount EdU labelling

10 µl of 10 mM 5-ethynyl-2’-deoxyuridine (EdU) (ThermoFisher Scientific; C10340) was administered via intraperitoneal injection to anaesthetised zebrafish and animals were returned to normal housing conditions for the desired time frame over which proliferation/EdU incorporation was analysed. Tissue was then harvested and fixed as described in section 2.3.1. EdU incorporation was detected using a Click-it® EdU Alexa Fluor® Imaging Kit (ThermoFisher Scientific; C10340) following immunostaining and prior to tissue clearing. Whole hearts were stained as described in the manufacturer’s instructions for tissue sections, however, incubation with the Click-it reaction cocktail was extended to 2 hours on a rotator.

2.3.5 Ce3D tissue clearing

Ce3D tissue clearing was performed using the method established by (Li et al., 2019). Tissues were protected from light at all stages. Tissues were prepared and fixed (section 2.3.1), and immunostained prior to clearing (section 2.3.3), if appropriate. To clear tissues, tissue samples were dabbed onto tissue to eliminate washing buffer then incubated in Ce3D clearing solution on a rotator at RT until clear (~1-2 days). An imaging chamber was formed by cutting 2 mm² apertures into a 1 mm deep silicone rubber sheet (ebay; 330788411590); cleared tissue was positioned within the apertures in Ce3D clearing solution and sandwiched between two glass coverslips (FisherScientific; 12363128) adhered with vacuum grease.

2.3.6 CUBIC tissue clearing

CUBIC tissue clearing was performed as previously described by (Susaki et al., 2015). Tissue was fixed and washed as described in section 2.3.1. Sample was immersed in 1:2 water diluted Reagent-1 (60 rpm) at 37 °C for 3-6 hours. Solution was replaced with 100% Reagent-1 and incubated at 37 °C (60 rpm), replacing solution every 2 days until clear. Tissue was washed in three changes of PBS O/N (RT, 60 rpm). Tissue was degassed then immersed in 1:2 PBS-diluted Reagent-2 at 37°C until the tissue sunk, after approximately 24 hours (60 rpm), then the solution was replaced and further incubated for 24 hours. Tissue was mounted in 4% low gelling agarose in Reagent-2 and the refractive index was adjusted to ~1.5.
Buffer | Constituents | Storage
---|---|---
Fixation buffer | 1x PBS, 4% paraformaldehyde | 4 °C for < 4 weeks; -20 °C for < 1 year.
Ce3D Washing buffer | 1x PBS, 0.3% (vol/vol) Triton X-100 and 0.5% (vol/vol) 1-thioglycerol. | 4 °C for 1 week
Ce3D Blocking buffer | 1x PBS, 0.5% (vol/vol) Triton X-100, 1% (vol/vol) BSA, and 1% (vol/vol) normal goat serum. | Make fresh
Ce3D clearing solution | 22% (wt/vol) N-methylacetamide, 86% (wt/vol) Histodenz, 0.01% (vol/vol) Triton X-100, 0.5% 1-thioglycerol. | RT for < 4 weeks
Reagent-1 (CUBIC) | 25 % urea (SLS; U5378-100), 25 % Quadrol (SLS; 122262-1L), 15 % Triton-X-100 in dH2O | RT for < 4 weeks
Reagent-2 (CUBIC) | 25 % urea, 50 % sucrose, 10 % triethanolamine in dH2O | RT for < 4 weeks
Citrate buffer | 10 mM (2.1 g) citric acid, 0.5% Tween-20 (SLS) | RT for 3 months
IF Blocking Buffer | PBS, 10% normal goat serum | 4 °C for 1 week
Mowiol Mountant | 2.4 g Mowiol 4-88, 6 g glycerol, 6 mL H2O, 12 ml 0.2 M Tris-Cl (pH 8.5), 2.5% DABCO | -20 °C

Table 2.3. Buffer and reagent used for immunofluorescence and tissue optical clearing.

<table>
<thead>
<tr>
<th>Target</th>
<th>Manufacturer</th>
<th>Cat. No.</th>
<th>Host Species</th>
<th>Dilution (sections)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-plastin</td>
<td>Gifted by (Cvejic et al., 2008)</td>
<td>N/A</td>
<td>Chicken</td>
<td>1:200</td>
</tr>
<tr>
<td>Collagen I</td>
<td>Abcam</td>
<td>ab23730</td>
<td>Rabbit</td>
<td>1:500</td>
</tr>
<tr>
<td>Tropomyosin, alpha-1 chain (CH1)</td>
<td>Developmental Studies Hybridoma Bank at the University of Iowa</td>
<td>-</td>
<td>Mouse</td>
<td>1:100</td>
</tr>
<tr>
<td>Phospho-Histone H3 (Ser10)</td>
<td>Cell Signaling Technology</td>
<td>3377</td>
<td>Rabbit</td>
<td>1:300</td>
</tr>
<tr>
<td>GFP</td>
<td>Abcam</td>
<td>ab13970</td>
<td>Chicken</td>
<td>1:100</td>
</tr>
<tr>
<td>mCherry</td>
<td>Invitrogen</td>
<td>M11217</td>
<td>Rat</td>
<td>1:100</td>
</tr>
<tr>
<td>Anti-rabbit AF568</td>
<td>Invitrogen</td>
<td>A10042</td>
<td>Donkey</td>
<td>1:500</td>
</tr>
<tr>
<td>Anti-mouse AF488</td>
<td>Invitrogen</td>
<td>A-11001</td>
<td>Goat</td>
<td>1:500</td>
</tr>
<tr>
<td>Anti-rat AF555</td>
<td>Invitrogen</td>
<td>A-21434</td>
<td>Goat</td>
<td>1:500</td>
</tr>
<tr>
<td>Anti-chicken AF488</td>
<td>Abcam</td>
<td>ab150169</td>
<td>Goat</td>
<td>1:500</td>
</tr>
<tr>
<td>Anti-chicken AF647</td>
<td>Invitrogen</td>
<td>A21449</td>
<td>Goat</td>
<td>1:500</td>
</tr>
</tbody>
</table>

Table 2.4. List of antibodies used for immunofluorescence staining.
2.4 *Ex vivo* culture and imaging

All buffers and media were filter sterilised with a 0.22 µm filter and pre-warmed to 28 °C prior to use. Dissecting instruments and area were sterilised and wiped down with 70% ethanol. Zebrafish were culled and heart extracted (preserving the ventricle, bulbus arteriosus and atrium) and placed in a petri-dish containing *Ex vivo* Culture Media (detailed in Table 2.5). Within a laminar flow hood, hearts were transferred to a 1.5 ml Eppendorf tube using a P1000 with a large aperture tip and washed three times with PBS.

For imaging, hearts were transferred to molten low gelling temperature agarose (37 °C) and mounted in 35 mm glass bottom CELLview culture dishes and submerged in *Ex vivo* Culture Media. Hearts were imaged on a Leica SP8 AOBS confocal laser scanning microscope with a 20x HC PL APO CS2 immersion objective or an Olympus IXplore SpinSR system with a Yokogawa CSU-W1 SoRa spinning disk unit and 30x/1.05NA silicone immersion objective. Imaging was performed in an environmental chamber at 28°C without CO₂ enrichment. For culturing, hearts were cultured in 12-well plates on an orbital rotator (150 rpm) at 28 °C, 5% CO₂. Media was exchanged every other day.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco’s Modified Eagle’s Medium – High Glucose (Sigma; D5796-500ml)</td>
<td>-</td>
</tr>
<tr>
<td>Cytiva HyClone™ Fetal Bovine Serum (Fisher Scientific; 11591821)</td>
<td>10%</td>
</tr>
<tr>
<td>MEM Non-Essential Amino Acids Solution (ThermoFisher Scientific; 11140050)</td>
<td>1%</td>
</tr>
<tr>
<td>Penicillin-Streptomycin</td>
<td>100U/ml/100ug/ml</td>
</tr>
<tr>
<td>2-mercaptoethanol (Fisher; 31350010)</td>
<td>50uM</td>
</tr>
<tr>
<td>Normocin (InvivoGen; ant-nr-1)</td>
<td>100ug/ml</td>
</tr>
</tbody>
</table>

Table 2.5. Constituents of *ex vivo* culture media.
2.5 **Flow cytometry (FC)/Fluorescence Activated Cell Sorting (FACS)**

2.5.1 **Tissue preparation for flow cytometry/FACS analysis**

Details of buffers are listed in Table 2.6. Cells and buffers were kept on ice throughout the preparation unless otherwise stated. All centrifugation steps were performed at 300 x g, 10 minutes, 4 °C.

2.5.1.1 **Heart**

Following dissection, the heart was transferred to PBS. The ventricle was separated from the atrium, bulbus arteriosus and any other surrounding non-cardiac tissue, then gently torn open to wash out any remaining blood. Tissue was digested in 250 µl Digest Buffer for approximately 1 hour (32°C, 800 rpm), with regular pipetting to aid digestion. Once fully dissociated, the cell suspension was mixed with one volume of Stopping Buffer. Cells were pelleted, washed once in 500 µl Suspension Buffer, filtered through a 40 µm Falcon® cell strainer (Fisher Scientific; 22363547) and resuspended in Suspension Buffer for analysis.

2.5.1.2 **Fin**

The caudal fin was resected, cut into small pieces to aid digestion and washed in PBS. The cell suspension was then prepared as previously described for the ventricle.

2.5.1.3 **Blood**

A ~4 mm incision was made through the skin and pericardial sack and the heart was quickly removed using forceps by pulling at the bulbus arteriosus. 3-10 µl blood was immediately collected from the chest cavity using a heparin-coated P20 pipette tip, mixed with 10 µl 500 U/ml Heparin (Alfa Aesar; A16198) in PBS then further mixed with Isolation Buffer and placed on ice for less than 20 minutes to avoid coagulation. Cells were pelleted, resuspended in 750 µl ACK Lysing Buffer (SLS; LZ10-548E) and incubated for 10 minutes at RT with gentle agitation to lyse red blood cells (RBCs). Lysis was terminated by addition of 750 µl Isolation Buffer. Cells were washed once and resuspended in Isolation Buffer for analysis.

2.5.1.4 **Spleen**

Spleens were dissected into Isolation Buffer, then mechanically dissociated by gently passing the tissue through a 40 µm cell strainer using a 1 ml syringe insert. RBCs were lysed and the cell suspension was prepared as previously described for blood.
<table>
<thead>
<tr>
<th>Buffer</th>
<th>Constituents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digest Buffer</td>
<td>1 X PBS, 5 mg/ml Collagenase II (Worthington Biochemical Corp; LS004176)</td>
</tr>
<tr>
<td>Stopping buffer</td>
<td>1 X PBS, 10% FBS (Fisher Scientific; 11591821)</td>
</tr>
<tr>
<td>Suspension Buffer</td>
<td>PBS, 2% FBS</td>
</tr>
<tr>
<td>Isolation Buffer</td>
<td>1 X PBS, 5% FBS</td>
</tr>
</tbody>
</table>

Table 2.6. Flow cytometry and FACS buffers.

2.5.2 Fluorescence associated cells sorting (FACS) and Flow Cytometry

All cells were kept on ice until analysis. Immediately prior to sorting, DRAQ7™ (Abcam; ab109202) was added to the cell suspension to stain dead cells. All flow cytometry analysis and FACS was carried out at 4°C using a BD Influx Fluorescence Associated Cell Sorter or a BD FACS ARIA™ II SORP Flow Cytometer Cell Sorter. For sorting and analysis, events were gated to select single, live cells (Figure 2.1). Cells were sorted into a 1.5 ml Eppendorf containing an appropriate buffer for downstream use. FCS files generated from sorts were analysed using FlowJo_v10.6.2 software.

![Figure 2.1. Gating strategy used to isolate, live single cells.](image-url)
2.6 **Cytology and Histology**

Details of stains and buffers are listed in Table 7.1, Appendix 7.1.

### 2.6.1 Cytology

FACS sorted cells were resuspended in 200 µl PBS and loaded into Shandon™ EZ Single Cytofunnels (Thermo Scientific; 11972345) and spun (1000 rpm, 5 min) onto coated Shandon Cytoslides (Thermo Scientific; 12026689) using a Cytospin Cytocentrifuge (Thermo Scientific). Slides were dried and fixed in 100% methanol for 5 minutes, stained in May-Grünwald’s stain for 5 minutes, then transferred to Giemsa stain for 10 minutes. Slides were rinsed in 3.3 mM Sorenson buffer (pH 6.8) for 3 minutes to differentiate then left to dry. Cytospins were imaged on an Olympus BX53 Upright Microscope with a 40X dry objective. Cell measurements were performed manually on Fiji by measuring the longest diameter of the cell body, excluding protrusions.

### 2.6.2 Paraffin embedding and sectioning

For wax sections, hearts were dissected with the bulbus arteriosus and atrium intact, fixed and washed as described in section 2.3.1. Hearts were then embedded in blocks of 1.5 % low gelling temperature agarose then passed through a series of 25%, 50%, 75% EtOH/PBS washes (50 rpm, RT, >1 hour per wash). Tissues were embedded in paraffin wax blocks by the Histology Services Unit (University of Bristol). Hearts were orientated to position the bulbous arteriosus above and atrium to the right of the ventricle and sectioned using Leica RM2125 microtome at a thickness of 7 µm/section. Sections were floated in a waterbath (50°C) and collected and dried on to Superfrost PLUS glass slides.

### 2.6.3 Acid Fuschin Orange G (AFOG) staining and image analysis

AFOG staining was performed on wax sections using the steps outlined in table 7.2. A sample of sections were stained for each heart to locate the injury, then additional sections within the injury area were then stained. Images were acquired on an Olympus BX53 Upright Microscope. 3 sections (one from each adjacent slide) were quantified and an average was calculated. Image analysis was performed using Fiji. All measurements were performed only on the ventricle. Injury size was calculated by drawing around the injury and measuring the
area. Collagen area was quantified by thresholding on the purple colour hues using the Fiji macro detailed in Appendix 7.5 and attached in Supplementary Files.

2.6.4 Immunofluorescence labelling tissue sections

Buffers listed in Table 2.3. Wax sections were dewaxed and rehydrated as outlined in steps 1-5 in table 7.2. For antigen retrieval, sections were incubated in 10 mM citrate buffer (Table 2.3) at 70 °C for one hour, then at RT for the following hour. Excess liquid was removed and sections were drawn around with hydrophobic pen. Sections were permeabilised with three washes in 0.5% Triton in PBS (PBST; RT, 5 minutes), and blocked with 10% goat serum in 0.5% PBST (RT, 1 hour). Sections were incubated with 15 µl primary antibody overnight in a humidified chamber (4 °C), washed 3 times in PBST, and incubated in the dark with secondary antibody (1 hour, RT). Sections were washed twice in PBST, stained with 15 µl 1/2000 DAPI in PBS (RT), washed twice in PBS.

2.6.5 EdU labelling tissue sections

Tissue sections were dewaxed and rehydrated as outlined in steps 1-5 in table 7.2. Following immunostaining, EdU incorporation was detected using a Click-it® EdU Alexa Fluor® Imaging Kit (ThermoFisher Scientific; C10340) following manufacturer’s instructions.

2.6.6 Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) labelling tissue sections

Tissue sections were dewaxed and rehydrated as outlined in steps 1-5 in table 7.2. Following immunostaining, TUNEL staining was performed using a ApopTag In Situ Apoptosis Detection Kit (EMD Millipore) following manufacturer’s instructions.

2.6.7 Mounting and imaging tissue sections

Following immunofluorescence staining, tissue sections were mounted with 60 µl Mowiol Mountant (Table 2.3). Slides were dried overnight at RT then stored at 4 °C. Slides were imaged on an Leica DMI6000 inverted epifluorescence microscope with a 10X HC PL Fluotar lens.
2.7 **Fluorescent image analysis**

2.7.1 **Wholemount imaging**

Images and videos were processed using Fiji (Schindelin *et al*., 2012). For manual cell counting, images were blinded and counted using the Cell Counter plugin on Fiji ([https://imagej.nih.gov/ij/plugins/cell-counter.html](https://imagej.nih.gov/ij/plugins/cell-counter.html)). For automated fluorescent cell analysis using macros, maximum intensity projections were made and cropped to the ventricle area. Analysis was then performed using the macros detailed in Appendix 7.5 and attached in the Supplementary Files. For the automated cell shape analysis, ‘Cell Shape Analysis’ plugin for the Modular Image Analysis (MIA) platform in Fiji was designed by Dr Stephen Cross (Wolfson Bioimaging Facility, University of Bristol). Analysis parameters and methodology is listed in Appendix 7.4.1.

2.7.2 **IMARIS**

3D projections were generated on IMARIS software using maximum intensity projections.

2.7.3 **Immunofluorescence on tissue sections**

Image analysis was performed using Fiji (Schindelin *et al*., 2012). Images were cropped to the ventricle area. For EdU, L-plastin, TUNEL and Collagen I analysis, one section within the injury area was analysed using the macros listed in Appendix 7.5 and attached in Supplementary Files.
2.8 RNA extraction and RT-qPCR

2.8.1 RNA extraction and cDNA synthesis

All steps were performed under RNA clean conditions.

2.8.1.1 Whole tissues

Tissues were dissected into RNase free PBS. For hearts, non-ventricular tissue was removed using forceps and by gently rolling the ventricle on tissue, then gently torn open and washed in PBS to remove remaining blood. Tissues were homogenised using a 22 gauge needle and syringe (720-2532, VWR) in 500-1000 µl TRI Reagent® (Sigma; T9424). A 1:5 volume of Chloroform (Sigma) was added and samples were shaken vigorously for 15 seconds then incubated at RT for 2 minutes. Samples were centrifuged (12,000 x g, 15 minutes, 4°C) to induce phase formation and the RNA-containing upper aqueous phase was removed and placed in a clean Eppendorf by careful pipetting. 10 µg Glycogen (Thermo Fisher Scientific) was added to act as a carrier and RNA was precipitated for 10 minutes at RT by addition of 1:2 volumes of Isopropanol (Sigma). RNA was pelleted (12,000 x g, 10 minutes, 4°C), washed in 1 volume of 75% Ethanol (7500 x g, 5 minutes, 4°C), the supernatant was removed and the RNA pellet allowed to dry at RT. Dried pellets were resuspended in RNase-free H2O (Qiagen) and the concentration determined by 260/280 absorption ratios using a NanoDrop™ 2000 Spectrophotometer (ThermoFisher Scientific). RNA was stored at -80°C or immediately DNase treated and reverse transcribed using a Maxima First Strand cDNA Synthesis Kit for RT-PCR with DNase (ThermoFisher Scientific) according to the manufacturer’s instructions.

2.8.1.2 FAC-sorted cells

Cells were resuspended in 50 µl PBS and lysed in 500 µl Trizol by vortexing for 1 minute, then RNA extraction was performed as above.

2.8.2 RT-PCR/qPCR

2.8.2.1 Primer design and validation

Primer sequences used for RT-PCR and qPCR (listed in Table 7.3) were obtained from published sources or designed from NCBI sequences in NCBI Primer-BLAST. Primers were designed to amplify a 70-300 bp region of cDNA, spanning exon-exon junctions where possible to avoid amplification of contaminating genomic DNA. Amplification efficiency of
qPCR primers was confirmed from the generation of a standard curve using a five-point serial dilution of cDNA. Primers were considered suitable for quantitative gene expression analysis if: i) the gradient of the standard curve ($r^2$) was within 3.1-3.4, indicating an amplification efficiency of 90-110%; ii) produced a single amplification product and did not produce primer dimers, determined by a single peak on a melting curve; iii) did not exhibit amplification in no template control (NTC) samples; iv) the PCR product was of the correct size, determined by gel agarose electrophoresis.

### 2.8.2.2 Quantitative gene expression analysis

For each 10 µl qPCR reaction, 10 ng, unless otherwise stated, of cDNA was mixed with 300-500 nM forward and reverse primer and 1X PowerUp SYBR™ Green Master Mix (ThermoFisher). Analysis was performed using a QuantStudio 3 Real-Time PCR System (ThermoFisher) using the fast-cycling mode (detailed in Table 2.7). Each reaction was performed in triplicate and the Ct value was averaged, following removal of outliers (Ct SD < 0.3). Expression of target sequences was calculated relative to the reference gene efla. To calculate relative fold-change, the Comparative Ct/$\Delta\Delta$Ct method was used. $\Delta\Delta$Ct was calculated by subtracting the mean $\Delta$Ct of the control condition (typically uninjured) from the mean $\Delta$Ct of the analysed timepoint. Fold change was then calculated relative to the control condition by $2^{-\Delta\Delta\text{Ct}}$. For a full list of primers used for PCR analyses, see Table 7.3, Appendix 7.3.

<table>
<thead>
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<tr>
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</tr>
</tbody>
</table>

Table 2.7. qPCR amplification and melt curve conditions.
2.9 **Statistics and power calculations**

In all cases n numbers refer to biological replicates; where pools of ventricles were used this is stated in the figure or figure legend. GraphPad Prism 8.2.1 was used to graph all data and perform statistical analysis. To determine statistical significance and reject the null hypothesis, an alpha value of 0.05 was used. In all cases, the null hypothesis indicates that there is no difference between conditions. Power calculations were performed using pilot data with GPower 3.1 software to determine the sample sizes required to achieve a statistical power of 80% (beta = 0.2).
3 Results I: Characterising mononuclear phagocyte populations in the adult zebrafish heart

Opening statement to Results Chapter I:

Some of the work included in this chapter has been published in the following article: Moyse, B. R. & Richardson, R. J. 2020. A Population of Injury-Responsive Lymphoid Cells Expresses mpeg1.1 in the Adult Zebrafish Heart. ImmunoHorizons, 4, 464. All data presented within the transcript was collected and analysed by myself and both myself and Rebecca Richardson contributed to the writing of the text.
3.1 **Chapter introduction**

3.1.1 **The Mononuclear Phagocyte System**

Cells of the mononuclear phagocyte system, namely monocytes, macrophage and DCs, are largely defined by their tissue distribution, function and morphology (Figure 3.1). Monocytes are defined by their localisation to the circulation and, in mammals, two main sub-populations of monocytes have been identified. Classical monocytes are short-lived cells within the circulation, and are recruited to sites of inflammation where they differentiate into MDMs or monocyte-derived DCs (Ginhoux and Jung, 2014). A second monocyte population are thought to be exclusively found within the vasculature and have macrophage-like characteristics, serving to patrol the endothelium and exhibit an anti-inflammatory profile (Ginhoux and Jung, 2014, Auffray et al., 2007).

Unlike monocytes, macrophages are found exclusively within tissues. Due to convergent morphology, functions and basal gene expression, the term ‘macrophage’ encompasses cells derived from both recruited monocyte populations and tissue resident populations. As previously described, these cells have an abundance of effector functions, having roles in phagocytosis, antigen presentation and the secretion of inflammatory and trophic factors (Reynolds and Haniffa, 2015). Despite the similarities possessed by these cells, macrophages describe a highly heterogeneous grouping of cells which can harness a diverse and plastic transcriptome, influenced by their environment, activation signals and ontogeny (Reynolds and Haniffa, 2015, Das et al., 2015).

DCs are the third and final population of MNPs and have unique and primary functions in immunomodulation. These highly phagocytic cells collect antigens from tissues and the lymphatic system and present them to adaptive immune cells within lymphoid tissues, serving to coordinate the innate and adaptive arms of the immune system (Reynolds and Haniffa, 2015, Hume, 2006). DCs also categorise cells with similar immunomodulatory functions and morphology, rather than origin, as both myeloid (monocyte-derived) and lymphoid precursors can give rise to DCs (MacDonald et al., 2005). However, as DCs share many functional, morphological and transcriptional characteristics to macrophages, separation of these cells can be complicated (Percin et al., 2018). Therefore, these cells have not been extensively studied and the contribution of these cells to tissue homeostasis and repair
remains somewhat elusive, although they have been assigned roles in modulating the inflammatory response (Reynolds and Haniffa, 2015, Das et al., 2015, Anzai et al., 2012).

Figure 3.1. The mononuclear phagocyte system and commonly used markers used to delineate populations in mice. Mammals possess two main types of monocytes within the blood which are derived from hematopoietic precursors (green). Classical monocytes (red) harness a predominantly pro-inflammatory profile and infiltrate into tissues upon infection and injury to produce MDMs (light blue) and monocyte-derived DCs (orange). Non-classical monocytes (yellow) have patrolling functions within the circulation. Homeostatic tissue resident macrophage populations (dark blue) are not usually derived from monocytes and are maintained by self-renewal. However, monocytes have been shown to contribute to these populations upon their depletion, and non-classical monocytes have been shown to give rise to a population of pro-reparative tissue macrophages (Nahrendorf et al., 2007, Ginhoux and Jung, 2014, Reynolds and Haniffa, 2015).
3.1.2 Studying the MNP system

The extensive and historical use of murine and human samples in immunological studies has established a wealth of species-specific cell surface and transcriptomic markers that can be used to stratify monocytes, macrophages, DCs, and sub-populations within these groupings (Figure 3.1; explored further in section 3.6) (Reynolds and Haniffa, 2015). This has enabled the identification of specific cell populations that have discrete functions and may facilitate the therapeutic targeting of such populations in various research fields, including tissue repair. On the contrary, although analogous populations of monocytes, macrophages and DCs have been defined in the zebrafish (Wittamer et al., 2011, Lugo-Villarino et al., 2010), due to its relative novelty as a model system for immune cell studies, there are currently limited tools available to delineate MNP populations (Rosowski, 2020). Despite considerable gene homology and conservation of the MNP system between mammals and zebrafish, identification of orthologous MNP markers is complex, largely due to evolutionary divergence and genome duplication (Howe et al., 2013, Sommer et al., 2020).

Nevertheless, some conservation has allowed for the transition of certain MNP markers to the zebrafish. Csf1r genes are highly conserved amongst vertebrates and due to the well-established roles of this gene in mammalian MNP homeostasis (Hume, 2006, Stanley and Chitu, 2014), csf1ra was one of the first genes to be characterised in zebrafish macrophages (Parichy et al., 2000). Yet, due to additional teleost-specific expression in pigment cells, this has compromised the use of csf1ra as an MNP-specific marker in zebrafish (Parichy et al., 2000). Alternative zebrafish MNP markers were identified by the knockdown of pu.1, which is a well-conserved transcription factor involved in myeloid and lymphoid cell differentiation (Zakrzewska et al., 2010). This led to the identification of high and specific expression of cxcr3.2, mfp4, mpeg1.1 (formerly known as mpeg1), marco and ptpn6 in macrophages of the larval zebrafish, thereby expanding the pool of markers that could be utilised (Zakrzewska et al., 2010).

Mpeg1 genes encode the highly conserved pore-forming protein, perforin-2, which has roles in the destruction of pathogens, and had been previously characterised to have macrophage-restricted expression in mice and humans (Spilsbury et al., 1995, Merselis et al., 2021). Characterisation by Ellett et al (2011) further confirmed specificity of mpeg1.1 to macrophage-
like cells in larval zebrafish and generated the first transgenic line to specifically label macrophages in zebrafish. *mpeg1.1* has since been the primary promoter to drive macrophage specific expression in transgenic lines, although more recent characterisation of *mfap4* has generated another armoury of transgenic lines to interrogate macrophage function in the zebrafish (Walton *et al.*, 2015).

By exploiting the genetic tractability and imaging capabilities of the transparent larval zebrafish, transgenic lines generated from these selected markers has greatly facilitated our understanding of the *in vivo* dynamics of macrophages and allowed isolation of these cells in adult studies. However, much of the initial characterisation of these markers was performed in larval zebrafish and although expression in tissue macrophages was confirmed, whether expression extends to monocytes and DCs has not been well defined.

Recent technological advancements and accessibility to RNA sequencing technology has facilitated the molecular characterisation of MNPs (Rougeot *et al.*, 2019, Tang *et al.*, 2017). However, this analysis is in its infancy, and few of the uncovered markers have been translated into tools which can be utilised to perform functional analysis of cells *in vivo*. Therefore, given the vast heterogeneity of MNPs, there is still much to be learnt about how to segregate such populations in zebrafish, which will undoubtedly help to reveal the mechanisms by which MNPs facilitate their regenerative capacity.

### 3.1.3 Chapter Aims

The aim of this chapter was therefore to elucidate potential methods which could stratify populations of macrophages and monocytes in the adult zebrafish. I then aimed to utilise these markers/methods to establish the dynamics of these populations following cardiac cryoinjury, which has not been previously dissected. My approach was to use cytological, imaging and gene expression analysis of existing transgenic lines, Tg(*mpeg1.1*:mCherry) and TgBAC(*csf1ra*:GFP) to establish whether these markers could stratify populations in both the steady state and following cardiac cryoinjury.
Part I: Characterising \textit{mpeg1.1} and \textit{csf1ra} expression in the adult zebrafish heart

3.2 \textit{mpeg1.1} and \textit{csf1ra} are differentially expressed by morphologically distinct cell populations in the uninjured adult zebrafish heart

\textit{mpeg1.1} and \textit{csf1ra} are commonly used as pan-macrophage markers in the larval and adult zebrafish (Ellett \textit{et al.}, 2011, Dee \textit{et al.}, 2016). However, the co-expression of these markers in adult tissues has not been fully interrogated. To characterise the distribution of MNPs expressing these macrophage markers in the unwounded zebrafish heart, confocal imaging of hearts from Tg(\textit{mpeg1.1}:mCherry); TgBAC(\textit{csf1ra}:GFP) adult zebrafish hearts was performed (Figure 3.2).

The majority of labelled cells expressed both transgenes (\textit{mpeg1.1+csf1ra+} cells), were evenly distributed on the surface of the heart and possessed a stellate morphology, typical of cardiac tissue macrophages (Figure 3.2AB) (Pinto \textit{et al.}, 2012, Bevan \textit{et al.}, 2020). However, cells expressing only \textit{csf1ra:GFP} with a similar stellate morphology (\textit{csf1ra+} cells) and a distinct population of smaller, rounded cells expressing only \textit{mpeg1.1:mCherry} (\textit{mpeg1.1+} cells) were also present. Analysis of fluorescence and cell shape within the ventricle showed that 49.5% of labelled cells were \textit{mpeg1.1+csf1ra+}, 18.2% \textit{csf1ra+} and 32.2% \textit{mpeg1.1+}, and indeed showed that \textit{mpeg1.1+} cells had a smaller diameter and volume, and increased circularity compared to \textit{csf1ra}-expressing cells (average sizes detailed in Figure 3.2C-E). Similar results were shown for cells within the atrium (34.4% of labelled cells were \textit{mpeg1.1+csf1ra+}, 11.0% \textit{csf1ra+} and 54.5% \textit{mpeg1.1+}), although all populations were slightly smaller and had an increased circularity (Figure 3.2F-H). This is possibly due to the inclusion of residual blood cells found within the thin structure of the atrium, which would not be included when imaging the surface of the ventricle.

This indicated that \textit{mpeg1.1} and \textit{csf1ra} were differentially expressed by MNPs within the heart and combined with their differing morphologies, may label subpopulations of MNPs. For example, the rounded morphology of \textit{mpeg1.1} cells could also be suggestive of monocytic origin, as these cells appear more rounded compared to their macrophage counterparts (Wittamer \textit{et al.}, 2011, Lugo-Villarino \textit{et al.}, 2010).
Figure 3.2. Mononuclear phagocyte populations in the adult Tg(mpeg1.1:mCherry); TgBAC(csf1ra:GFP) zebrafish heart. A-B) Wholemount imaging of mpeg1.1:mCherry csf1ra:GFP fluorescence. A) Image of the whole zebrafish heart. Dotted line demarcates the ventricle (V), atrium (A) and bulbus arteriosus (BA). Boxes (i, ii) outline enlarged regions shown in B. Scale bar = 200 µm. B) Zoomed regions within the ventricle (i) and atrium (ii), as identified in A, showing the morphology of mpeg1.1± csf1ra± cells. Arrows identify mpeg1.1+ cells; white arrowheads identify mpeg1.1+csf1ra+ cells; black arrowheads identify csf1ra+ cells. Scale bar = 20 µm. C-H) Automated cell shape analysis of mpeg1.1± csf1ra± cells from a 388 µm² Z-stack imaged within the ventricle (C-E) and atrium (F-H). Cells were classified by their expression of mpeg1.1:mCherry and/or csf1ra:GFP and individual cell volume (CF), circularity (DG), and diameter (EH) was measured. Statistical analysis was performed by Brown-Forsythe and Welch’s ANOVA test following removal of outliers by the ROUT outlier test (Q = 1%). One experimental replicate of one fish.
Similarly, flow cytometry indicated that 71.0% ± 6.3% SD of labelled cells from unwounded ventricles were mpeg1.1+csf1ra+ but 18.0% ± 6.7% SD were mpeg1.1+, and a minority of cells were csf1ra+ cells (1.2% ± 0.8% SD) (Figure 3.3A-C). The discrepancy in proportions between flow cytometry and imaging data is likely due to differences in fluorescence thresholds used to separate these populations, but demonstrates that labelled cells differentially express csf1ra and mpeg1.1. The forward (FSC) and side scatter (SSC) profile of these cells which respectively give an indication of cell size and granularity, also supported the differing morphologies of these cell populations observed by imaging. Intriguingly, although csf1ra+ and mpeg1.1+csf1ra+ cells had a relatively large FSC and SSC as expected for myeloid cells, mpeg1.1+ cells had a very low FSC and SSC profile, more typical of lymphoid cells (Figure 3.3A).

To further investigate the morphology of these three cellular sub-types, the cell populations were FAC-sorted and cytological analysis was performed (Figure 3.3D-E). Indeed, csf1ra-expressing cells had characteristics of MNPs, with many cells having a kidney bean shaped nucleus, typical of monocytes, and many dendrites and granules typical of macrophages and DCs (Lugo-Villarino et al., 2010, Wittamer et al., 2011, Traver et al., 2003). Interestingly, a large proportion of csf1ra+ cells had a monocyte-like appearance, whereas many mpeg1.1+csf1ra+ cells had protrusions. Nevertheless, consistent with their scatter profile, mpeg1.1+ cells appeared lymphocytic, with a large, rounded nucleus and little cytoplasm. Cell populations also differed by cell diameter, with mpeg1.1+ cells having an average cell body diameter of 8.1 µm ± 2.4 SD compared to 18.0 µm ± 4.0 SD for mpeg1.1+csf1ra+ cells (Figure 3.3E).
Figure 3.3. Flow cytometry and cytology of cardiac csf1ra± and mpeg1.1± populations. A) Flow cytometry plot of csf1ra:GFP and mpeg1.1:mCherry expression in the ventricle of unwounded hearts (left plot) and corresponding FSC and SSC of these populations (right plot). B-C) Graphical representation of the relative proportions of labelled cells (C) and average number of MNPs isolated from a single ventricle (D) expressing mpeg1.1- and csf1ra- transgenes. Graphs shows mean ± SD from two independent experiments. N = 15. D) May-Grünwald Giemsa staining of csf1ra± and mpeg1.1± sub-populations FAC-sorted from the uninjured ventricle. Cells were isolated from 6 pooled ventricles, one experimental replicate. Scale bar = 20 µm. E) Quantification of cell diameter from cytology images. Statistical analysis performed using one-way ANOVA with Tukeys’s multiple comparisons tests.
3.3 **Distinct mpeg1.1±csf1ra± populations are also observed in hematopoietic tissues and the skin**

To identify whether the distinct csf1ra± and mpeg1.1± populations were unique to cardiac tissue, imaging and flow cytometry of the caudal fin, blood and spleen of adult Tg(mpeg1.1:mCherry); TgBAC(csf1ra:GFP) zebrafish was performed. By imaging, mpeg1.1+, mpeg1.1+csf1ra+, and csf1ra+ cells were identified in all tissues, with mpeg1.1+ cells having a smaller and more rounded morphology compared to csf1ra+ cells in all tissues (Figure 3.4A; Video 1, Appendix 7.6). The one exception to this was within the fin, where some mpeg1.1+ cells appeared large and stellate. This was supported by flow cytometry of the fin, where mpeg1.1+ cells had a higher forward and side scatter profile (Figure 3.4C). Cytological analysis of cells isolated from the fin (Figure 3.4D), also showed that although the majority of cells had a size and lymphoid appearance similar to ventricular mpeg1.1+ cells, some cells had a unique large, rounded morphology (Figure 3.4E). Due to similarities in morphology, these cells are likely to be the recently described non-myeloid cell type, metaphocytes, which perform antigen processing functions in the skin of adult zebrafish (Lin et al., 2019b, Kuil et al., 2020), suggesting that two populations of mpeg1.1+ cells that do not express csf1ra exist in adult skin.

Interestingly, the fluorescence distribution of csf1ra:GFP and mpeg1.1:mCherry determined by flow cytometry was very distinct in the different tissues (Figure 3.4B). In the spleen and blood, mpeg1.1+ cells comprised the largest proportion of labelled cells (76%, single experiment of pool of 5 samples; 85.7% ±5.2% SD, respectively) and there were relatively more csf1ra+ cells compared to mpeg1.1+csf1ra+ cells than within the heart and fin (Figure 3.4B). Furthermore, although the FSC/SSC profile of csf1ra+ cells in the blood and spleen was characteristic of myeloid cells, csf1ra+ cells isolated from the fin possessed a very high side scatter (Figure 3.4C). This is likely because of the inclusion of xanthophores, pigment producing cells of the skin, which are also known to express csf1ra (Parichy et al., 2000, Parichy and Turner, 2003).

This analysis demonstrates that in addition to the heart, mpeg1.1+ lymphoid-like cells are found in the fin and are particularly abundant in the spleen and blood. It also demonstrates that the skin likely possesses additional populations of mpeg1.1+csf1ra- metaphocytes and mpeg1.1-csf1ra+ xanthophores, highlighting the disadvantage of utilising these transgenic lines to study macrophages in the skin.
A Caudal fin (i) Blood (ii) Spleen (iii)

Tg(mpeg1.1:mCherry)

TgBA/Csf1r(mGFP)

Merge

B

C

D mpeg1.1+ csf1ra+ mpeg1.1+ csf1ra+

E

Cell body diameter (um)
Figure 3.4. Characterisation of csf1ra± and mpeg1.1± populations in the fin, blood and spleen. A) Confocal imaging of the caudal fin (column i), circulation/blood (ii) and spleen (iii) in Tg(mpeg1.1:mCherry); TgBAC(csf1ra:GFP) fish. Arrowheads identify mpeg1.1+ cells. Imaging in the caudal fin and spleen are on fixed tissues. Imaging of the circulation is from a single frame from live imaging (Video 1, Appendix 7.6); dotted line outlines the vessel. Scale bar = 20 µm. B-C) Representative flow cytometry plots of labelled cells within each tissue showing mpeg1.1 and csf1ra reporter intensity (B) and the corresponding FSC and SSC of cell populations (C). D-E) Cytospin analysis of csf1ra± and mpeg1.1± populations isolated from the caudal fin. D) Representative cells from each population. Arrowheads identify larger cells that were not observed in cytopspins of the ventricle. Scale bar = 20 µm. E) Quantification of cell body diameter. Average cell body diameter: mpeg1.1+ = 11.1 µm ± 3.5 µm SD, csf1ra+ 18.1 µm ± 4.5 µm SD, mpeg1.1+csf1ra+ = 18.0µm ± 3.3 µm SD. One experimental replicate of 6 pooled fins; n indicates the number of cells analysed. Statistical analysis performed using one-way ANOVA with Tukey’s multiple comparisons tests.
3.4 *csf1ra*-expressing cells show a transcription profile typical of MNPs, yet *mpeg1.1*+ cells express B cell and natural killer-like (NKL) cell markers

The analysis performed thus far indicated that *csf1ra*-expressing cells were indeed MNPs whereas *mpeg1.1*+ cells appeared to be lymphoid-like, with the exception of the skin. To further elucidate the identity of these populations, cells were FAC-sorted and probed for the expression of monocyte/macrophage, NK/NKL cell and B cell markers by RT-PCR. These markers were chosen as *mpeg1.1* expression was recently detected in B cells and NKL cells in a single cell transcriptomics study (Tang et al., 2017). In order to gain a large number of cells for analysis, which was not possible from hearts, and to avoid collection of the proposed labelled non-lymphoid cells in the fin, cells were sorted from blood isolated from fish at 3 dpi.

Due to highly variable cell counts and relatively low RNA yields obtained from cell sorts, RNA quantification could not be performed to normalise the cDNA inputted into the RT-PCR reaction, using the tools available. cDNA was therefore produced from the total cells isolated for each population (indicated in the legend of Figure 3.5) and diluted by the same amount for each reaction. Resultantly, variations in expression levels are difficult to determine from RT-PCR gels yet indicate whether expression is present or absent. Despite this, variations in the reference gene (*ef1a*) give an indication of relative inputted RNA and band intensity does somewhat correlate with cell number.

Analogous with their MNP-like identity, *mpeg1.1*+*csf1ra*+ and *csf1ra*+ cells showed clear expression of *csf1ra*, *mpeg1.1*, *mpeg1.2*, *mfap4* and *marco*, which have been defined to be expressed by monocytes and macrophages in zebrafish (Zakrzewska et al., 2010) (Figure 3.5). In contrast, *mpeg1.1*+ cells demonstrated low expression of MNP genes, yet showed distinct expression of B cell (*cd79a*, *ighm*) (Liu et al., 2017) and NKL cell (*nkl.3*, *nkl.4*) (Tang et al., 2017) markers, which were absent in both *csf1ra*-expressing populations. This analysis further confirmed that *mpeg1.1*+ cells were not MNPs and likely comprise a population of B cells and NKL cells, whilst simultaneously confirming the identity of *mpeg1.1*+*csf1ra*+ and *csf1ra*+ cells as MNPs.

Interestingly, endogenous expression of *mpeg1.1* was detected in *csf1ra*+ cells which were classified to be negative for *mpeg1.1*:mCherry reporter expression by FACS. However, taking their low frequency in tissues compared to *mpeg1.1*+*csf1ra*+ cells, but higher abundance in
monocyte-rich sites of the blood and spleen (Figure 3.4), in addition to their monocyte-like morphology, this could support a hypothesis that csf1ra+ (mpeg1.1- or mpeg1.1low) cells represent a population of monocytes transitioning into mature mpeg1.1+csf1ra+ MNPs. Their classification of mpeg1.1- by FACS gating may also be as a result of a delay in mCherry fluorophore synthesis.

Figure 3.5. Expression of MNP, B cell and NK/NKL cell markers in csf1ra± and mpeg1.1± blood cells. csf1ra± and mpeg1.1± populations were isolated from the whole blood of three fish at 3 dpi and probed for the expression of lineage markers by RT-PCR. Cell numbers: csf1ra+ = 11800, mpeg1.1+ = 2300, mpeg1.1+csf1ra+ = 4800.
3.5 \textit{csf1ra±} and \textit{mpeg1.1±} populations show different patterns of expansion following cardiac cryoinjury

The previous characterisation importantly demonstrated that \textit{mpeg1.1+csf1ra−} cells comprise a significant proportion of all \textit{mpeg1.1:mCherry} labelled cells, and should therefore be excluded from the analysis of MNPs when using \textit{mpeg1.1} reporters. Despite this, the variation in \textit{mpeg1.1} expression by \textit{csf1ra}-expressing cells and the differing morphologies of these populations suggested that \textit{mpeg1.1} may serve as a useful method to segregate monocytes and mature macrophages. Furthermore, as the \textit{mpeg1.1+} population can be neatly differentiated by the absence of \textit{csf1ra} expression and striking differences in morphology, the utilisation of Tg(\textit{mpeg1.1:mCherry}); TgBAC(\textit{csf1ra:GFP}) enables the study of this elusive population in parallel, without interfering with the analysis of \textit{csf1ra}-expressing MNPs.

Therefore, to dissect the responses of these newly identified \textit{mpeg1.1±csf1ra±} subpopulations in response to our cardiac cryoinjury model, flow cytometry of cells harvested from Tg(\textit{mpeg1.1:mCherry}); TgBAC(\textit{csf1ra:GFP}) pooled ventricles and whole blood at 1, 3, 7 and 14 days post cardiac cryoinjury (dpi), in addition to uninjured controls, was performed. Similar to previous observations (Bevan \textit{et al.}, 2020), an accumulation of \textit{mpeg1.1+csf1ra+} MNPs was seen within the ventricle at 3 and 7 dpi, and this population remained the predominant population throughout the timecourse (Figure 3.6A-B). Both \textit{csf1ra}-expressing MNP populations also exhibited an increase in size and granularity at 1 and 3 dpi in both the ventricle and the blood, although this was slightly less pronounced in the \textit{csf1ra+} population (Figure 3.6A, C). This increased FSC/SSC is indicative of an activated phenotype which is expected of the pro-inflammatory monocytes and macrophages that attend the injury site during the initial inflammatory phase.

However, the dynamics of the \textit{csf1ra+} population differed to that of \textit{mpeg1.1+csf1ra+} cells, only showing an increased frequency at 3 dpi (Figure 3.6B). Nevertheless, at 1 and 3 dpi the relative proportion of \textit{csf1ra+} cells increased, as a result of decreased \textit{mpeg1.1+csf1ra+} and \textit{mpeg1.1+} frequency (Figure 3.6B). This loss of the \textit{mpeg1.1+csf1ra+} and \textit{mpeg1.1+} cells is likely due to the death of resident cells within the injury region but suggests that similar numbers of \textit{csf1ra+} cells expand as are lost, which could suggest that these putative monocytes are being rapidly recruited, unlike the other populations. Furthermore, the fluorescence profile of \textit{csf1ra+} cells
at 1 and 3 dpi appear to be transitioning into the $mpeg1.1+csf1ra+$ population in both the ventricle and the blood (Figure 3.6A, C). Interestingly, the blood also appeared to have two distinct populations of $mpeg1.1+csf1ra+$ cells, which showed variable levels of both $csf1ra$ and $mpeg1.1$ (Figure 3.6C), which was also evident in the earlier analysis of the fin and spleen (Figure 3.4B). The fluorescence profile indicated the $csf1ra+$ cells are transitioning into the population with high $csf1ra$ expression and appeared to expand at early stages post-injury suggesting, that this population is analogous to the $mpeg1.1+csf1ra+$ population in the ventricle. However, an additional population of $mpeg1.1+csf1ra^{dim}$ cells was also observed within the blood. Curiously, this population could also be seen sporadically in the ventricle analysis following injury (Figure 3.6A). Unfortunately, due to the large variability of blood cell counts between fish, trends of the $mpeg1.1\pm csf1ra\pm$ populations within the blood could not be established to quantify the dynamics of these populations but warrants further investigation.

Nevertheless, the fluorescence distribution, FSC/SSC profile and transient expansion of the $csf1ra+$ population further suggests that this population encompasses newly recruited monocytes that are responsive to tissue injury. Their presence in the steady state (Figure 3.2, Figure 3.3) may also indicate that monocyte populations do contribute to the cardiac MNP pool, which has been shown in mammals (Epelman et al., 2014) or represent intraluminal monocytes. Collectively, this analysis also confirms that $csf1ra+$ and $mpeg1.1+csf1ra+$ populations within the heart respond to cardiac injury with the expected dynamics of MNPs.

Interestingly, although $mpeg1.1+$ cell frequency showed similar dynamics to $mpeg1.1+csf1ra+$ cells at 1 and 3 dpi, levels peaked at 3 dpi and declined thereon (Figure 3.6B). $mpeg1.1+$ cells also remained small and non-granular at all timepoints, further supporting their lymphoid origin (Figure 3.6A, C).
Figure 3.6. Flow cytometry analysis of csf1ra± and mpeg1.1± populations following cardiac cryoinjury. A) csf1ra:GFP vs. mpeg1.1:mCherry flow cytometry plots showing the distribution of fluorescence in unwounded ventricles and at 1, 3, 7 and 14 dpi, with corresponding FSC/SSC plots of each subpopulation. B) Quantification of csf1ra± and mpeg1.1± cell frequency, expressed as a percentage of live, single cells, at timepoints post-injury. Mean frequency per ventricle ± SD is shown for the uninjured samples as two uninjured control ventricles were analysed at each timepoint to control for day to day variation. N numbers: Uninjured = 4 pools of 2; 1, 3, 7 dpi = pools of 4, 14 dpi = pool of 3 ventricles. One independent experiment. C) csf1ra:GFP vs. mpeg1.1:mCherry flow cytometry plots showing the distribution of fluorescence of cells isolated from whole blood of uninjured fish and at 1, 3, 7 and 14 dpi, with corresponding FSC/SSC plots of each subpopulation. One independent experiment.
Part II: Exploring the specificity and conservation of MNP markers

Given the discovery that mpeg1.1 expression was not restricted to cells of the MNP system, I wanted to further explore the specificity of other existing zebrafish MNP markers. I also wanted to investigate the presence and expression of orthologs to commonly used mammalian MNP-associated genes and possibly identify new markers that may enable the stratification of MNP populations.

3.6 **Zebras have few orthologs of mammalian MNP markers**

Firstly, I wanted to summarise the predicted presence or absence of zebrafish orthologs to mouse genes which are commonly used to define populations of monocytes and macrophages. Through literature searches, genes of interest were compiled (listed in Table 3.1) and investigated using the Alliance of Genome Resources database (The Alliance; Version 5.0.0; [https://www.alliancegenome.org/](https://www.alliancegenome.org/)). The Alliance compiles genetic information from six model organism databases (including the Mouse Genome Database and the Zebrafish Information Network) and the Geno Ontology Consortium and provides orthology predictions using the DRSC Integrative Ortholog Prediction Tool (DIOPT). The DIOPT integrates multiple orthology databases\(^1\) and summarises the gene orthology predicted by these databases for each species. A score of how many of these databases identify a particular gene to be an ortholog to the input gene is given; therefore, the higher the score, the more likely it is an ortholog. Table 3.1 provides a summary of the genes that were searched alongside their specificity and function in mice and the orthologs detected in humans and zebrafish.

This confirmed that Csf1r signalling is highly conserved within zebrafish, as csf1ra was detected to be the main ortholog to murine Csf1r homolog in 10/11 databases (Table 3.1). The paralog of csf1ra, csf1rb was only detected in 2/11 databases. However, as expected, few orthologs of many of the other murine genes were robustly detected. For example, no orthologs of the commonly used monocyte marker Ly6c1 are predicted, and only 3 out of 10 databases identified an ortholog for the tissue macrophage marker Agre1 (F4/80) (Nahrendorf et al., 2007, Waddell et al., 2018). Despite this, zebrafish ccr2 was detected to be the Ccr2

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\(^1\) Ensembl Compara, HGNC, Hieranoid, InParanoid, OMA, OrthoFinder, OrthoInspector, PANTHER, PhylomeDB, Roundup, TreeFam, and ZFIN
ortholog in 5/11 databases, but interestingly, was also detected to be the only potential ortholog of Cx3cr1, although this was only predicted by one database. This expression of ccr2 is particularly interesting considering the use of this cell surface receptor to identify monocytes/MDMs that have been repeatedly shown to elicit negative effects on tissue repair, as described in section 1.7.3.1 (Lavine et al., 2014, Dick et al., 2019, Nahrendorf et al., 2007) and suggests that analogous populations may be present in zebrafish.

The reverse orthology analysis was also performed on commonly used zebrafish genes (Table 3.2) and showed that human and mouse had commonly identified orthologs to marco and mfap4. Intriguingly, MPEG1 and Mpeg1 were predicted to be an ortholog to mpeg1.2 in 10/11 databases, yet only an ortholog to mpeg1.1 in 2/11 databases. Accordingly, the gene expression analysis in Figure 3.5 indicated specific mpeg1.2 expression in csf1ra-expressing MNPs, but was completely lacking in the mpeg1.1+ lymphoid population. It is likely, therefore, that mpeg1.2 may be superior to mpeg1.1 in specifying MNP-enriched expression.

The expression of mfap4, apoeb, acod1 (irg1), mhc2dab and mertka were also explored, as these genes have been shown to be expressed in specific macrophage populations in the zebrafish, and various transgenic lines have been created (Walton et al., 2015, Peri and Nüsslein-Volhard, 2008, Sanderson et al., 2015, Wittamer et al., 2011). Orthologs for these genes were commonly identified, indicating high conservation of these genes (Table 3.2). One exception of this analysis is MHC II. MHC II genes encode antigen presenting molecules and are therefore commonly used to identify MNPs in zebrafish and in mammals (Reynolds and Haniffa, 2015). Due to the complexity and multiple copies of these genes, direct orthologous genes are challenging to detect. However, mhc2dab has been identified to be an MHC II gene in zebrafish and has been shown to be expressed by MNPs (Wittamer et al., 2011, Sambrook et al., 2005).

Collectively, this confirmed that very few murine markers are conserved in zebrafish, highlighting the evolutionary divergence of the genes utilised by mice and zebrafish for MNP homeostasis. However, this indicates that csf1ra expression and function is likely to be highly similar in zebrafish, supporting previous work suggesting that it comprehensively labels both monocyte and macrophage populations. This also informed the subsequent analysis of potential candidate markers.
# Mouse Gene Expression and Function

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression</th>
<th>Function</th>
<th>Human</th>
<th>Zebrafish</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Csfr1r</strong></td>
<td>- Expressed by all cells of the MNP system</td>
<td>Cell surface receptor for M-CSF/CSF-1 which is required for the survival, proliferation and differentiation of MNPs</td>
<td><strong>CSF1R (11/11)</strong></td>
<td><strong>csf1ra (10/11)</strong></td>
</tr>
<tr>
<td><strong>Ccr2</strong></td>
<td>- High expression by <strong>classical monocytes</strong></td>
<td>Required for monocyte mobilisation and recruitment to tissues expressing its ligand, Ccl2.</td>
<td><strong>CCR2 (5/11)</strong></td>
<td><strong>ccr2 (5/11)</strong></td>
</tr>
<tr>
<td><strong>Cx3cr1</strong></td>
<td>- Highly expressed by <strong>tissue-resident macrophages</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- High expression on <strong>non-classical monocytes</strong>; low expression by classical monocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ly6C1</strong></td>
<td>- High expression by <strong>classical monocytes</strong> which differentiate into monocyte derived macrophages. - Low expression by non-classical monocytes</td>
<td>Largely unknown. Reported to have acetylcholine receptor binding activity.</td>
<td><strong>LY6H (3/11)</strong></td>
<td>-</td>
</tr>
<tr>
<td><strong>Adgre1 (F4/80)</strong></td>
<td>- <strong>Monocytes and macrophages</strong></td>
<td>Cell adhesion molecule</td>
<td><strong>ADGRE1 (11/11)</strong></td>
<td><strong>adgre10 (3/10)</strong></td>
</tr>
<tr>
<td><strong>Itgam (Cd11b)</strong></td>
<td>- Expressed on all <strong>MNPs</strong> in addition to granulocytes and natural killer cells</td>
<td>Integrin, cell adhesion molecule</td>
<td><strong>ITGAM (9/11)</strong></td>
<td><strong>itgam.1 (8/11)</strong></td>
</tr>
<tr>
<td><strong>Cd68</strong></td>
<td>- <strong>Macrophages</strong></td>
<td>Scavenger receptor</td>
<td><strong>CD68 (9/11)</strong></td>
<td><strong>cd68 (2/11)</strong></td>
</tr>
<tr>
<td><strong>Sell (Cd62l)</strong></td>
<td>- Expressed by <strong>classical monocytes</strong></td>
<td>L-selectin, cell adhesion molecule</td>
<td><strong>SELL (11/11)</strong></td>
<td>-</td>
</tr>
<tr>
<td><strong>Itgax (Cd11c)</strong></td>
<td>- <strong>Conventional DCs</strong></td>
<td>Integrin, cell adhesion molecule</td>
<td><strong>ITGAX (11/11)</strong></td>
<td><strong>itgam.1 (10/11)</strong></td>
</tr>
<tr>
<td><strong>Mrc1 (Cd206)</strong></td>
<td>- Highly expressed by <strong>classically activated/M1 macrophages</strong> - Low expression on alternatively activated/M2 macrophages</td>
<td>Pattern recognition receptor</td>
<td><strong>MRC1 (11/11)</strong></td>
<td><strong>mrc1a (10/11)</strong></td>
</tr>
<tr>
<td><strong>Mertk</strong></td>
<td>- Highly expressed by <strong>macrophages</strong></td>
<td>Involved in phagocytosis</td>
<td><strong>MERTK (10/11)</strong></td>
<td><strong>Mertka (10/11)</strong></td>
</tr>
<tr>
<td><strong>Timd4</strong></td>
<td>- Expressed by populations of <strong>resident cardiac macrophages</strong></td>
<td>Involved in apoptotic cell clearance</td>
<td><strong>TIMD4 (11/11)</strong></td>
<td><strong>timd4 (2/11)</strong></td>
</tr>
<tr>
<td><strong>Lyve1</strong></td>
<td>- Expressed by populations of <strong>resident cardiac macrophages</strong></td>
<td>Glycoprotein receptor</td>
<td><strong>LYVE1 (11/11)</strong></td>
<td><strong>lyve1a (8/11)</strong></td>
</tr>
</tbody>
</table>

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Table 3.1. Summary of human and zebrafish orthologs to commonly used murine MNP markers. Classical murine marker genes of MNP populations were compiled from a range of published literature (Epelman et al., 2014, Dick et al., 2019, Nahrendorf et al., 2007, Shiraishi et al., 2016, Reynolds and Haniffa, 2015) were searched using The Alliance database (https://www.alliancegenome.org/) and orthologs to these genes in humans and zebrafish were recorded. Numbers in brackets indicate the number of total databases searched that identified the gene as an ortholog to the inputted gene. Dash indicates where no ortholog was identified.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression</th>
<th>Function</th>
<th>Human</th>
<th>Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>csf1ra</td>
<td></td>
<td>As shown in Table 3.1</td>
<td>MPEG1</td>
<td>Mpeg1</td>
</tr>
<tr>
<td>mpeg1.1</td>
<td>- Macrophages, unknown in other MNP populations</td>
<td>Pore-forming perforin involved in pathogen responses</td>
<td>MPEG1</td>
<td>Mpeg1</td>
</tr>
<tr>
<td>mpeg1.2</td>
<td>- MNPs</td>
<td>Pore-forming perforin involved in pathogen responses</td>
<td>MPEG1</td>
<td>Mpeg1</td>
</tr>
<tr>
<td>mfap4</td>
<td>- Expressed in monocytes and macrophages, unknown expression in DCs</td>
<td>Predicted antigen binding activity</td>
<td>MFAP4</td>
<td>Mfap4</td>
</tr>
<tr>
<td>marco</td>
<td>- Expressed in macrophages</td>
<td>Scavenger receptor</td>
<td>MARCO</td>
<td>Marco</td>
</tr>
<tr>
<td>mhc2dab</td>
<td>- Highly expressed by MNP</td>
<td>Antigen presentation molecule</td>
<td>Functional orthologs are annotated</td>
<td></td>
</tr>
<tr>
<td>apoeb</td>
<td>- Highly expressed by microglia</td>
<td>Involved in lipid homeostasis</td>
<td>APOE</td>
<td>Apoe</td>
</tr>
<tr>
<td>acod1 (irg1)</td>
<td>- Activated macrophages</td>
<td>Bactericidal roles</td>
<td>ACOD1</td>
<td>Acod1</td>
</tr>
<tr>
<td>mertka</td>
<td>- Phagocytic cells</td>
<td>Involved in signalling pathways required for phagocytosis</td>
<td>MERTK</td>
<td>Mertk</td>
</tr>
</tbody>
</table>

Table 3.2. Summary of human and murine orthologs to commonly used zebrafish MNP markers. Classical zebrafish marker genes of MNP populations were compiled from a range of published literature (Zakrzewska et al., 2010, Benard et al., 2015, Sanderson et al., 2015, Walton et al., 2015, Peri and Nüsslein-Volhard, 2008, Wittamer et al., 2011) and the analysis was performed as described in Table 3.1.
3.7 **Zebrafish MNP markers show differential expression in monocytes, and following cardiac cryoinjury**

Next, I utilised existing transcriptomics datasets containing RNA sequencing data from MNPs and adult zebrafish hearts to explore the expression of the zebrafish MNP-markers listed in Table 3.2 to establish whether expression of these markers was restricted to tissue macrophages or whether monocyte precursors also express these markers. Furthermore, I also probed these datasets for the orthologs to mammalian markers (Table 3.2) to identify if these genes could be detected.

Three publicly available transcriptomics datasets were therefore analysed (Appendix 7.7). The first two datasets were derived from RNA sequencing on cells isolated from the adult kidney. As the kidney is the site of definitive hematopoiesis in zebrafish, these datasets would be enriched for the gene expression of monocytes. The third dataset was derived from RNA sequencing of ventricles isolated from uninjured fish and at early timepoints post-injury, where recruited monocytes would be abundant and MNP markers are likely to be dynamic.

### 3.7.1 ‘Dissecting hematopoietic and renal cell heterogeneity in adult zebrafish at single-cell resolution using RNA sequencing’ (Tang et al., 2017)

In an attempt to elucidate new markers of haematopoietic cells, Tang *et al* (2017) performed single cell RNA-sequencing on kidney marrow and thymic T cells isolated from a range of adult transgenic zebrafish lines that have been reported to identify specific haematopoietic cell lineages. This included lines that labelled hematopoietic stem progenitor cells (HSPCs), thrombocytes, neutrophils/myeloid cells, B cells and T cells. Cells were sorted from wild type transgenic fish, in addition to the isolation of *lck:GFP*+ cells from *rag1*−/− fish, which allowed the collection of NK cells due to the absence of *lck:GFP*+ T cells which are not viable in *rag1* mutants. The RNA sequencing data acquired from these lineages were used to establish trajectories of gene expression analysis of different lineages. Subsequently, unlabelled hematopoietic cells from wild type kidneys underwent single cell sequencing. Informed by the gene expression profiles acquired from the transgenic lines, *t* stochastic neighbour embedding (tSNE) clustered cells into the main hematopoietic lineages in addition to kidney

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2 Tg(*Runx1*+23:GFP) (HSPCs), Tg(*cd41:GFP*) (HSPCs and thrombocytes), Tg(*mpx:GFP*) (neutrophils/myeloid), Tg(*rag2:GFP*) (B cells) and Tg (*lck:GFP*) (T cells)
stromal cell types. From this, they generated a list of genes that were enriched in the following groups: HSC thrombocytes, blood progenitors, erythroid cells, neutrophils, macrophages, macrophages/myeloid cells, B cells, T cells, NK cells, NKL cells, and other kidney cells.

Utilising this information, I probed this dataset for the previously described genes (Table 3.1 and Table 3.2). As stated in section 3.4, this analysis identified mpeg1.1 expression in not only the macrophage/myeloid group, but also in the B cell and NKL cell group. Conversely, mpeg1.2, csf1ra, marco, mfap4, apoeb, mertka and irg1 only localised to the macrophage group, further confirming their restricted expression to monocytes and macrophages. The expression of the csf1ra paralog, csf1rb, was also explored and was identified in the macrophage/myeloid group but also in blood progenitors, suggesting less specific expression within the monocyte/macrophage lineage, in line with recent reports (Hason et al., 2022, Ferrero et al., 2021). As expected, mhc2dab was also expressed in macrophages/myeloid cells, but also showed expected expression in B cells and NKL cells (Wittamer et al., 2011).

Interestingly, timd4, which has been shown to identify resident murine cardiac macrophages (Dick et al., 2019), was also restricted to macrophages. ccr2 was not identified in any group, however, as this receptor is upregulated following mobilisation to the circulation following inflammation, expression may be low in the analysed population (Fujimura et al., 2015). As expected, itgam was identified in the myeloid/macrophage group, but also in neutrophils and NKL cells, consistent with this expression profile in mammals (Kawai et al., 2005).

Furthermore, I also searched this database for other commonly used lineage specific genes to further clarify their expression to hematopoietic lineages, such as markers for T cells, NK cells, neutrophils (Carmona et al., 2017, Langenau et al., 2004, Kitaguchi et al., 2009). Using this information, in combination with information acquired from various literature searches (such as metaphocyte-specific genes (Kuil et al., 2020), I generated Venn diagram shown in Figure 3.7 to map the specificity of commonly used markers. This also provides a valuable reference to generate primers to interrogate the identity of cell populations, as was performed in Figure 3.5.
3.7.2 ‘Single-cell RNA-sequencing uncovers transcriptional states and fate decisions in hematopoiesis’ (Athanasiadis et al., 2017)

Similar to Tang et al (2017), Athanasiadis et al (2017) performed single cell RNA-sequencing on cells sorted from kidney of 8 different transgenic zebrafish lines\(^3\) that have been reported to label different haematopoietic cell lineages (namely MNPs, neutrophils, T cells, thrombocytes, erythrocytes, and HSPCs), in addition to cells sorted from a non-transgenic, wild type control. RNA sequencing data was then pooled and the Monocle2 algorithm was used to identify five transcriptional cell states or cell types in which all the cells fell into: HSC

\(^3\) Tg(mfap4:tdTomato) (monocytes/macrophages), Tg(cd4:mCherry) (T cells, macrophages), Tg(lyz:DsRed2) (neutrophils), Tg(mpx:EGFP) (neutrophils, myeloid lineages), Tg(cd41:EGFP) (HSPCs, thrombocytes), Tg(runx1:Cherry) and Tg(tal1:EGFP) (HSPCs), Tg(gata1a:GFP) (erythrocytes)
homeostasis (HSPCs), antigen processing (monocytes), leukocyte migration (neutrophils), erythocyte differentiation (erythrocytes) and blood coagulation (thrombocytes). For each cluster, a list of highly/differentially expressed genes was produced. In addition, an online interactive trajectory of cell differentiation from HSPCs to each of the differentiated cell type/cluster has been created, called BASiCz (accessed at https://www.sanger.ac.uk/tool/basicz/basicz/). This was used to localise each of the genes of interest to the monocyte differentiation state (Figure 3.8). mpeg1.1, mpeg1.2, csf1ra, mfap4, marco and mertka expression localised to the monocyte lineage and generally showed higher expression in more differentiated cells indicating that precursors likely do not express these markers. Conversely, csf1rb, itgam and mhc2dab expression was widely detected in all lineages, as expected. ccr2 expression was sporadic, but did localise to HSPCs and monocytes, supporting conserved expression in zebrafish. An additional gene, monocyte to macrophage differentiation factor (mmd) was also mapped due to its proposed roles in differentiation (Lin et al., 2021), and did show some enrichment within the monocyte lineage.

This analysis provides further detail to the findings identified in section 3.7.1 and suggests that the zebrafish markers localise to monocytes and macrophages. However, this also suggests that mpeg1.1 and marco expression increases upon monocyte/macrophage differentiation and supports the hypothesis that csf1ra+ and mpeg1.1+csf1ra+ cells may represent populations of monocytes and macrophages, respectively.
Figure 3.8. Expression of MNP-associated genes in single cells organised on a trajectory of their differentiation state, using transcriptomics data from Athanasiadis et al (2017). The trajectory shows cells in the centre which have an expression profile characteristic of HSPCs, and branches off to cells with profiles typical of monocytes, neutrophils, thrombocytes and erythrocytes. Using the BASiCz platform (https://www.sanger.ac.uk/tool/basicz/), genes of interest were searched to visualise their expression within cells on the trajectory. Details of analysis files are in Appendix 7.7.
3.7.3 ‘Reciprocal analyses in zebrafish and medaka reveal that harnessing the immune response promotes cardiac regeneration’ (Lai et al., 2017)

Finally, I wanted to associate the expression of these MNP markers to their dynamics following cardiac cryoinjury. Lai et al (2017) published a comparative study of the dynamics of whole ventricle transcriptome following cardiac regeneration in zebrafish and medaka. Despite being a teleost fish that is closely related to the zebrafish, medaka (Oryzias latipes) are unable to undergo cardiac regeneration, therefore this study aimed to identify species-specific differences in gene expression following cardiac cryoinjury that may be central to mediating their different regenerative responses. RNA sequencing was performed on ventricles from each species at uninjured, sham-injured (thoracic incision only), 6 hpi and 1, 3, and 5 days post cardiac cryoinjury timepoints. Log2 fold changes (Log2FC) were calculated to demonstrate changes in gene expression levels compared to uninjured and sham injured controls at each timepoint (Lai et al., 2017).

Utilising the zebrafish Log2FC values of injury timepoints versus uninjured controls, I plotted the expression patterns of the MNP-associated genes of interest (Figure 3.9). Interestingly, mpeg1.1, mpeg1.2 and timd4 were not detected in this dataset, but csf1ra and mfap4 showed rapid upregulation, particularly at 1 and 2 dpi, indicating the influx of macrophages into the injury site (Figure 3.9A). csf1rb did not follow this trend, showing minimal changes until 5 dpi when expression increased, further supporting that csf1ra and csf1rb paralogs are differentially expressed. marco and apoeb were also increased at 1 dpi, with marco upregulation being much more modest compared to apoeb, but levels were similarly maintained throughout the duration of the analysis (Figure 3.9B). ccr2 expression was also upregulated from 1-5 dpi (Figure 3.9C), which could suggest an influx of ccr2-expressing cells, like that observed in mammals (Yan et al., 2013). Surprisingly, mertka expression only showed modest changes, whereas mhc2dab showed a gradual increase throughout the timecourse.

This dataset was also probed to identify the pattern of collagen expression, which may help investigate the scarring dynamics of zebrafish (Figure 3.9D). Interestingly, the majority of these genes increased throughout the timecourse, indicating that multiple collagen types are deposited during this phase.
3.7.4 Summary of transcriptomics analysis

In summary, this analysis of transcriptomics datasets has confirmed that the majority of commonly used zebrafish MNP-associated genes are likely to be expressed in monocytes, in addition to macrophages, which hasn’t been confirmed for many genes. However, this analysis also demonstrates that levels of these genes may be able to stratify different populations of macrophages and monocytes. Candidates include marco and mpeg1.1, as hypothesised in Part I. Analysis of these markers following cryoinjury also indicates that these markers are differentially upregulated in response to injury and suggests that different populations may be expressing different markers, which may help to elucidate different subpopulations going forward.

Figure 3.9. Dynamics of MNP-associated and collagen genes in the adult zebrafish following cardiac cryoinjury. Plots were generated using publicly available Log2FC values generated from transcriptomic analysis of ventricles following cardiac cryoinjury (Lai et al., 2017). RNA sequencing was performed on four whole pooled ventricles for each timepoint post cardiac cryoinjury and genes were normalised prior to relative expression analysis. Log2FC values represent the fold change relative to uninjured controls. A-C) Expression patterns of zebrafish MNP-associated genes. Genes are segregated into three graphs to aid visualisation. D) Expression of collagen genes. Details of analysis files are in Appendix 7.7.
3.8 *csf1ra*-expressing MNPs have distinct expression profiles

Following on from the transcriptomics analysis, I next wanted to expand the characterisation of MNPs in the adult zebrafish heart. I also wanted to further dissect the mpeg1.1+csf1ra+ population, as a distinct population of mpeg1.1+csf1ra\textsuperscript{dim} cells were also visible within flow cytometry plots of all tissues, including the ventricle when large cell numbers were analysed (Figure 3.6). Similarly, the analysis of other tissues in Figure 3.4 showed a similar mpeg1.1+csf1ra\textsuperscript{dim} population. FACS of the adult kidney and heart (ventricle and atrium cells were combined to maximise cell collection) from Tg(mpeg1.1:mCherry); TgBAC(csf1ra:GFP) cells was therefore performed to isolate populations of csf1ra+, mpeg1.1+csf1ra+, mpeg1.1+csf1ra\textsuperscript{dim} and background control cells and the expression of MNP genes of interest were analysed (Figure 3.10).

mpeg1.1+csf1ra\textsuperscript{dim} cells had an overlapping FSC/SSC profile with the other two MNP population in both tissues, suggesting a similar myeloid origin (Figure 3.10A-B). Interestingly, the mpeg1.1+csf1ra\textsuperscript{dim} population comprised the largest population within the kidney. Unfortunately, many of the genes failed to amplify within the cardiac MNP populations particularly within the csf1ra+ and mpeg1.1+csf1ra\textsuperscript{dim} populations, likely due to insufficient RNA yields. Despite this, csf1ra, mpeg1.1, mpeg1.2, mfap4, marco, itgam and il1b were detected in the mpeg1.1+csf1ra+ population (Figure 3.10D). Furthermore, all genes amplified within the kidney populations, except ccr2, which suggests that these genes were below detection limits in heart cells and also confirms the MNP profile of these cells. Although it is difficult to determine relative expression levels from this analysis, some interesting differences were observed. In the kidney, csf1ra, marco and mpeg1.2 expression was highest in the mpeg1.1+csf1ra+ population, despite having the lowest number of cells. mmd expression was also high in the csf1ra+ population, as was il1b. Interestingly, mertka was expressed by both mpeg1.1+csf1ra+ and csf1ra+ populations, but not by mpeg1.1+ csf1ra\textsuperscript{dim} cells. csf1rb was also highly expressed by csf1ra+ cells as this gene has been repeatedly reported to be expressed by myeloid progenitor cells (Ferrero et al., 2021, Hason et al., 2022) this further adds to the hypothesis that csf1ra+ cells could be monocytes.
Figure 3.10. Characterisation of mpeg1.1+csf1ra dim cells. A-B) Flow cytometry plots of labelled cells within the ventricle and atrium (A) and kidney (B) of unwounded Tg(mpeg1.1:mCherry); TgBAC(csf1ra:GFP) fish. Plots show gating for csf1ra+, mpeg1.1+csf1ra+ and mpeg1.1+ csf1ra dim populations and the associated FSC and SSC profile of each population. Analysis was performed on pooled tissues harvested from 9 fish. One experimental replicate. C) RT-PCR analysis of the sorted populations from the heart and kidney shown in A and B. Background cells consist of live, single, unlabelled cells, which have been excluded from plots A and B to better show the distribution of labelled populations. Variations in gel intensity are due to the requirement for PCR products to be run on separate gels and differences in the autoexposure during acquisition. Cell counts (kidney/heart): csf1ra+ = 61,000/3382; mpeg1.1+ csf1ra dim = 240,000/11,500; mpeg1.1+csf1ra+ = 29,000/38,000, background = 300,000/300,000.
3.9 **ccr2 expression is detected within the newly cryoinjured heart**

To further investigate the expression of ccr2, FAC-sorted cells from uninjured and 1 dpi Tg(mpeg1.1:mCherry); TgBAC(csf1ra:GFP) ventricles shown in Figure 3.6 were probed for the expression of ccr2 (Figure 3.11) to see if this gene was expressed during the pro-inflammatory phase. In line with previous analysis, csf1ra-expressing cells showed characteristic expression of MNP markers, whereas the mpeg1.1+ lymphoid population completely lacked expression of these markers, further validating the findings shown in Figure 3.5 in heart cells. However, very low expression of ccr2 was also detected in mpeg1.1+csf1ra+ cells isolated from uninjured hearts, with stronger expression shown at 1 dpi (Figure 3.11). Surprisingly, putative csf1ra+ monocytes did not show expression of ccr2, however cell counts were very low in this population, therefore expression levels may be below the detection limit. This analysis therefore supports the presence of ccr2+ monocytes which respond to injury in the zebrafish.

![Figure 3.11. ccr2 expression in mpeg1.1+csf1ra+ cells within the ventricle at 1 dpi. RT-PCR analysis of MNP markers from FAC-sorted cells isolated from uninjured and 1 dpi Tg(mpeg1.1:mCherry); TgBAC(csf1ra:GFP) ventricles (shown in Figure 3.6). Cell counts (uninjured/1 dpi): mpeg1.1+csf1ra+ = 6000/7900; mpeg1.1+ = 1700/1800; csf1ra+ = NA/712. The low abundance of csf1ra+ cells within the uninjured heart prevented analysis of this population.](image-url)
3.10 *csf1ra, csf1ra* and *mfap4* show different patterns of upregulation following cardiac cryoinjury

To further explore the selected MNP-associated genes following cardiac cryoinjury, in particular *csf1rb* and *mmd* which showed interesting expression in *csf1ra*+ cells, qPCR was performed on cDNA extracted from whole ventricles at timepoints over 21 days post injury.

Analysis of both *csf1r*- paralogues was performed to identify whether their expression was similar over the initial stages of MNP recruitment. At 6 hpi and 1 dpi, *csf1ra* appeared to show a small but gradual increase in expression, whereas *csf1rb* marginally decreased (Figure 3.12AB). However, whereas *csf1ra* remained approximately two-fold elevated at timepoints between 1 to 7 dpi, *csf1rb* expression gradually increased at 3 and 7 dpi, peaking at 7 dpi. By 14 and 21 dpi, both paralogues returned to uninjured levels. However, at all timepoints the expression of the genes was highly variable, and no significant change was determined at any point.

*mfap4* expression showed a similar trend to *csf1ra*, exhibiting a gradual increase in the first 7 days post-injury. Expression peaked and was statistically significant at 3 and 7 dpi, showing a 5.4 ± 2.7 SD and 5.7 ± 1.9 SD fold-change compared to uninjured levels, respectively. By 14 to 21 dpi, expression remained at approximately a three-fold increase compared to uninjured levels (Figure 3.12C). *mmd* expression was also analysed but this displayed very subtle but variable changes across the injury time course (Figure 3.12D). Interestingly, expression was highest at 7 dpi, showing a two-fold increase, coinciding with peak levels of all other genes.

*ccl2* expression, which encodes the ligand to CCR2, was also assessed by qPCR, but showed high variability and couldn’t consistently be detected in samples, suggesting that expression of this gene was low. Due to limited sample availability, qPCR could not be performed on all other markers, however, *marco, itgam, timd4, mertka* and *ccr2* were also analysed by RT-PCR analysis on a single set of the cDNA samples. This showed that *marco* and *cd11b* were expressed in the ventricle, however *timd4* expression only showed weak expression at 7 dpi, and no *mertka* or *ccr2* expression was detected, suggesting that expression is very low in whole hearts. However, despite loading the same quantity of cDNA for each sample, the expression of the reference gene (*ef1a*) was highly variable therefore the relative expression of these genes cannot be determined.
Similarly to the expression analysis performed by Lai et al (2017), this analysis indicated that csf1ra and mfap4 expression vary, indicating that different populations of MNP likely express different levels of these markers. mfap4 expression also appears to be very high and therefore is likely to be a useful marker of monocytes and macrophages. The expression of csf1rb also indicates that this is not strongly expressed by monocytes which would be prevalent at the early stages of injury.
Figure 3.12. Expression of MNP markers in whole ventricles following cardiac cryoinjury. A-D) qPCR analysis of csf1ra (A), csf1rb (B), mfap4 (C) and mmd (D) presented as relative fold change compared to uninjured levels (i) and as dCt values compared to reference gene ef1a (ii). Numbers within (i) indicates number of ventricles/replicates analysed per timepoint. Two independent experiments. E) RT-PCR analysis of additional MNP-associated genes. N = 1.
3.11 *mfap4* labels macrophages within the adult zebrafish

The previous analysis indicated that *mfap4* was a robust marker of monocytes and macrophages, showing stable expression in transcriptomics analysis of monocytes, sorted cell populations and showed clear upregulation following injury. Multiple *mfap4* driven transgenics have been established and have been reported to have stable expression in macrophages (Walton *et al.*, 2015). To investigate the specificity of *mfap4* expression to MNPs, Tg(*mfap4:Turquoise2*) and Tg(*mfap4:tdTomato*) fish were characterised (Figure 3.13). Transgenic fluorescence appeared localised to macrophage like cells in 5 dpf larvae and this was also seen in the adult hearts and fin in Tg(*mfap4:Turquoise2*) fish. Surprisingly, macrophage-specific fluorescence could not be seen in the adult Tg(*mfap4:tdTomato*) fish and this was further confirmed by flow cytometry (Figure 3.13B, D). This is likely due to dim expression or silencing within our transgenic line. However, *mfap4:mTurquoise2*+ cells were detected in the ventricle, fin and blood of adult fish (Figure 3.13C). Gene expression analysis of *mfap4*+ sorted cells from the fin also confirmed co-expression of *marco* in these sorted cells but the absence of neutrophil, B cell, NK/NKL cell specific markers, although low *lck* (a marker of T cells) expression was detected (Figure 3.13E).

Collectively, this indicated that the Tg(*mfap4:Turquoise2*) transgenic could assist the analysis of monocytes and macrophages in the adult zebrafish, although *lck* expression in the sorted cell population requires further investigation. Combining the *mfap4* transgenic with the *csf1ra* transgenic would be interesting to establish if these markers were differentially expressed in MNPs, as was established for *mpeg1.1*, however, due to the combination of similar fluorophores (*csf1ra:GFP* and *mfap4:mTurquoise2*) this analysis could not be performed in adult fish.
Figure 3.13. Characterisation of *mfap4* transgenic zebrafish. A-B) Confocal imaging of (Tg(*mfap4*:mTurquoise2) (A) and Tg(*mfap4*:tdTomato) transgenic fluorescence (B) in 5 dpf zebrafish (i), the adult ventricle (ii) and adult fin (iii). C-D) FACS analysis from Tg(*mfap4*:mTurquoise2) (C) and Tg(*mfap4*:tdTomato) (D) zebrafish, in addition to control/non-transgenic (non-Tg) fish to show gating strategy for positive cells. E) RT-PCR analysis of lineage markers in *mfap4*:mTurquoise2+ cells isolated from the fin (*mfap4+*), background/*mfap4*:mTurquoise2- cells (*mfap4-*). cDNA from whole 5 dpf larvae was used as a positive control.
3.12 Live imaging the adult zebrafish heart

The analysis thus far indicated that \textit{mpeg1.1±csf1ra±} monocytes and macrophages likely respond to cardiac cryoinjury in the adult zebrafish heart and represent distinct populations. I have also postulated that \textit{csf1ra+} monocytes may transition into \textit{mpeg1.1+csf1ra+} macrophages in response to injury and may potentially also maintain homeostatic cardiac MNP populations.

Imaging of fluorescently labelled fixed hearts and histologic specimens provides useful snapshots into the cellular microenvironment, yet these methods prevent the observation of cell behaviour, migration and changes in the phenotype of cells (such as the potential upregulation of \textit{mpeg1.1} in \textit{csf1ra+} cells) which can provide important insights into cellular function during homeostasis and tissue repair. Despite the advantages of the zebrafish model to study heart regeneration, due to the size, location, beating, and overlying skin opacity, imaging the adult heart \textit{in vivo} is not yet possible. Although methods are being developed to image beating hearts in adult zebrafish \textit{in vivo} (Taylor \textit{et al.}, 2019), there are significant challenges that need to be overcome before we can use it to address questions about heart regeneration.

Multiple studies have shown that zebrafish hearts, amongst other species’, can be cultured \textit{ex vivo} for prolonged periods (up to one month) without the requirement for complex perfusion systems (Pieperhoff \textit{et al.}, 2014, Cao and Poss, 2016, Kikuchi \textit{et al.}, 2011). Generally, this culture procedure involves dissection of the ventricle (with or without the atrium and bulbus arteriosus intact) and incubation in a serum-supplemented culture media at 28 °C + 5% CO\textsubscript{2} in multiwell plates. Recent studies have also shown that cultured heart slices are viable and enable proliferation of cardiomyocyte proliferation to be studied (Honkoop \textit{et al.}, 2021). Strikingly, cultured hearts have also been shown to retain their ability to regenerate the epicardium and vasculature following cardiac cryoinjury (Cao and Poss, 2016, Yip \textit{et al.}, 2020), indicating that the \textit{ex vivo} environment supports regenerative capacity to some extent. Thus far, this system has not been utilised to study the dynamics of leukocytes within the zebrafish heart, therefore, the potential to live-image cardiac MNP dynamics \textit{ex vivo} was explored.
3.13 **Leukocytes undergo shape changes and interactions *ex vivo*, but not migration**

I first wanted to establish whether this system could be used to study cell dynamics immediately following extraction of the zebrafish heart, thereby limiting potential artefacts caused by prolonged culture. The *ex vivo* culture media recipe and preparation of the hearts was adapted from Cao and Poss (2016), due to the reported maintenance of tissue health for up to 2 weeks. However, media was supplemented with 30 mM 2,3-butanedione monoxime (BDM) during imaging to prevent contraction of the cardiomyocytes, as preliminary studies showed that the heartbeat prevented acquisition of a stable Z-stack due to limited acquisition speed. BDM has also been repeatedly reported to improve the viability of cardiomyocytes in *in vitro* culture (Thum and Borlak, 2001, Kivistö *et al.*, 1995). Following dissection and wash steps under sterile conditions, Tg(*mpeg1.1:*mCherry); TgBAC(*csf1ra:*GFP) hearts were mounted in 1.5% low-gelling temperature agarose in a glass bottom dish with overlying culture media.

Hearts were imaged at 28 °C with ambient CO₂ levels using a confocal microscope and a series of xyz images was acquired over several hours within a region of interest on the ventricle (Figure 3.14; further detail in Materials and Methods, section 2.4).

![Figure 3.14. Schematic of *ex vivo* imaging platform.](image)

Uninjured or injured hearts, with the ventricle, atrium and bulbus arteriosus intact, were dissected, washed and mounted in 1.5% low-gelling agarose in a sterile glass bottom dish and submerged in culture media. Culture media was supplemented with 30 mM BDM to prevent cardiomyocyte contraction to aid stable image acquisition. xyzt imaging was performed on a confocal microscope at 28 °C.
To determine whether the *ex vivo* imaging set up allowed the visualisation of MNP migration, a 1 dpi heart was analysed, as activated MNPs would be responding to the injury at this timepoint (Figure 3.15A). The field of view was chosen to image the border zone of the injury to visualise potential migration of leukocytes within the uninjured tissue to the injury site, and also enable visualisation of cells within the injury site itself (Figure 3.15; Video 2, Appendix 7.6).

Labelled cells underwent shape changes throughout the 5-hour imaging duration and could be seen to extend protrusions and interact with each other (Column i, Figure 3.15), but unexpectedly, showed no or very limited migration (Column ii, Figure 3.15). Furthermore, several cells gradually seemed to disappear between frames (Column iii, Figure 3.15); this didn’t appear to be due to photobleaching, as there was no characteristic fading of fluorescence prior to disappearance and it wasn’t consistent across the image. There was also no blebbing or rounding of cells suggestive of apoptosis, and cells didn’t show signs of migrating out of the field of view.
Figure 3.15. *Ex vivo* imaging in a 1 dpi Tg(*mpeg1.1:mCherry*); TgBAC(*csf1ra:GFP*) ventricle. Right of the dashed line is the injury area. Large panels show maximum projections of single channel (*csf1ra:GFP* and *mpeg1.1:mCherry*) and merged channel images at hourly intervals during image acquisition. Stacks were acquired every 6 minutes for 5 hours. Insets identify regions i-iii enlarged in the small panels to the right for each timepoint. Column i) follows the protrusion/shape change of a *csf1ra*+ cell. Column ii) demonstrates the migration of a *csf1ra*+ cell. Column iii) identifies a cell that ‘disappears’ throughout the timecourse. Single heart from one experimental replicate. Scale bars: Large panels = 50 µm; small panels = 20 µm. See Video 2, Appendix 7.6.
3.14 **BDM inhibits MNP motility**

To firstly determine whether the disappearance of cells was due to the presence of an injury, which may cause cell death of some leukocytes, or because of the *ex vivo* conditions, imaging was repeated in unwounded hearts. Laser power was also reduced and scanning speed was increased to minimise potential phototoxicity caused by imaging. Nevertheless, similar disappearance of selected cells and no migration was observed indicating that this was independent of injury (Figure 3.16; Video 3, Appendix 7.6).

![Figure 3.16. Ex vivo imaging in the unwounded Tg(mpeg1.1:mCherry); TgBAC(csf1ra:GFP) ventricle. Maximum projections of csf1ra:GFP, mpeg1.1:mCherry, and merged channel images at the start and end of image acquisition. Stacks were acquired every 6 minutes throughout the duration of imaging. Arrows identify cells that disappear throughout the timecourse. Images were acquired from a single heart from one of two experimental replicates. Scale bar = 100 µm. See Video 3, Appendix 7.6.](image-url)
These results indicated that the culture environment or protocol was not supportive of MNP migration and may induce cell toxicity. The initial approach involved imaging cells immediately after dissection and wash steps to minimise effects of prolonged culture. However, to investigate whether the rapid change of microenvironment was adversely affecting the cells, 7 dpi hearts were acclimatised to the *ex vivo* conditions by incubating them in culture media in a well plate overnight prior to mounting and imaging.

As previously shown, MNPs could be seen to extend protrusions and interact with other cells during the timeframe suggesting that the cells were viable and active after culture. Furthermore, fewer cells seemed to disappear potentially indicating that cell survival was improved, yet cell migration was still absent (Figure 3.17).

![Image](image.png)

**Figure 3.17.** *Ex vivo* imaging of 7 dpi + 1 day cultured Tg(*mpeg1.1:mCherry*); TgBAC(*csf1ra:GFP*) ventricle. Hearts were cultured in culture media in a 12-well dish for 24 hours prior to imaging. Maximum projections of *csf1ra:GFP*, *mpeg1.1:mCherry*, and merged channel images at the start and end of image acquisition. Stacks were acquired every 9 minutes for 2 hours. Arrowheads identify cells that have undergone shape changes throughout the timecourse. Scale bar = 20 µm. See Video 4, Appendix 7.6.
To posit why leukocyte migration may have been inhibited, the experimental set-up was re-evaluated. This led to consideration of adding BDM to the culture media which was required to prevent the heart beating using the initial confocal set-up. BDM is routinely added to in vitro cardiomyocyte cultures, including in a recent publication by Honkoop et al (2021) which shows live-imaging of cardiomyocyte proliferation in ex vivo heart slices. Regrettably, we neglected to consider the effect this may have on other cell types. Literature searches identified that although BDM was formally predicted to be selective to inhibiting muscle myosin (Garcia-Dorado et al., 1992), it also affects myosin in other cells, including leukocytes (Urwyler et al., 2000). BDM concentrations within the range used in the culture media, have been shown to induce constant shape changes in resting human spherical polymorphonuclear leukocytes, largely in the form of small bleb-like protrusions on the cell surface, and inhibit locomotion and cell polarity to chemokines (Urwyler et al., 2000). This is highly reminiscent of what was observed in Figures 3.15-3.17 and demonstrates the incompatibility of using BDM with addressing the biological questions at hand in this ex vivo system.
3.15 **Cardiac MNPs are highly motile**

The feasibility of imaging the contracting heart by making modifications to the imaging set up was therefore investigated. Fortunately, this was assisted by the availability of a new spinning disk confocal microscope system, which is advantageous to standard confocal microscopy for live cell imaging due to faster acquisition time and reduced laser exposure (Gräf et al., 2005). Imaging was therefore repeated using the set up outlined in Figure 3.14, however no BDM was added to the culture media. Acquisition time of each Z-stack was also minimised to approximately 1 minute and frequency was increased to once every 2.5 minutes. Increasing the agarose density to 4% was also required to partially restrain the contraction and prevent the agarose dislodging due to beating.

Repeating the imaging in an unwounded Tg(mpeg1.1:mCherry); Tg(csflra:GFP) ventricle showed a striking difference in cellular behaviour compared to that shown in Figure 3.16. Characteristic of macrophage motility (Lam and Huttenlocher, 2013), many mpeg1.1+csflra+ cells could be seen to migrate large distances via the extension of large filopodial-like protrusions and commonly retaining their stretched morphology, suggesting that they are migrating within the interstitium of the dense striated myocardium or along the vasculature (yellow outlined cells, Figure 3.18; Video 5; Appendix 7.6). Some cells showed directional travel, moving out of the field of view, whereas others seemed to migrate back and forwards along the same path. However, some cells displayed limited motility throughout the duration of the timecourse but underwent constant shape changes and extended protrusions (blue and green outlined cell, Figure 3.18). mpeg1.1+ lymphoid cells also showed a pronounced difference in their migration characteristics compared to MNPs. Most of these cells remained stationary, undergoing slight shape changes and extending small protrusions, including to interact with MNPs, although several cells did rapidly migrate by the extension of small pseudopodial-like protrusions (orange outlined cell, Figure 3.18).

In an attempt to quantify the speed, directionality and cell interactions, analysis of this imaging data using a 3D tracking module on Fiji (designed by Dr Stephen Cross, University of Bristol) was established. Unfortunately, segmentation of cells proved difficult due to the abundance of overlapping cells and due to the spindly morphology of the MNPs, which confused the cell tracking software (Video 6, Appendix 7.6). Further optimisation of this
software is therefore required and may require the use of a nuclear marker to assist the separation of cells.

Due to time constraints, further repeats of this analysis were not possible and visualisation of migration in injured hearts could not be performed. However, this illustrates the extensive motility of cardiac MNPs and \textit{mpeg1.1}+ lymphoid cells and that these cells form extensive interactions with one another. This work therefore demonstrates the exciting potential of this system to interrogate the dynamics of leukocytes within the zebrafish heart.
Figure 3.18. *Ex vivo* imaging the unwounded Tg(*mpeg1.1:mCherry*); TgBAC(*csf1ra:GFP*) ventricle using spinning disk confocal microscopy. Images show max projection images at the defined time. Stacks were acquired every 2.5 minutes for 2 hours. Outlines identify single cells to demonstrate their migration/movement throughout the timecourse. Scale bar = 20 µm.
3.16 **Ex vivo culture does not maintain normal cardiac MNP dynamics**

In addition to the imaging capabilities, *ex vivo* culturing of adult zebrafish hearts would enable the study of resident cell populations without the influence of newly recruited populations. This may help to establish the dynamics of the maintenance of tissue resident populations, such as whether these cells divide and populate the tissue niche and whether these cells migrate into the tissue to maintain internal populations.

To begin to establish whether this system could support such studies, MNPs were characterised within hearts cultured for 6 hours, 1, 3 and 7 days then tissue cleared (technique described more in Results II, section 4.4) and imaged. Heart rate was also recorded as a proxy for health of the tissue. Interestingly, the MNP distribution appeared largely normal following 6 hours in culture (hc) and after 1 day in culture (dc), although populations looked slightly more sparse (Figure 3.19). Yet, from 3 dc, MNP morphology and distribution appeared vastly abnormal. Cells commonly appeared large, highly fluorescent and a matrix like structure visible in the mCherry channel appeared to cover the heart. Despite the gross aberration in MNP morphology, the hearts continued to beat indicating that cardiomyocytes were still viable, although heart rate was highly variable between individual hearts (Figure 3.19C-D).

The original publication also reported similar aberrations in heart rate, but showed that cardiac morphology is largely retained within two weeks of culture and the epicardium was unaffected by culturing (Cao and Poss, 2016). However, previous studies have not assessed the maintenance of leukocyte populations within *ex vivo* heart cultures over prolonged periods. This analysis may therefore suggest that although *ex vivo* cultures maintain selected cell populations, including cardiomyocytes, epicardial and endothelial cells (Cao and Poss, 2016, Honkoop *et al.*, 2021, Yip *et al.*, 2020) they do not support the maintenance of resident leukocyte populations.
Figure 3.19. MNP distribution in adult hearts following prolonged ex vivo culture. Whole hearts were extracted from Tg(mpeg1.1:mCherry); TgBAC(csflra:GFP) fish and maintained by ex vivo culture for up to 7 days. Hearts were fixed following 6 hours and 1, 3 and 7 days in culture, immunostained with anti-GFP and anti-mCherry antibodies used to enhance transgene fluorescence and imaged following tissue clearing. Heart rates were also recorded. A-B) Max projection of whole hearts (A) and a region within the ventricle (B) showing the morphology of GFP and mCherry fluorescence and merged channel images. C-D) Individual heart rates (C) and average heart rate (D), expressed as beats per minute (bpm) of ex vivo cultured hearts at each timepoint analysed. Star symbols indicate arrhythmic beating. Scale bars: panel A = 200 µm; panel B = 20 µm.
3.17 Chapter summary

The aim of this chapter was the to better distinguish monocytes and macrophages in the adult zebrafish. Using a combination of flow cytometry, imaging and gene expression analysis of Tg(mpeg1.1:mCherry); TgBAC(csf1ra:GFP) fish, populations of mpeg1.1+, csf1ra+, mpeg1.1+csf1radim, mpeg1.1+csf1ra+ cells were identified with distinct morphological characteristics.

Further inspection of the mpeg1.1+ population, revealed that these cells are not cells of the MNP system, rather, they show characteristics of lymphocytes and additional molecular analyses suggests they are a mixed population of B cells and NKL cells. This therefore indicates that mpeg1.1 is not exclusively a marker of MNPs in the adult zebrafish but surprisingly, the paralog of mpeg1.1, mpeg1.2, showed consistent expression in MNP populations.

Nevertheless, mpeg1.1 expression level can be used to distinguish subpopulations of csf1ra-expressing cells. Through characterisation of the dynamics of mpeg1.1±csf1ra± cells to cardiac cryoinjury, cytological analysis and interrogation of published transcriptomics data, I have proposed that mpeg1.1 expression can be used to stratify populations of csf1ra+ monocytes and mpeg1.1+csf1ra+ mature macrophages. Expression of ccr2 was also detected, indicating that a population of ccr2+ monocytes analogous to those identified in mammals, may be present. A population of mpeg1.1+csf1radim cells was also identified, and appear to show some distinction in gene expression, notably mertka, which warrants further investigation. Furthermore, I have also confirmed that mfap4 expression likely localises to global monocyte and macrophage populations, whereas marco expression may also be a determinant of macrophage differentiation.

Finally, I have also established an ex vivo imaging platform in which cardiac MNP migration can be assessed. This enabled the visualisation of cTM dynamics within the uninjured adult zebrafish heart and will facilitate the future analysis of cellular interactions that could not be performed in vivo.
4 Results II. – The role of *csf1ra* in cardiac MNP dynamics

4.1 Chapter introduction

4.1.1 Colony stimulating factor 1 receptor (Csf1r) in MNP function and maintenance

As explored in Results I, the *Csf1r* signalling pathway is highly conserved in mononuclear phagocytes of vertebrates and acts as a valuable marker of these cells (Sasmono *et al.*, 2003, Hume, 2006). *Csf1r* encodes the tyrosine kinase receptor CSF1R and has two ligands: CSF-1/M-CSF, which is found within the circulation and also produced by MNPs themselves, and IL-34, which is localised to tissues (Hume *et al.*, 2020, Stanley and Chitu, 2014). In mammals, signalling through CSF1R promotes survival, proliferation, and differentiation to the monocyte/macrophage lineages, but also promotes macrophage function, production of cytokines, chemotaxis and polarization to an M2 phenotype (Stanley and Chitu, 2014, Tushinski *et al.*, 1982, Stanley *et al.*, 1983, Stanley *et al.*, 1978).
4.1.2 CsF1r-mutation in mice

In humans, loss of CSF1R leads to neurological disorders and skeletal impairments due to the loss of tissue macrophages, and dysregulated activity can be carcinogenic and cause inflammatory disorders (Stanley and Chitu, 2014, Hume et al., 2020). To elucidate the function of CSF1R signalling, many mutant mouse lines with disrupted CSF1R signalling have been generated (Dai et al., 2002, Marks and Lane, 1976, Wiktor-Jedrzejczak et al., 1990). Due to the importance of CSF1R in normal MNP development and homeostasis, the loss of CSF1R depletes many populations of MNPs (Hume et al., 2020). However, studying the effect of mutations in Csf1, Il34 and CsF1r mutants has shown that MNPs in different tissue niches have a differential requirement for CSF1R-ligands and CSF1R itself. Unsurprisingly, complete loss of CSF1R signalling in CsF1r-/- mice elicits the most detrimental loss of MNP populations, reducing peripheral blood monocytes and the majority of tissue macrophages and DC populations. Yet some MNPs do remain, highlighting that CSF1R is not required for their generation (Dai et al., 2002, Percin et al., 2018). Furthermore, although loss of csf1 or il34 results in similar MNP deficiencies, these mutants retain selected tissue populations that are absent in CsF1r-/- mice, such as within the skin, lymphoid organs and selected DC populations, including Langerhans cells (Dai et al., 2002, Wiktor-Jedrzejczak et al., 1982, Cecchini et al., 1994, Wang et al., 2012). This demonstrates the differential reliance on CSF-1 and IL-34 for the maintenance of MNPs in different sites. However, these studies have not characterised the defects observed in cTM populations. In addition, although the remaining MNPs in CsF1r-/- mice have been shown to appear smaller and more rounded (Dai et al., 2002), the effect on cell function has not been extensively explored.

Interestingly, disrupted CSF1R signalling also causes lymphopenia, slight increases in granulocytes and anatomical defects including low body weight, impaired development and survival, osteopetrosis and deafness, which may be partly due to secondary effects caused the loss of MNPs (Marks and Lane, 1976, Dai et al., 2002, Wiktor-Jedrzejczak et al., 1982).
4.1.3 Csf1r- function in zebrafish

Due to gene duplication, zebrafish have two orthologues of Csf1r: csf1ra and csf1rb, which share approximately 50% exonic and 44% protein sequence identity with one another (Ensembl, https://www.ensembl.org/index.html), but as shown in Table 3.1 (Results I), csf1ra is predicted to be the main ortholog. Indeed, as my data and transcriptomics analysis showed, csf1ra expression is restricted to MNPs, as observed in mammals (Hume, 2006), whereas csf1rb expression is widespread across many hematopoietic lineages. Interestingly, however, zebrafish csf1ra has acquired additional expression in neural crest progenitors and mediates roles in pigmentation which is not present in mammals (Caetano-Lopes et al., 2020, Parichy et al., 2000, Parichy and Turner, 2003).

To study the contribution of these genes to zebrafish MNP homeostasis, several mutant zebrafish lines have been generated (listed in Table 7.4, Appendix 7.8). This has shown that although neither paralog is essential for the emergence of primitive macrophages (Ferrero et al., 2021), signalling through Csf1rα is required for their migration and colonisation of peripheral tissues (Herbomel et al., 2001, Caetano-Lopes et al., 2020, Wu et al., 2018). Furthermore, although csf1ra mutation only results in a slight reduction of MNPs within the CHT of larval zebrafish (thereby representing monocytes generated by definitive myelopoiesis), it results in a significant reduction in peripheral tissue macrophages and microglia, indicating that csf1ra is important for the maintenance of tissue macrophage populations (Morales and Allende, 2019, Pagán et al., 2015, Oosterhof et al., 2018, Wu et al., 2018). Despite this, microglial deficiencies observed during larval stages have been shown to be less pronounced in adult tissues, indicating recovery of these populations (Oosterhof et al., 2018). However, csf1rb mutation has been shown to cause a more deleterious effect on adult microglia than csf1ra mutation, leading to ~60% and ~20% reduction, respectively (Oosterhof et al., 2018).

More recent studies have, therefore, begun to establish to role of csf1rb in MNP homeostasis to identify its distinct role to csf1ra in zebrafish myelopoiesis. Unlike csf1ra, csf1rb is not expressed by primitive macrophages, rather showing expression in the developing heart and otic vesicle at this developmental stage (Caetano-Lopes et al., 2020). However, stable
expression is detected in sites of definitive hematopoiesis and in microglia from 48 hpf and 72 hpf, respectively (Ferrero et al., 2021). This reflects its distinct role from csf1ra as a regulator of definitive myelopoiesis (Hason et al., 2022). Thus, csf1rb has been shown to be essential for the emergence of definitive microglia that subsequently colonise the CNS and replace primitive macrophages during juvenile and adult stages (Ferrero et al., 2018), thereby providing a mechanism by which these different mutants affect microglia populations at different developmental stages (Ferrero et al., 2021). Therefore, it is thought that together, csf1ra and csf1rb fulfil the roles of mammalian Csf1r. Indeed, absence of both genes in csf1rdm mutants leads to almost complete loss of macrophages in most organs, including the CNS and skin, in both larval and adult zebrafish, mimicking the phenotype observed in Csf1r-/- mice (Kuil et al., 2020, Oosterhof et al., 2018).

4.1.4 csf1ra and tissue macrophages

As described in section 1.7, primitive macrophages have been shown to be important for seeding tissue macrophage populations, including within the heart (Hashimoto et al., 2013, Epelman et al., 2014). Due to requirement of csf1ra for the colonisation of tissues by primitive macrophages, the study of csf1ra mutants therefore provides an opportunity to study zebrafish that lack that these populations, and has the potential to elucidate how tissue macrophage populations are maintained and the requirement of these populations. Consequently, csf1ra mutants have been repeatedly used to study the effect of macrophage deficiencies in tissue repair, although largely in larval studies. This has shown that due to csf1ra mutation, macrophage recruitment to fin injury is delayed and results in impaired tail fin regeneration due to increased apoptosis and reduced cell proliferation (Morales and Allende, 2019). Similarly, csf1ra mutants display impaired angiogenesis following wounding (Gurevich et al., 2018). However, it is likely that csf1ra mutation also has effects on macrophage function, and it is hard to disentangle this from the effects of macrophage deficiency. This is supported by the observation that the remaining macrophages are less responsive to injury, more rounded, less migratory and have proliferative defects (Pagán et al., 2015, Wu et al., 2018, Morales and Allende, 2019). Interestingly, csf1ra-/- macrophages also express less tnfα compared to wild type fish following injury (Gurevich et al., 2018, Bevan et al., 2020) but show
elevated ROS and *il1b* levels and lower levels of *tgfb1a* (Morales and Allende, 2019). This could suggest that a dysregulated inflammatory environment in the absence of normal tissue macrophage frequency and function, contributes to the inefficient regeneration.

### 4.1.5 Chapter aims

Within this chapter, I therefore wanted to perform a detailed characterisation to ascertain if, and how, *csf1ra* mutation causes an MNP deficiency within the adult zebrafish heart, which has not been previously performed. It was also proposed that this may elucidate if primitive macrophage populations contribute to self-renewing cTM populations, which may have analogous pro-regenerative roles to those populations identified in mammals (Lavine *et al.*, 2014, Dick *et al.*, 2019) and be central to the favourable macrophage response seen in zebrafish.
4.2 Establishment of the \textit{csf1raj4e1/j4e1; Tg(mpeg1.1:mCherry); TgBAC(csf1ra:GFP)} line

To begin to interrogate the importance of \textit{csf1ra} in cardiac MNP homeostasis, I crossed zebrafish harnessing a \textit{csf1raj4e1} mutation to \textit{Tg(mpeg1.1;mCherry); TgBAC(csf1ra:GFP)} double transgenic fish to generate homozygous-mutant \textit{csf1raj4e1/j4e1; Tg(mpeg1.1;mCherry); TgBAC(csf1ra:GFP)} fish (Parichy et al., 2000). The \textit{j4e1} mutation is a G>A point mutation early within the first kinase domain of \textit{csf1ra} which causes a valine to methionine substitution, and has been reported to cause loss of function of Csf1r (Figure 4.1) (Parichy et al., 2000). This was confirmed by the presence of the reported pigment defect in homozygous mutant fish reported by Parichy et al (2000) (observations not shown). Although \textit{csf1raj4e1/j4e1} fish will have a loss of endogenous \textit{csf1ra} function, TgBAC\textit{(csf1ra:GFP)} expression will still be possible, however the number of GFP-expressing cells and relative GFP expression is likely to be altered by the mutation.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{signal_sequence.png}
\caption{Exonic gene sequence of zebrafish \textit{csf1ra}, identifying the location of the \textit{j4e1} point mutation. This mutation results in a valine to methionine substitution at position 614 in Csf1r protein. Figure adapted from Parichy et al (2000).}
\end{figure}
4.3 **Adult csf1ra^{4e1/j4e1} zebrafish retain MNPs on the surface of the ventricle**

Various studies have documented dramatic differences of the effect of *csf1ra* mutation on tissue specific MNP populations in larval and adult zebrafish (Morales and Allende, 2019, Pagán *et al.*, 2015, Oosterhof *et al.*, 2018), and *csf1ra^{4e1/j4e1}* mutants have been visualised to have fewer *mpeg1.1*-expressing cells within the heart following cardiac cryoinjury (Bevan *et al.*, 2020). However, no in-depth characterisation has been performed on the adult zebrafish heart.

Whole mount imaging of Tg(*mpeg1.1:mCherry*); TgBAC(*csf1ra:GFP*) ventricles in the wild type and *csf1ra^{4e1/j4e1}* background revealed *csf1ra*+ and *mpeg1.1+csf1ra*+ cells with a stellate morphology typical of MNPs on the surface of the ventricle of all fish examined (Figure 4.2AB), as previously described for wild type ventricles (Figure 3.2; Results I). Quantification of *csf1ra:GFP*-expressing MNPs, which I have previously established to be an inclusive marker of MNPs unlike *mpeg1.1:Cherry*, showed that there was only a small, but non-significant, reduction of MNPs on the surface of *csf1ra^{4e1/j4e1}* ventricles compared to wild type controls (Figure 4.2C).
Figure 4.2. Analysis of uninjured csf1ra^{j4e1/j4e1}; Tg(mpeg1.1:mCherry); TgBAC(csft1ra:GFP) adult ventricles. A) Maximum projections of endogenous csf1ra:GFP; mpeg1.1:mCherry fluorescence in wild type and csf1ra^{j4e1/j4e1} adult zebrafish hearts. Dashed lines demarcate the ventricle. Scale bar = 100 µm. B) Representative zoomed images of labelled cells within the ventricle. C) Automated quantification of csf1ra:GFP+ cell number per 1000 µm² of ventricle area. N = 5 per genotype, one experimental replicate. Statistical analysis was performed by Kruskal-Wallis test.
4.4  *csf1ra*<sup>jke1/jke1</sup> ventricles lack the majority of cTMs in the centre of the heart

As *csf1ra* mutation has been shown to only cause slight overall reductions of microglia in the adult zebrafish brain, but causes abnormalities in the microglia distribution (Oosterhof et al., 2018), the distribution of cTMs within the deep myocardium was assessed. As these cells would not be visualised by conventional wholemount imaging due to limited imaging depth (as shown in Figure 4.2) methods to optically clear zebrafish were explored to image these MNP's throughout the heart.

To establish the best method to preserve integrity of the tissue and transgenic fluorescence, two previously described methods were trialled: the Clear, Unobstructed Brain/Body Imaging Cocktails and Computational analysis (CUBIC) method (Susaki et al., 2015) and Clearing-enhanced 3D (Ce3D) (Li et al., 2019). Trials of these methods on Tg(*mpeg1.1:mCherry*); Tg(*fli1:GFP*) hearts (*fli1:GFP* labels the vasculature) showed that transgenic fluorescence was preserved using both methods, however, Ce3D rendered the heart much more transparent than CUBIC, enabling precise visualisation of the fluorophores throughout the myocardium.

Figure 4.3. Trial of CUBIC and Ce3D tissue clearing on adult zebrafish hearts. Tg(*mpeg1.1:mCherry*); Tg(*fli1:GFP*) hearts were optically cleared using Ce3D and CUBIC methodologies. A) Single channel and merged channel images of a single optical section showing a cross-section of the heart. B) Maximum intensity projection of the cleared heart. Scale bar = 200 µm.
Utilising Ce3D tissue clearing to assess hearts from Tg(mpeg1.1;mCherry); TgBAC(csf1ra:GFP) and csf1raj4e1/j4e1; Tg(mpeg1.1;mCherry); TgBAC(csf1ra:GFP) fish, MNPs could be seen on the surface of the heart and distributed throughout the ventricle in wild type hearts (Figure 4.4A; Video 7, Appendix 7.6). As observed in Figure 4.2, MNPs were also present on the surface of csf1raj4e1/j4e1 ventricles (Figure 4.4B, 0-100 µm), yet, there was striking absence of csf1ra+mpeg1.1± MNPs at depths of 100-400 µm (Figure 4.4BC; Video 8, Appendix 7.6). mpeg1.1:mCherry expression was also lower in MNPs of csf1raj4e1/j4e1 fish: 8.4 ± 5.26 % of total csf1ra:GFP expressing cells were classified as mpeg1.1+ cells in wild type fish compared to 67.9 ± 17.0 % in csf1raj4e1/j4e1 mutants (Figure 4.4C). Thus, total counts throughout the ventricle showed that csf1raj4e1/j4e1 ventricles had a 76% increase in csf1ra+ cells (wild type = 15.7 ± 7.8 cells per region; csf1raj4e1/j4e1 = 27.8 ± 9.4), but a 76% decrease in mpeg1.1+ csf1ra+ cells (wild type = 184.0 ± 34.9 cells per region; csf1raj4e1/j4e1 = 20.0 ± 20.0). Interestingly, mpeg1.1+ lymphoid cells retained strong expression of mpeg1.1:mCherry and frequency was only reduced by 27%, but cell counts were highly variable in both groups (wild type = 140.3 ± 65.0 cells per region; csf1raj4e1/j4e1 = 102.0 ± 27.8). This suggests that the maintenance of mpeg1.1 populations is not affected by csf1ra mutation or affected to a lesser extent.

It is worth noting, that there were significantly less mpeg1.1+ csf1ra+ and total csf1ra-expressing cells at all depths including on the surface of the heart (Figure 4.4C), which was not observed in Figure 4.2. This is likely to be due to the low resolution and tissue penetration of images in Figure 4.2 which only captures the cells on the very surface of the ventricle, whereas Figure 4.4 captures cells within the first 100 µm. Furthermore, images in Figure 4.4 were also acquired in tissue where the fluorescence signal was amplified with the use of fluorescent antibodies, unlike in Figure 4.2. It is also interesting that mpeg1.1+ lymphoid cells showed the inverse distribution compared to MNPs, showing increased frequency with increased depth in both wild type and csf1raj4e1/j4e1 mutants. This could suggest that these cells are preferably distributed within the trabeculae, or this could be as a result of more cells being within the lumen within deep regions of the heart.

Collectively, this shows that although csf1ra mutants retain MNPs on the surface of the heart, they have a striking absence of MNPs deep within the myocardium and within the trabeculae.
This could indicate that csf1ra is required for the establishment of trabecular cTM populations by primitive macrophages, but surface MNPs are retained independently of these populations, perhaps by constant replenishment by monocytes. Fittingly, as the remaining csf1ra<sup>j4e1/j4e1</sup> MNPs show an increased proportion of csf1ra+ cells, which I have proposed to be monocytes/MDMs, this could also support this hypothesis. Interestingly, embryonic and definitive cTM populations are spatially distinct in the murine heart. Embryonic CCR2- tissue resident and definitive CCR2+ MDMs populations have been identified to localise to the compact myocardium and the trabecular myocardium, respectively (Leid et al., 2016). As csf1ra mutants appear to lack trabecular MNPs, this may suggest that zebrafish have similar, spatially distinct sub-populations which have distinct ontogenies and possess unique functions. However, due to the loss of trabecular cTMs in the csf1ra<sup>j4e1/j4e1</sup> mutants, this distribution is the inverse of what is observed in mice.

Nevertheless, as csf1ra mutation may also affect other aspects of MNP homeostasis, including migration, survival or proliferation (Stanley and Chitu, 2014), further investigation of the cause of this deficiency is required.
Figure 4.4. Whole mount imaging in cleared, uninjured wild type and csf1ra/j4e1 Tg(mpeg1.1:mCherry); TgBAC(csfr1a:GFP) ventricles. Transgenic fluorescence was detected using fluorescently labelled anti-GFP and anti-mCherry antibodies. Z-stacks were acquired in the middle of the ventricle starting from the surface of the ventricle to a depth of 400 µm within the myocardium, reaching the trabeculae but not the lumen. A-B) Images are a single, representative Z-slice within the defined depth. Insets show enlarged regions of boxed cells. Scale bar = 20 µm. C) The Z-stack was divided into 200 x 200 x 100µm (xyz) sub-stacks and csf1ra:GFP± mpeg1.1:mCherry± cells were manually counted using the Cell Counter plugin on Fiji following blinding of datasets. Graph shows mean frequency ± SD of each population per 100 µm sub-stack. Analysis of 3 fish per genotype, one experimental replicate. Statistical analysis was performed on mean frequency per fish by two-way ANOVA with Šidák’s multiple comparisons test. Graphs show statistically significant p values.
4.5 Few cells are actively proliferating within the adult heart

To determine if MNP proliferation was affected in \textit{csf1ra} mutants, wild type and \textit{csf1ra^{kle/jcl}} Tg\textit{(mpeg1.1:mCherry)}; TgBAC\textit{(csf1ra:GFP)} hearts were examined for phospho-histone H3 (PH3) expression to detect cells that were actively dividing throughout the ventricle. Proliferative cells within the ventricle were rare and no PH3+ MNPs were observed in either wild type or \textit{csf1ra^{kle/jcl}} fish (Figure 4.5). This indicates that the proliferation of all cells is very low within the ventricle, including within the MNP compartment.

Interestingly, preliminary data in wild type Tg\textit{(mpeg1.1:mCherry)}; TgBAC\textit{(csf1ra:GFP)} fish treated with EdU for 24 hours showed that MNPs near the surface of the ventricle were labelled for EdU incorporation, suggesting they had proliferated within 24 hours prior to harvesting (Figure 4.6). The majority of these EdU+ MNPs were \textit{mpeg1.1+csf1ra+} cells, rather than \textit{csf1ra+}, and appeared ramified, indicating that they were within the tissue. This suggests that either these cells had proliferated within the tissue, or had recently been recruited from proliferating monocytes (Zhu \textit{et al.}, 2009). Unfortunately, due to time constraints, further repeats and quantification of this analysis in both wild type and \textit{csf1ra} mutants could not be performed, but this demonstrates that labelling technique would help establish whether \textit{csf1ra} mutants have a proliferation defect.

In an attempt to investigate whether increased apoptosis contributed to the absence of MNPs in \textit{csf1ra^{kle/jcl}} hearts, I trialled the use of cleaved caspase-3 antibodies, phospho gamma H2A.X antibodies and TUNEL staining in attempt to quantify cell death. However, I was not able to successfully use these techniques in wholemount staining therefore this could not be assessed.
Figure 4.5. Proliferating cells in cleared, uninjured wild type and *csf1ra*<sup>j4e1/j4e1</sup> ventricles. Unwounded wild type and *csf1ra*<sup>j4e1/j4e1</sup> Tg(*mpeg1.1:mCherry); TgBAC(*csf1ra:GFP*) ventricles were stained with anti-GFP, anti-mCherry and anti-phospho-histone H3 antibodies to label cells undergoing mitosis and Z-stacks were acquired through a 400 µm depth into ventricle. Image shows a single optical plane where a proliferating cell was visible. Inset show rare PH3+ cells, however these did not co-localise with *mpeg1.1±csf1ra±* cells. Representative of n = 2. One experimental replicate. Scale bars: 20 µm; inset = 5 µm.
Figure 4.6. Proliferating cTMs in the wild type ventricle determined by EdU labelling. Uninjured wild type Tg(mpeg1.1:mCherry); TgBAC(csf1ra:GFP) fish were treated with EdU 24 hours prior to harvesting. EdU incorporation was labelled by AF647 and transgenic fluorescence was enhanced with anti-GFP and anti-mCherry antibodies. Z-stacks were acquired through a 300 µm depth into ventricle. Image shows a maximum projection of the defined depth. Arrowheads identify EdU+ mpeg1.1+csf1ra+ cells; arrow shows an example of an EdU+csf1ra+ cell. Inset shows an enlarged example of an EdU+ MNPs. Scale bars: 20 µm; inset = 5 µm.
4.6 \textit{csf1ra}^{j4e1/j4e1} MNPs migrate during \textit{ex vivo} imaging

Next, to establish whether \textit{csf1ra}^{j4e1/j4e1} MNPs had an overt migration defect which may impair distribution, I utilised the \textit{ex vivo} imaging platform to visualise MNP migration in unwounded \textit{csf1ra}^{j4e1/j4e1} ventricles (Figure 4.7; Video 9, Appendix 7.6). As observed in Figure 4.4, a large proportion of the labelled cells were \textit{csf1ra}+, unlike in wild type fish whereby the majority of cells were \textit{mpeg1.1}+\textit{csf1ra}+ (Figure 3.3, Results I), although \textit{mpeg1.1}+\textit{csf1ra}+ MNPs and \textit{mpeg1.1}+ cells were also abundant (Figure 4.7). Similar to wild type hearts, cells underwent constant shape changes and/or migration, however, the extent of which was highly cell-specific; some cells migrated considerable distances (pink and green cell, Figure 4.7) whereas others did not migrate at all but continuously extended protrusions (yellow cell, Figure 4.7). Interestingly, several \textit{csf1ra}+ cells had a more rounded appearance potentially supporting their monocytic origin, however further repeats would be required to confirm this.

This demonstrated that \textit{csf1ra}^{j4e1/j4e1} MNPs could migrate laterally across the ventricle as observed in wild type fish. Transverse migration, which would be required to colonise the myocardium, was not observed, however this was also not observed in wild type fish (Figure 3.18, Results I). Therefore it is not possible to discern from this limited dataset how this may be affected. Further optimisation and validation of this platform, alongside repeats and longer timecourses, is therefore required to establish this \textit{ex vivo} system, but does provide evidence that \textit{csf1ra} MNPs similarly patrol the surface of the ventricle as observed in wild type fish.
Figure 4.7. *Ex vivo* imaging in the unwounded *csf1ra*<sup>iklikie</sup>; Tg(*mpeg1.1:mCherry); TgBAC(*csf1ra*:GFP) ventricle. Imaging was performed immediately after extraction of the heart using the optimised, BDM-free protocol on the spinning disk system (section 3.15). Panel shows snapshots of the single and merged channels at the defined timepoint throughout a 2 hour timelapse. Coloured outlines each follow a single cell to demonstrate that cells were moving and migrating. Pink and green outlines highlight a *csf1ra*+ MNP, whereas yellow and blue outlines highlight a *mpeg1.1*+*csf1ra*+ MNP. One single heart from one experimental replicate. Scale bar = 20 µm.
4.7 **csf1ra-mutants have a diminished MNP response to cardiac injury**

From the analysis in uninjured fish, it was difficult to ascertain how *csf1ra* mutation might be causing the altered MNP distribution. I therefore sought to establish whether MNP expansion and distribution was also affected in *csf1ra* mutants following cardiac injury, which may help elucidate how MNP dynamics are impaired. Therefore, to firstly assess the precise dynamics of MNPs in *csf1raj4e1/j4e1* fish following cardiac cryoinjury, flow cytometry of wild type and *csf1raj4e1/j4e1* Tg(*mpeg1.1:mCherry*); TgBAC(*csf1ra*:GFP) ventricles was performed on ventricles in the uninjured state and over the two weeks post-injury, the timeframe in which MNPs accumulate and dissipate in wild type fish (Figure 3.6, Results I). As expected from imaging data, analysis of uninjured wild type and *csf1raj4e1/j4e1*; Tg(*mpeg1.1:mCherry*); TgBAC(*csf1ra*:GFP) fish demonstrated that *csf1raj4e1/j4e1* ventricles had a pronounced deficiency of *csf1ra*-expressing MNPs (Figure 4.8A). *mpeg1.1+csf1ra+* cells comprised 2.7 ± 2.6 SD percent of live cells in wild type ventricles but only 0.18 ± 0.12 SD in *csf1raj4e1/j4e1* ventricles, although a similar frequency of *csf1ra+* cells was observed in wild type and *csf1raj4e1/j4e1* ventricles (0.13 ± 0.16 SD and 0.11 ± 0.12 SD percent of live cells, respectively). The relative proportions of *csf1ra+* and *mpeg1.1+csf1ra+* cells also supported that *mpeg1.1* expression is reduced upon loss of *csf1ra* (Figure 4.8B). Only 28.8 % ± 15.7 SD of labelled cells were *mpeg1.1+ csf1ra+* in *csf1ra* mutants compared to 68.3 % ± 7.7 SD in wild type fish (Figure 4.8B, Figure 4.9A). The frequency of *mpeg1.1+* lymphoid cells was unchanged in *csf1raj4e1/j4e1* ventricles.
Figure 4.8. Analysis of uninjured \textit{mpeg1.1±} and \textit{csf1ra±} populations of the uninjured wild type and \textit{csf1ra^{j4e1/j4e1}} ventricle. Data also represented in Figure 4.9. A) Frequency of each cell population expressed as a percentage of total, live, single cells. B) Relative proportions of each population expressed as a percentage of total labelled cells. Statistical significance determined by Multiple Mann-Whitney tests. Graphs show p values. Wild type: n = 5; \textit{csf1ra^{j4e1/j4e1}}: n = 4.
Following injury, csf1ra± mpeg1.1± cell populations in wild type fish displayed a similar profile to the previous analysis presented in Figure 3.6 (Results I) (Figure 4.9A-D). At 3 dpi, there was an increase in csf1ra+ and mpeg1.1+csf1ra+ cells, with mpeg1.1+csf1ra+ cells remaining elevated at 7 dpi, then returning to uninjured levels by 14 dpi. Interestingly, csf1ra+ cells in csf1raj4e1/j4e1 fish showed a similar frequency and trend to wild type controls throughout the timecourse (Figure 4.9A, D-E). However, although csf1ra mutants did show a slight increase in mpeg1.1+csf1ra+ cells at 3 dpi, cell numbers did not continue to increase, remaining at approximately 1.5% of live cells throughout the timecourse. The frequency of mpeg1.1+csf1ra+ cells in csf1raj4e1/j4e1 fish therefore remained between 4-6 fold lower than that of wild type fish between 1 and 7 dpi and showed a significantly reduced frequency at 7 dpi (7.84 ± 1.02 SD percent of live cells in wild type fish compared to 1.41 ± 0.91 SD in csf1raj4e1/j4e1). This is concordant with previous data from the lab which showed that csf1ra mutants have fewer mpeg1.1+ cells on the surface of the ventricle at 3 and 7 dpi (Bevan et al., 2020). Collectively, this indicates that csf1raj4e1/j4e1 fish have an impaired ability to expand MNP populations following cardiac injury and a larger proportion of MNPs attending the wound site are mpeg1.1-.

Looking to the mpeg1.1+ lymphoid population, csf1ra mutants showed a reduced frequency compared to wild type fish at 1 dpi, but numbers expanded at 3 and 7 dpi. However, by 14 dpi, levels declined, unlike in wild type controls. This could indicate that loss of csf1ra may directly or indirectly, via the loss of MNPs which are important for priming B cell function and expansion (Craxton et al., 2003), affect the response of mpeg1.1+ lymphoid cells to tissue injury. Nevertheless, further repeats of this data would be required to confirm the exact dynamics of the mpeg1.1+ lymphoid population as counts were highly variable at some timepoints.
Figure 4.9. Ventricular mpeg1.1±csf1ra± dynamics in wild type and csf1ra<sup>ie<sub>1j<sub>1</sub></sup> following cardiac cryoinjury determined by flow cytometry. A-C) Frequency of each subpopulation expressed as a percentage of live, single cells in wild type and csf1ra<sup>ie<sub>1j<sub>1</sub></sup></sup> ventricles. Multiple Mann-Whitney tests with Bonferroni-Dunn multiple comparisons test was used to determine significant differences between wild type and mutant fish at each timepoint for each subpopulation. Graphs show adjusted p values. D-E) Representative plots of GFP and mCherry expression (top row) and the associated FSC and SSC profiles of each population (bottom row) in wild type (D) and csf1ra<sup>ie<sub>1j<sub>1</sub></sup></sup> ventricles at each timepoint analysed. Each plot is the analysis of a single ventricle. One experimental replicate for each genotype. N numbers (wild type/cs<sup>ie<sub>1j<sub>1</sub></sup></sub>): Uninjured = 5/4; 1 dpi = 3/4; 3 dpi = 8/3; 7 dpi = 3/7; 14 dpi = 2/3. Uninj = uninjured.
4.8 **Distribution of injury associated MNPs is altered in csf1raj4e1/j4e1 mutants**

To assess whether MNP distribution and proliferation was affected in csf1ra mutants following injury, PH3 staining and tissue clearing was repeated in ventricles at 1 and 7 dpi. These timepoints were chosen to visualise the initial recruitment of monocytes and at the timepoint where peak MNP frequency is observed within the wild type ventricle, respectively.

At 1 dpi, many MNPs could be visualised across the surface of the wild type ventricle, with more cells present within a localised region, indicating the site of injury (Figure 4.10Ai; Video 10, Appendix 7.6). An accumulation of MNPs was also observed at the site of the ventricle in csf1ra mutants, however, fewer MNPs were observed on the uninjured surface of the ventricle making the injury site very distinct, unlike in wild type fish (Figure 4.11Bi; Video 11, Appendix 7.6).

To visualise the distribution of MNPs within the myocardium, both within the injury site and in the adjacent uninjured tissue, higher resolution imaging at the border zone to the injury was performed (Figure 4.10ABii, iii). This showed that MNPs were infiltrating the wound in both wild type and csf1raj4e1/j4e1 ventricles, however, the majority of csf1raj4e1/j4e1 MNPs appeared on the surface of the injury. This difference was further accentuated at 7 dpi. In wild type ventricles, a dense infiltration of MNPs was observed throughout the wound site, whereas MNPs remained within the upper myocardium of csf1raj4e1/j4e1 ventricles, with a very sparse infiltration within the tissue (Figure 4.10C-D; Video 12-13, Appendix 7.6). Reduced mpeg1.1 expression in csf1raj4e1/j4e1 fish was also evident at both 1 and 7 dpi, with the majority of mpeg1.1-expressing cells resembling the lymphoid population, unlike the numerous stellate mpeg1.1+csf1ra+ MNPs visible within injury of the wild type fish. Strikingly, a uniform distribution of MNPs was retained in the uninjured myocardium of wild type fish at both 1 and 7 dpi, and did not appear to be proliferating, indicating that these cTMs do not migrate and redistribute to the injury site (Figure 4.10AC).

Unlike in uninjured hearts, where actively proliferating cells were virtually absent (Figure 4.5), numerous proliferating cells, including MNPs, were present in both wild type and csf1raj4e1/j4e1 ventricles (Figure 4.10C). PH3 labelling was identified in both csf1ra+ and mpeg1.1+csf1ra+ MNPs, but was rare in mpeg1.1+ cells. A similar frequency of proliferating
csf1ra+ cells was observed in both genotypes, yet although proliferating mpeg1.1+csf1ra+ cells were present in wild type ventricles, few were present in csf1ra mutants. Due to the abundance of cells within the region, it was challenging to accurately quantify MNP number from these images. However, comparing this data to flow cytometric quantification at these timepoints (Figure 4.9), where we see a similar frequency of csf1ra+ cells, yet a deficiency of mpeg1.1+csf1ra+ cells in csf1ra<sup>del/del</sup> ventricles, could suggest that csf1ra<sup>del/del</sup> MNP cells have a similar ability to proliferate, yet lower cell numbers are responsible for the reduced proliferation rate observed.
Figure 4.10. Distribution of *csf1ra±* and *mpeg1.1±* cells in wild type and *csf1raj4e1/j4e1* ventricles at 1- and 7-days post cardiac cryoinjury. Injured Tg(*mpeg1.1*:mCherry); TgBAC(*csf1ra*:GFP) hearts were fluorescently stained with anti-GFP, anti-mCherry and anti-PH3 antibodies and optically cleared to visualise the distribution of labelled cells throughout the ventricle. 500 µm Z-stacks were acquired for the whole heart and at the border zone of the injury. A-D) Representative single channel and merge channel images of wild type (A, C) and *csf1raj4e1/j4e1* (B, D) hearts at 1 (A-B) and 7 (C-D) dpi. Row i) Maximum intensity projection of whole ventricle. Dashed line identifies the ventricle. Inset shows region shown in row ii and iii. Row ii, iii) Maximum intensity projection acquired at the border zone of the injury showing the whole stack (ii) and at a 200-400 µm depth to show cells within the myocardium. Insets show examples of proliferating MNPs identified by the boxes. E) Quantification of proliferating (PH3+) cells within each genotype at each timepoint. The total number of PH3+ cells were manually counted, grouped by their co-expression with *mpeg1.1* and *csf1ra*, per 580 µm x 580 µm x 500 µm (xyz) stack acquired at the border zone. Statistical analysis was performed by multiple Mann Whitney tests for each population at each timepoint but showed no significant difference. One experimental replicate. N = 3 per condition. Scale bars: row i) = 200 µm; row ii, iii) = 100 µm.
4.9 $\text{Csf1ra}^{\text{Het}/\text{Het}}$ MNPs retain markers of MNPs

Given the distribution and $\text{mpeg1.1}$ expression defects presented by $\text{Csf1ra}^{\text{Het}/\text{Het}}$ MNPs, the expression of typical MNP and pro-inflammatory markers was assessed in $\text{csf1ra}^{\text{Het}/\text{Het}}$ MNPs to identify whether these cells showed any overt differences in characteristic MNP gene expression which may indicate a differentiation defect. $\text{csf1ra}^+$, $\text{mpeg1.1}+\text{csf1ra}^\text{dim}$ and $\text{mpeg1.1}+\text{csf1ra}^+$ populations were sorted from pooled wild type and $\text{csf1ra}^{\text{Het}/\text{Het}}$ ventricles and atria (in order to gain the maximum cell yields) for RT-PCR analysis.

The expression profile of $\text{csf1ra}^{\text{Het}/\text{Het}}$ MNPs cells was highly similar to that of wild type cells (Figure 4.11C). All populations showed some expression of $\text{csf1ra}$, $\text{csf1rb}$, $\text{mpeg1.2}$, $\text{mfap4}$, $\text{marco}$ and $\text{mmd}$. As expected $\text{mpeg1.1}$ was not expressed by the $\text{csf1ra}^+$ populations. Interestingly, $\text{marco}$ and $\text{mpeg1.1}$ expression, which is suggested to be a marker of more differentiated macrophages, was highly expressed by $\text{mpeg1.1}+\text{csf1ra}^+$ populations in both wild type and $\text{csf1ra}$ mutants, suggesting that these cells establish a mature phenotype. As observed in Figure 3.10 (Results I), the phagocytosis marker, $\text{mertka}$, was expressed by $\text{csf1ra}^+$ and $\text{mpeg1.1}+\text{csf1ra}^+$ cells but was again absent in the $\text{mpeg1.1}+\text{csf1ra}^\text{dim}$ population in both genotypes, perhaps suggesting a distinct function and profile of this population. Expression of the cell adhesion molecule $\text{itgam}$ was also considerably higher in all $\text{csf1ra}$ mutant populations compared to wild type. Due to the defective tissue infiltration phenotype observed, this may be a compensatory mechanism to increase cell adhesion. Oosterhof et al (2018) similarly show upregulation of leukocyte migration and chemotaxis genes in $\text{csf1ra}$ mutant adult microglia, although interrogation of their transcriptomics data showed no increase in $\text{itgam}$. $\text{timd4}$ expression was also detected in $\text{mpeg1.1}+\text{csf1ra}^+$ populations from wild type and mutants. As previously described, $\text{timd4}$ has been shown to be a marker of resident cardiac macrophages in mice (Dick et al., 2019), showing that some conservation of this marker is seen in zebrafish and requires further investigation.

Intriguingly, all MNP populations also showed significant expression of $\text{il1b}$ and $\text{tnfa}$, known MNP-expressed pro-inflammatory genes, suggesting that resident cardiac MNPs are capable of producing pro-inflammatory mediators, both in wild type and $\text{csf1ra}$ mutants. $\text{csf1ra}$ mutants have been repeatedly shown to produce lower levels of $\text{tnfa}$ (Bevan et al., 2020,
Gurevich et al., 2018, Tsarouchas et al., 2018). The high expression of these mediators in unwounded tissue is also intriguing, although we cannot disregard that the cell sorting process may induce cell stress that may alter such responsive genes.

Collectively, this analysis indicates that \textit{csf1ra}^{j4e1/j4e1} cardiac MNPs possess a largely similar MNP-expression profile to wild type controls and indicates that \textit{csf1ra} mutation does not completely abrogate MNP differentiation of the remaining cells. However, relative gene expression analysis by qPCR or transcriptomic analysis would be essential to determine whether slight alterations are observed.

It is also worth noting that \textit{csf1ra} expression was detected in all \textit{csf1ra}^{j4e1/j4e1} MNP populations, despite the presence of the mutation. Cross-checking of the primer binding site shows that the amplification occurs downstream of the mutation site and is within the first kinase domain (Figure 4.1). This indicates that \textit{csf1ra} transcripts may not be completely degraded in \textit{csf1ra}^{j4e1/j4e1} fish as a result of the mutation, which is surprising given that significantly reduced \textit{csf1ra} expression was reported in larval fish from this mutant strain by Parichy et al (2000). However, as it is only a single substitution, this could suggest that the mutation manifests at the protein level. Alternatively, it is possible that a transcript could be produced from the \textit{csf1ra} transgene, which is generated from a BAC, although this is unlikely considering the insertion of \textit{gfp} likely disrupts downstream transcription.
Figure 4.11. Characterisation of MNP and inflammatory marker expression in MNPs isolated from unwounded wild type and csf1ra\textsuperscript{4e1/j4e1} Tg(mpeg1.1:mCherry); TgBAC(csf1ra:GFP) hearts. A-B) FACS plots of fluorescence intensity of labelled cells showing the gating strategy for csf1ra\textsuperscript{+}, mpeg1.1\textsuperscript{+} csf1ra\textsuperscript{dim} and mpeg1.1\textsuperscript{+} csf1ra\textsuperscript{+} populations and their associated FSC/SSC profile in wild type (A) and csf1ra\textsuperscript{4e1/j4e1} (B) hearts. C) RT-PCR analysis of sorted populations using the MNP-specific markers explored in Results I. cDNA generated from whole kidney marrow was used as a positive control; water was used for the negative control. Cells were sorted from pools of 15 hearts for each group. One experimental replicate. Cell numbers (WT/csf1ra\textsuperscript{4e1/j4e1}): csf1ra\textsuperscript{+} = 3300/3400; mpeg1.1\textsuperscript{+} csf1ra\textsuperscript{dim} = 4900/2400; mpeg1.1\textsuperscript{+} csf1ra\textsuperscript{+} = 30,000/12,000; background cells = 250,000.
4.10 **Injury size is reduced in \textit{csf1ra}^{j4e1/j4e1} hearts**

Macrophages have important roles in activating collagen secretion by fibroblasts and have also recently been shown to directly contribute to scar deposition (Simões \textit{et al.}, 2020, Frangogiannis, 2012). Furthermore, the Richardson lab have also shown that \textit{csf1ra}^{j4e1/j4e1} fish have reduced collagen I deposition following cardiac cryoinjury (Bevan \textit{et al.}, 2020). Given the drastic deficiency and reduced infiltration of MNPs into the injury site observed in \textit{csf1ra}^{j4e1/j4e1} fish, their scarring response was further investigated.

To assess collagen deposition more broadly, Acid Fuschin Orange G (AFOG) histological staining, which stains all collagen types, was performed in wild type and \textit{csf1ra}^{j4e1/j4e1} zebrafish at 14 and 21 dpi (Figure 4.12). At 14 dpi, regeneration and scar removal have been shown to begin (Chablais and Jaźwińska, 2012), therefore it was hypothesised that assessment at these timepoints may identify whether \textit{csf1ra}^{j4e1/j4e1} hearts have less collagen deposition initially, and/or whether scar resolution is altered. At 14 dpi, an infiltration of purple collagen staining was observed in the injury site, lined by a layer of orange-stained fibrin on the exterior in both wild type and \textit{csf1ra}^{j4e1/j4e1} hearts (Figure 4.12), as expected (Chablais \textit{et al.}, 2011). The injury site had a similar composition at 21 dpi in wild type hearts, although injury size appeared contracted (Figure 4.12A), indicating that regeneration was occurring. However, fibrin was only visible in 25% of \textit{csf1ra}^{j4e1/j4e1} hearts, with a collagenous scar still visible. No significant difference in injury size between wild type and \textit{csf1ra}^{j4e1/j4e1} fish was observed, although results were variable and \textit{csf1ra}^{j4e1/j4e1} fish showed a slight reduction in injury size (Figure 4.12B). Quantification of collagen area was also performed by thresholding on the purple hues within the ventricle using Fiji software. However, due to low n numbers and high variability (discussed further below), it was difficult to discern whether there was a quantifiable difference between groups.

Nevertheless, visual comparison and similar injury sizes indicated that there was no obvious phenotype in \textit{csf1ra}^{j4e1/j4e1} fish at these timepoints and confirmed that \textit{csf1ra} mutation, and the resulting MNP deficiencies, does not abrogate bulk scar deposition within the injury site. An additional timepoint at 28 dpi was therefore performed to assess the continued progression of cardiac regeneration and collagen resolution in \textit{csf1ra}^{j4e1/j4e1} mutants. Interestingly, injury
size was similar to that at 21 dpi in csf1ra mutants, yet wild type controls showed an unexpected increase in injury size and severity, indicating a failure of regeneration and/or adverse response to injury (Figure 4.12AB). As injury size appeared to reduce from 14 to 21 dpi, it was thought that underlying health problems with the wild type control fish, which were age-matched but not siblings of csf1ra mutants, was likely the cause of this adverse result. Injuries were therefore repeated in wild type fish at 28 dpi and plotted alongside the original results. Unfortunately, this could not be repeated in csf1raj4e1/j4e1 fish due to the lack of appropriately aged fish. Injury size remained similar to that observed at 21 dpi in wild types, likely as a result of the unavoidable biological variation and slightly bigger injury size in the repeats. Nevertheless, csf1raj4e1/j4e1 mutants did consistently have a smaller injury size at all timepoints, although, due to weak staining intensity, collagen measurements also could not be accurately measured from the repeats. Additional replicates would therefore be required to confirm that csf1ra mutants have altered scar dynamics and injury size, but this analysis does fit with previous observation of reduced collagen I deposition (Bevan et al., 2020).

However, although analysis of regeneration by histological means can provide valuable qualitative insight into the tissue morphology, reliable quantification of this analysis can be challenging. Slight variations in the orientation of the injury site and angle of the heart during sectioning can result in large alterations in the visualisation/representation of the injury site within sections. This could be partly overcome by the quantification of many sections throughout the injury site to build a 3D representation of the injury size but introduce practical challenges and requires a large amount of sections to be analysed. Furthermore, batch to batch variation can also make it difficult to compare samples. Slight, unavoidable differences in the success of tissue preservation (e.g. contraction or wrinkling of the tissue during processing) or staining intensity can cause large variations in the measurement of collagen staining. Therefore slight alterations in phenotype may not be possible to resolve using this readout. Utilising immunofluorescent staining and 3D imaging of collagen on cleared tissue may therefore help to establish whether there is a later collagen defect in csf1ra mutants.
Figure 4.12. Histological staining for collagen and fibrin deposition in wild type and csf1ra mutant hearts at 14, 21 and 28 dpi. A) Representative images of an AFOG stained section in each group showing the composition of the injury site. Collagen staining is shown in purple, fibrin staining is shown in orange/yellow, myocardium is stained in light orange and nuclei are stained black. B-C) Quantification of injury area (B) and collagen area (C), represented as a percentage of the ventricle area. For 14 and 21 dpi timepoints, values were averaged from 3 slides whereas measurements were only taken from a single slide for 28 dpi. Repeats for the 28 dpi timepoint are shown by triangles. N numbers (wild type/csfr1ra^J4e1J4e1): 14 dpi = 3/6; 21 dpi = 4/4; 28 dpi = 8/6.
4.11 Chapter summary

This work has shown that *csf1ra* mutation causes a profound reduction in cardiac MNPs in the adult zebrafish, both within the intact heart and following cardiac cryoinjury. This was characterised by a large deficiency within the trabecular compartment and the remaining macrophages also show a striking reduction in *mpeg1.1* expression, potentially indicating a differentiation defect. My analysis is indicative that the altered distribution is due to impaired migration, as although MNPs accumulate at the wound site on the surface of the injury, they fail to invade the injury site as observed in wild type fish. Analysis of proliferation also suggests that this is unaffected in *csf1raj4e1/j4e1* fish, but due to the inability to quantify cell death, reduced cell survival could also contribute to MNP deficiency. Interestingly, the remaining MNPs in *csf1raj4e1/j4e1* fish expressed typical MNP markers, however more sensitive analysis would need to be performed to establish whether changes in expression levels were observed.

3D imaging analysis in the wild type heart has also provided new insights into how cTMs are maintained and how monocyte populations reach the wound site. The distribution of cells deep within the myocardium appear to be replenished by cells on the surface of the heart, both in the steady state and following injury. Furthermore, cardiac MNPs within the trabecular myocardium adjacent to the injury maintain their distribution throughout the myocardium and were not observed to proliferate, suggesting that these cells do not significantly contribute to the accumulation of cells at the injury site. From this analysis we cannot determine whether the impaired colonisation of tissues by primitive macrophages during larval stages in the *csf1ra* mutant contributes to the observed MNP deficiency, therefore further lineage tracing would be required to establish this.

Finally, *csf1ra* mutants did also appear to have a slightly altered regenerative and scarring response to cardiac injury. Scarring in the *csf1ra* mutant appeared to be more compact, with a reduced fibrin content compared to wild type controls, suggesting that the altered macrophage distribution may affect scarring.
5 Results III: Interleukin-1β in zebrafish heart regeneration

5.1 Chapter introduction

5.1.1 Pro-inflammatory signalling of IL-1β following tissue damage

As outlined in section 1.2.1, the release of inflammatory mediators following tissue injury is important for the activation and recruitment of inflammatory cells to clear debris and prime downstream repair (Frangogiannis, 2012). Interleukin 1 beta (IL-1β) is a potent pro-inflammatory cytokine central to the amplification of pro-inflammatory mediators, recruitment of inflammatory cells, and initiating matrix production by fibroblasts (Bujak and Frangogiannis, 2009). However, despite being necessary for regeneration in some models (Tsarouchas et al., 2018, Hasegawa et al., 2017), IL-1β has also been repeatedly shown to elicit deleterious effects during cardiac repair (Szekely and Arbel, 2018).

5.1.2 Activation of IL-1β signalling

Following tissue damage, such as that caused by MI, the release of DAMPS or cytokines (such as TNFα, IL-18, and IL-1α and IL-1β itself) stimulates IL-1β production, primarily by MNPs (Figure 5.1) (Fields et al., 2019, Dinarello et al., 2012, Epelman et al., 2015). These signals drive the upregulation of IL-1β transcription and translation and simultaneously lead to activation of the inflammasome (Epelman et al., 2015, Bujak and Frangogiannis, 2009). The inflammasome is an intracellular complex that is required to generate active caspase-1, which subsequently cleaves IL-1β precursors to their active form (Fields et al., 2019, Bujak and
Frangogiannis, 2009). Following activation, IL-1β binds IL-1 receptor type 1 (IL-1RI) which is expressed on the majority of cell types (Bujak and Frangogiannis, 2009, Dinarello, 2009).

IL-1RI signalling is further augmented by the release of active IL-1α from necrotic or damaged cells (Fields et al., 2019, Bujak and Frangogiannis, 2009, Lugrin et al., 2015). Unlike IL-1β, IL-1α is constitutively produced in an active precursor form within the cytoplasm and can therefore immediately activate IL-1 signalling following tissue damage (Lugrin et al., 2015, Kaneko et al., 2019). IL-1α and IL-1β have functionally similar signalling domains and provoke the same responses when binding IL-1RI, which is the sole signalling receptor for both IL-1 molecules (Fields et al., 2019).

Due to the widespread expression of IL-1RI on multiple cell types, IL-1β mediates a plethora of responses via activation of the NF-κB pathway, leading to the secretion of chemokines, cytokines and other stimulatory factors (Bujak and Frangogiannis, 2009). Furthermore, the presence of IL-1RI on MNPs elicits autocrine activity and induces positive feedback of the production of IL-1β and other pro-inflammatory cytokines. Collectively, this amplifies the inflammatory environment and generates a chemokine gradient to the ischaemic site which attracts further leukocytes, predominantly neutrophils and monocytes (Bujak and Frangogiannis, 2009, Kaneko et al., 2019). IL-1β also facilitates the recruitment of leukocytes by the upregulation of leukocyte adhesion molecules VCAM-1 and ICAM-1 on endothelial and mesenchymal cells, respectively (Dinarello, 2009). Systemic IL-1β activity also induces monocytopoiesis in the spleen, producing monocytes which are mobilised to sites of inflammation (Leuschner et al., 2012). The neutrophilic response is also promoted by IL-1, by driving the expansion of circulating neutrophil populations and prolonging neutrophil survival within tissues. This, in turn, leads to further activation of extracellular IL-1β by neutrophil proteases which can cleave precursor molecules (Dinarello et al., 2012, Sadatomo et al., 2017).
Figure 5.1. Interleukin-1 signalling following cardiac damage. Necrotic cell death releases active precursor IL-1α and stimulates the release of pro-inflammatory cytokines and chemokines from resident cells within the injury site. Resident macrophages produce IL-1β precursors which become activated by caspase-1 mediated cleavage and secreted. Autocrine signalling via IL-1RI drives further production of pro-inflammatory cytokines and IL-1β, which upregulate leukocyte adhesion molecules (VCAM-1) on endothelial cells and lead to the extravasation of neutrophils and monocytes into the infarct site, which further contribute to the production of IL-1β. Neutrophil proteases can also cleave IL-1 molecules to activate extracellular cytokines (Bujak and Frangogiannis, 2009, Fields et al., 2019, Dinarello et al., 2012).
5.1.3 Resolution of IL-1β signalling

Due to the autoinflammatory nature of IL-1β production, tight regulation is required to control signalling. Transcription and secretion are therefore highly regulated and the requirement for inflammasome-mediated cleavage conveys extrinsic control of potent IL-1β activity (Dinarello, 2009, Bujak and Frangogiannis, 2009). Antagonistic cytokine IL-1Ra and the decoy receptor IL-1RⅡ, also quench and outcompete IL-1 proteins to modulate IL-1β activity (Fields et al., 2019, Dinarello, 2009, Kaneko et al., 2019). MNPs also have intrinsic mechanisms to downregulate il1b expression: phagocytic clearance of cell debris and apoptotic neutrophils induces the production of anti-inflammatory cytokines such as TGFβ which promote downregulation of il1b (Tsarouchas et al., 2018, Morales and Allende, 2019, Das et al., 2014, Bujak and Frangogiannis, 2009).

Timely induction of these regulatory mechanisms is therefore essential to terminate pro-inflammatory signalling and advance to tissue repair (section 1.2). This is, in part, coordinated by the inhibitory effects of IL-1β signalling for fibroblast to myofibroblast differentiation (Talman and Ruskoaho, 2016).

5.1.4 Paradoxical roles of IL-1β in cardiac repair and regeneration

IL-1β activity in the immediate phase post-MI has been shown to be beneficial in the deposition of a collagenous scar that is required to prevent rupture of the infarcted heart (Hwang et al., 2001) and has also been shown to be necessary to prime cells for fin and axonal regeneration in zebrafish (Hasegawa et al., 2017, Tsarouchas et al., 2018). Yet, IL-1β has also been repeatedly documented to exacerbate tissue injury and impede subsequent repair (Mirza et al., 2013, Abbate et al., 2008, Abbate et al., 2010, Sadatomo et al., 2017). IL-1β has been reported to directly harm cardiomyocytes, causing reduced and arrhythmic contractility, alongside increased apoptosis and hypertrophy (Weisensee et al., 1993, Dinarello et al., 2012, Bujak and Frangogiannis, 2009). Extensive pro-inflammatory cell infiltration caused by IL-1β can also lead to further damage (Bujak and Frangogiannis, 2009, Nahrendorf et al., 2010).

Blockade of IL-1 signalling post-MI has therefore been shown to be beneficial to wound healing in experimental and clinical settings. Treatment with a recombinant IL-1Ra biologic, Anakinra, (which outcompetes IL-1α and IL-1β) following MI in rodents significantly
improves survival, characterised by reduced infarct size, apoptosis and ventricular remodelling (Abbate et al., 2008). Furthermore, IL-1RI−/− mice show less reactive fibrosis (Hwang et al., 2001) and similar prevention of cardiac remodelling is observed upon specific blockade of IL-1β, suggesting that IL-1β mediates the deleterious effects of IL-1R signalling (Abbate et al., 2010). Reduced ventricular remodelling and consequent incidence of heart failure has also been observed during a clinical trial whereby Anakinra was administered to patients following MI, highlighting the exciting therapeutic potential of manipulating this pathway (Dinarello et al., 2012).

Common to these studies is a suppressed inflammatory response, notably by reduced monocyte and neutrophil recruitment, which is unsurprising given the multiple roles IL-1β in leukocyte recruitment (Saxena et al., 2013). Due to their roles in both amplifying and suppressing IL-1β signalling, MNPs are commonly implicated in the adverse regulation of IL-1 signalling and have been attributed to adverse healing outcomes mediated by monocytes and macrophages (as discussed in Introduction section 1.7.3). For example, excessive IL-1β signalling through pro-inflammatory macrophages has been shown to be responsible for impaired healing of diabetic wounds due to the maintenance of an M1-like environment that is inhibitory to repair (Mirza et al., 2013). Contrastingly, the absence of anti-inflammatory macrophages during zebrafish fin regeneration has also been shown to prolong inflammation resulting in increased apoptosis and decreased cell proliferation (Hasegawa et al., 2017, Morales and Allende, 2019). Therefore, timely modulation by MNPs seems to be central to avoid the deleterious effects of IL-1β signalling.

Aberrant IL-1β signalling thereby serves as a mechanism by which MNPs elicit adverse effects following cardiac injury (Lavine et al., 2014, Nahrendorf et al., 2007, Panizzi et al., 2010). Indeed, CCR2+ MDMs exhibit pronounced inflammasome activation and express significant levels of Il1b in postnatal mouse models of MI (Epelman et al., 2014, Mezzaroma et al., 2011). Given the deleterious effects of monocytes on cardiac healing and the association of the inflammasome with adverse remodelling (Mezzaroma et al., 2011), IL-1β may be central to the MNP associated pathology of heart failure.
Chapter aims

The aim of this chapter was therefore to establish whether zebrafish MNPs show a similar expression of \textit{il1b} to mammalian MNPs and whether \textit{il1b} is required for normal regeneration and scarring. My approach was to utilise an existing TgBAC(\textit{il1b}:GFP); Tg(\textit{mpeg1.1}:mCherry) reporter line to assess the localisation of \textit{il1b} expression to macrophages subpopulations and characterise the inflammatory and regenerative response in \textit{il1b} mutant fish.
5.3 **Cardiac cryoinjury induces a rapid and strong upregulation of \( \textit{il1b} \) expression within the ventricle**

To begin to characterise the role of \( \textit{il1b} \) in the context of zebrafish cardiac inflammation and repair, the expression of \( \textit{il1b} \) was analysed following cardiac cryoinjury. Returning to the transcriptomics dataset described in section 3.7.3 (Lai et al., 2017), the expression of \( \textit{il1b} \) at injured timepoints relative to uninjured and sham-injured levels in zebrafish was plotted (Figure 5.3A). This showed that \( \textit{il1b} \) expression is strongly and rapidly induced by 6 hpi following cardiac cryoinjury (Log2FC = 7.7), then gradually declines in subsequent days, remaining slightly elevated by 5 dpi (Log2FC = 2.7). Interestingly, sham injury also caused upregulation of \( \textit{il1b} \) within the ventricle, however this was delayed and less strongly induced compared to cryoinjured hearts (Figure 5.3A).

To confirm this pattern of \( \textit{il1b} \) expression in our own cryoinjury model, the cDNA samples from uninjured and 6 hpi and 1, 3, 7, 14 and 21 dpi ventricles used in Figure 3.12 were also assessed for the expression of \( \textit{il1b} \) by qPCR (Figure 5.3C-D). Uninjured hearts showed constitutive low-level expression of \( \textit{il1b} \), but this was significantly upregulated at 6 hpi (21.8 ± 2.6 SD fold) and, to a lesser extent, at 1 dpi (6.7 ± 2.1 fold). Between 3 dpi and 21 dpi, \( \textit{il1b} \) expression showed a downward trend, returning to within uninjured levels during this timeframe.
Figure 5.3. Ventricular *il1b* expression following cardiac cryoinjury. A) Relative *il1b* expression of whole ventricles at timepoints following cardiac cryoinjury compared to uninjured and sham-injured controls. Log2FC values were obtained from publicly available RNA sequencing data published by Lai *et al.*, 2017 (Appendix 7.7). 4 whole ventricles were pooled per condition and genes were normalised prior to relative expression analysis. B, C) Whole ventricle *il1b* expression determined by qPCR in uninjured fish and at timepoints following cardiac cryoinjury. Analysis was performed on individual ventricles. B) Delta Ct values determined by subtracting the Ct value for *il1b* (gene of interest/GOI) from the Ct value for *ef1a* (reference gene). C) Mean *il1b* fold change relative to uninjured controls. Error bars show SD. Statistical analysis was performed on delta Ct values, comparing the expression at each timepoint to all other timepoints by Kruskal-Wallis with Dunn’s multiple comparisons test. Graph shows adjusted p values. N numbers: Uninjured = 6, 6 hpi = 4, 1 dpi = 6, 3 dpi = 5, 7 dpi = 5, 14 dpi = 6, 21 dpi = 5.
5.4  *il1b* is expressed by *mpeg1.1*+ MNPs and other cell types

As MNPs have been shown to express *il1b* following MI in mice and humans (Lavine *et al.*, 2014, Bajpai *et al.*, 2018), the localisation of *il1b* expression to MNPs was investigated in the ventricle during the first week post-injury (Figure 5.4, Figure 5.5). Whole mount imaging of hearts from Tg(*mpeg1.1:mCherry*); TgBAC(*il1b:GFP*) fish showed that uninjured hearts were almost completely devoid of localised GFP fluorescence, whereas at 6 hpi to 3 dpi, *il1b:GFP*+ cells were identifiable near the injury site (Figure 5.4A, Figure 5.5). By 7 dpi, however, there were few *il1b:GFP*+ cells remaining, and these appeared very dim and localised to the injury site. Quantification of mean *il1b*:GFP intensity within the ventricle mimicked the RNA expression pattern observed in Figure 5.3BC, peaking at 6 hpi then gradually declining (Figure 5.4B). Assessment of the standard deviation of GFP pixels, which indicates how localised and bright *il1b*:GFP expression was, peaked at 1 dpi, indicating that positive cells were strongly expressing the transgene (Figure 5.4C).

To assess whether MNPs were the main expressors of *il1b*:GFP, higher magnification imaging was performed (Figure 5.5). This revealed that at 6 hpi, although there were many *il1b*:GFP+ cells present, the majority of these cells were not *mpeg1.1:mCherry*+ (*il1b*+ cells) and appeared very rounded. At 1 and 3 dpi, there were many cells that were double positive for the transgenes (*mpeg1.1*+*il1b*+ cells) and appeared larger and more stellate compared to *il1b*+ cells observed at 6 hpi, and were likely to be macrophages. *il1b*+ cells were also observed, but many of these cells appeared less rounded compared to *il1b*+ cells observed at 6 hpi. *mpeg1.1*+*il1b*+ cells were also present; *mpeg1.1*+ lymphoid-like cells were identifiable but many had a macrophage-like morphology. Interestingly, by 7 dpi, the majority of *il1b*:GFP+ cells were *mpeg1.1*:mCherry- (Figure 5.5).

Collectively, this indicated that MNPs express *il1b* at early stages of injury but expression is largely downregulated by 7 dpi. Furthermore, *il1b* is also expressed by a significant amount of non-*mpeg1.1* expressing cell types, particularly at the immediate and late timepoints.
Figure 5.4. Confocal imaging of Tg(mpeg1.1:mCherry); TgBAC(il1b:mCherry) adult zebrafish hearts following cardiac cryoinjury. A) Exemplar max projection images of ventricles at each timepoint. Dashed lines demarcate the ventricle. Scale bar = 200µm. B) Mean il1b:GFP intensity within the ventricle. C) Average pixel standard deviation within the ventricle, which represents the intensity localisation of GFP expression. Statistical analysis was performed by Kruskal-Wallis test with Dunn’s multiple comparison test, comparing the values to uninjured values. Graph shows adjusted p values. Uninjured, 6 hpi, 3 dpi, 7 dpi: n = 3; 1 dpi: n = 2. One experimental replicate.
Figure 5.5. Co-localisation of \textit{il1b}:GFP and \textit{mpeg1.1}:mCherry in the ventricle following cardiac injury. Z-stack projections of whole-mount Tg(\textit{mpeg1.1}:mCherry); Tg(\textit{il1b}:GFP) ventricles at unwounded and post-cardiac cryoinjury timepoints. Insets show enlarged regions identified by the boxes. Green boxes identify single positive \textit{il1b}:GFP+ cells; red boxes identify single positive \textit{mpeg1.1}:mCherry+ cells; yellow boxes identify double positive \textit{il1b}:GFP+; \textit{mpeg1.1}:mCherry+ cells.

Although imaging analysis informs the distribution of \textit{mpeg1.1} and \textit{il1b} localisation, it is difficult to accurately quantify relative co-expression throughout the ventricle. To better discern this, the injuries were repeated at the same timepoints in adult Tg(\textit{mpeg1.1}:mCherry); TgBAC(\textit{il1b}:GFP) fish and ventricles were FAC-sorted. Unfortunately, due to the logistics of performing the injury and FACS, the 6 hpi timepoint could not be analysed. To avoid inclusion of \textit{mpeg1.1}+ lymphoid cells within the quantification and from collection, events were gated to exclude FSC\textsuperscript{low} and SSC\textsuperscript{low} events that corresponded to \textit{mpeg1.1}+\textit{csf1ra}- events.

As expected, there were virtually no \textit{il1b}+ events in uninjured ventricles. However, at 1 and 3 dpi, there was an increase in \textit{il1b}+ (3.53 ± 3.13 and 5.38 ± 6.58 SD, percent of live cells, respectively) and \textit{il1b}+\textit{mpeg1.1}+ events (1.94 ± 1.45 SD and 6.60 ± 5.16 SD) (Figure 5.6C). By 7 dpi, the frequency of \textit{il1b}+ events returned to that of uninjured fish. Interestingly, the average GFP fluorescence intensity of \textit{mpeg1.1}+\textit{il1b}+ cells slightly decreased between 1 and 3 dpi (Figure 5.6D), suggesting \textit{il1b} expression was decreasing in MNPs or may represent degradation of the fluorophore following cessation of \textit{il1b} expression. In accordance with imaging data, the analysis showed that many \textit{il1b}-expressing cells were not \textit{mpeg1.1}+ MNPs, with \textit{mpeg1.1}+ MNPs comprising 41.3 ± 14.5 % and 62.3 ± 10.4 % of \textit{il1b}-expressing cells at 1 and 3 dpi, respectively (Figure 5.6E). Conversely, a significant proportion of MNPs did not express \textit{il1b}, with approximately 22.0 ± 14.6 % and 28.4 ± 17.0 % of \textit{mpeg1.1}+ cells expressing \textit{il1b} at 1 and 3 dpi, respectively (Figure 5.6E). This is likely, in part, because only the cells at the injury are expressing \textit{il1b}, as observed in Figure 5.4.
Figure 5.6. FACS analysis of Tg(mpeg1.1:mCherry); TgBAC(iil1b:mCherry) adult zebrafish hearts following cardiac cryoinjury. A-B) Exemplar plots of mpeg1.1:mCherry and iil1b:GFP co-expression (A) with the corresponding FSC/SSC profile of each subpopulation (B) in live, single, labelled cells from a single heart at each timepoint. Plots show percentages of labelled cells. C) Mean frequency of each cell population as a percentage of live, single cells. D) Geometric mean GFP intensity of each cell population ± SD. E) Percentage of mpeg1.1+ iil1b+ cells that comprise total iil1b-expressing and mpeg1.1-expressing population ± SD. N numbers are detailed on C. Statistical analysis performed by Kruskal-Wallis test with Dunn’s multiple comparison’s test, comparing each timepoint to uninjured values. One experimental replicate.
5.5  *il1b*<sup>sh446</sup> mutation abolishes *il1b* expression in zebrafish

To understand the importance of IL-1β signalling for normal cardiac regeneration in the zebrafish, I next wanted to assess the inflammatory, scarring and regenerative response to cardiac cryoinjury in zebrafish that have a loss of function mutation in *il1b* (Ogryzko *et al.*, 2019). *il1b*<sup>sh446/sh446</sup> mutant zebrafish have a 44 bp deletion in exon 4 creating a premature stop codon that results in a truncated protein (Figure 5.7AB).

The *il1b*<sup>sh446</sup> mutation was confirmed in our zebrafish line by the presence of a smaller PCR product in homozygous and heterozygous mutant fish (Figure 5.7C). Absence of *il1b* expression in the ventricle of *il1b*<sup>sh446/sh446</sup> was also confirmed at 6 hours post cardiac cryoinjury (6 hpi) using qPCR (Figure 5.7D). Relative *il1b* expression was also analysed in *il1b<sup>+/sh446</sup>* ventricles and was 0.5-fold that of wild type fish, suggesting that expression is proportional to allele number (Figure 5.7D). Due to limited adult fish numbers, further replicates could not be performed to confirm this expression pattern. However, a 0.5-fold reduction in *il1b* expression was also recorded in larval *il1b<sup>+/sh446</sup>* fish compared to wild type controls at 6 hours post tail fin resection, thereby supporting this hypothesis (Figure 5.7E).
Figure 5.7. Annotation and confirmation of il1b<sup>sh446</sup> mutation. A) Exonic sequences of il1b genomic DNA showing the 44 bp deletion in exon 4 in il1b<sup>sh446</sup> mutants. The location of genotyping and qPCR primer binding sites are also shown. B) Sequence alignment of wild type (upper sequence) and truncated, mutant (lower sequence) Il-1β protein. C) Agarose gel showing the differential PCR product size in il1b<sup>sh446/+</sup>, il1b<sup>+/-</sup> and il1b<sup>+/+</sup> fish, confirming the presence of the mutation. D-E) qPCR analysis of relative il1b expression in wild type, il1b<sup>sh446/+</sup> and il1b<sup>sh446/sh446</sup> adult ventricles at 6 hours post cardiac cryoinjury (D) and in 5 dpf larval tails at 6 hours post tail fin resection (E). D) Individual ventricles were analysed over one experimental replicate. Animals were age-matched and co-housed following injury. Wild type and il1b<sup>sh446/+</sup>: n = 3, il1b<sup>sh446/sh446</sup>: n = 2. Graphs show mean with SD. E) Pools of 5 larval tails were used per condition. One experimental replicate using siblings.
5.6 *il1b*KO*sh446* fish have a normal distribution of ventricular MNPs

To ascertain whether *il1b* mutation affects homeostatic cardiac MNP populations, whole mount imaging was performed on wild type, *il1b+*/*sh446* and *il1b*KO*sh446* Tg(*mpeg1.1:mCherry*) hearts to visualise MNP distribution, with the caveat that *mpeg1.1*+ lymphoid cells would also be labelled. *mpeg1.1*-expressing cells could be seen distributed evenly on the surface of the ventricle with a MNP-like morphology, as observed in previous chapters, in all genotypes (Figure 5.8AB). Quantification of *mpeg1.1*-expressing cells showed that *il1b*-mutation had no effect on the number or size of cells on the surface of the ventricle (Figure 5.8C).

Higher power imaging of optically cleared *il1b*KO*sh446* Tg(*mpeg1.1:mCherry*) hearts was also performed to determine whether the distribution of internal and trabecular *mpeg1.1*-expressing MNPs was normal (Figure 5.9). This also allowed the separation and quantification of *mpeg1.1*+ MNPs and *mpeg1.1*+ lymphoid cells using differences in the size and circularity characteristics determined previously (Figure 3.2, Results I). This showed that *mpeg1.1*+ MNPs and lymphoid cells were observed throughout the depth of the wild type and *il1b*KO*sh446* ventricles with no significant difference in frequency between genotypes.

Collectively, this indicates that *il1b* is not required for the normal distribution of *mpeg1.1*-expressing MNPs throughout the ventricle. Nevertheless, the analysis shown in Figure 5.9 was only performed in duplicate per genotype to identify if there was a stark phenotypic difference of MNP distribution, as observed in *csf1r*KO*sh446* ventricles. Interestingly, the counts were highly variable between the two wild type samples analysed, therefore more replicates would be required to establish whether the frequency was different.
Figure 5.8. Homeostatic cardiac MNP distribution in wild type, *il1b*+/sh446 and *il1b*sh446/sh446 Tg(*mpeg1.1:mCherry*) hearts. A-B) Maximum intensity projections of *mpeg1.1:mCherry* fluorescence in whole mount hearts. Dashed line identifies the ventricle. Scale bars = 200 µm (A); 20 µm (B). C-D) Automated analysis of *mpeg1.1*+ cell counts per 1000 µm² ventricle area (C) and average *mpeg1.1*+ cell area within the ventricle (D) (representative examples of analysed images shown in A. Statistical analysis was performed using Kruskal-Wallis test with Dunn's multiple comparisons test but revealed no significant difference in either metric. N = 7 for all genotypes. Two experimental replicates.
Figure 5.9. Distribution of mpeg1.1-expressing cells throughout the myocardium of wild type and il1b<sup>sh446/sh446</sup> ventricles. A) Representative max projections of 100 µm sub-stacks throughout a 500 µm depth of a wild type and il1b<sup>sh446/sh446</sup> ventricle. Arrows in panel i show examples of cells that would be classified as MNPs (arrow) and lymphoid cells (arrowhead). Scale bar = 20 µm. B) Max projections of whole wild type and il1b<sup>sh446/sh446</sup> cleared hearts. Dashed line identifies the ventricle. Scale bar = 200 µm. C) Quantification of mpeg1.1-expressing MNPs, lymphoid-like cells and total mpeg1.1-expressing cells at defined depths in wild type and il1b<sup>sh446/sh446</sup> ventricles. Counts were performed manually using the Cell Counter Plugin on Fiji from a 400 x 400 x 100 µm (x, y, z respectively) region. One experimental replicate. n = 2 per genotype.
5.7  *il1b*-mutation does not affect injury size and gross collagen scarring

The preceding findings indicate that *il1b*<sup>sh446/sh446</sup> fish have a normal distribution of cardiac MNPs, but these cells, amongst other *il1b*-expressing cell types, will be unable to produce the strong wave of *il1b* expression that is observed during the first 3 days post cardiac cryoinjury in wild type fish (Figure 5.3). As II-1β secretion by tissue macrophages has been shown to be important for instigating cellular responses to infection and injury (Bujak and Frangogiannis, 2009), the effect of *il1b*-mutation on the tissue damage caused by cardiac cryoinjury, and the resulting inflammatory and regeneration response, was assessed.

Wild type and *il1b*<sup>sh446/sh446</sup> fish were cardiac cryoinjured and hearts were harvested and processed at 3 and 14 dpi for histological analysis. These timepoints were chosen as at 3 dpi the frequency of MNPs and other leukocytes within the ventricle are expanding (Figure 3.6, Results I) and as II-1β signalling is important for the recruitment of inflammatory cells (Dinarello <i>et al.</i>, 2012), perturbing this may directly affect tissue repair. By 14 dpi, MNP levels have returned to uninjured levels and tissue regeneration is underway (Chablais <i>et al.</i>, 2011), therefore disruption to *il1b* signalling that acts at early stages of tissue repair may affect the course of regeneration at later timepoints.

Survival of *il1b*<sup>sh446/sh446</sup> fish following cardiac cryoinjury did not show any noticeable difference to that of wild type fish. To visualise the injury area and assess the deposition of fibrin and collagen, tissue sections of injured hearts were stained with AFOG histological stain (Figure 5.10A). As expected, red-stained fibrin could be seen throughout the injury site at 3 dpi, which acts to rapidly seal the wound site, and little collagen scarring was present. No significant difference in injury size was observed between wild type and *il1b*<sup>sh446/sh446</sup> fish, accounting for 28.0 ± 15.2 % SD and 23.1 ± 10.3 % SD of ventricular area, respectively (Figure 5.10AB). As discussed in section 4.10, this high variability in relative injury size is likely to be due to differences in heart size and location of injury throughout the serial sections, in addition to biological variability.

By 14 dpi, the injury area was largely infiltrated with purple-stained collagen, with some fibrin remaining at the exterior of the wound in both wild type and *il1b*<sup>sh446/sh446</sup> ventricles (Figure 5.10A). Quantification of collagen area showed no significant difference between the genotypes, however, again, this was highly variable due to differences in the staining
intensity. Despite this, no observable difference in the morphology of scarring could be identified. Injury area also decreased similarly in both genotypes (18.5 ± 13.6% SD and 20.5 ± 15.7% SD of ventricle area, respectively), thereby identifying no differences in tissue repair or collagen scarring in the \textit{il1b}\textsuperscript{sh446/sh446} fish. Unfortunately, however, many specimens had to be excluded from analysis as many \textit{il1b}\textsuperscript{sh446/sh446} hearts had large fat depositions surrounding the heart and no injury was visible within sections indicating that this prevented correct cryoinjury of the heart. Further repeats at 14 dpi would therefore be required to confirm these findings.

5.8 \textit{il1b}-mutants have faster collagen I resolution

Collagen I has been shown to be a key component of scar tissue (Mathew-Steiner \textit{et al.}, 2021) and was shown to be upregulated from the transcriptomics analysis in Figure 3.9 (Results I). As AFOG staining identifies regions of scar tissue composed from all collagen types, the dynamics of collagen I cannot be discerned from this analysis. Adjacent sections were therefore immunostained for collagen I, alongside tropomyosin to identify intact cardiomyocytes and consequently identify the injury area. At 3 dpi, a layer of collagen I surrounded the ventricles of both wild type and \textit{il1b}\textsuperscript{sh446/sh446} fish, as previously described (Bevan \textit{et al.}, 2020, Simões \textit{et al.}, 2020), whereas at 14 dpi, the majority of collagen I was localised to the wound site (Figure 5.10A). Interestingly, quantification of collagen I area showed significantly reduced collagen I within the ventricles of \textit{il1b}-mutants (Figure 5.10D), with collagen staining appearing to be less intense and showing a reduced infiltration within the injury site.

Collectively, this suggested that \textit{il1b} mutants do not demonstrate any differences in injury size, regeneration or bulk collagen scarring, but they appear to resolve collagen I scarring faster than wild type controls.
Figure 5.10. Injury size and collagen scarring in wild type and *il1b*<sup>h446h446</sup> cryoinjured ventricles.

A) Representative tissue sections following AFOG staining (column 1) and immunofluorescence staining of DAPI (column 2), tropomyosin (column 3) and collagen I (column 4) in adjacent sections within the injury area. AFOG staining (Column 1) shows fibrin in red, collagen in blue/purple, muscle in orange and nuclei in black. Scale bar = 20 µm. B) Quantification of injury area determined from AFOG stained sections, expressed as percentage of total ventricle area. C) Total collagen area within the ventricle, determined from AFOG stained sections. D) Quantification of collagen I area. Groups were age-matched and co-housed following cardiac injury. N numbers for each analysis are represented by the number of data points; one experimental replicate. Statistical analysis was performed by Mann-Whitney test.
5.9 **Leukocyte infiltration within the wound site is reduced in *il1b*-mutants at the early stages of tissue repair**

To identify whether *il1b*-mutation affects immune cell recruitment and infiltration into the wound site, sections were also immunostained for L-plastin, a pan-leukocyte marker (Kell *et al.*, 2018). At 3 dpi, leukocytes could be seen throughout the injury site in both wild type and *il1b*<sup>sh446/sh446</sup> ventricles (Figure 5.11A). Nevertheless, leukocytes appeared to be more sparsely distributed within the injury area of *il1b*<sup>sh446/sh446</sup> hearts and labelled cells occupied a lower percentage area of the ventricle compared to wild type controls (7.2 ± 3.9 % SD and 13.0 ± 5.1 % SD of ventricle area respectively) (Figure 5.11B-D). As expected, leukocytes were less abundant by 14 dpi and their frequency and distribution appeared similar in both genotypes, comprising approximately 3% of ventricle area (Figure 5.11ACD).
Figure 5.11. Leukocyte infiltration into the injury site. A) Immunofluorescence staining of tissue sections within the injury site of wild type and il1b<sup>sh446/sh446</sup> hearts at 3 and 14 dpi, showing DAPI (column 1), tropomyosin (column 2), L-plastin (column 3) and merged images (column 4). Scale bar = 20 µm. B) Zoomed images of insets in A (i, ii), showing leukocyte infiltration (L-plastin+) within the injury site of 3 dpi wild type and il1b<sup>sh446/sh446</sup> ventricles. C) Quantification of L-plastin/leukocyte area, shown as a percentage of ventricle area. Statistical analysis was performed by Mann-Whitney test. N numbers for each analysis are represented by the number of data points.
5.10 **Proliferation is unchanged in *il1b*<sup>sh446/sh446</sup> fish but show slightly increased levels of apoptosis**

To assess whether *il1b*-mutation affected cell proliferation following injury, fish were also treated with EdU 24 hours prior to harvesting. Detection of EdU incorporation showed no significant difference in the number of proliferating cells between wild type and *il1b*<sup>sh446/sh446</sup> fish at 3 or 14 dpi, although there were slightly fewer EdU+ cells in *il1b*<sup>sh446/sh446</sup> fish at 3 dpi (Figure 5.12AB). However, despite counterstaining cardiomyocytes with tropomyosin, it was not possibly to accurately determine whether the proliferative cells were cardiomyocytes or other cells infiltrating the wound. Despite this, as there did not appear to be a regenerative defect shown by injury size (Figure 5.10), this collectively supports that the absence of *il1b* does not affect regeneration, at least at the timepoints analysed.

As the absence of IL-1 signalling has been associated with decreased levels of apoptosis (Suzuki *et al.*, 2001, Sadatomo *et al.*, 2017), TUNEL staining was also performed in *il1b*-mutant fish to assess the levels of apoptotic cells (Figure 5.12A, C). Apoptosis in wild type fish were highly consistent and showed a reduction between 3 and 14 dpi, as expected. Conversely, *il1b* mutants showed highly variable levels, and but showed a small but non-significant increase in apoptosis at both 3 and 14 dpi, suggesting that apoptosis may be enhanced upon loss of *il1b*. 

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Figure 5.12. Cell proliferation and apoptosis in wild type and *il1b*^sh446/sh446^ ventricles at 3 and 14 dpi.

A) Tissue sections showing staining for nuclei (DAPI) (column 1), tropomyosin (column 2) and incorporated EdU (column 3) and merged channel images (column 4). TUNEL staining (column 5) was also performed in adjacent section. Dashed lines identify the ventricle. Scale bar = 20 µm. B) Quantification EdU+ proliferating cells within the ventricle, normalised to ventricle area. C) Quantification apoptotic, TUNEL+ cells within the injury site, normalised to injury size. Statistical analysis was performed by Mann-Whitney test. N numbers for each analysis are represented by the number of data points.
Chapter summary

To establish the dynamics of \textit{il1b} expression and assess the contribution of MNPs to this response following cardiac cryoinjury, I utilised gene expression analysis, and imaging and flow cytometric analysis of a TgBAC(\textit{il1b}:GFP); Tg(\textit{mpeg1.1}:mCherry) double transgenic line. This showed \textit{il1b} is rapidly expressed in the ventricle within 6 hpi, however, expression promptly returns to homeostatic levels within 3 dpi. \textit{il1b}:GFP expression was observed in a proportion of \textit{mpeg1.1}+ MNPs, indicating that these cells contribute to the induction of this response. To establish the importance of \textit{il1b} expression on the key phases following cardiac injury, cardiac cryoinjury was performed on \textit{il1b}-mutant zebrafish. Injury size was not affected by \textit{il1b} mutation, however, increased and highly variable levels of cell death were observed. Despite this, proliferation was unaffected and \textit{il1b} mutants appeared to regenerate the injury area similarly to wild type controls. In line with other MI models (Bujak \textit{et al.}, 2008, Saxena \textit{et al.}, 2013), analysis of the leukocyte infiltrate at 3 dpi indicated that the recruitment of leukocytes was impaired in \textit{il1b}-mutants in the initial pro-inflammatory phase, but resolution of inflammatory cells was unaffected. Interestingly, \textit{il1b} mutants also appeared to resolve collagen I scarring faster than wild type controls, although bulk collagen deposition was unaffected at the timepoints analysed.

Collectively, this indicates that \textit{il1b} is important for the normal pro-inflammatory response in zebrafish, which is partly mediated by the expression of \textit{il1b} by MNPs, but it is not essential for the induction of the scarring and regenerative response in zebrafish.
6 Discussion

6.1 Introduction and rationale

Heart failure poses a massive clinical burden due to the increasing prevalence of non-fatal MI that result in irreversible damage to cardiac muscle (Murphy et al., 2020). Although much investigation has been underway to identify and overcome the mechanisms that restrict cardiomyocyte regeneration and compensate for these either by engraftment of exogenous cells or by the manipulation of endogenous cardiac cells, these methods have shown limited success (Cahill et al., 2017). Research is therefore shifting to look beyond cardiomyocytes to the complex microenvironment that may be inhibiting the regenerative potential of cardiac muscle (Ryan et al., 2020). This includes a new appreciation of the effect that inflammation and scarring has on the regenerative environment and how this mediates adverse remodelling of the tissue, which is central to the pathology of heart failure. Understanding the mechanisms by which non-myocytes can mediate both successful and unsuccessful tissue repair or regeneration in model organisms will therefore help to identify the roadblocks to regeneration in mammals and consequently lead to the development of new therapeutic approaches.

The appropriate induction of an inflammatory response and recruitment of immune cells to sites of injury is essential for the progression of tissue repair and regeneration, and MNPs have been repeatedly documented to be pivotal to this response (Gao et al., 2021). The secretion of inflammatory mediators by tissue macrophages and MDMs following tissue injury is essential to mediate clearance of cell debris and orchestrate the actions of many cell types responsible for resolving tissue damage (Frangogiannis, 2014). However, MNPs have a dichotomous involvement in tissue repair as they have been implicated in mediating the
adverse scarring phenotype that is observed in postnatal mammals, which underlies the pathological remodelling that causes heart failure (Frantz and Nahrendorf, 2014, Nahrendorf et al., 2010). Consequently, there is an ever-increasing appreciation that MNPs are a highly heterogenous grouping of cells and the unique functions and regulation of distinct subpopulations mediate their opposing roles. Identifying and enhancing the activity of beneficial cells and modulating the activity of harmful cells may therefore help to promote a regenerative outcome.

Evidence from regenerative and reparative mouse models suggest that recruited pro-inflammatory monocyte populations have deleterious effects on tissue repair whereas embryonically derived tissue resident macrophages foster pro-regenerative mechanisms, characterised by low-level scarring and increased cardiomyocyte proliferation (Lavine et al., 2014, Dick et al., 2019). Although zebrafish are known to have extensive cTM populations and expand MNP populations following cardiac injury with strikingly similar dynamics to that of mammals (Bevan et al., 2020, Lai et al., 2017), we have yet to establish the ontogeny of these populations and their relative contribution to tissue regeneration. I therefore sought to better define MNP populations and begin to address the overarching question of how different zebrafish MNP populations respond to cardiac cryoinjury in a manner that facilitates cardiac regeneration, particularly in respect to the mediation of transient scarring.

6.2 Defining MNP markers in the adult zebrafish

Unlike in mammals, markers to segregate populations of monocytes, macrophages and DCs have not been well-defined in zebrafish. Despite this, studies of ‘macrophages’ in zebrafish have been plentiful, and have most commonly utilised transgenic fluorescent reporter lines driven by mpeg1.1 which was one of the first macrophage specific genes determined by larval zebrafish studies (Ellett et al., 2011). Multiple mpeg1.1 transgenic lines have therefore been generated to interrogate macrophage dynamics, with currently 55 transgenic lines and 147 citations listed on the Zebrafish Information Network (https://zfin.org/). Transgenic lines have also been generated using the csf1ra promoter which shows high conservation with mammalian Csf1r, which labels all cells of the MNP system. However, the relative expression of these markers in different MNP populations has not been documented in adult fish,
therefore I utilised these markers in combination to establish if their differential expression could be used to segregate populations of monocytes and macrophages.

Unexpectedly, via the characterisation of cell morphology, gene expression, and spatiotemporal dynamics to cryoinjury, this analysis identified a population of mpeg1.1+csf1ra-lymphoid cells which comprises B cells and NKL cells. This supported simultaneous findings by Ferrero et al (2020) that mpeg1.1 is expressed by B cells, and provided additional functional evidence presented by Tang et al (2017) that expression extends to a population of cells with a transcriptional profile of NKL cells. The recent identification of mpeg1.1 expression in metaphocytes further compounds the promiscuity of mpeg1.1 as an MNP marker (Kuil et al., 2020, Lin et al., 2019b). This expression in other, non-MNP cell types is likely as a result of the initial characterisation of mpeg1.1 being performed in larval (<5 dpf) zebrafish in which, as exemplified in Figure 1.5, macrophage ontogeny is diverse and a comprehensive and mature immune system is not yet present. Accordingly, these findings by myself and others emphasise that mpeg1.1 transgenes must be used with caution and previous studies that have utilised this marker to isolate and track macrophage populations in the adult zebrafish must be re-evaluated. Despite this, I have demonstrated that these mpeg1.1+ lymphoid cells can be distinguished by the absence of csf1ra-transgenic expression, or by their size, morphology and distinct FSC/SSC profile.

The considerable frequency and activity of these mpeg1.1+ lymphoid cells (characterised by flow cytometry and live imaging) within the heart and other tissues also warrants further investigation into the identity and function of these cells, which was beyond the scope of this work. Although the presence of NK and NKL cells in zebrafish has been determined by the identification of orthologous mammalian NK specific genes by single cell sequencing and morphological studies (Hernández et al., 2018, Tang et al., 2017, Moss et al., 2009, Moore et al., 2016), functional studies of these cells have not been performed. mpeg1.1 transgenics may therefore aid the characterisation of these cells. The presence of the mpeg1.1+ B cells and NKL cells in the steady state, and their expansion of the following cardiac injury, also suggests an active involvement of these cells in tissue homeostasis and repair. Interestingly, resident B cells and NK cells have also been identified in the murine heart (Pinto et al., 2012). In addition, considering our studies into the regulation of MNP populations, mature B cells have been
shown to be involved in recruiting harmful monocyte populations (Zouggari et al., 2013, Adamo et al., 2018) and NK cells regulate monocyte maturation during cardiac repair (Ayach et al., 2006, Ong et al., 2015, Ong et al., 2017). The dual csf1ra and mpeg1.1 reporter transgenic line may therefore be beneficial to study these populations in parallel to establish whether different regulatory mechanisms are present within the zebrafish.

My analysis of mpeg1.1±csf1ra± cells within various tissues has also highlighted differences in relative expression levels of the mpeg1.1 in MNPs and demonstrates that it may serve as a useful marker to separate subpopulations of monocytes and macrophages by their differentiation state. Although co-expression of high levels of mpeg1.1 and csf1ra was common within the heart, the blood had a higher ratio of single positive csf1ra+ cells, indicating that monocytes generally have a lower mpeg1.1 expression compared to mature tissue macrophages. Furthermore, the expansion of cardiac csf1ra+ cells, which harnessed a monocyte-like morphology, in the early stages post-cardiac cryoinjury also supported that these cells represent a transitory population of recruited monocytes within the tissue. Transcriptomics analysis of hematopoietic cell lineages also indicates that mpeg1.1 is more highly expressed by more differentiated monocytes/macrophages. In addition, csf1ra mutants, which potentially have a macrophage differentiation impairment (Lin et al., 2019a, Stanley and Chitu, 2014), showed a striking reduction in mpeg1.1 expression. Therefore, I have proposed that csf1ra+mpeg1.1- cells identify a population of monocytes and potentially immature macrophages, and therefore mpeg1.1 is not a comprehensive marker of MNPs. However, further characterisation of the ontogeny and gene expression of these cells would be required to confirm this. In addition, as csf1ra+ monocytes likely transition to a mpeg1.1+csf1ra+ profile also presented by cTMs, this labelling technique is not able to permanently label this monocyte derived population to trace their function within tissues. However, this does allow for the isolation of an injury-responsive monocyte population in which new monocyte markers could be identified, as discussed in section 6.6.

I have also shown that mpeg1.2 is consistently detected in csf1ra-expressing MNP populations, yet was not expressed within the mpeg1.1+ lymphoid population. There are three reported paralogs of mpeg1 in the zebrafish which have been previously described to have distinct roles and be expressed at different stages of development (Benard et al., 2015). In the larval
zebrafish, mpeg1.1 and mpeg1.2 have been shown to have macrophage specific expression, whereas mpeg1.3 is a pseudogene (Benard et al., 2015). However, my orthology analysis showed that mpeg1.2 is predicted to be the primary ortholog to mammalian Mpeg1, which has been determined to be macrophage- and DC-specific (Spilsbury et al., 1995, Merselis et al., 2021). Collectively, this indicates that mpeg1.2 represents a more specific and reliable MNP marker compared to mpeg1.1. This observation, therefore, also raises questions about the function of these genes in their respective cells, given that this is poorly documented despite their frequent use as markers.

My analysis has also further clarified the expression pattern of csf1ra. Interestingly, although the consistent expression of csf1ra:GFP of MNPs in all tissues suggests that zebrafish csf1ra-transgenes mimic the labelling of global MNP cells observed in mammals (Sasmono et al., 2003), distinct populations of mpeg1.1+csf1ra+ and mpeg1.1+csf1ra\textsuperscript{dim} cells were also identified. This indicates that further stratification of MNP populations may be possible using csf1ra expression levels. Firstly, my identification of three distinct mpeg1.1\pm csf1ra\pm populations within the blood is suggestive of multiple monocyte subpopulations within the adult zebrafish, which has not been previously described. In line with my hypothesis that csf1ra+ monocytes transition into an mpeg1.1+csf1ra+ MDM population, the blood mpeg1.1+csf1ra+ population also appeared to expand in response to injury, suggesting that this population may represent more differentiated precursors to tissue MDMs. However, an additional population of mpeg1.1+csf1ra\textsuperscript{dim} cells, was also prevalent within the blood of uninjured fish and constituted a minor population of tissues (Figure 3.4B Figure 3.10A, B). Recent reports from the Richardson lab have visualised intravascular mpeg1.1-expressing cells which adhere to the luminal endothelium (Scott et al., 2021). This observation may have captured a monocyte prior to extravasation (and therefore could be representative of a mpeg1.1+csf1ra+ cell) however, it could also suggest that zebrafish have an analogous population of non-classical monocytes that patrol the endothelium in mammals (Cros et al., 2010). Intriguingly, the mpeg1.1+csf1ra\textsuperscript{dim} population consistently showed no expression of mertka, when isolated from either the heart or the kidney (Figure 3.10D, Figure 4.11C). MERTK/Mertka is a receptor involved in phagocytosis and clearance of apoptotic cells and interestingly, has been shown to be important for the homeostatic functions of cTMs within the heart, in mammals (Nicolás-Ávila
et al., 2020). This indicates that the $mpeg1.1+csf1ra^{dim}$ populations have distinct phagocytic properties. Similarly, populations of classical and non-classical monocytes have been shown to have different phagocytic properties. Classical monocytes are highly phagocytic due to their roles in pathogen and debris clearance, whereas non-classical monocytes have been shown to be specialised for Fc receptor mediated phagocytosis (Zhao et al., 2009). Therefore, although it is highly speculative, this $mpeg1.1+csf1ra^{dim}$ population could represent a population analogous to non-classical monocytes that are distinct from the $csf1ra$-expressing populations which represent classical, injury-responsive monocytes. Although $mpeg1.1+csf1ra^{dim}$ cells were detected within tissues by flow cytometry, this could be due to their presence within residual blood and the vasculature, rather than the tissue itself. However, non-classical monocytes have also been shown to give rise to pro-reparative macrophages at late stages of injury, including within the murine heart (Olingy et al., 2017, Nahrendorf et al., 2007, Saxena et al., 2013). More detailed analysis of these cells is therefore warranted to establish whether they do indeed represent a distinct population and whether they have roles in tissue regeneration.

It is also worth addressing that the contribution of DCs to MNPs was not investigated in my analysis. Mammalian myeloid DCs have been shown to express both $Csf1r$ and $Mpeg1$ (Merselis et al., 2021, MacDonald et al., 2005) and zebrafish have been shown to possess DCs (Lugo-Villarino et al., 2010, Wittamer et al., 2011). This suggests zebrafish DCs likely fall within the $mpeg1.1+csf1ra^{+}$ population of tissues. Indeed, many of the $mpeg1.1+csf1ra^{+}$ cells isolated from the heart and fin were highly dendritic (Figure 3.2), however, the similar ramified morphology of DCs and cTMs prevents segregation of these populations by histology alone. Interestingly, absent or low $Mertk$ expression has been shown in some murine DC populations (Gautier et al., 2012, Seitz et al., 2007), and considering that DCs are found within the blood and tissues (Miles et al., 2014), this could also be a potential identity of the $mpeg1.1+csf1ra^{dim}$ population. It would therefore be interesting to elucidate the relative contribution of DCs to the cardiac MNP pool in zebrafish. In rodents, resident populations of DCs have been identified (Hart and Fabre, 1981) and these expand in response to MI, although the frequency of myeloid DCs is only ~4% that of total macrophages (Yan et al., 2013). Despite this, these sparse populations of DCs have been attributed important immunomodulatory roles.
following MI and their depletion resulted in enhanced ventricular remodelling, accompanied by prolonged \textit{il1b} expression and increased infiltration of pro-inflammatory monocytes and macrophages (Anzai \textit{et al.}, 2012). Parallel populations in the zebrafish heart are therefore likely to have similar roles in favourably modulating \textit{il1b} expression and monocyte recruitment.

The adventitious finding that \textit{mpeg1.1} is not exclusively expressed by MNPs also highlights the requirement for more rigorous identification of cell-specific markers in zebrafish. To explore the specificity of other existing markers of zebrafish and murine MNP markers, and identify whether they could potentially be used to stratify populations, I probed existing transcriptomics databases performed on adult kidney derived hematopoietic cells. This indicated that \textit{mfap4} and \textit{marco} expression is restricted to all monocytes and macrophages, but with increased \textit{marco} expression likely giving a good representation of macrophage differentiation. However, in mammals, in addition to expression on macrophages and DCs, \textit{marco} has been shown to be expressed on pro-inflammatory monocytes (Xu \textit{et al.}, 2017), suggesting that this may not be able to stratify between these populations.

An orthology search also confirmed that many key murine markers do not have robust orthologs in zebrafish. This includes \textit{Ly6C1}, which is commonly used to stratify populations of monocytes with differential reparative phenotypes during cardiac repair (Nahrendorf \textit{et al.}, 2007) and \textit{Cx3cr1} which identifies populations of pro-reparative, tissue resident macrophages (Dick \textit{et al.}, 2019, Burgess \textit{et al.}, 2019). However, zebrafish do have an ortholog to \textit{Ccr2}, which is commonly used to identify recruited monocytes and macrophages in mice, and these populations have been repeatedly attributed roles in mediating adverse remodelling (Vagnozzi \textit{et al.}, 2020, Lavine \textit{et al.}, 2014, Dick \textit{et al.}, 2019, Dewald \textit{et al.}, 2005). In mammals, \textit{Ccr2} encodes a chemokine receptor that is important for mobilisation of inflammatory monocytes to the blood and their CCL2-mediated recruitment to injury, but is also expressed by B cells, T cells and NK cells (Dewald \textit{et al.}, 2005, Fujimura \textit{et al.}, 2015). There is evidence to suggest that this mechanism is conserved in zebrafish, as \textit{Ccr2} has been reported to have roles in monocyte recruitment to \textit{Mycobacterium} infection in the larval zebrafish (Cambier \textit{et al.}, 2017, Cambier \textit{et al.}, 2014). Xu \textit{et al} (2018) also showed that \textit{ccl2} was upregulated at 1 dpi where they observe the greatest influx of macrophages in their cardiac cryoinjury model. \textit{ccr2} expression was also concomitantly upregulated at 1 dpi in transcriptomics analysis performed
by Lai et al. (2017), with similar dynamics to that observed in mammals (Dewald et al., 2005). My own investigations into this marker similarly showed low ccr2 expression by RT-PCR in the mpeg1.1+csf1ra+ population isolated from 1 dpi ventricles (Figure 3.11). Surprisingly, it was not expressed by the putative csf1ra+ monocyte population, although this could be due to insufficient RNA yields from this sparse population. Expression was also not detected in whole injured hearts at any timepoint (ccl2 expression was also inconsistent in this analysis), or within sorted populations isolated from the uninjured ventricle or kidney. Therefore, it is likely that ccr2 does identify an analogous pro-inflammatory monocyte population to that observed in mammals, which differentiate into mpeg1.1+csf1ra+ MDMs within the heart following cardiac cryoinjury. However, ccr2 expression appears to be low compared to other MNP markers and requires the use of more sensitive analysis. Single cell transcriptomics of MNPs isolated at early timepoints post-injury would identify whether expression is restricted to a subset of cells and would also help to elucidate other monocyte markers. Comparison with the transcriptome of murine monocytes may also identify underlying differences which mediate the different regenerative outcome observed between these species (Rizzo et al., 2020).

The characterisation of their dynamics and relative contribution to the cardiac injury response would also be aided by the generation of a ccr2 reporter line and use of CCR2 inhibitors, which have recently been demonstrated to be efficacious in larval zebrafish (Sommer et al., 2021).

In summary of this section of work, my characterisation of MNP gene expression and dynamics following cardiac injury has added further evidence that zebrafish undergo a similar MNP expansion to that of mammals following MI, involving the activation and recruitment of monocyte populations. This indicates that subtle differences in the regulation and gene expression of these populations is the cause of their adverse tissue repair outcomes in mammals. I have also provided further clarity of the specificity of commonly used MNP markers which can aid future analysis of these populations within the heart, blood and other tissues. However, this analysis also highlights the difficulty of stratifying MNP subpopulations using a few select markers, due to the intrinsic plasticity and similarities between populations, which still remains a challenge in mammalian studies (Reynolds and Haniffa, 2015). Despite this, advancing technologies and the advantages of using the adult
zebrafish as a model system, most notably the ease of generating mutant and transgenic reporter lines, promises to help facilitate future analysis within the field.

6.3 Studying macrophages by ex vivo imaging

To further elucidate the dynamics of $\text{mpeg1.1}^\pm \text{csf1ra}^\pm$ MNPs in the uninjured and injured heart and investigate the hypothesis that $\text{csf1ra}^+$ cells transition into $\text{mpeg1.1}^+\text{csf1ra}^+$ cells, I explored the potential to live-image hearts. The amenability of zebrafish hearts to ex vivo culture and availability of transgenic lines has already provided valuable insights into epicardial regeneration (Cao and Poss, 2016, Kikuchi et al., 2011, Wang et al., 2015), revascularisation (Yip et al., 2020) and cardiomyocyte proliferation (Honkoop et al., 2021) and posed the exciting potential to extend this analysis to leukocyte dynamics.

Establishment and optimisation of the ex vivo imaging platform allowed, for the first time, the visualisation of MNP dynamics in the adult zebrafish heart. This demonstrated that cTMs are highly motile in the uninjured heart and constantly patrol the tissue, as observed in larval studies (Kaveh et al., 2020, Herbomel et al., 2001, Lam and Huttenlocher, 2013). In the murine heart, cTMs have been established to have an M2-like profile and play important roles in phagocytosing antigens and maintaining tolerance, therefore this behaviour is characteristic of trophic surveillance (Pinto et al., 2012). $\text{mpeg1.1}^+\text{csf1ra}^+$ cells also seem to form extensive interactions with $\text{mpeg1.1}^+$ lymphoid cells via protrusions, indicating that these cells are likely exchanging signals.

Unfortunately, due to time constraints, repetition of optimised ex vivo imaging platform in injured hearts could not be performed to establish how MNPs responded to tissue injury or visualise the potential upregulation of mpeg1.1. Yet, this work demonstrates the potential of this system to interrogate the dynamics of leukocytes within the zebrafish heart to address such questions. This system may also be useful to interrogate the cellular mechanics of migration through a dense, complex, and pressurised tissue. The stretched morphology of migrating cells indicated that cell may be migrating between the cardiomyocytes, endothelial cells and fibroblasts that comprise the main structure of the tissue (Talman and Ruskoaho, 2016), although a transgenic reporter line for these cell types would be required to confirm this. This inevitably requires extensive remodelling of the ECM and cTM nucleus to enable
such migration, fields of research which are still actively being explored (Wolf et al., 2013, Calero-Cuenca et al., 2018).

The observation that BDM impairs MNP migration is also an important finding. BDM is a myosin II ATPase inhibitor, which is commonly used in cardiomyocyte cultures to increase cell viability (Thum and Borlak, 2001, Kivistö et al., 1995) and immobilising cardiac muscle in heart slice cultures (Brandenburger et al., 2012, Honkoop et al., 2021) by preventing cell contraction. However, the effects on leukocyte migration observed by myself and Urwyler et al (2000) indicate this treatment is incompatible with survival and maintenance of cTMs within these cultures and its use should be avoided, if possible. Furthermore, preliminary investigations into the preservation of MNPs within the ex vivo culture indicates that the current published protocols used in explant cultures also do not support normal macrophage homeostasis over prolonged periods, likely because of insufficient growth factors derived from the cardiac tissue. However, increased cell death (compared to in vivo controls) has been reported in cultured hearts, even after 1 dc (Honkoop et al., 2021), therefore MNPs are likely to be responding to this, and may be partly responsible for the abnormal phenotype observed at 3 dc (Figure 3.19). Similarly, macrophages within human heart slice cultures have been shown to upregulate pro-inflammatory markers such IL-1β after 24 hours in culture in response to cell death (Bajpai et al., 2018). Curiously, Honkoop et al (2021) also showed that cardiomyocyte proliferation declined at 3 dc indicating that the ex vivo environment does not fully support sustained cardiomyocyte proliferation (Honkoop et al., 2021). It would therefore be interesting to determine whether this is a result of dysregulated macrophage activity.

Collectively, these observations highlight the caveats of the system in that the ex vivo conditions will inevitably have other implications on cell viability and production of signalling molecules, which may have implications on macrophage activity. Future investigation into the timeframe in which cells remain viable in our own hands, and/or optimisation of the culture conditions (e.g. by addition of growth factors or a perfusion system) would therefore be required to use this system over long periods. Furthermore, validation of findings using these systems would also be required using in vivo analysis, which, although technically challenging, is possible in the juvenile heart using synchronised imaging techniques (Taylor et al., 2019). However, upon validation, this system has the
potential to investigate many aspects of leukocyte biology which would not be possible in vivo, such as drug treatments or high throughput analysis.

6.4 Dynamics of zebrafish cardiac MNPs

Macrophages are highly plastic cells and their adaptive inflammatory profile and unique tissues-specific functions are significantly governed by environmental cues (Das et al., 2015). However, given the distinct functions of cTM populations of embryonic lineage compared to recruited populations, it has been suggested that lineage may intrinsically program functionality, including roles during tissue repair (Epelman et al., 2014, Dick et al., 2019, Lavine et al., 2014, Leid et al., 2016). The ontogeny of tissue resident macrophages is beginning to be established in zebrafish and has identified interesting differences in their maintenance within different tissue niches and compared to mammals. Recent lineage tracing has shown that although primitive microglia colonise and populate the central nervous system initially, these cells are gradually replaced by HSC-derived cells (Ferrero et al., 2018), thereby differing from mammals whereby self-maintaining primitive yolk-sac derived microglia persist throughout the lifetime of the organism (Ginhoux et al., 2013). Similarly, Langerhans cells have been shown to be solely HSC-derived in the zebrafish as opposed to some contribution from primitive and EMP-derived macrophages in mammals (He et al., 2018). The ontogeny of cardiac macrophages has yet to be extensively studied in zebrafish, although He et al (2018) also proposed that brain, liver and heart macrophages are likely to be HSC-derived using evidence from lineage tracing and due to the timing of their colonisation. Unpublished data from the Richardson lab also indicates that macrophages do not colonise the heart until 17-21 dpf. This significant delay from the emergence of primitive macrophages (detailed in section 1.7.1) therefore also supports a HSC origin. This differs from mammalian systems in which the majority of tissue resident macrophages are seeded during embryonic development (Epelman et al., 2014, Bajpai et al., 2018). Furthermore, these embryonic macrophages have been attributed pro-regenerative functions compared to harmful recruited populations (Lavine et al., 2014, Bajpai et al., 2019). If zebrafish cTMs are indeed HSC-derived and maintained by monocyte recruitment, this may suggest that lineage is a less important determinant of a pro-regenerative macrophage phenotype in the zebrafish compared to mammals. Further work is therefore required to establish how cTMs are maintained in the
adult zebrafish and establish whether they have important and undiscovered contributions to eliciting the regenerative response.

Such questions that I attempted to address were, firstly, whether cTMs migrate to the injury, as observed to sites of injury within the skin (Lin et al., 2019b), and, secondly, how monocytes are recruited to the injury site. I therefore explored the dynamics of cTMs using detailed imaging techniques of wild type and csf1ra mutant fish, which have been shown to have deficiencies in tissue resident macrophage populations to begin to address these questions (Caetano-Lopes et al., 2020, Morales and Allende, 2019, Kuil et al., 2020).

Previous analysis of cellular distribution within the heart has been via whole mount imaging and histological sections, both of which have limited spatial resolution. The establishment of Ce3D tissue clearing therefore facilitated three-dimensional analysis of the cardiac architecture and gleaned new insights into the distribution of MNPs within the tissue. In uninjured wild type ventricles, I identified that MNPs are evenly distributed throughout the myocardium and on the surface of the ventricle, likely beneath the epicardium. Labelling of mitotic cells with anti-PH3 antibodies showed very limited proliferation of MNPs within the uninjured state. However, preliminary analysis of EdU pulse labelling over a 24-hour period prior to harvesting identified many MNPs on the surface of the heart that had recently undergone proliferation, but few were present deep within the myocardium. Thus, this suggests that these surface cells are being actively replenished, either by self-renewal or by recruitment from monocytic sources that have emerged from highly proliferative precursor populations within the timeframe of EdU treatment (Zhu et al., 2009). Conversely, the lack of proliferative cTMs within the trabeculae indicates that these cells are either long-lived and undergo limited proliferation, or alludes to a mechanism by which these cells are constantly replenished by migration of the surface macrophages into the myocardium. The analysis of csf1ra mutants may also inform the origin and maintenance of cTMs. Repeating 3D imaging and flow cytometric analysis in csf1ra mutant ventricles demonstrated that csf1ra is essential for the maintenance of cTMs within the trabecular myocardium but is less important for cTM distribution within the endocardium or upper compact myocardium. This could be due to the requirement of csf1ra for, i) the migration, ii) survival and/or iii) proliferation of cTMs within
the trabeculae, as Csf1r signalling has been shown to be important for all of these processes (Oosterhof et al., 2018, Herbomel et al., 2001, Wu et al., 2018).

Nevertheless, recent studies have demonstrated that csf1ra and csf1rb have distinct roles in mediating primitive and definitive hematopoiesis, respectively (Ferrero et al., 2021). Oosterhof et al (2018) have shown that normal colonisation of the CNS by microglia is dependent on csf1ra during larval stages of development due to the requirement of csf1ra for the migration of primitive macrophages to the CNS. However, they showed that at adult stages, csf1ra mutation has little effect on the frequency of adult microglia populations, but that csf1rb mutation caused a massive loss of microglia due to the requirement of csf1rb in mediating definitive hematopoiesis, which is the source of adult microglia (Ferrero et al., 2018, Oosterhof et al., 2018, Xu et al., 2015). Therefore, despite evidence to suggest that cTMs are HSC-derived, the striking cTM deficiency observed in csf1ra mutants could also be due to the requirement of csf1ra for, iv) colonisation of the heart by primitive macrophages that seed lifelong cTMs. If this is the cause, then the remaining cTMs on the surface of the csf1ra mutant heart may represent cells of monocytic lineage, which would also be supported by their csf1ra+ profile.

As it was difficult to dissect the cause of this altered distribution in the unwounded heart, the distribution of cardiac MNPs was also assessed following injury. 3D imaging of wild type hearts at 1 dpi showed that MNPs were present on the ventricular surface and infiltrating the myocardium at the injury site. An accumulation of MNPs was also observed at the injury site in csf1ra-mutants, however in corroboration with imaging and flow cytometry data, many of these cells were csf1ra+ and few cells infiltrated deep within the injury site. At 7 dpi, differences in MNP localisation were even more pronounced, with a dense infiltration of mpeg1.1+csf1ra+ cells within the injury site in wild type fish, whereas cells remained on the surface of the ventricle in csf1ra mutants. Collectively, these observations could suggest that a migration impairment is the cause of altered MNP distribution in csf1ra mutants. Given the accumulation of MNPs at the injury site on the surface of the heart, and ex vivo imaging of csf1ra+clj/clj MNP migration demonstrates that the remaining cells can migrate laterally across the ventricle and/or extravasate from vessels on the surface of the heart. However, as these csf1ra+clj/clj MNPs fail to infiltrate into the myocardium, this is indicative of an inability to invade the tissue. This could also further support the hypothesis that proliferating cells on the
surface of the heart are important for maintaining trabecular cTMs, and that recruited monocytes reach the wound site by extravasating out of vessels on the surface of the heart and migrating transversely into the tissue (Figure 6.1). Interestingly, although csf1ra mutation has been shown to only cause a slight reduction in microglia frequency in the adult brain, distribution of microglia was affected (Oosterhof et al., 2018). They found that fewer cells were present in the dorsolateral region of the optic tectum, but unchanged numbers within the dorsal and ventral regions. Therefore, once the ex vivo imaging and analysis pipeline has been optimised, 3D spatial tracking of cells in injured wild type and csf1ra mutant hearts may help to elucidate whether the csf1ra mutants do indeed have an impaired ability to migrate into the myocardium.

The mechanism of impaired migration and requirement of csf1ra for the colonisation of tissues by primitive macrophages, but not definitive macrophages, has not been established. However, the observation that cardiac MNPs in csf1ra mutants also show reduced expression of mpeg1.1, also indicates that csf1ra mutation does cause a differentiation impairment. Similarly, expression of classical MNP markers, including mpeg1.1, marco and mhc2dab are reduced in microglia of csf1ra mutant microglia, although they suggest that these cells don’t have a differentiation defect (Oosterhof et al., 2018). However, our own studies have shown that csf1raj4e1/j4e1 mutants have a lower proportion of tnfa-expressing mpeg1.1+ cells at 3 dpi (Bevan et al., 2020), as has been shown in larval studies (Gurevich et al., 2018). This similarly suggests that macrophage function is impaired in csf1ra mutants and there are therefore many reasons why migration may be affected. It would therefore be interesting to assess the expression of adhesion molecules within csf1ra mutants to establish whether these are altered, especially as itgam appeared to be more highly expressed in csf1ra mutant MNPs (Figure 4.8). Chemokines have been shown to be upregulated in csf1ra mutant microglia, and it was suggested that this may be a compensatory mechanism due to the altered distribution observed (Oosterhof et al., 2018). Furthermore, the expression of MMPs and other matrix remodelling proteinases in csf1ra mutant MNPs may also be worthy of inspection, as secretion of these molecules is required for migration and recruitment of macrophages to the injured heart (Xu et al., 2018). Interestingly, murine cTMs have been shown to enriched expression of Mmp13 compared to other macrophages (Pinto et al., 2012); given the loss of trabecular cTMs
in csf1ra mutants, it is possible that this alters the ECM environment, making it less permissile to MNP infiltration.

Additional impairments in proliferation and survival cannot be disregarded as a cause of MNP deficiency in csf1ra mutants, however. Proliferation of csf1ra+ cells was unaffected by csf1ra mutation, yet far fewer proliferating mpeg1.1+csf1ra+ cells were observed. Due to the vast reduction of mpeg1.1+csf1ra+ cells in csf1ra mutants, it is difficult to establish whether this low proliferation is as a result of low numbers, or the proliferation defect is the cause of the mpeg1.1+csf1ra+ deficiency. Nevertheless, as my analyses suggest that csf1ra+ monocytes differentiate into mature mpeg1.1+csf1ra+ macrophages, this may collectively indicate that the reduction in mpeg1.1+csf1ra+ cells is as a result of impaired differentiation and survival of MNPs, rather than reduced proliferation to maintain the population. Similarly, although csf1ra has been shown to be less important for the establishment of adult microglia populations (Oosterhof et al., 2018), recent reports suggest that csf1ra is important for proliferation and maintenance of microglia in the adult zebrafish (Ferrero et al., 2021, Wu et al., 2018). Intriguingly, Ferrero et al (2021) also showed that adult csf1ra mutants do have a severe deficiency of microglia, comparable to that of csf1rb mutants, thereby differing to observations by Oosterhof et al (2018). This may be due to different methods of quantification. Oosterhof et al (2018) quantified microglia by L-plastin staining, whereas Ferrero et al (2021) quantified microglia by imaging mpeg1.1 reporter expression. Given the reduction of mpeg1.1 expression observed in my analysis and that by Oosterhof et al (2018), this could account for a reduction in the cells that were quantified by Ferrero et al (2021) and requires further confirmation.

Together, this analysis indicates that csf1ra is critical for the normal maintenance and function of cardiac MNPs in both the steady and injured state, and may suggest that it is more important than in the maintenance of adult microglia. Similarly, osteoclasts are severely diminished in adult csf1ra mutants (Caetano-Lopes et al., 2020) and a reduction of splenic and liver macrophages has also been reported, suggesting that this is observed in multiple tissues (Pagán et al., 2015). Further analysis of apoptosis and proliferation (using EdU labelling to label more comprehensively proliferating cells) would be required to elucidate whether these factors also contribute to the deficiencies observed. Furthermore, lineage tracing of cardiac cTMs is required to assess if primitive populations do contribute to the adult cTM pool. In an
attempt to address this, I began to create an inducible macrophage colour switch line Tg(ubi:creERT2); Tg(mfap4:loxP-mCherry-loxP-EGFP) which would allow the temporal labelling of macrophages by treating embryos with 4-hydroxytamoxifen at different periods during development. Although I successfully generated the Tg(ubi:creERT2) line, transgene expression under the control of the mfap4 promoter could not be detected and, due to lack of other suitable cloning vectors to drive expression in pan-MNPs, I no longer pursued this. However, future use of cell lineage tracing techniques presented by Ferrero et al. (2018) and He et al. (2018) would provide valuable insights into the origin of cTMs and injury responsive MNPs, and support our understanding of cardiac MNP dynamics in the zebrafish. This, combined with the study of csf1rb+/- and csf1ra+/-; csf1rb+/- double mutant fish, would help to elucidate whether cTMs in the adult heart are maintained by HSCs, as has been established for other zebrafish tissues (He et al., 2018, Ferrero et al., 2018). A question that remains in these studies, however, is whether zebrafish tissue macrophage populations are maintained solely by self-renewal or whether constant monocyte input significantly contributes to the maintenance of tissue macrophages populations, which is not commonly observed in mammals (Hashimoto et al., 2013).

![Figure 6.1. Distribution and dynamics of cTMs and recruited populations in the steady state and following injury in wild type and csf1ra mutant fish.](image)

My analysis suggests that colonisation of the trabecular myocardium and infiltration of the injury site required the migration of MNPs from the surface of ventricle, which is impaired in csf1ra^{401/401} fish.
The significant macrophage deficiency in csf1ra mutants also presented an excellent opportunity to investigate how scarring was affected in the absence of normal macrophage frequency and dynamics. The Richardson lab had previously demonstrated that collagen I deposition was reduced in csf1ra mutants compared to wild type controls, but could be rescued by LPS treatment, suggesting that a blunted inflammatory phenotype of csf1ra<sup>ie1/jie1</sup> MNPs may be responsible (Bevan et al., 2020). Interestingly, I have shown that despite the loss of infiltrating MNP populations, csf1ra mutants are still able to deposit a significant scar, indicating that these cells are not essential for scar deposition. However, injury size and scarring did appear to be more compact in csf1ra<sup>ie1/jie1</sup> fish with a reduced fibrin infiltrate at later timepoints. Due to difficulties in accurately quantifying scarring, as described in section 4.10, further analysis would be required to confirm a difference in regeneration.

### 6.5 Interleukin 1β and cardiac regeneration

Multiple studies have shown that the abrogation of IL-1 signalling minimises infarct size and adverse scar deposition in rodent models of MI (Bujak et al., 2008, Abbate et al., 2010, Abbate et al., 2008) but early expression of il1b is also advantageous to regeneration in other zebrafish tissues (Hasegawa et al., 2017, Tsarouchas et al., 2018). Collectively, this indicates that zebrafish favourably modulate IL-1β activity in response to injury in a way which is permissible to appropriate, transient scarring, unlike in mammalian systems. I therefore explored the dynamics of il1b expression and MNPs following cardiac cryoinjury to assess the contribution of these cells to il1b induction, and subsequently examined the effect of its absence on the inflammatory and early regeneration response.

#### 6.5.1 il1b dynamics and the role of MNPs

Assessment of ventricular il1b expression following cardiac cryoinjury showed a similar induction and resolution pattern to that following zebrafish resection and cryoinjury models, and mammalian MI models (Huang et al., 2013, Dewald et al., 2004, Simões et al., 2020, Lai et al., 2017). By 6 hpi, il1b showed profound upregulation, but this was significantly attenuated by 1 dpi and remained similar to uninjured levels for the remainder of the timecourse. Although the quantity of active Il-1β protein cannot be extrapolated from il1b expression due to its highly regulated translation, cleavage and secretion (Dinarello et al., 2012), this analysis demonstrates that zebrafish mount a similar il1b expression response to mammals in the
context of cardiac injury. This also emphasises that il1b cannot be completely inhibitory to successful tissue repair and it is rather the timely modulation of Il-1β protein activity or cellular distribution that may cause the reported adverse effects in mammalian systems.

Il-1β has been associated with pro-inflammatory profile of harmful recruited CCR2+ monocytes and MDMs (Lavine et al., 2014, Bajpai et al., 2019). To assess the contribution of MNPs to il1b expression following cardiac cryoinjury, the expression of csf1ra and mfap4 was simultaneously assessed to establish how these macrophage markers correlated with il1b expression (Figure 3.12, Figure 5.3). Expression profiles for mfap4 and csf1ra across multiple injury time points showed a similar pattern of MNP expansion to that established by flow cytometry, but qPCR also indicated increased expression of these markers at 6 hpi and 1 dpi. Our previous characterisation of MNP frequency by flow cytometry at various timepoints post-injury indicated little expansion of total ventricular MNP frequency by 1 dpi (Figure 3.6) (Bevan et al., 2020), but imaging analysis shows that there is an infiltration of MNPs at the wound site by 1 dpi. Collectively, this suggested that the loss of resident cTMs by injury is balanced by the recruitment of monocyte-derived MNPs before 1 dpi, and therefore indicates that an influx of recruited monocytes and/or cTMs may contribute to the abrupt upregulation of il1b at 6 hpi.

Flow cytometric analysis of TgBAC(il1b:GFP); Tg(mpeg1.1:mCherry) double transgenic fish following cardiac cryoinjury indeed showed that mpeg1.1+ MNPs comprised approximately 40-60% of il1b-expressing cells at 1 and 3 dpi, respectively. Nevertheless, a large proportion of il1b expressing cells were not mpeg1.1 expressing MNPs (Figure 6.2). Many of these cells are likely to be neutrophils, which are known to be another main source of il1b expression (Sadatomo et al., 2017, Dinarello, 2009, Tsarouchas et al., 2018) and infiltrate the ventricle between 6 hpi to 3 dpi in our cryoinjury model (Bevan et al., 2020). Importantly, the csf1ra+ monocyte population infiltrate the injury site at early stages post-injury where we see the peak expression of il1b and would not have been included in this analysis due to the use of the mpeg1.1 reporter. It would therefore be necessary to confirm whether the il1b+mpeg1.1+population include these cells and assess their relative expression of il1b. Unexpectedly, numbers of il1b:GFP+ cells determined by flow cytometry showed a lag compared to that observed by qPCR, with levels peaking at 3 dpi. This could be as a result of the delay of
transcription, translation and folding of the transgene compared to rapid induction of endogenous *il1b*. Notably, this also differs from the *il1b*:GFP fluorescence profile which was observed by wholemount imaging whereby peak GFP fluorescence and localisation occurred between 6 hpi to 1 dpi. However, wholemount imaging only accounts for cells on the surface of the ventricle, and collectively suggests that *il1b*+ cells may continue to accumulate within the injury site between 1 and 3 dpi by the transverse migration mechanism that has been hypothesised. Interestingly, expression of *il1b* was also detected in sorted *mpeg1.1*±*csf1ra*± populations from uninjured ventricles (Figure 3.10, Results I) indicating that resident cardiac macrophages likely contribute to this *il1b* expression.

This analysis confirms that MNPs produce *il1b* in our cryoinjury model but may contribute to a lesser extent than the influx of pro-inflammatory neutrophils at the immediate phase post-injury. Assessment of relative *il1b* expression by qPCR or transcriptomics of sorted *csf1ra*+ and *mpeg1.1*+*csf1ra*+ populations at early timepoints post-injury would be required to establish this, in addition to the identification of the cell types and relative expression levels in the *il1b*+*mpeg1.1*- population. It will also be important to assess if zebrafish MNPs may play more significant roles in attenuating IL-1β production, resulting in a more transient pro-inflammatory phase, rather than amplification of the signal which is observed in mammals (Dinarello *et al.*, 2012). Indeed, the absence of macrophages to produce anti-inflammatory cytokines has been shown to prolong *il1b* expression and impair regeneration (Hasegawa *et al.*, 2017, Morales and Allende, 2019, Tsarouchas *et al.*, 2018). Assessing the expression of modulatory cytokines, such as TGFβ and IL-1Ra by MNPs, in addition to inflammasome activation, in our cardiac cryoinjury model would therefore be interesting to determine the role of MNPs in the *il1b* response. Furthermore, given the significant MNP deficiency displayed by *csf1ra* mutants, in addition to their migration defects and inability to generate normal *tnfa*+ and *mpeg1.1*+ MNPs (Bevan *et al.*, 2020), how both the induction and resolution of *il1b* expression is affected in these mutants warrants further investigation. This may also provide a link to the possible altered scarring dynamics observed.

Future assessment of the dynamics and distribution of *il1b*-expressing cells would also benefit from the use of more inclusive and responsive transgenic lines, given the caveats of the *mpeg1.1* transgenic and possible lag of *il1b*:GFP expression compared to endogenous
expression. Unfortunately, due to the combination of fluorescent transgenic lines available, I could not utilise our existing TgBAC(csf1ra:GFP) line with the TgBAC(il1b:GFP) line and to our knowledge, non-GFP lines have not been generated for either of these genes. I therefore sought to cross the TgBAC(il1b:GFP) line to a Tg(mfap4:TdTOMato) line, which from gene expression analysis and other reports is likely to be a faithful marker of monocytes and macrophages (Walton et al., 2015), but in our hands, transgenic expression was not retained during adulthood. Generation of a non-GFP il1b transgenic line which is more representative of active gene expression, such as the use of fluorophores that have short half lives (Li et al., 1998, He et al., 2019), in combination with the TgBAC(csf1ra:GFP) line, would enable the tracking and analysis of il1b-expressing MNPs. In combination with more in-depth 3D and ex vivo imaging analysis at earlier timepoints post-injury, this would help to elucidate the origin and fate of these cells. Furthermore, analysis of cleaved IL-1β protein via Western Blot or proteomics analysis would be required to assess how il1b expression correlates with active protein in zebrafish.
Figure 6.2. Summary of the dynamics of \textit{il1b} expression and potential \textit{il1b}-expressing cell populations following cardiac cryoinjury in the adult zebrafish. The analysis performed by myself and others (Simões \textit{et al.}, 2020), shows that \textit{il1b} is rapidly expressed within hours following cardiac cryoinjury and correlates with the influx of neutrophils (Lai \textit{et al.}, 2017, Xu \textit{et al.}, 2018, Bevan \textit{et al.}, 2020). At 1 dpi, \textit{il1b} expression is significantly reduced and MNPs are observed at the injury site, which largely appear \textit{mpeg1.1+csf1ra+} but there is also a notable increase in \textit{csf1ra+} cells compared to uninjured and late stage timepoints (i.e. 7 dpi). MNPs (\textit{csf1ra±mpeg1.1±}) continue to accumulate within the ventricle, peaking at 3-7 dpi then returning to uninjured levels by 14 dpi. Interestingly, there is lag between peak \textit{il1b} expression and peak \textit{il1b+} cells, which occurs at 3 dpi, possibly indicating that initial short-lived, pro-inflammatory cells, notably neutrophils, contribute to high \textit{il1b} expression, whereas many cells have low level expression at later timepoints and/or GFP fluorescence does not represent active \textit{il1b} expression due to delayed synthesis and degradation of the fluorophore. Bevan \textit{et al} (2020) have also shown that there is an accumulation of \textit{tnfa+} cells at the initial timepoints post-injury.
6.5.2 Heart regeneration in il1b mutants

Given the requirement for il1b to prime regenerative responses in other zebrafish tissues (Hasegawa et al., 2017, Tsarouchas et al., 2018) and the apparent involvement of il1b in enhancing adverse scarring responses (Abbate et al., 2010, Abbate et al., 2008, Saxena et al., 2013) I next investigated how the absence of il1b affects cardiac inflammation, scarring and regeneration.

Similar to what has previously been established for IL-1RI-/- mice (Bujak et al., 2008), MNP distribution was unaffected by il1b mutation in the uninjured ventricle suggesting that il1b is not important for the maintenance of cTMs. Yet, there was a slight reduction in the number of leukocytes within the ventricle of il1bsh446/sh446 fish at 3 dpi compared to wild type controls, with no difference observed by 14 dpi. This mimics a similar suppressed inflammatory response observed in mouse models of infarction (Sadatomo et al., 2017, Bujak et al., 2008, Saxena et al., 2013) and demonstrates the importance of Il-1β in inflammatory cell recruitment in the initial phase post-injury in the zebrafish. However, in some infarction models, reduced or absent IL-1 signalling has been shown to reduce infarct size due to reduced apoptosis (Sadatomo et al., 2017, Suzuki et al., 2001, Hasegawa et al., 2017), which was not observed in our model. Rather, there was a small but non-significant increase in apoptosis at both 3 and 14 dpi in il1b-mutants compared to wild type. From the immunofluorescence staining performed, it is difficult to ascertain the cells undergoing apoptosis. However, Il-1β promotes the survival of neutrophils, and absence of il1b has been shown to induce apoptosis of regenerative cells (Hasegawa et al., 2017). Closer examination of the dynamics of neutrophils and cardiomyocytes may therefore be interesting; optimisation of TUNEL staining on whole mount tissues, in combination with tissue clearing and nuclear-tagged transgenic lines would be an excellent strategy to assess this. This would also be useful in determining whether specific cell types show differences in proliferation, which was not observed when assessing gross proliferation within the mutants.

Due to the observation that interference with IL-1 signalling can also result in reduced scar deposition, both within and distal to the infarct zone (Bujak et al., 2008), collagen scarring was also assessed in il1b-mutants. Analysis of gross collagen deposition by histological staining showed no difference between il1b-mutants and wild type controls. As previously characterised (Bevan et al., 2020), at 3 dpi collagen was restricted to the exterior of the heart,

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whereas a dense collagen matrix infiltrated the injury site at 14 dpi. Interestingly, staining for collagen I, which was shown to be upregulated in the transcriptomics analysis (Figure 3.9) and is known to be deposited within the heart following injury (Simões et al., 2020), showed a similar localisation to the surface of the ventricle at 3 dpi in both groups. Interestingly, Bevan et al (2020) showed that ablation of macrophages within the first 3 dpi leads to significantly lower collagen I at 3 dpi, meanwhile, increasing the initial pro-inflammatory response leads to increased collagen I deposition. Comparing these analyses, as a defect was not observed at 3 dpi in il1b mutants, this may suggest that loss of IL-1β does not affect macrophage recruitment significantly enough to impair collagen I deposition. Segregation of the immune cells that are impaired by il1b mutation would therefore be useful to establish this. Analysis of later timepoints would be essential to confirm how this may affect scar resolution and success of complete regeneration.

However, il1b-mutants exhibited weaker and less extensive collagen I deposition at 14 dpi compared to wild type controls and did not infiltrate the wound site as extensively. Firstly, the different distribution of scar tissue detected by AFOG staining and collagen I staining highlights how scar tissue is comprised of multiple collagen types (Sánchez-Iranzo et al., 2018). In addition, this indicates that the scar composition of il1b-mutants may be altered, and as different collagen types have unique properties (Mathew-Steiner et al., 2021), this is likely to effect the stability of the scar tissue. In combination with supportive factors delivered by MNPs, IL-1β is important for priming and inducing timely collagen deposition by fibroblasts (Frangogiannis, 2014). The absence of IL-1β signalling and reduced immune cell infiltration into the wound site therefore likely contributes to the altered collagen distribution observed.

In addition, macrophages have recently been shown to directly secrete collagen, including collagen I, during cardiac repair and this macrophage derived collagen appears to be largely localised to the exterior of the heart (Simões et al., 2020). Therefore, this may be a direct mechanism by which reduced MNP infiltration into the wound may contribute to this altered scar phenotype. 3D imaging and flow cytometry analysis to determine how macrophage dynamics are affected by il1b-mutation during the first week post-injury would help to establish whether altered macrophage dynamics are responsible for the differences in collagen I deposition observed.
In conclusion, my analysis shows that in the absence of il1b, zebrafish maintain the ability to deposit a collagenous scar but appear to show a different distribution of collagen I. Assessing scar dynamics in addition to the assessment of the regeneration of cardiac muscle will be important to establish whether il1b signalling is essential to full cardiac regeneration in the zebrafish.

6.6 Concluding remarks and future work

Through the completion of my PhD research, I have broadened our knowledge of cardiac MNP dynamics in the intact and cryoinjured zebrafish heart and their contribution to the regenerative response. My characterisation of commonly used MNP-markers will help segregate populations of monocytes and macrophage in future studies which will be required to elucidate the distinct properties that cells may have. Furthermore, I have identified putative populations of csf1ra+ and ccr2+ monocytes. Further investigation into the properties and regulation of these cells may help to elucidate how these populations differ in zebrafish to avoid the deleterious effects mediated by analogous populations in mammals. This includes the regulation of their inflammatory phenotype, including il1b expression, which has been associated with the pro-inflammatory profile of these harmful mammalian populations. Use of the ex vivo imaging and tissue clearing protocols will also help to perform more detailed characterisation of MNP populations, which was not previously possible. In addition, my results show that the alteration of MNP dynamics by csf1ra mutation and abrogation of Il-1β signalling by MNPs, and other cells, does not result in overt changes to the regenerative ability of zebrafish. Nevertheless, assessment of complete regeneration and scar resolution in these mutants is required to confirm that the relative importance of these genes for natural heart regeneration the adult zebrafish, which may show delayed effects that could not be identified with the course of this analysis.

My characterisation of MNP dynamics has also informed the design of a transcriptomics study that would produce novel and valuable insights into how zebrafish MNPs respond to cardiac cryoinjury in a manner that favours a regenerative outcome. Although transcriptomic analysis of monocytes and macrophages isolated from the blood and uninjured/injured ventricle may have helped to elucidate markers of these populations that could have been interrogated, we initially had a poor understanding of the dynamics of these populations, and how and when
would be the optimum time point to isolate them. If bulk transcriptomics had been performed on \textit{mpeg1.1}+ cells, which has been the favoured macrophage marker for similar studies within the heart (Sanz-Morejón \textit{et al.}, 2019, Simões \textit{et al.}, 2020), our analysis would have been contaminated with the \textit{mpeg1.1}+ lymphoid cell population and likely missed important populations of \textit{mpeg1.1}- monocytes.

Single cell sequencing analysis of \textit{csf1ra}-expressing MNPs isolated from the ventricle and blood at 1-3 dpi (when monocyte/MNP expansion is underway) is likely to identify distinct transcriptomic profiles representing monocytes, MDMs, cTMs and DCs. Analysis at this early stage, particularly at 1 dpi when \textit{il1b} expression is still high, would also enable the contribution of MNPs to \textit{il1b} expression and regulation to be assessed. This would indicate whether distinct populations of MNPs are enriched for \textit{il1b} expression, and whether MNPs are important producers of IL-1 regulatory molecules, such as IL-1Ra, which are required to prevent amplification of \textit{il1b} production by MNPs themselves. As \textit{il1b} is such a potent modulator of the timing of the inflammatory response and induction of tissue repair responses, this could identify if zebrafish MNPs, or perhaps distinct populations, modulate this response preferentially to mammals, facilitating a regenerative outcome.

In summary, the work presented here has further demonstrated the importance of inflammatory cell populations in the response to cardiac tissue damage and has paved the way for further classification of precise subtypes in adult zebrafish. This promises to help elucidate the mechanisms by which zebrafish mediate their fascinating ability to regenerate cardiac tissue and lead to new insights into how this could be promoted in mammalian systems.
References


Wang, Y., Szretter, K. J., Vermi, W., Gilfillan, S., Rossini, C., Cella, M., Barrow, A. D., Diamond, M. S. & Colonna, M. 2012. IL-34 is a tissue-restricted ligand of CSF1R required for the development of Langerhans cells and microglia. *Nature Immunology*, 13, 753-760.


### Appendix

#### 7.1 Histological Stains and buffers

<table>
<thead>
<tr>
<th>Stain/buffer</th>
<th>Constituents and preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>20X Sorenson buffer (pH 6.8)</td>
<td>0.066 M KH₂PO₄, 0.066M Na₂HPO₄</td>
</tr>
<tr>
<td>May-Grünwald’s stain</td>
<td>0.25% May Grunwald solution in methanol. Dilute 1:1 with 1X Sorenson buffer</td>
</tr>
<tr>
<td>Giemsa’s stain</td>
<td>Dissolve 1g Giemsa powder in to 54ml Glycerine. Heat this to 60 °C for 1.5-2 hours then cool to RT. Add 84ml methanol. Put in tightly stoppered bottle in the dark for a week. Filter before use. Dilute 1 in 9 with 1X Sorenson buffer for staining.</td>
</tr>
<tr>
<td>Weirgert iron haemotoxylin</td>
<td>Solution A: 1g Haematoxylin, 100ml 100% ethanol. Solution B: 4ml 30% aqueous ferric chloride (anhydrous), 1ml concentrated hydrochloric acid, 95ml distilled water. Solutions A and B are made up separately and equal volumes are combined immediately before use</td>
</tr>
<tr>
<td>1% HCl-alcohol</td>
<td>Hydrochloric acid in 70% industrial methylated spirits</td>
</tr>
<tr>
<td>Scott’s Tap Water</td>
<td>3.5g of sodium bicarbonate and 20g of magnesium sulphate dissolved in 1000ml of tap water</td>
</tr>
<tr>
<td>1% phosphomolydbolic acid</td>
<td>10 g molybdophosphoric acid powder in 1000 ml distilled water.</td>
</tr>
<tr>
<td>AFOG solution (pH 1.09)</td>
<td>Boil 2.5 g Aniline Blue in distilled water. Once dissolved, allow to cool. Once cool, add 5 g Orange G and 7.5 g Acid Fuschin. Adjust the pH to 1.09 with HCl.</td>
</tr>
<tr>
<td>Bouin’s fixative</td>
<td>Mix together 750ml Saturated aqueous Picric Acid, 250ml Formalin, 50ml Acetic Acid</td>
</tr>
</tbody>
</table>

Table 7.1. Details of histological stains and buffers.
### 7.2 Dewaxing and AFOG staining wax sections

<table>
<thead>
<tr>
<th>Step</th>
<th>Solution</th>
<th>Incubation time</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Histoclear (National Diagnostics; NAT1330)</td>
<td>30 minutes</td>
<td>Dewaxing</td>
</tr>
<tr>
<td>2</td>
<td>100% Ethanol</td>
<td>5 minutes</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>90% Ethanol</td>
<td>5 minutes</td>
<td>Rehydration</td>
</tr>
<tr>
<td>4</td>
<td>70% Ethanol</td>
<td>5 minutes</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Tap water</td>
<td>Rinsing</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Bouin’s fixative</td>
<td>2 hours at 60 °C, 22 hours at RT</td>
<td>Refixation</td>
</tr>
<tr>
<td>7</td>
<td>Tap water</td>
<td>Rinse until slide is clear</td>
<td>Rinse</td>
</tr>
<tr>
<td>8</td>
<td>Weigert iron haemotoxylin</td>
<td>8 minutes</td>
<td>Nuclear stain</td>
</tr>
<tr>
<td>9</td>
<td>Tap water</td>
<td>Run under tap water until clear</td>
<td>Wash step</td>
</tr>
<tr>
<td>10</td>
<td>1% HCl-alcohol</td>
<td>10 seconds</td>
<td>Differentiates nuclei</td>
</tr>
<tr>
<td>11</td>
<td>Tap water</td>
<td>10 seconds</td>
<td>Wash step</td>
</tr>
<tr>
<td>12</td>
<td>Scott’s tap water</td>
<td>30 seconds</td>
<td>Blue</td>
</tr>
<tr>
<td>13</td>
<td>Tap water</td>
<td>Rinse</td>
<td>Wash step</td>
</tr>
<tr>
<td>14</td>
<td>Check differentiation of nuclei staining.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Distilled water</td>
<td>5 seconds</td>
<td>Wash step</td>
</tr>
<tr>
<td>16</td>
<td>1% phosphomolydbolic acid</td>
<td>5 minutes</td>
<td>Stops aniline blue staining other tissue structures</td>
</tr>
<tr>
<td>17</td>
<td>Distilled water</td>
<td>5 seconds</td>
<td>Wash step</td>
</tr>
<tr>
<td>18</td>
<td>AFOG solution</td>
<td>5(-8) minutes</td>
<td>AFOG staining</td>
</tr>
<tr>
<td>19</td>
<td>Tap water</td>
<td>Rinse 3 times</td>
<td>Wash step</td>
</tr>
<tr>
<td>20</td>
<td>Check staining.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>70% Ethanol</td>
<td>10 seconds</td>
<td>Dehydration</td>
</tr>
<tr>
<td>22</td>
<td>90% Ethanol</td>
<td>10 seconds</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>100% Ethanol</td>
<td>10 seconds</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>Dry in wind tunnel O/N or proceed to mounting</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>Histoclear</td>
<td>5 minutes</td>
<td>Clears tissue/removes alcohol</td>
</tr>
<tr>
<td>26</td>
<td>Coverslip slides with DPX Mountant and allow to dry</td>
<td>O/N</td>
<td>Mounting</td>
</tr>
</tbody>
</table>

**Table 7.2. Protocol for AFOG staining.**
7.3 RT-PCR and qPCR primer details

<table>
<thead>
<tr>
<th>Target</th>
<th>Oligonucleotide sequence of forward (F) and reverse (R) primers (5′-3′)</th>
</tr>
</thead>
</table>
| eef1a1l1 (ef1a) | F: CGTGGTATCACCATTCAGACATTGC  
 R: TCACGCCCATCAGATCTCCGGAAGTGA |
| csf1ra4 | F: ATGGCCTACACCAATTTCCC  
 R: AGTTTGCTGCTGGAATGCTG |
| csf1rb | F: ATGGCGACCTCCTCAACTTC  
 R: ACGGTGTGTCCCTAAAGCTG |
| mfap4 | F: ACGCTTCTCTCTGCTGGAACCA  
 R: CCCCTCACATCCAGGAATCC |
| mpeg1.1 | F: CGGGTTCAAGTCCGTAACCA  
 R: TGGCGTCCAGGATTTCTCTT |
| mpeg1.2 | F: CAGAGAAGCTCCAGTCAGG  
 R: CAGGAATCAGGCTGGAAGGTC |
| marco | F: ACATGAAAACTCAGGAGGCGC  
 R: TGAGTCCACATCCAGGACAGG |
| il1b | F: TGAACGTACATGCAGGACAGG  
 R: AAGACGCCGACTGAATCCACC |
| tnfa | F: GCCCTTTTCTGATACTCACAAG  
 R: TGCCCAAGTGTCTTCTTCTT |
| il10 | F: GCCCTTTTCTGATACTCACAAG  
 R: TGCCCAAGTGTCTTCTTCTT |
| mmd | F: GCTGCTTCTTGTGATGGATGG  
 R: ACCCTGCCAGTAGTGGAGTAG |
| ighm | F: TCAGAGTGAGCAGGGCTGTAAG  
 R: GTGCTGCGGCTGATCATTCTT |
| cd79a | F: TTCAGGCTGTCTTCTTCTTCTG  
 R: TGAGTCCAGAGAGGAGACTCT |
| nkl.3 | F: CTGCTCAACCTGTACATTCTCTC  
 R: CAAGCAGGACATGATCCAGG |
| nkl.4 | F: CCAATGATGCACTCAGGATG  
 R: TCACAAATTCCTGCAGAACAG |
| ltgam (cd11b) | F: TTCAGGCTGTCTTCTTCTTCTG  
 R: TGAGTCCAGAGAGGAGACTCT |
| mertka | F: CATCTGCTACATCGGGTCAG  
 R: GTTGTACCTGACCCATGC |
| il1b | F: TGAACGTCATCATCGGCTCTC  
 R: AAGACGGACTGAATCCACC |
<p>| ccr2 | F: TGAGCTTTATAACCAAGAGAGA |</p>
<table>
<thead>
<tr>
<th>Gene</th>
<th>F:</th>
<th>R:</th>
</tr>
</thead>
<tbody>
<tr>
<td>timd4</td>
<td>CCCGGTGTTTGTCTCTGTCG</td>
<td>TGCTACATGCCAAGTTTCGT</td>
</tr>
<tr>
<td>actb2</td>
<td>CACTGTGGCCCATCTACGAG</td>
<td>TCCACTGGTCTCTTGTGCG</td>
</tr>
<tr>
<td>mhc2dab</td>
<td>GCACGCCAGCTTAACTCAAC</td>
<td>CCCGGTGTTGTCTCTGTCG</td>
</tr>
<tr>
<td>cd45</td>
<td>CAAGGAGCCCGAGAGACCCAG</td>
<td>TCCACTGGTCTCTTGTGCG</td>
</tr>
<tr>
<td>mpx</td>
<td>TGATGGTGTGATGAGGTGTG</td>
<td>CACCTGCACTGAGAGTGGG</td>
</tr>
<tr>
<td>gata2a</td>
<td>GGTACCGCAATCCGCTGTCGC</td>
<td>GCACGCCAGCTTAACTCAAC</td>
</tr>
<tr>
<td>lck</td>
<td>TGGGCCACGATTGATATCCG</td>
<td>TCCACTGGTCTCTTGTGCG</td>
</tr>
</tbody>
</table>

Table 7.3. Oligonucleotides sequences of RT-PCR and qPCR primers. All primers were used at an annealing temperature of 60 °C and amplified over 35-40 cycles.
7.4 Fiji Modular Image Analysis

The Modular Image Analysis (MIA) tool is designed by Dr Stephen Cross (University of Bristol). The supplementary files detailed below can be found at https://drive.google.com/drive/folders/1cBxcHQ_eFaKtmSyS4shjhBpk7bCEBKXI?usp=sharing.

7.4.1 Cell shape analysis plugin

Processed using MIA v0.21.8 for Fiji (Schneider et al., 2012, Schindelin et al., 2012). Cells detected in both channels using same method: 2D median filter to reduce image noise whilst retaining sharp object edges; image binarised using global Otsu threshold (Otsu, 1979); applied threshold was systematically reduced to 75% of the calculated value to increase the segmented areas; binarised image was subject to distance-based watershed transform to separate adjacent regions that had become merged; 2D holes (enclosed background pixel regions) in binarised images filled; objects detected as contiguous regions of foreground-labelled pixels (Legland et al., 2016); detected cells subject to minimum volume threshold. Any cells smaller than this threshold were discarded from further analysis. Cells in contact with the 2D image edge (i.e. not considering Z-axis) were also removed from further analysis as their areas could not be accurately determined. Cells from channels 1 and 2 were related based on their mutual overlap. Any cells with at least 25% overlap with another cell from the opposite channel were classed as being related. The number of related (overlapping) cells was calculated. 2D ellipses were fit to each cell and used to calculate eccentricities (Domander et al., 2021). MIA and plugin files are attached in Supplementary Files.

7.4.2 3D tracking plugin

Processed using MIA v0.21.8. Plugin for the analysis of xyzt images acquired using the ex vivo platform. The plugin segments cells detected in two channels and tracks their migration in 3D. Plugin attached in Supplementary Files.
7.5 **Fiji macros for image analysis**

List of macros used for image analysis using Fiji software. Macros are attached in the Supplementary Files found at:

https://drive.google.com/drive/folders/1cBxcHQ_eFaKtmSyS4shjhBpk7bCEBKXI?usp=sharing.

- Macro 1: Cell counting macro to quantify *csf1ra*+ cells in a maximum projection of whole mount imaging.
- Macro 2: Macro to quantify collagen area from AFOG stained tissue sections.
- Macro 3: Cell counting macro to quantify *mpeg1.1*+ cells in a maximum projection of whole mount imaging.
- Macro 4: Macro for the quantification of Collagen I area of immunofluorescence sections.
- Macro 5: Macro for the quantification of L-plastin area of immunofluorescence sections.
- Macro 6: Macro for the quantification of EdU+ cells in immunofluorescence sections.
- Macro 7: Macro for the quantification of TUNEL+ cells in immunofluorescence sections.

7.6 **Legends for Video files**

All video files can be found within the Supplementary Files, found at:

https://drive.google.com/drive/folders/1cBxcHQ_eFaKtmSyS4shjhBpk7bCEBKXI?usp=sharing.

**Video 1. Live imaging of a superficial vessel on the flank of an adult Tg(*mpeg1.1:mCherry*); TgBAC(*csf1ra:GFP*) zebrafish.** Panels show *mpeg1.1:mCherry* fluorescence (A), *csf1ra:GFP* fluorescence (B) and merge + brightfield (C). Scale bar: 20µm.

**Video 2. Ex vivo imaging in a 1 dpi Tg(*mpeg1.1:mCherry*); TgBAC(*csf1ra:GFP*) ventricle.** Video shows maximum intensity projections of *csf1ra:GFP* fluorescence (green), *mpeg1.1:mCherry* (red) and merged channels. Frames are at 6 minute intervals acquired over a 5 hour period. Scale bar = 100 µm.
**Video 3.** *Ex vivo* imaging in an unwounded Tg(mpeg1.1:mCherry); TgBAC(csf1ra:GFP) ventricle. Video shows maximum intensity projections of csf1ra:GFP fluorescence (green), mpeg1.1:mCherry (red) and merged channels. Frames are at 6 minute intervals over a 1 hour period. Scale bar = 100 µm.

**Video 4.** *Ex vivo* imaging of 7 dpi + 1 day cultured Tg(mpeg1.1:mCherry); TgBAC(csf1ra:GFP) ventricle. Hearts were cultured in culture media in a 12-well dish for 24 hours prior to imaging. Video shows maximum projections of csf1ra:GFP, mpeg1.1:mCherry, and merged channel images. Frames are at 9 minute intervals acquired over a 2 hour period. Scale bar = 20 µm.

**Video 5.** *Ex vivo* imaging the unwounded Tg(mpeg1.1:mCherry); TgBAC(csf1ra:GFP) ventricle using spinning disk confocal microscopy. Video shows maximum projections of csf1ra:GFP, mpeg1.1:mCherry, and merged channel images. Frames are at 2.5 minute intervals for 2 hours. Scale bar = 20 µm.

**Video 6.** Video showing tracks of migrating cells shown in Video 5, using the 3D Tracking Plugin detailed in Appendix 7.4.2.

**Video 7.** 3D projection of a cleared, uninjured wild type Tg(mpeg1.1:mCherry); TgBAC(csf1ra:GFP) ventricle. mpeg1.1 is shown in red; csf1ra is shown in green and PH3 staining is shown in magenta.

**Video 8.** 3D projection of a cleared, uninjured wild type Tg(mpeg1.1:mCherry); TgBAC(csf1ra:GFP) ventricle. mpeg1.1 is shown in red; csf1ra is shown in green and PH3 staining is shown in magenta.

**Video 9.** *Ex vivo* imaging the unwounded csf1ra<sup>het/het</sup>; Tg(mpeg1.1:mCherry); TgBAC(csf1ra:GFP) ventricle using spinning disk confocal microscopy. Video shows maximum projections of csf1ra:GFP, mpeg1.1:mCherry, and merged channel images. Frames are at 2.5 minute intervals for 2 hours. Scale bar = 20 µm.

**Video 10.** 3D projection of a cleared, 1 dpi wild type Tg(mpeg1.1:mCherry); TgBAC(csf1ra:GFP) ventricle. mpeg1.1 is shown in red; csf1ra is shown in green and PH3 staining is shown in magenta.
Video 11. 3D projection of a cleared, 1 dpi csf1ra<sup>het/het</sup>; Tg(mpeg1.1:mCherry); TgBAC(csf1ra:GFP) ventricle. mpeg1.1 is shown in red; csf1ra is shown in green and PH3 staining is shown in magenta.

Video 12. 3D projection of a cleared, 7 dpi wild type Tg(mpeg1.1:mCherry); TgBAC(csf1ra:GFP) ventricle. mpeg1.1 is shown in red; csf1ra is shown in green and PH3 staining is shown in magenta.

Video 13. 3D projection of a cleared, 7 dpi csf1ra<sup>het/het</sup>; Tg(mpeg1.1:mCherry); TgBAC(csf1ra:GFP) ventricle. mpeg1.1 is shown in red; csf1ra is shown in green and PH3 staining is shown in magenta.

7.7 Analysis files for transcriptomics data

Raw analysis files can be found within the Supplementary Files, found at: https://drive.google.com/drive/folders/1cBxcHQ_eFaKtmSyS4shjhBpk7bCEBKXI?usp=sharing.


### 7.8 *csf1r* Zebrafish mutants:

<table>
<thead>
<tr>
<th>Line</th>
<th>ZFIN ID</th>
<th>Type of mutation</th>
<th>Mutation</th>
<th>Protein</th>
<th>Founding lab</th>
<th>Publications</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>csf1ra</em>&lt;sup&gt;Δ&lt;sub&gt;1&lt;/sub&gt;&lt;/sup&gt;</td>
<td>ZDB-ALT-001205-14</td>
<td>Point mutation</td>
<td>G&gt;A at position 1949</td>
<td>V614M</td>
<td></td>
<td>(Parichy et al., 2000) (Wu et al., 2018, Parichy et al., 2000)</td>
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<tr>
<td><em>csf1ra</em>&lt;sup&gt;Δ&lt;sub&gt;2&lt;/sub&gt;&lt;/sup&gt;</td>
<td>ZDB-ALT-001205-12</td>
<td>Point mutation</td>
<td>T&gt;A at position 820</td>
<td>N237K</td>
<td></td>
<td>(Parichy et al., 2000)</td>
</tr>
<tr>
<td><em>csf1ra</em>&lt;sup&gt;Δ&lt;sub&gt;α&lt;/sub&gt;&lt;/sup&gt;</td>
<td>ZDB-ALT-001205-16</td>
<td>Small deletion</td>
<td>2648-2652del</td>
<td>L847Δ Early truncation/premature stop</td>
<td></td>
<td>(Parichy et al., 2000)</td>
</tr>
<tr>
<td><em>csf1ram&lt;sup&gt;Δ5&lt;/sup&gt;</em></td>
<td>ZDB-ALT-170811-1</td>
<td>Point mutation</td>
<td>G&gt;T at position 1466 (ENU mutagenesis)</td>
<td>E454X (Truncation)</td>
<td>Harris lab</td>
<td>(Caetano-Lopes et al., 2020)</td>
</tr>
<tr>
<td><em>csf1rb</em>&lt;sup&gt;Δ&lt;sub&gt;8&lt;/sub&gt;&lt;/sup&gt;</td>
<td>ZDB-ALT-200427-8</td>
<td>Indel</td>
<td>c.479-482delinsTGAATTAT (Frameshift) (CRISPR-Cas9)</td>
<td>Early truncation</td>
<td>Harris lab</td>
<td>(Caetano-Lopes et al., 2020)</td>
</tr>
<tr>
<td><em>csf1rb</em>&lt;sup&gt;Δ&lt;sub&gt;12&lt;/sub&gt;&lt;/sup&gt;</td>
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<td>Small deletion</td>
<td>c.476-488del (Frameshift) CRISPR-Cas9</td>
<td>Early truncation</td>
<td>Harris lab</td>
<td>(Caetano-Lopes et al., 2020)</td>
</tr>
<tr>
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<td>Small deletion</td>
<td>Exon 3 (TALEN)</td>
<td>Early truncation</td>
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<td>(Oosterhof et al., 2018) (Kuil et al., 2020)</td>
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<td><em>csf1rb</em>&lt;sup&gt;Δ25&lt;/sup&gt;</td>
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<td>Point mutation</td>
<td>G&gt;A (splice site)</td>
<td>Splice site?</td>
<td></td>
<td>(Kuil et al., 2020, Ferrero et al., 2021)</td>
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</tbody>
</table>

Table 7.4. List of published *csf1ra* and *csf1rb* mutants with details of the mutation and publications in which these lines have been used.