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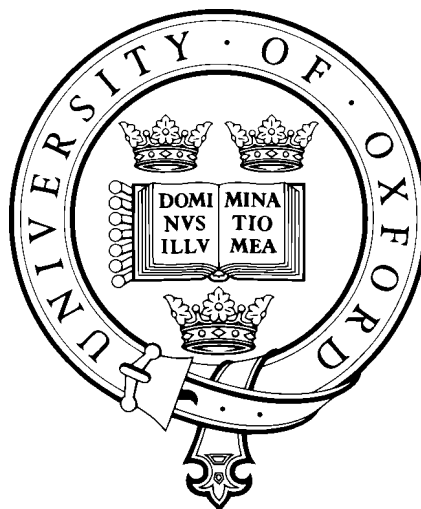
Characterisation of the development of cardiac lymphatic vessels and their response to myocardial infarction

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Abstract

The lymphatic vasculature is a blind-ended network, present in all mammals, which is responsible for immune surveillance, tissue fluid regulation and absorption of lipid-containing chylomicrons into the gut. During pathological conditions lymphatic vessels expand via the process of lymphangiogenesis in order to aid the clearance of inflammation and interstitial fluid. The systemic lymphatic vasculature begins to develop in the mouse embryo around E9-9.5. This process is initiated at three venous sites within the embryo where lymphatic endothelial cells (LECs) migrate away from veins to form lymphatic structures. Since the 17th century it has been known that the lymphatic vasculature is present in the heart, however, until recently little was known regarding its developmental origins or its function during cardiovascular disease. The aims of this thesis were to characterise the development of cardiac lymphatic vessels and their response to adult heart injury, as a means to identify whether the lymphatics reactivate embryonic programs during pathological insult and to establish a potential basis for future therapeutic targeting towards improved repair.

I have characterised the spatiotemporal development of the murine cardiac lymphatics and have performed lineage tracing to determine their cellular origins. This revealed that cardiac LECs are not solely derived from veins, which was confirmed by analysis of conditional PROX1 knockout mouse hearts. Collectively these data refute the previous dogma that all lymphatic vessels are venous-derived. I have also performed studies to assess the roles of lymphatic vessels following myocardial infarction (MI). This revealed that expression of the pro-lymphangiogenic growth factor VEGF-C is reactivated in the heart post-MI, driving cardiac lymphangiogenesis. Treatment with additional recombinant VEGF-C further enhanced the lymphangiogenic response and resulted in improvements in cardiac function. Furthermore, VEGF-C treatment resulted in reduced numbers of macrophages within the heart by day 7 post-MI. This suggests that enhancing lymphangiogenesis leads to increased clearance of the post-MI inflammatory cells, which may modulate levels of inflammation and fibrosis, resulting in an improved outcome. I have also utilised genetic mouse models (LYVE-1 knockout and PROX1 heterozygous lines) with defects in their lymphatic vasculature. Following MI in LYVE-1 knockout animals, where transmigration of leucocytes into lymphatic vessels is impaired, neutrophils and macrophages were found to accumulate within the heart at day 7 post-injury. Together these data demonstrate that therapeutic targeting of the cardiac lymphatic vasculature may have a significant impact on the resolution of inflammation and the post-MI healing process.

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List of abbreviations

4-OHT	4-hydroxytamoxifen
AGM	Aorta-gonad-mesonephros
BEC	Blood endothial cell
BMC	Bone marrow-derived cell
BSA	Bovine serum albumin
CCV	Common cardinal vein
cDNA	Complementary DNA
DAB	3,3'-Diaminobenzidine
DAMPs	Damage-associated molecular patterns
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EMPs	Erythro-myeloid progenitors
ER	Oestrogen receptor
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FMO	Fluorescence minus one
H&E	Hematoxylin and eosin
HBSS	Hank's balanced salt solution
HSCs	Haemopoietic stem cells
HSPCs	Haemopoietic stem and progenitor cells
I.P.	Intraperitoneal
ISV	Intersomitic vessel
JLS	Jugular lymph sacs
JV	Jugular vein
LAD	Left anterior decending artery
LEC	Lymphatic endothelial cell
LV	Left ventricle
LVEF	Left ventricular ejection fraction
MHC	Major histocompatibility complex
MI	Myocardial infarction
MLN	Mediastinal lymph node
MMP	Matrix metalloproteinases
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
PACs	Paracordal cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PRRs	Pattern recognition receptors
qRT-PCR	Real time quantitative PCR
RBC	Red blood cell
RNA	Ribonucleic acid
S.E.M.	Standard error of the mean
SV	Sinus venosus

sVP	Superficial venous plexus
TIMP	Tissue inhibitor of metalloproteinase
Tregs	Regulatory T cells
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

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1. Introduction

1.1. The lymphatic vasculature

The lymphatic vasculature is composed of a network of capillaries, collector vessels and lymph nodes (Alitalo, Tammela et al., 2005). Single layers of oak-leaf shaped lymphatic endothelial cells (LECs) connect together by “button-like junctions” to form a blind-ended network of lymphatic capillaries (Baluk, Fuxe et al., 2007, Koltowska, Betterman et al., 2013). This comprehensive network extends throughout the entire body in all mammals and is essential for the maintenance of tissue fluid homeostasis, absorption of dietary lipids and immune responses. Historically lymphatic vessels have often been overlooked, having simply been considered to be the body’s sewerage system, however, they do play significant roles in many diseases. They provide the primary route for cancer metastasis, undergo substantial changes during inflammation and contribute directly to conditions such as lymphedema and lymphangioma. Given these important roles, research focussing on the development of lymphatic vessels and their functions in disease has attracted increasing interest in recent years. Interestingly it was only last year that lymphatic vessels were found to be present within the meninges of the brain (Aspelund, Antila et al., 2015, Louveau, Smirnov et al., 2015) giving rise to many new questions regarding their potential role in neurological diseases.

1.2. Development of murine lymphatic vessels

In mammals blood vessels initially form through the process of vasculogenesis, where angioblasts form clusters which differentiate into endothelial cells (Carmeliet & Jain, 2011). Expansion of the vascular plexus then occurs through angiogenesis and key signalling molecules act to specify vessels as arteries or veins (Swift & Weinstein, 2009). In the mouse embryo lymphatic vessels first begin to form at around embryonic day (E)

9-9.5 after these initial processes of blood vessel development have taken place. The first LECs bud from three venous sites in the embryo. These sites, as shown in **Figure 1.1**, are the anterior wall of the cardinal vein (Wigle & Oliver, 1999), intersomitic vessels (ISVs) (Yang, García-Verdugo et al., 2012) and the superficial venous plexus (sVP) (Hägerling et al., 2013). Expansion of the network then occurs through lymphangiogenesis, forming a primitive systemic lymphatic plexus throughout the embryo by E14.5. Further growth and remodelling of this plexus occurs through to postnatal stages in order to form a mature lymphatic network (Norrmen, Ivanov et al., 2009). A diagrammatic representation of this process is shown in **Figure 1.2**. The elucidation of this mechanism has relied upon the identification of a number of genes/proteins which are specifically expressed in LECs and play key roles in the developmental process. Furthermore, the deletion of these genes in mouse models has provided further insights into the molecular pathways which control the specification and expansion of the lymphatic network. These key genes/proteins will be introduced in more detail in the following sections.

Figure 1.1

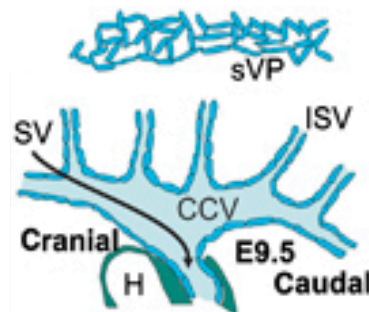


Figure 1.1: Schematic of venous sites from which lymphatic endothelial cells arise

Lymphatic endothelial cells, which form the systemic lymphatic vasculature, bud from three main venous sites in the embryo. These are the common cardinal vein (CCV), surface venous plexus (sVP) and intersomitic vessels (ISVs). SV = sinus venosus, H = heart. Figure adapted from (Hägerling, Pollmann et al., 2013).

Figure 1.2

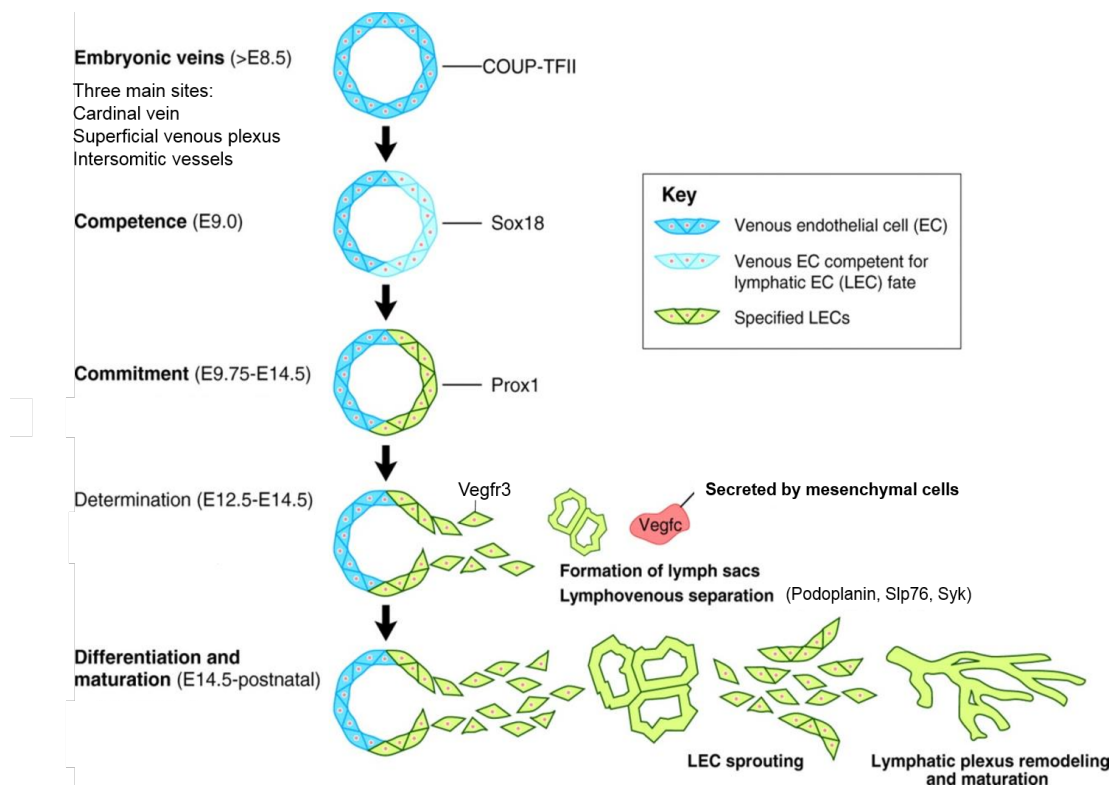


Figure 1.2: Development of the systemic lymphatic vasculature

Lymphatic development begins at E9.5 in the mouse after vasculogenesis and primary sprouting of venous blood endothelial cells. There are three main COUP-TFII⁺ venous sites where this process is initiated: the dorsolateral wall of the cardinal vein, surface venous plexus (sVP) and intersomitic vessels. SOX18 is expressed in a subset of the venous cells and, together with COUP-TFII, binds to the *Prox1* promoter initiating its transcription. PROX1 is considered to be the master regulator as it is essential for the determination and maintenance of LEC fate. From E12.5 onwards PROX1⁺ LECs migrate away from veins to form primitive lymphatic structures. This process is driven by the growth factor VEGF-C which binds to VEGFR-3 expressed on LECs. The budding of LECs away from veins is also dependant on lymphovenous separation in which the protein PODOPLANIN and the haematopoietic factors SLP76 and SYK play key roles. By E14.5 a primitive lymphatic plexus is present throughout the embryo which continues to undergo remodelling and maturation through to postnatal stages. LEC = lymphatic endothelial cell. Figure adapted from (Oliver & Srinivasan, 2010).

1.2.1. Key proteins for the initial specification of LECs

1.2.1.1. PROX1

The transcription factor prospero-related homeobox gene 1 (PROX1) was discovered in 1999 to be essential for the development of lymphatic vasculature (Wigle & Oliver, 1999). PROX1 was the first protein which was found to be expressed in the lymphatic vasculature and not the blood vasculature during embryonic development. Generation of *Prox1* null mice revealed E14.5 embryos that presented with a complete lack of lymphatic vasculature, as LECs failed to bud from the cardinal vein. This resulted in severe oedema and at later stages embryonic lethality, suggesting that PROX1 is essential for the development of the lymphatic vasculature. Further insight into the role of PROX1 during lymphatic development came in 2002 when it was demonstrated that addition of *Prox1* cDNA adenovirus to blood endothelial cells prompted these cells to become LECs (Hong, Harvey et al., 2002). It has also been revealed that *mirR-181a* provides negative regulation of PROX1 levels in endothelial cells (Kazenwadel, Michael et al., 2010). This may be important for the silencing of *Prox1* in blood vessels to prevent a switch to LEC fate. Together these data have provided evidence that PROX1 is the ‘master regulator’ of lymphatic development as it is essential for the specification and maintenance of LEC fate.

Interestingly PROX1 is also expressed during cardiac development (Oliver, Sosa-Pineda et al., 1993) and is involved in the maintenance of cardiomyocyte sarcomeric structure and growth (Risebro, Searles et al., 2009). This was demonstrated in PROX1 knockout mice which have abnormal heart development; these mutants also presented with cardiac oedema, a potential indicator of defective lymphatic vessels.

1.2.1.2. COUP-TFII

COUP transcription factor 2 (COUP-TFII or NR2F2) is expressed in all venous cells (You, Lin et al., 2005) and also in LECs. Within LECs it directly binds to PROX1 and acts a co-regulator to further increase PROX1 expression in venous sites of LEC sprouting and to maintain the LEC fate (Lee, Kang et al., 2009, Srinivasan, Geng et al., 2010, Yamazaki, Yoshimatsu et al., 2009)

1.2.1.3. SOX18

SRY-related HMG box 18 (SOX18) has been shown to be a molecular switch which is able to activate LEC fate (Francois, Caprini et al., 2008). SOX18 is transiently expressed in a subset of cells within the cardinal vein which later express PROX1 and bud off to form lymphatic vessels. It was discovered that the *Prox1* promoter has two putative Sox binding sites (SoxA and SoxB) to which SOX18 is able to bind and initiate the switch towards LEC fate by prompting the transcription of PROX1. Characterisation of *Sox18* null embryos revealed a similar phenotype to *Prox1* null embryos at E14.5, including failure of LECs to differentiate from the cardinal vein, oedema and ultimately lethality. This work indicated that SOX18 acts upstream of PROX1 in the pathway to establish LEC fate, however, elucidation of the signalling factors which act upstream of SOX18 still remain to be established.

1.2.2. Key proteins for separation of lymphatic vessels from veins and expansion of the network

1.2.2.1. PODOPLANIN

PODOPLANIN is a transmembrane glycoprotein which is first expressed in the lymphatic endothelium from around E11.0-11.5 (BreitenederGeleff, Matsui et al., 1997, Schacht, Ramirez et al., 2003, Yang et al., 2012). *Podoplanin* knockout mice do not

survive postnatally and display severe oedema in addition to dilated lymphatic vessels which are blood-filled (Bertozzi, Schmaier et al., 2010, Schacht et al., 2003). These studies have highlighted the crucial role PODOPLANIN plays in the process of lymphovenous separation, which is required as developing lymphatic vessels migrate away from veins. Binding of PODOPLANIN to C-type lectin-like receptor 2 (CLEC-2) results in activation of the haematopoietic signalling factors SLP-76 and SYK, enabling platelet aggregation and lymphovenous separation (Abtahian, Guerriero et al., 2003, Bertozzi et al., 2010, Schacht et al., 2003, Sebzda, Hibbard et al., 2006).

1.2.2.2. VEGFR-3

Vascular endothelial growth factor receptor-3 (VEGFR-3) (also known as fms-like tyrosine kinase Flt4) was the first LEC marker to be discovered in 1995 (Kaipainen, Korhonen et al., 1995). It is expressed within angiogenic sprouts throughout the embryonic blood vasculature, but is largely restricted to lymphatic endothelium by E14.5 (Hägerling et al., 2013, Kaipainen et al., 1995). However, it is important to note that there are some sites of postnatal VEGFR-3 activity within blood vessels. VEGFR-3 is expressed at the angiogenic front within the retina during early postnatal stages and is required for angiogenesis which occurs in adults in response to disease or injury (Tammela, Zarkada et al., 2008).

VEGFR-3 is a transmembrane tyrosine kinase receptor and is closely related to VEGFR-1 and VEGFR-2 which are involved in blood vessel angiogenesis (Olsson, Dimberg et al., 2006). Vascular endothelial growth factors C and D (VEGF-C and VEGF-D) act as the main signalling proteins during lymphangiogenesis by binding to, and activating VEGFR-3 (Haiko, Makinen et al., 2008). VEGF-C is secreted by the mesenchyme in the region of sprouting LECs and then binds to VEGFR-3 expressed on LECs, activating the

receptor and promoting lymphangiogenesis (Karkkainen, Haiko et al., 2004, Kukkk, Lymboussaki et al., 1996). *Vegfc*^{-/-} embryos have also been observed to have reduced numbers of PROX1⁺ LEC progenitors and lack lymphatic vessels (Hägerling et al., 2013, Srinivasan, Escobedo et al., 2014). Creation of a *Vegfr3-LacZ* knock in mouse revealed further insights into the role of VEGFR-3 in development. *Vegfr3* null embryos were embryonic lethal by E12.5 and at E10 embryos displayed an underdeveloped yolk sac blood vasculature in addition to cardiac defects, highlighting the importance of VEGFR-3 signalling for early blood vessel and cardiac development. *Vegfr3*^{LacZ/+} heterozygous embryos were found to be phenotypically normal and displayed VEGFR-3 expression throughout the developing lymphatic network (Dumont, 1998). Recent evidence has revealed that there are five PROX1 binding sites within *Vegfr3* regulatory regions. This allows direct dosage dependant targeting of the *Vegfr3* gene and the establishment of a positive feedback loop between PROX1 and VEGFR-3 (Srinivasan et al., 2014).

1.2.2.3. CCBE1 and ADAMTS3

Collagen and calcium-binding EGF domain-containing protein 1 (CCBE1) is expressed in the heart and in close proximity to developing lymphatic vessels where it acts to enhance the activity of VEGF-C, thus promoting lymphangiogenesis. *Ccbe1* was first discovered to be essential for lymphangiogenesis in zebrafish forward-genetic screens (Hogan, Bos et al., 2009). *Ccbe1* knockout mouse embryos also displayed a complete lack of definitive lymphatic structures due to the failure of PROX1⁺ LECs to bud from the cardinal vein (Bos, Caunt et al., 2011, Hägerling et al., 2013). CCBE1 acts to promote proteolytic cleavage of pro-VEGF-C into the fully active form by activating the ADAM metalloproteinase with thrombospondin type 1 motif 3 (ADAMTS3) metalloprotease (Bos et al., 2011, Jeltsch, Jha et al., 2014, Le Guen, Karpanen et al., 2014). This proposed mechanism has been supported by the generation of *Adamts3*^{-/-} embryos which

displayed severe oedema and a complete lack of lymphatic vessels (Janssen, Dupont et al., 2016).

1.2.2.4. LYVE-1

Lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) was discovered in 1999 to be specifically expressed in LECs (Banerji, Ni et al., 1999) and later also in liver blood sinusoids and macrophages (Carreira, Nasser et al., 2001). Despite LYVE-1 being expressed on lymphatic vessels it does not appear to be required for their formation as *Lyve-1^{-/-}* embryos develop lymphatic vessels normally and *Lyve-1^{-/-}* adult animals display normal tumour lymphangiogenesis (Gale, Prevo et al., 2007). Recent work has revealed that although LYVE-1 is not required for lymphatic vessel development, it does in fact have an important functional role in the migration of macrophages across the inflamed lymphatic endothelium (Lawrance, Banerji et al., 2016), (Bhattacharjee & Jackson, unpublished data).

1.2.3. Development of cardiac lymphatic vessels

The presence of an extensive lymphatic network within the heart was first described by Rudbeck in 1653 (Rudbeck, 1653). Studies in various animal models and on human samples have revealed that the mammalian heart possesses an extensive lymphatic capillary network (Aagaard, 1924, Bullon & Huth, 1972, Johnson & Blake, 1966, Lupinski, 2009) which allows the drainage of lymph to sub-epicardial pre-collector vessels that ultimately drain to the mediastinal lymph nodes (Lupinski, 2009, Miller, 1982, Weirather, Hofmann et al., 2014).

Prior to the work presented in this thesis some basic descriptive studies had been published on the development of murine cardiac lymphatic vessels. These studies,

which utilised immunohistochemistry and ink injections, revealed that cardiac lymphatics are present within the mouse heart from E11.0-E12.0 and later expand through the process of lymphangiogenesis (Flaht, Jankowska-Steifer et al., 2012, Flaht-Zabost, Gula et al., 2014, Juszynski, Ciszek et al., 2008, Karunamuni, Yang et al., 2010). Two pre-collector vessels were also found to form between E15 and E18 on the sub-epicardial surface (Ciszek, Skubiszewska et al., 2007, Flaht-Zabost et al., 2014). Whilst these studies provide basic anatomical descriptions of the cardiac lymphatics they do not provide comprehensive analysis of the full developmental process with multiple time points and markers. The work presented in later chapters of this thesis provides further insights into the mechanisms by which the cardiac lymphatics develop and the cellular origins from which they derive.

1.2.4. Cellular origins of lymphatic vessels

Scientific debate over the cellular origins of lymphatic vessels dates back to the early 20th century when two main opposing theories were suggested. The first theory, known as the “centrifugal model” was put forward by Florence Sabin in 1902. Sabin performed ink injections on pig embryos which led to the conclusion that lymphatics bud from the veins (Sabin, 1902). This was followed in 1910 by Huntingdon and McClure’s “centripetal” model which suggested that lymphatics arise from distinct cells originating from mesenchymal spaces, but do not derive from veins and was based on studies of serial sections from cat embryos (Huntingdon G, 1910).

Since these historical studies, immunofluorescent staining and lineage tracing have been employed to determine the precise origin of lymphatic vessels. The Cre-lox system (Hoess, Ziese et al., 1982) has been used by many lineage tracing studies in order to label a specific population of cells and their progeny as shown in **Figure 1.3**. A mouse

in which Cre is expressed under the control of a tissue specific promoter is crossed with a Cre-dependent *lacZ* (Soriano, 1999) or fluorescent reporter strain (R26R) (Srinivas, Watanabe et al., 2001). Recombination then occurs and a *STOP* cassette which is flanked by two *LoxP* sites is excised leading to constitutive expression of the reporter in these cells and their progeny.

Figure 1.3

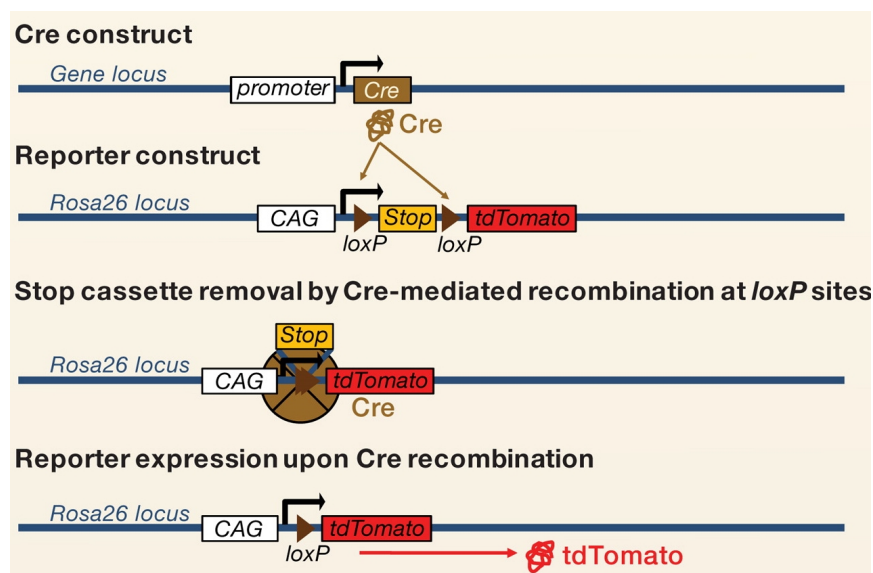


Figure 1.3: Cre-lox system for lineage tracing

This schematic shows the genetic elements in the Cre-lox system. Cre recombinase is constitutively expressed in a specific set of cells, under to the control of a tissue specific promoter. When crossed with a *R26R-tdTomato* mouse recombination occurs at the *loxP* sites in the cells in which Cre is expressed. This results in the excision of the *STOP* cassette and expression of the tdTomato reporter in these cells and all of their daughter cells. Figure adapted from (Kretzschmar & Watt, 2012).

Using the Cre-lox system *Tie2-Cre;R262R-LacZ* embryos were generated in which the venous endothelium and its derivatives are labelled. This revealed PROX1⁺ cells in the lymph sacs which were labelled by *LacZ*. In addition *Tie2-Cre;Prox1^{flox}/LacZ* conditional knockout embryos were generated and were found to lack most PROX1⁺ LECs within the cardinal vein (Srinivasan, Dillard et al., 2007). *Runx1*^{-/-} embryos, which lack definitive haematopoiesis, presented with lymph sacs which were morphologically normal. The lymph sacs were, however, blood filled, indicating that although haematopoietic cells do not give rise to LECs they do play a role in the process of lymphovenous separation (Srinivasan et al., 2007). Together the findings of this study indicated that systemic lymphatic vessels are solely venous derived.

More recently studies have been performed to investigate the cellular origins of organ-based lymphatic vessels. Linda Klotz, a previous student in the Riley group, observed that not all cardiac LECs are labelled by *Tie2-Cre* and *Pdgfrb-CreER* lines which target the blood vasculature. Further lineage tracing studies performed by Klotz and by myself were undertaken to investigate whether a population of cardiac LECs may originate from a haematopoietic origin. This work is presented in chapters 3 and 4 of this thesis. Studies in other organ-based lymphatic beds, published at a similar time to the work presented in this thesis, also support our finding that not all LECs derive exclusively from veins. Within both the mesentery and skin a proportion of LECs are non-venous in their cellular origin (Mahadevan, Welsh et al., 2014, Martinez-Corral, Ulvmar et al., 2015, Stanczuk, Martinez-Corral et al., 2015). The non-venous progenitor which gives rise to 33% of skin based LECs has not yet been discovered (Martinez-Corral et al., 2015). Two studies have reported non-venous origins of LECs within the mesentery. One demonstrated that arteriogenesis is required for formation of lymphatic vessels specifically within the dorsal mesentery (Mahadevan et al., 2014).

Another found that a proportion of mesenteric LECs derive from ckit⁺ cells which were reported to arise from the haemogenic endothelium (Stanczuk et al., 2015).

1.2.5. Haemogenic endothelium

The haemogenic endothelium represents a specialised subset of endothelial cells which are able to give rise to haematopoietic stem and progenitor cells (HSPCs) during primitive and definitive haematopoiesis, reviewed recently by (Gritz & Hirschi, 2016, Swiers, Rode et al., 2013). Only a small number (1-3%) of endothelial cells within sites of haemogenic endothelium have the ability to give rise to haematopoietic cells (Goldie, Lucitti et al., 2008, Marcelo, Sills et al., 2013). RUNX1 is a key regulator which is expressed at high levels within these sites and appears to be required for haematopoietic activity (Chen, Yokomizo et al., 2009).

Primitive haematopoiesis occurs prior to the onset of circulation within the embryonic yolk sac from E6.5, forming CSF1R⁺ erythro-myeloid progenitors (EMPs) which give rise to adult tissue-resident macrophages in multiple organs (Gomez Perdiguero, Klapproth et al., 2015). It has been demonstrated a large proportion of EMPs originate from Tie2⁺ cells (Frame, Fegan et al., 2016, Gomez Perdiguero et al., 2015, Hoeffel, Chen et al., 2015, Palis, Robertson et al., 1999). Tie2 was originally defined as an endothelial specific promoter (Schlaeger, Bartunkova et al., 1997) which has also been reported to be expressed in sites of haematopoietic activity including yolk sac progenitors, the aorta-gonad-mesonephros (AGM) region, fetal liver and haematopoietic stem cells (HSCs) (Cumano & Godin, 2007). Analysis using a constitutive *Tie2-Cre* line revealed that 88% of yolk-sac haematopoietic progenitors derive from a Tie2⁺ origin (Tang, Harrington et al., 2010). The remaining unlabelled 12% may be a result of inefficient

Cre recombination or could represent a population of cells which derive from a Tie2⁺ source.

Definitive haematopoiesis and the formation of CD31⁺ CD41⁺ c-Kit⁺ SCA-1⁺ HSCs, which have the ability to differentiate into all types of blood cell, takes place post-circulation. This occurs within the placenta from E9-9.5 (Alvarez-Silva, Belo-Diabangouaya et al., 2003, Gekas, Dieterlen-Lievre et al., 2005, Ottersbach & Dzierzak, 2005, Rhodes, Gekas et al., 2008) and then within the AGM region and the endocardium of the embryo proper (de Bruijn, Speck et al., 2000, Medvinsky & Dzierzak, 1996, Muller, Medvinsky et al., 1994, Nakano, Liu et al., 2013). HSCs populate the foetal liver by E11-12 (Kumaravelu, Hook et al., 2002, Morrison, Hemmati et al., 1995) and the bone marrow by E16.5 (Coskun, Chao et al., 2014).

1.3. Functions of the lymphatic vasculature and its roles in during homeostasis and disease

1.3.1. Maintenance of tissue fluid balance

One of the key functions of the lymphatic system is to maintain tissue-fluid homeostasis; the gaps between LECs at the ends of capillaries allow extravasated fluid to enter into the lymphatics for drainage (Tammela & Alitalo, 2010). This fluid then moves into larger collecting vessels which have a thin basement membrane, intraluminal valves, some pericyte coverage, and a smooth muscle lining (Oliver, 2004, Tammela & Alitalo, 2010). The transported lymph drains to regional lymph nodes before ultimately reaching the thoracic duct and where it is returned to the venous system at the subclavian vein. Given the crucial role of the lymphatic vasculature in maintenance of tissue-fluid homeostasis it is unsurprising that oedema, caused by an accumulation of

fluid, can result from lymphatic defects. Many mouse models in which lymphatic development is impaired display severe oedema and embryonic lethality (Karkkainen et al., 2004). Genetic mutations in humans can cause lymphedema, where fluid retention and tissue swelling occurs in a localized area. For example a mutation in VEGFR-3 results in Milroy's disease in which defective lymphatic drainage presents most commonly in the legs (Mellor, Hubert et al., 2010). Lymphedema can also result from surgical damage, for example around 20-30% of post-breast cancer surgery patients develop this condition following axillary lymph node dissection (McLaughlin, Wright et al., 2008, Stanton, Modi et al., 2009).

1.3.2. Immune cell trafficking

The lymphatic vasculature is important for immune surveillance during normal physiology. Lymph, which contains cellular debris, antigens and antigen-loaded dendritic cells, is drained to the lymph nodes where adaptive immune responses are elicited. This provides information to prime lymphocytes or to modulate immune tolerance for self-antigens (Card, Yu et al., 2014). During local tissue inflammation lymphatic flow is enhanced, which is driven by an increase in the production of lymph and enhanced pumping efficiency of collecting vessels (Miteva, Rutkowski et al., 2010, Zawieja, 2009). The increased demand which is imposed on the lymphatic network at sites of inflammation triggers an expansion of the network; a diagrammatic representation of this process is shown in **Figure 1.4**. Examples of when this takes place include wound healing (Zampell, Yan et al., 2012), transplant rejection (Kerjaschki, Regele et al., 2004) and states of chronic inflammation such as rheumatoid arthritis and psoriasis (Cueni & Detmar, 2008). The main accepted mechanism of growth is budding of new vessels from existing ones through the process of lymphangiogenesis (Kim, Kataru et al., 2014). This process is driven by VEGF-C and VEGF-D which are both key

lymphangiogenic factors released by macrophages (Ji, 2012, Schoppmann, Birner et al., 2002, Zumsteg & Christofori, 2012). Several studies have revealed that suppression of VEGF-C/VEGFR-3 signalling impairs the lymphangiogenic response, resulting in an increase in the severity of inflammation (Baluk, Tammela et al., 2005, Guo, Zhou et al., 2009, Wirzenius, Tammela et al., 2007). Interestingly several studies have also reported a direct lineage contribution from macrophages, which undergo direct transdifferentiation into LECs during both inflammation and tumour induced lymphangiogenesis (Hall, Volk-Draper et al., 2012, Kataru, Jung et al., 2009, Kerjaschki, Huttary et al., 2006, Kerjaschki et al., 2004, Maruyama, Li et al., 2005, Zumsteg, Baeriswyl et al., 2009). Post-inflammation the lymphatic network regresses in response to anti-lymphatic signalling pathways. The main drivers of this process are T lymphocytes which release the JAK/STAT modulator interferon gamma (IFN- γ) which has a strong anti-lymphatic effect by causing a downregulation of PROX1, LYVE-1 and PODOPLANIN in LECs, in addition to repressing sprouting of new vessels (Kataru, Kim et al., 2011).

At sites of inflammation there are large numbers of immune cells which either die *in situ* or are cleared by lymphatic vessels. Macrophages (Bellingan, Caldwell et al., 1996, Bellingan, Xu et al., 2002, Lawrance et al., 2016), neutrophils (Beauvillain, Cunin et al., 2011, Rigby, Ferguson et al., 2015) and dendritic cells (Randolph, Angeli et al., 2005) are all able to enter lymphatic capillaries between discontinuous endothelial junctions in order to be cleared to draining lymph nodes. A wide range of cytokines are expressed on LECs which drive this process (Card et al., 2014). One example is C-C chemokine receptor 7 (CCR7) which is present on particular subsets of leucocytes and T cells, and drives attraction of these cell types towards C-C chemokine ligand 21 (CCL21) expressing LECs (Forster, Davalos-Misnitz et al., 2008). It has also been shown that

Macrophage-1 antigen (Mac-1) expression (Cao, Lawrence et al., 2005) and the cleavage of the integrin alpha M beta 2 ($\alpha M\beta 2$) by metalloproteinases (MMPs) (Gomez, Tang et al., 2012) is required for macrophage efflux to lymph nodes.

Once antigens and antigen-presenting cells arrive at the draining lymph nodes they activate adaptive immune responses. The main effectors of adaptive immunity are T- and B-cells which provide antigen specific responses. Dendritic cells, monocytes, macrophages and B-cells process antigens within their internal lysosomes and then express antigen derived peptides on major histocompatibility complex (MHC)-encoded antigen presenting molecules (Janeway, Bottomly et al., 1985, Janeway Jr & Jason, 1980, Villadangos & Schnorrer, 2007). Antigen presentation to B- and T-cells within the lymph nodes provides information to ensure the appropriate priming and maturation of these cells, before their departure from the lymph nodes to evoke an adaptive immune response.

Figure 1.4

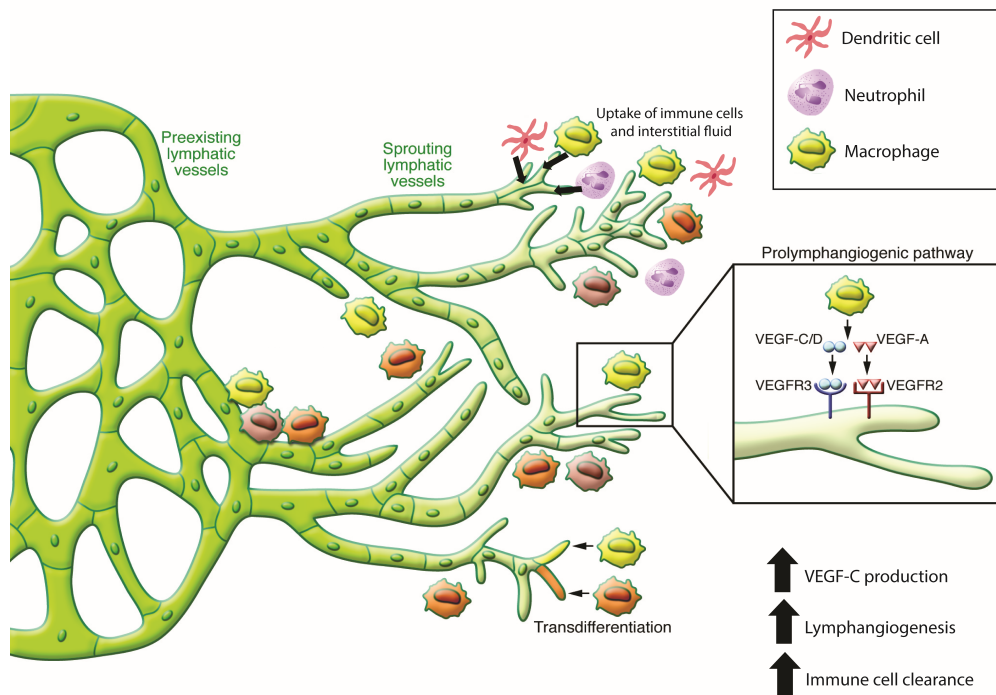


Figure 1.4: Response of lymphatic vessels to inflammation

In response to increased levels of inflammation lymphatic vessels grow via the process of lymphangiogenesis. This occurs in response to the increased demand imposed on the lymphatics for the clearance of interstitial fluid and immune cells such as neutrophils, macrophages and dendritic cells. Macrophages play a key role in this process as they secrete VEGFs including VEGF-C and VEGF-D which bind to VEGFR-3 and drive the lymphangiogenic process. There is also some evidence that macrophages are able to contribute to the formation of additional lymphatics by direct transdifferentiation into LECs. Figure adapted from Kim et al (2014).

Despite the strong lymphangiogenic response which occurs in various inflammatory diseases, chronic inflammation, which is associated with poor outcomes, still persists. Several studies have reported that addition of the pro-lymphangiogenic growth factors VEGF-C and VEGF-D, via adenoviral delivery or administration of recombinant protein, can promote increased lymphangiogenesis, resulting in additional clearance of inflammation and interstitial fluid (Aspelund, Robciuc et al., 2016). One such study revealed that addition of VEGF-C in a murine model of inflammatory bowel disease resulted in a reduction in the severity of chronic inflammation, due to enhanced drainage of inflammatory cells to the lymph nodes, this ultimately resulted in improved

clinical measurements (D'Alessio, Correale et al., 2014). Other examples of the benefits of pro-lymphangiogenic therapy on reducing oedema and promoting immune cell clearance have been observed in models of polycystic kidney disease (Huang, Woolf et al., 2016), lung allografts (Cui, Liu et al., 2015) and skin inflammation (Huggenberger, Siddiqui et al., 2011).

1.4. Myocardial infarction and ischaemic heart disease

During myocardial infarction (MI), stenosis of a coronary artery results in a region of the heart being starved of oxygen and metabolites, leading to cardiomyocyte death. As a result the function of the heart is impaired which may result in sudden death, cardiac rupture or formation of a scar and ventricular remodelling, leading to progressive contractile dysfunction and ultimately heart failure. Whilst survival rates for MI have been improving, a growing number of survivors are living with impaired left ventricular function. In the UK there are currently over 7 million people who have some form of cardiovascular disease and 0.5 million people with heart failure (BHF, 2016). Whilst there are therapies such as angiotensin converting enzyme inhibitors and diuretics which aid the symptoms of heart failure to some extent, the only cure for this debilitating condition is heart transplantation. On top of the huge impact cardiovascular disease can have on individuals lives there is also a large financial burden. The cost to the UK economy due to premature death and disability is estimated at over £15 billion per year and up to £11 billion per year is spent on healthcare for these conditions (BHF, 2016).

1.4.1. Cardiac regenerative medicine

To address the issue of ischaemic heart disease much research is currently ongoing in the field of cardiac regenerative medicine with the hope of developing therapies which

will replace the lost muscle with new cardiomyocytes. The adult mammalian heart is incapable of large scale regeneration to replace these cells; in fact it is estimated that in humans only 0.5 to 1% of cardiomyocytes are replaced annually (Bergmann, Bhardwaj et al., 2009). This is due to a loss of active proliferation and the inability of mature cardiomyocytes to re-enter the cell cycle (Lin & Pu, 2014). During the first week of life in neonatal mice cardiac regeneration can occur (Porrello, Mahmoud et al., 2011) and recently a case of human neonatal cardiac regeneration following severe MI has also been documented (Haubner, Schneider et al., 2016). Investigations into the extension of this 'regenerative window' have begun to focus on promoting adult cardiomyocytes to re-enter the cell cycle. Autologous stem cell therapies have also been heavily investigated, however, bone marrow-derived cells have shown little efficacy in some clinical trials (Sanganalmath & Bolli, 2013). Current work is ongoing with a focus on embryonic stem cells and induced pluripotent cells which can be differentiated into the various cell types which populate the heart. Other strategies include enhancing the activity of endogenous cardiac progenitor cells, reactivation of foetal gene programmes and direct reprogramming of non-cardiomyocytes such as fibroblasts into cardiomyocytes (Lin & Pu, 2014). Whilst many of these approaches seek to replace the cell types lost during MI they do not address the excessive fibrosis and adverse remodelling which occurs and can lead to impaired functional outcomes. Interestingly differences in the post-injury immune response have been observed between regenerative and non-regenerative models (Sattler & Rosenthal, 2016, Talman & Ruskoaho, 2016), suggesting that the status of inflammation in the heart may be highly influential on the repair process.

1.4.2. Inflammatory response to myocardial infarction

An extensive immune response takes place following MI. This is initially triggered by cardiomyocytes which undergo necrosis and release danger associated molecular patterns (DAMPs); small fragments of intracellular components or extracellular matrix which are released from the damaged tissue. DAMPs bind to pattern recognition receptors (PRRs) on the surface of tissue resident macrophages, prompting their activation and the release of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF α) and interleukin 6 (IL-6) and chemokines which activate neutrophil recruitment (Yamasaki, Ishikawa et al., 2008). Tissue resident macrophages, which are defined as C-C chemokine receptor type 2 negative (CCR2⁻), populate the heart during embryogenesis and provide immune surveillance during homeostasis by sampling the local inflammatory environment (Epelman, Lavine et al., 2014, Pinto, Paolicelli et al., 2012).

By 24 hours post-MI large numbers of neutrophils are present within the infarcted region which breakdown necrotic tissue and can also potentially damage surrounding cardiomyocytes (Nahrendorf, Swirski et al., 2007). There is evidence from animal models that, post-MI, neutrophil depletion can reduce the extent of injury (Romson, Hook et al., 1983) and in humans high levels of circulating neutrophils are associated with increased adverse remodelling and poor outcomes (Akpek, Kaya et al., 2012, Ma, Yabluchanskiy et al., 2013). Once neutrophils have performed their function they largely undergo apoptosis and are cleared by macrophage efferocytosis (Fox, Leitch et al., 2010), a phagocytotic process in which apoptotic cells are cleared (Martin, Peters et al., 2014). Some evidence suggests this promotes macrophages to switch from a pro- to anti-inflammatory phenotype, causing them to upregulate the secretion of pro-resolution factors such as interleukin 10 (IL-10) and transforming growth factor

beta (TGF- β) (Fadok, Bratton et al., 1998, Ortega-Gomez, Perretti et al., 2013, Thorp, 2012). There is also now evidence that CCR7 expressing neutrophils are able to directly exit areas of inflammation via the lymph and migrate to lymph nodes (Beauvillain et al., 2011, Rigby et al., 2015).

The release of pro-inflammatory cytokines and chemokines stimulates the recruitment of monocytes via the bloodstream which enter the infarcted heart and differentiate into macrophages (Frangogiannis, 2007). Many studies which have investigated the inflammatory response that occurs during wound healing have classified macrophages as either classically activated M1 macrophages or alternatively activated M2 macrophages (Mosser & Edwards, 2008). It is important to note that although it can be useful to broadly group macrophages as being either M1 or M2 this classification system can be misleading as the functional profiles of macrophages *in vivo* often lie somewhere on a spectrum between the two extremes (Mosser & Edwards, 2008). Therefore, it has been suggested that use of the terms inflammatory/reparative is more appropriate to describe the macrophages present within the heart following MI (Hulsmans, Sam et al., 2016). Inflammatory macrophages have phagocytic and proteolytic properties which allow them to clear necrotic cell debris and apoptosed neutrophils. They also release a range of pro-inflammatory factors including TNF α , IL-6 and interleukin 1 beta (IL-1 β) (Pinto, Godwin et al., 2014). Reparative macrophages secrete anti-inflammatory cytokines such as interleukin 10 (IL-10), pro-angiogenic factors such as VEGF, and pro-fibrotic factors such as insulin-like growth factor 1 (IGF-1) and TGF- β (Pinto et al., 2014). These signals promote angiogenesis and the formation of extra-cellular matrix (ECM) which stabilizes the infarcted left-ventricular (LV) wall. The release of TGF- β promotes increased collagen production by stimulating the differentiation of fibroblasts into collagen-releasing myofibroblasts. TGF- β also

signals to increase the expression of tissue inhibitors of metalloproteinases (TIMPs) which block the breakdown of ECM (Murray & Wynn, 2011, Roberts, Sporn et al., 1986). It has been noted that inflammatory macrophages may also promote angiogenesis to some extent as they are highly fibrolytic and release TNF α , and IL-1 β which are both pro-angiogenic (Pinto et al., 2014).

Following the initial detection of damaged cells by tissue resident macrophages the response of monocytes and macrophages to MI can be divided into two phases. During phase I which occurs within the first few days following injury, pro-inflammatory Ly-6C^{hi} monocytes are recruited and give rise to inflammatory macrophages (Nahrendorf et al., 2007). When inflammation begins to resolve, from around day 5 post-MI, the population shifts towards Ly-6C^{lo} monocytes/macrophages which are fibrotic/reparative macrophages (Nahrendorf et al., 2007). It was initially thought that a second wave of infiltrating towards Ly-6C^{lo} monocytes gives rise to these reparative macrophages, however, there is now evidence to suggest changes in the environment can cause Ly-6C^{hi} macrophages to undergo a phenotypic switch into Ly-6C^{lo} macrophages (Hilgendorf, Gerhardt et al., 2014). By 2-3 weeks post-MI the majority of macrophages have died or departed from the infarct. At this time point the granulation tissue begins to undergo maturation into a collagen rich scar (Blankesteyn, Creemers et al., 2001).

Dendritic cells are also recruited to the infarcted heart and provide the link between the innate and adaptive immune responses through antigen presentation to T-cells at lymph nodes (Anzai, Anzai et al., 2012, Dobaczewski, Xia et al., 2010). Numbers of both T and B lymphocytes have been noted to increase in the heart by day 7 post-MI (Yan, Anzai et al., 2013). Regulatory T cells (Tregs) are a subpopulation of CD4⁺ cells known

to shape and co-ordinate immune responses by expressing anti-inflammatory cytokines including IL-10, TGF- β and interleukin 35 (IL-35) (Sattler & Rosenthal, 2016). Interestingly therapeutic Treg activation post-MI has been found to promote the production of M2 macrophages from monocytes; resulting in an increase in collagen deposition. The result of this was reduced rupture of the left-ventricular (LV) wall and improved healing. Conversely, depletion of Tregs, using *Foxp3^{DTR}* mice, resulted in increased numbers of pro-inflammatory macrophages, which coupled with reduced levels of pro-resolution factors, resulted in increased left-ventricular (LV) dilation and a poorer outcome (Saxena, Dobaczewski et al., 2014, Weirather et al., 2014).

Administration of immunosuppressant therapy post-MI has been found to have deleterious effects on post-MI patients in clinical trials (Chung, Packer et al., 2003, Roberts, DeMello et al., 1976), demonstrating that the inflammatory response is essential for the healing process. However, it may be possible that the inflammatory response can be modulated to achieve an optimum balance between the factors which govern the early clearance of cellular debris and the later reparative process, with the hope of ultimately reducing the amount of non-contractile scar formation. There is now increasing evidence that shifting the balance of the inflammatory profile within the heart away from inflammatory macrophages and the pro-inflammatory factors they release, and towards increased levels of pro-reparative mediators may be beneficial (Frangogiannis, 2012, Pinto et al., 2014). CCR2 is required for the migration of monocytes into the heart which give rise to inflammatory macrophages. Genetic deletion or RNA knockdown of *Ccr2* has been found to improve post-MI cardiac healing, indicating that reduced levels of inflammatory macrophages may be beneficial (Kaikita, Hayasaki et al., 2004, Leuschner, Dutta et al., 2011, Majmudar, Keliher et al., 2013). Conversely depletion of reparative macrophages following MI results in impaired

healing (Shiraishi, Shintani et al., 2016), whereas increasing the number of reparative macrophages or delivering the factors they release, including IGF-1 and IL-10, has been found to improve the post-MI outcome (Burchfield, Iwasaki et al., 2008, Santini, Tsao et al., 2007, Shiraishi et al., 2016). Overexpression of IGF1-Ea has been found to reduce the accumulation of innate immune cells in the heart at day 3 post-MI and at day 7 there is a shift in the balance of inflammation towards an increase in the reparative macrophage population, resulting in improved post-injury recovery (Gallego-Colon, Sampson et al., 2015, Santini et al., 2007, Vinciguerra, Santini et al., 2010). However, prolonged activation of reparative macrophages also has the potential to promote excessive fibrosis, adverse cardiac remodelling and diastolic dysfunction (Hulsmans et al., 2016). The use of RNA interference (RNAi) to reduce the overall numbers of leucocytes in the infarct post-MI has been found to result in significant improvements in left-ventricular ejection fraction (Sager, Dutta et al., 2016). This suggests that a reduction in the overall load of inflammatory cells in the heart post-MI may be sufficient to prevent excessive levels of inflammation and fibrosis, thus providing an improved environment for cardiac healing.

1.4.3. Myocardial oedema

Myocardial oedema, which is an accumulation of fluid in the heart, is a common feature in many post-MI patients where it has been observed to persist for up to 6-12 months (Manrique, Gerbaud et al., 2009, Nilsson, Nielsen et al., 2001). Oedema occurs when fluid accumulates at a greater rate than which it is cleared by the lymphatics. The heart is particularly sensitive to increases in interstitial fluid volume which can rapidly result in impaired function. For example an increase of 3.5% in myocardial water content has been found to reduce cardiac output by up to 40% (Laine & Allen, 1991). Myocardial oedema can have a severe impact on cardiac function and post-MI recovery. The

accumulation of fluid creates stiffening of the myocardium, impairing contractility and resulting in systolic and diastolic dysfunction (Dongaonkar, Stewart et al., 2010). It can also disrupt collagen fibres (Weber, Pick et al., 1990), compromising the structural integrity of the heart and creating a greater distance for the diffusion of oxygen from capillaries to cardiomyocytes, which could result in increased infarct sizes post-MI (Ziegler & Goresky, 1971). The lymphatic vessels are essential for the prevention of cardiac oedema and the associated deleterious functional consequences. This has been demonstrated in surgical models where obstruction of the cardiac lymphatics has been found to result in impaired cardiac function (Cui, 2010, Kline, Miller et al., 1963, Ludwig, Schertel et al., 1997). The movement of lymph into the cardiac lymphatics is driven by increased interstitial fluid pressure and also by the beating of the heart (Laine, Mehlhorn et al., 1995, Mehlhorn, Allen et al., 1995a, Mehlhorn, Davis et al., 1995b). In fact, cardiac lymph flow is dramatically reduced upon cardiac arrest or during ventricular fibrillation (Mehlhorn et al., 1995a, Mehlhorn et al., 1995b, Taira, Yamashita et al., 1979, Ullal, Kluge et al., 1972). Furthermore, increasing cardiac contractility has been found to improve myocardial lymphatic function (Allen, Geissler et al., 1997, Taira, Morishita et al., 1977). Targeting the cardiac lymphatics to improve the removal of interstitial fluid in the heart has proved beneficial in animal models. Treatment with hyaluronidase, which increases lymphatic flow in the heart, has been shown to reduce infarct size (Maclean, Fishbein et al., 1976). Moreover, a study published earlier this year revealed that treatment with the pro-lymphangiogenic growth factor VEGF-C in a rat model may be beneficial in reducing post-MI cardiac oedema and improving functional outcomes (Henri, Pouehe et al., 2016).

1.4.4. Role of cardiac lymphatics following myocardial infarction

Despite the potentially important physiological functions lymphatic vessels play in the clearance of post-MI immune cells and interstitial fluid, until recently there were only a handful of anecdotal reports on this topic. One study employed D2-40 (PODOPLANIN) staining to detect lymphatic vessels in human post-mortem post-MI samples which revealed an increase in lymphatic density by the later stages of tissue granulation. VEGF-C expression was also detected in the infarcted region in what were assumed to be cardiomyocytes, however, co-staining to confirm this was not performed (Ishikawa, Akishima-Fukasawa et al., 2007). Another study which assessed lymphatic density using immunohistochemistry in a range of post-mortem human samples revealed an increase in lymphatic density in ischaemic and inflamed tissue (Kholova, Dragneva et al., 2011). In post-MI rat heart sections staining for PODOPLANIN demonstrated an enlargement and increased number of lymphatic vessels in the infarcted region at days 7, 14, 21 and 28 post-MI (Sun, Wang et al., 2012). Together these data provided some evidence for the occurrence of lymphangiogenesis post-MI, however, further investigations into the extent, time course and significance of this response were required. Prior work undertaken in the Riley lab by Linda Klotz provided further insight into this process and demonstrated that the murine cardiac lymphatic vasculature undergoes significant lymphangiogenesis by day 7 after permanent ligation of the left-anterior descending (LAD) coronary artery, which was assessed by whole-mount immunostaining, staining on sections and gene expression changes at multiple time points. These observations are introduced in chapter 5 of this thesis as they provided the basis for much of the work included there.

1.5. Hypotheses and aims

The major aims of this thesis were to fully investigate two preliminary observations previously made in the Riley lab. The first observation was that a proportion of embryonic cardiac LECs appeared to derive from a non-venous source and the second was that cardiac lymphatic vessels undergo lymphangiogenesis in response to myocardial infarction.

Based on these observations the following hypotheses were established:

- 1) A proportion of the cardiac lymphatic vasculature is non-venous in origin
- 2) PROX1 is essential for the development of cardiac lymphatic vessels
- 3) Administration of the pro-lymphangiogenic growth factor VEGF-C will promote enhanced lymphangiogenesis and aid the clearance of inflammation post-MI
- 4) Impaired lymphangiogenesis or disrupted uptake of post-MI immune cells by cardiac lymphatic vessels will result in impaired clearance of immune cells and a poor outcome post-MI

To test the above hypotheses the following aims were established:

- 1) Characterise the spatiotemporal development of cardiac lymphatic vessels during embryonic development and early postnatal stages
- 2) Perform lineage tracing to further investigate whether a proportion of cardiac LECs are derived from a non-venous origin
- 3) Utilise Cre-lox genetic models to determine whether PROX1 is required in cardiac LECs which derive from venous and non-venous origins
- 4) Further characterise the response of the cardiac lymphatics to myocardial injury and investigate the effect of recombinant VEGF-C therapy on the resolution of post-MI inflammation and healing

- 5) Employ models in which lymphangiogenesis or clearance of immune cells by lymphatic vessels is genetically perturbed to further determine the importance of cardiac lymphatics in the clearance of post-MI inflammation

2. Materials and methods

2.1. Mice

2.1.1. Strains

The following mouse strains were used for these studies: *Csf1r-CreER* (Qian, Li et al., 2011), *LYVE-1^{-/-}* (Gale et al., 2007), *Prox1^{loxP/+}* (Iwano, Masuda et al., 2012), *R26R-EYFP* (Srinivas et al., 2001), *R26R-tdTomato* (Madisen, Zwingman et al., 2010), *Tie2-Cre* (Koni, Joshi et al., 2001), *Vav1-Cre* (de Boer, Williams et al., 2003), *Vegfr3^{LacZ/+}* (Dumont, 1998). Breeding was carried out using only Cre⁺ males for all Cre strains except the *Vav1-Cre* where Cre⁺ females were used. Mice were cared for and housed by Oxford University Biomedical Services. For experiments where wild type mice were required C57BL/6 animals were used (Charles River Laboratories). All animal experiments were carried out according to UK Home Office project licences PPL 30/2987, PPL30/3155 and were compliant with the UK Animals (Scientific Procedures) Act 1986.

2.1.2. Timed matings

In order to generate embryos females were paired with male studs and were checked for vaginal plugs each morning. Embryonic staging was determined by the day of vaginal plug (E0.5).

2.1.3. Tamoxifen preparation and injection

4-hydroxytamoxifen (4-OHT; Sigma) was dissolved in peanut oil (Sigma) at a concentration of 10 mg/ml by 2 hours of vortexing, followed by shaking at 37°C until fully dissolved. Aliquots were stored at -20°C and were freshly thawed before being pre-heated to 37°C for injections. In order to induce Cre activity 2 mg 4-OHT was administered to pregnant females by intraperitoneal injection at the required

embryonic stage using a 1ml syringe (Beckton Dickinson) and a 25G needle (BD Microlance). 100 µl progesterone (10 mg/ml) was co-administered to reduce abortion rates.

2.1.4. VEGF-C (Cys156Ser) preparation and injection

25 µg carrier free recombinant human VEGF-C (Cys156Ser) (R&D systems) was dissolved in 2.5ml sterile Phosphate Buffered Saline (PBS; Thermo Fisher Scientific) to give a 10 µg/ml solution which was stored at 4°C for up to one month. 100 ng/g VEGF-C (Cys156Ser) was administered to mice via intraperitoneal injection on each treatment day. For experiments where VEGF-C (Cys156Ser) was administered at multiple time points during the first 7 days post-MI animals were injected at days 0, 2, 4 and 6. For some experiments, as stated in the results sections, an additional dose was administered at day 3.

2.1.5. Embryo dissection

Embryos were harvested from pregnant females at the required embryonic stage. Following removal from the female, the uterus was placed in ice cold PBS (Sigma) and opened to remove the embryos. Hearts and dorsal skins, when required, were collected from embryos for subsequent experimental analysis. Tails were also taken for genotyping.

2.1.6. Adult heart dissection

Adult mice were sacrificed by schedule 1 cervical dislocation and the heart was removed. Hearts collected for immunostaining were washed in ice cold PBS prior to fixation. Hearts for flow cytometry were washed in ice cold Hank's Balanced Salt Solution (HBSS; Life Technologies) prior to tissue digestion. Hearts for subsequent RNA

extraction were removed and immediately placed in a cryo tube (Nunc Thermo Scientific) which was submerged in liquid nitrogen. For some experiments post-MI hearts for RNA extraction were dissected into infarct and remote regions before snap freezing.

2.2. Myocardial infarction model

Myocardial infarction surgery was performed by Mala Rohling, a senior research technician in the Riley group. For experiments which required genetically modified mice both males and females aged 10-12 weeks were used for surgery. C57BL/6 females (Charles River) aged 10-12 weeks were used for experiments that did not require genetically modified animals. Mice were anesthetized in an induction chamber with 4% isoflurane, 1L/minute oxygen. Buprenorphine (buprenorphine hydrochloride; Vetergesic) was delivered as a 0.015 mg/mL solution via intraperitoneal injection to provide analgesia. Mice were then moved onto a heat pad, connected to a nose-cone providing 2.5% isoflurane to maintain anaesthesia and a rectal temperature probe was inserted for monitoring. An endotracheal tube was then inserted and mice were placed under assisted external ventilation. Fur was shaved from the chest region and the skin was disinfected. An incision was made in the chest region in order to expose the ribcage. A slide retractor was used to separate the intercostal muscle between the 2nd and 3rd ribs (females) or 3rd and 4th ribs (males) which exposed the heart. The pericardium was removed from the heart and a suture was used to permanently ligate the left anterior descending coronary artery. The ribcage was then closed using sutures and surgical glue, and pectoral muscles were moved back into place. The chest incision was then closed with sutures. 100 µl fluids were administered per mouse. Mice were then taken off anaesthesia and recovered in a heated chamber at 37°C. Once active and well-recovered mice were transferred to a cage and kept in an incubator overnight at 26°C with a wet diet and jelly for the first 24 hours after surgery.

Following this period of time mice were transferred back to a normal cage at room temperature. Mice were monitored regularly during the first 24 hours and then once every day until they were sacrificed.

2.3. Molecular methods

2.3.1. DNA extraction

Ear biopsies from mice and tissue from embryos were collected for genotyping. Tissue was dissolved overnight at 55°C in 500 µl tail lysis buffer (**Table 2.6**). Following this DNA was precipitated with 500 µl of isopropanol and samples were centrifuged at 13,000 rpm for 15 minutes. The supernatant was removed and the remaining pellet was washed with 1 ml of 70% ethanol. The samples were centrifuged again at 13,000 rpm for 5 minutes. The remaining pellet containing DNA was resuspended in 200 µl TE Buffer (**Table 2.6**). Samples were incubated at 37°C and vortexed in order to dissolve the DNA pellet.

2.3.2. Polymerase chain reaction (PCR)

Extracted DNA was used for genotyping in PCRs. Primers used are displayed in **Table 2.1**. The following reagents were used to set up the PCR reactions: Illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare), 0.05 pmol/ml of each primer, 2 µl DNA, sterile H₂O was added to give a total reaction volume of 25 µl. Thermal cycling was carried out in a 96 well Veriti™ thermal cycler (Applied Biosystems) using the programs shown in **Table 2.2**. A positive control sample of known genotype was included for each genotype being tested.

Table 2.1. PCR primers for genotyping

Gene	Primer 1	Primer 2	Primer 3	Primer 4	PCR program
<i>Cre</i>	GTTCGCAAGAACCTGATGGACA	CTAGAGCCTGTTTTGCACGTTC	N/A	N/A	Taqgenom2
<i>iCre</i>	AGATGCCAGGACATCAGGAAC CTG	ATCAGCCACACCAGACACAGAG ATC	N/A	N/A	iCre
<i>Prox1- flox</i>	CAGCCCTTTTGTCTGTTGGC CAG	GCAGATGCTGTCCCTACCGTCC	N/A	N/A	Taqgenom2
<i>R26r- eyfp</i>	AAGTCGCTCTGAGTTGTTAT	GCGAAGAGTTTGTCTCAACC	GGAGCGGGAGAAATGGAT ATG	N/A	R26R-EYFP
<i>R26r- tdtomato</i>	AAGGGAGCTGCAGTGGAGTA	CCGAAAATCTGTGGGAAGTC	GCATTAAAGCAGCGTATCC	CTGTTTCCTGTAC GGCATGG	R26R- tdTomato
<i>Vegfr3- lacZ</i>	TCACTCCCAGCCTAGAGCTGC	CGAGGCAGAGCCACAGGCGC	GACGACAGTATCGGCCTCAG	N/A	Vegfr3/ LacZ

Table 2.2. PCR thermal cycling programs

Taqgenom2

Stage	Temperature (°C)	Duration (minutes.seconds)	Cycles
1	94	2.00	1
2	94	0.30	35
	60	0.30	
	72	0.45	
3	72	5.00	1
4	4	∞	1

iCre

Stage	Temperature (°C)	Duration (minutes.seconds)	Cycles
1	94	1.30	1
2	94	0.30	35
	64	0.45	
	72	0.45	
3	72	2.00	1
4	4	∞	1

Vegfr3-LacZ

Stage	Temperature (°C)	Duration (minutes.seconds)	Cycles
1	95	5.00	1
2	95	0.20	35
	63	0.20	
	72	0.20	
3	72	5.00	1
4	4	∞	1

R26R-EYFP

Stage	Temperature (°C)	Duration (minutes.seconds)	Cycles
1	94	2.00	1
	55	1.30	
2	72	0.45	29
	94	0.30	
	55	0.45	
3	72	3.00	1
4	4	∞	1

R26R-tdTomato

Stage	Temperature (°C)	Duration (minutes.seconds)	Cycles
1	94	5.00	1
2	94	0.30	35
	61	0.30	
	72	0.30	
3	72	7.00	1
4	4	∞	1

2.3.3. Agarose gel electrophoresis

Following PCR amplification DNA samples to be genotyped were separated on 2% agarose gels. 2 g of agarose (Sigma Aldrich, UK) was dissolved in 100 ml of TBE buffer (**Table 2.6**) by heating in a microwave. To allow UV visualisation of DNA 5 µl of GelRed (VWR) was added to the agarose gel before it was poured into moulds and allowed to set. PCR products were mixed with 5 µl DNA Gel Loading Dye (6X) before being loaded into the wells of the gel. 120 V was passed through the gel for 45 minutes which allowed separation of bands to distinguish between genotypes. Gels were visualised in a UV box.

2.3.4. RNA extraction from tissue

RNA was isolated from snap frozen tissue samples using TRIzol reagent (Thermo Fisher Scientific). Tissue was homogenized in 1 ml of TRIzol solution using a manual homogenizer and a 21G sterile needle (Becton Dickinson). After being incubated for 5 minutes at room temperature 200 µl of chloroform was added to the samples. Samples were then vortexed and were incubated again for 10 minutes at room temperature. Following incubation samples were centrifuged at 11,000 rpm for 15 minutes at 4°C which allowed separation of the samples into an aqueous RNA containing layer and an organic DNA and protein containing layer. The aqueous layer was removed and transferred into a 1.5 ml tube, the organic layer was discarded. In order to precipitate RNA isopropanol was added to the samples which were then incubated on ice for 20 minutes. Following incubation the samples were centrifuged at 11,000 rpm for 15 minutes at 4°C which produced an RNA containing pellet. The pellet was washed with 75% EtOH before another spin at 9,000 rpm for 10 minutes at 4°C. The pellet was air-dried for 10 minutes and resuspended in RNase-free H₂O. A Nanodrop2000 (Thermo Fisher Scientific) was used to measure RNA quality and concentration.

2.3.5. cDNA synthesis

cDNA was synthesized from extracted RNA for use in real-time quantitative PCR (qRT-PCR). Reactions were prepared in RNase free 0.2 ml tubes (Thermo Fisher Scientific) using the following reagents: 1 µg RNA made up to a volume of 8.5 µl with DEPC H₂O, 0.5µl of random primers (20 µg/ml; Promega), 1 µl dNTPs (from 10 mM, GE Healthcare), 2 µl MgCl₂ (25 mM, Thermo Scientific), 2 µl Dithiothreitol (DTT, 0.1 M, Life Technologies), 1 µl RNasin® plus RNase inhibitor (Promega), 4 µl 5X FS Buffer (Life Technologies) and 1 µl of SuperScript® III Reverse Transcriptase (Life Technologies). A negative control without the SuperScript® III Reverse Transcriptase was included for each set of reactions. A 96 well Veriti™ thermal cycler (Applied Biosystems) was used to cycle the reactions at 25°C for 10 minutes, 42°C for 50 minutes and 70°C for 15 minutes, before cooling to 4°C. Following cDNA synthesis samples were diluted to 4 ng/µl in DEPC water and stored in the short-term at 4°C prior to use in qRT-PCR experiments.

2.3.6. RNA extraction from sorted cells

An RNAaqueous-Micro Kit (Thermo Fisher Scientific) was used to extract RNA from macrophages sorted by fluorescence-activated cell sorting (FACS). The kit was used according to manufacturer's instructions. The first step was lysis of the cells which was performed by adding a guanidinium thiocyanate-containing lysis solution. Ethanol was then added to the lysate which was then passed through a silica filter column that binds RNA. Several washes were performed and RNA was then eluted from the column into 11 µl of RNase free water. A final DNase treatment step was performed in order to remove any remaining contaminant genomic DNA. A Nanodrop2000 (Thermo Fisher Scientific) was used to measure RNA quality and concentration.

2.3.7. cDNA synthesis from RNA extracted from sorted cells

cDNA was prepared from RNA extracted from FACS-isolated macrophages using a QuantiTect Whole Transcriptome kit (Qiagen). This kit was used as it allows small amounts of RNA to be amplified to yield large amounts of cDNA, thus allowing a large number of genes to be profiled with qRT-PCR. Qiagen state that “The kit amplifies cDNA derived from all regions of RNA transcripts, including 5’ ends, but does not provide amplified cDNA corresponding to full-length RNA transcripts”. Three main steps were performed: reverse transcription, ligation and whole transcriptome amplification. For the reverse transcription step 10 µg of each RNA sample was added to a reverse transcription mix in order to generate cDNA by heating at 30 minutes at 37°C and then for 5 minutes at 95°C. This was followed by a ligation step where a high-efficiency ligation mix was added to the cDNA and then incubated at 22°C for 2 hours. Finally the whole transcriptome amplification step was performed by adding an amplification mix to the ligated cDNA and incubating the mixture for 8 hours at 30°C. The 8 hour incubation generated up to 40 µg cDNA from 10 µg RNA.

2.3.8. Real-time quantitative PCR

Relative mRNA expression levels of genes of interest were quantified using qRT-PCR. Primer sequences are displayed in **Table 2.3**. MicroAmp Fast Optical 96-well 0.1 ml reaction plates (Thermo Fisher Scientific), were used to set up reactions which were composed of the following reagents: 8 ng cDNA, 13 µl Fast SYBRGreen Master Mix (Thermo Fisher Scientific), 6.5 µl DEPC H₂O, and 0.05 pmol/ml of each primer. All samples were run in triplicate and a no template control well was included for each gene analysed. Reactions were run on a ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific) with thermal cycles of 95°C for 15 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Melt curves were also included to confirm that no unspecific

amplification products such as primer-dimers were produced with the various primer sets used. Cycle Threshold (Ct) values were obtained from the ViiA™ 7 and were exported to Microsoft Excel for analysis. The comparative cycle threshold method ($2^{-\Delta\Delta Ct}$) (Livak & Schmittgen, 2001) was used to calculate relative quantification of gene expression. This was determined by first calculating ΔCt values where the values were normalized by subtracting the Ct values of a housekeeping gene from the Ct values of genes of interest. The housekeeping genes used were *18s*, *Actb*, *Gapdh* and *Hprt*. For each experiment all housekeeping genes were run for a selection of samples and the housekeeping gene with the most consistent expression between experimental groups was selected for calculation of ΔCt values. $\Delta\Delta Ct$ values were calculated by calibrating values to the average ΔCt of internal control group samples e.g. values from treated samples were calibrated relative to the average of the vehicle treated group.

Table 2.3. Primers for qRT-PCR

Gene	Forward	Reverse
<i>18s</i>	CATTCTGAACGTCTGCCCTAT	GTTTCTCAGGCTCCCTCTCC
<i>Actb</i>	TTCTACAATGAGCTGCGTGTG	GGGGTGTGAAGGTCTCAAA
<i>Cd206</i>	CAGGTGTGGGCTCAGGTAGT	TGTGGTGAGCTGAAAGGTGA
<i>Cd80</i>	GGCAAGGCAGCAATACCTTA	CTCTTTGTGCTGCTGATTCTG
<i>Cd86</i>	CTTACGGAAGCACCCATGAT	CCCATTGAAATAAGCTTGCG
<i>Chi3l3</i>	AGGAGCAGGAATCATTGACG	TTTCTCCAGTGTAGCCATCCTT
<i>Col1a1</i>	GCAAGAGGCGAGAGAGGTTT	GACCACGGGCACCATCTTTA
<i>Col1a2</i>	CTGGAACAAATGGGCTCACTG	CAGGCTCACCAACAAGTCCTC
<i>Col3a1</i>	ACGTAGATGAATTGGGATGCAG	GGGTTGGGGCAGTCTAG
<i>Col4a1</i>	ACGTGGCCGAGAATTTCA	ACGTGGCCGAGAATTTCA
<i>Col6a1</i>	CTGCTGCTACAAGCCTGCT	CCCCATAAGGTTTCAGCCTCA
<i>Gapdh</i>	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
<i>Hprt</i>	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG
<i>Il10</i>	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT
<i>Il1b</i>	AAAGAATCTATACCTGTCTGTGTAATGAAA	GGTATTGCTTGGGATCCACACT
<i>Il6</i>	CTTCCATCCAGTTGCCTTCTTG	AATTAAGCCTCCGACTTGTGAAG
<i>Lyve1</i>	GGCTTTGAGACTTGCAGCTATG	GCAGGAGTTAACCAGGTG
<i>Prox1</i>	GAAGGGCTATCACCCAATCA	TGAACCACTTGATGAGCTGC
<i>Tgfb1</i>	CCGCTGCATATCGTCCTGTG	AGTGGATGGATGGTCCTATTACA
<i>Tnf</i>	CTGTAGCCCACGTCGTAGC	TTGAGATCCATGCCGTTG
<i>Vegfr3</i>	CCATCGAGAGTCTGGACAGC	CCGGGATGGTGGTCACATAG

2.4. Staining

2.4.1. Whole-mount X-Gal staining

Following extraction, hearts were washed in ice cold PBS containing 2mM MgCl₂ and then fixed in X-Gal fixation solution (**Table 2.6**) for 2 hours at room temperature. Hearts were then washed in PBS 2mM MgCl₂ and incubated in 5-bromo-4-chloro-3-indolyl- β -D-galactosidase (X-Gal) staining solution (**Table 2.6**) overnight at room temperature. Once staining had developed hearts were rinsed in PBS and imaged using a Zeiss stereomicroscope.

2.4.2. Hematoxylin and eosin (H&E) staining

Hearts collected for H&E staining were fixed overnight in 4% paraformaldehyde (PFA) at 4°C. Hearts were then dehydrated by performing serial washes, each for 2 hours in the following dilutions of EtOH: 50%, 70%, 80%, 90%, 96%, 100% and, 100%. Hearts were then washed in butanol overnight before being placed in 50:50 butanol and paraffin wax (Histoplast, Fisher) at 56°C for 1 hour. Hearts then underwent two washes in 100% paraffin at 56°C prior to embedding in moulds with paraffin. A microtome was used to cut 8 µm sections which were transferred onto Superfrost Plus slides (VWR). Slides were washed in PBS and transferred to Haematoxylin (Sigma Aldrich, UK) for 5 minutes. Slides were then washed again in H₂O and immersed in Eosin staining solution (Sigma, UK) for 1 minute which was followed by another wash. The sections were then rehydrated through serial EtOH washes prior to mounting of coverslips with DPX mounting media (Sigma, UK). Slides were imaged using a Zeiss stereomicroscope.

2.4.3. Immunofluorescence on cryosections

Whole embryos and dissected embryonic hearts were fixed in 2% PFA overnight at 4°C. Whole adult hearts were fixed overnight in 4% PFA also at 4°C. Following fixation samples were washed three times for 10 minutes in PBS and then transferred to 30% sucrose overnight at 4°C. Following equilibration in sucrose samples were embedded in OCT and frozen at -80°C. 8-10 µm slices were cut using a cryostat and transferred onto Superfrost Plus slides (VWR). Slides were dried on a slide dryer for 5 minutes before being rinsed with PBS. Sections then underwent permeabilization with 0.5% Triton X-100 (Sigma, UK) for 5 minutes, followed by another rinse in PBS for 5 minutes. Sections were then blocked in blocking solution, composed of 10% goat/donkey serum, 1% bovine serum albumin (BSA) and 0.1% Triton X-100, for 1 hour. Blocking was followed by a 4°C overnight incubation in primary antibody which was diluted in

blocking solution. A list of primary antibodies and the dilutions used are included in **Table 2.4**. For each primary antibody used one section was incubated without primary antibody as a secondary antibody alone control. Following primary antibody incubation sections were washed several times with 0.1% Triton X-100 and incubated with Alexa Fluor secondary antibodies for 1 hour at room temperature. All secondary antibodies were diluted in PBS; a list of the secondary antibodies used is included in **Table 2.5**. Following incubation in secondary antibody the slides were washed three times in 0.1% Triton X-100 for 5 minutes, with DAPI included in the final wash. A small amount of 50:50 Glycerol:PBS was then added to slides and a 22x50 mm coverslip (Fisherbrand) was placed on top and sealed with nail varnish. Immunofluorescent staining was imaged using an Olympus FV1000 confocal microscope. Z-stack and tiling functions were employed when required. Images were processed using ImageJ software.

2.4.4. Immunofluorescence on embryonic hearts and whole embryos

Whole embryonic hearts and whole E10.5 embryos were permeabilized for 10 minutes in 0.3% Triton X-100/PBS and subsequently blocked in blocking solution (1% BSA, 0.3% Triton X-100 in PBS). Samples were incubated with primary antibodies (**Table 2.4**) diluted in blocking solution overnight at 4°C, then washed three times for 1 hour in 0.3% Triton X-100/PBS. Samples were then incubated with secondary antibodies (**Table 2.5**) diluted in PBS overnight at 4°C, protected from light and then washed with PBS. Nuclei were then stained with DAPI. For mounting of samples glass slides (VWR) were covered in 3-4 layers of electrical tape. Windows were cut into the tape to create shallow wells into which the samples were placed and covered with a small amount of 50:50 Glycerol:PBS. A coverslip (Fisherbrand) was then placed on top of the slides which were then sealed with nail varnish. Immunofluorescent staining was imaged using an Olympus FV1000 confocal microscope. Z-stack and tiling functions were

employed to obtain maximum intensity Z-projections of whole hearts and embryos. Images were processed using ImageJ software. Analysis of vessels and branching calculations were performed using Angiotool software (Zudaire, Gambardella et al., 2011).

2.4.5. Immunofluorescence on whole adult mouse hearts

A novel method was developed for immunofluorescent staining and imaging on whole adult mouse hearts. This was adapted from a previously published method (Brede, Friedrich et al., 2012). Whole adult hearts were fixed in 4% PFA overnight at 4°C and then washed several times in PBS. Hearts were then bleached for several hours in 15% hydrogen peroxide/PBS until pale in colour. Hearts were then rinsed with PBS and permeabilized in 1% Triton X-100 for 1 hour. This was followed by a 24 hour incubation in blocking solution (2% BSA/PBS) at 4°C. Primary antibodies (**Table 2.4**), diluted in blocking solution were then added and left on the sample at 4°C for 2-3 days. Hearts were then washed several times in PBS and secondary antibodies (**Table 2.5**) diluted in PBS were added. All primary and secondary antibodies were used at a 1 in 100 dilution. Hearts were incubated in secondary antibody overnight at 4°C and were then washed in PBS several times. When required DAPI was added to the final wash for 30 minutes. Hearts were stored in PBS prior to imaging. Whole hearts were mounted onto the end of a bent needle for imaging using a Zeiss Z1 light sheet microscope. Light sheet imaging enabled an overall view of lymphatic network to be obtained. Small biopsies of heart were also dissected and mounted onto slides for imaging using an Olympus FV1000 confocal where higher resolution images were required. All images were post-processed using Image J in order to create maximum intensity Z-projections.

2.4.6. 3, 3'-diaminobenzidine (DAB) whole-mount staining

Hearts were fixed in 4% PFA overnight at 4°C and then washed several times in PBS. Hearts were bleached in 5% H₂O₂/PBS for several hours until pale in appearance. This was followed by several washes in PBST (0.1% Tween 20 (Sigma) in PBS). Hearts were then washed twice in PBTD (0.1% Tween 20, 1% DMSO (Sigma) in PBS) before being transferred into blocking solution for 2 hours (10% sheep serum in PBTD). Hearts were then incubated in primary antibody (**Table 2.4**) overnight at 4°C. Following this hearts were washed for 30 minutes in PBTD, 10 times, before overnight incubation with the secondary rabbit-anti-goat HRP antibody which was diluted at 1:200 in blocking solution. The following day hearts were washed 3 times for 15 minutes with PBTD before being placed in Vector Elite ABC Reagent (Avidin:Biotin:Peroxidase complex) for 1 hour. Several more washes in PBTD were performed before equilibration with DAB developing buffer (Vector labs) for 15 minutes. DAB, H₂O₂ and nickel from the DAB Peroxidase Substrate Kit (Vector labs) were added to the developing buffer in order to develop the staining. Once staining had developed hearts were washed in H₂O and were then imaged using a Zeiss stereomicroscope.

Table 2.4. List of primary antibodies

Primary antibody	Company/Cat no.	Tissue expression/marker	Dilution	Species raised in
ENDOMUCIN	Santa Cruz Biotechnology (SC-53941)	Venous endothelium and capillaries	1 in 50	Rat
PECAM (CD31)	BD Pharmingen (553370)	Endothelium	1 in 50	Rat
PROX1	Angiobio (11-002)	Lymphatic endothelial cells, cardiomyocytes	1 in 200	Rabbit
PROX1	R&D Systems (AF2727)	Lymphatic endothelial cells, cardiomyocytes	1 in 200	Goat
LYVE-1	Angiobio (11-034)	Lymphatic endothelium	1 in 200	Rabbit
LYVE-1	Novus Biologicals (NBP1-43411)	Lymphatic endothelium	1 in 500	Rat
VEGFR-3	R&D Systems (AF743)	Lymphatic vessels and angiogenic blood vessels	1 in 50	Goat
GFP	Abcam (ab13970)	GFP	1 in 2000	Chicken
TER119	BD Biosciences (550565)	Erythrocytes	1 in 50	Rat
PODOPLANIN	Strattech (10R-P155A)	Lymphatic vessels, epicardium	1 in 500	Hamster
VEGF-C	Abcam (ab9546)	VEGF-C	1 in 200	Rabbit
F4/80	Abcam (6640)	Macrophages	1 in 500	Rat
CD68	Bio-Rad (MCA1957)	Macrophages	1 in 500	Rat

Table 2.5. List of secondary antibodies

Antibody	Supplier	Dilution
AlexaFluor goat α -chicken 488	Invitrogen	1 in 200
AlexaFluor goat α -hamster 488	Invitrogen	1 in 200
AlexaFluor donkey α -goat 488	Invitrogen	1 in 200
AlexaFluor donkey α -goat 594	Invitrogen	1 in 200
AlexaFluor donkey α -goat 647	Invitrogen	1 in 200
AlexaFluor donkey α -rabbit 488	Invitrogen	1 in 200
AlexaFluor donkey α -rabbit 594	Invitrogen	1 in 200
AlexaFluor donkey α -rabbit 647	Invitrogen	1 in 200
AlexaFluor donkey α -rat 488	Invitrogen	1 in 200
AlexaFluor goat α -rat 594	Invitrogen	1 in 200
AlexaFluor chicken α -rat 647	Invitrogen	1 in 200

Table 2.6. Solutions

Solution	Ingredients
Tail lysis buffer	100mM Tris pH8.5, 5mM EDTA, 0.2% SDS, 200mM NaCl with 0.05mg/ml Proteinase K
Tris-borate-EDTA (TBE) buffer	40mM Tris pH8.0, 20mM Boric acid, 1mM EDTA
Tris-EDTA (TE) buffer	10mM Tris pH8.0, 1mM EDTA
X-gal fixative	2.7ml of 37% Formaldehyde; 0.8ml of 25% Glutaraldehyde; 0.2ml of 2% NP40 substitute; make up to 100ml with PBS + 2mM MgCl ₂)
X-gal staining solution	5mM K ₃ Fe(CN) ₆ , 5mM K ₄ Fe(CN) ₆ (0.823g and 1.056g, respectively in 500ml H ₂ O)+ 1.25mg/ml X-Gal substrate in DMSO to make 50ml staining solution

2.5. Flow cytometry

2.5.1. Heart digestion

Digestion of hearts was based on a previously published protocol (Pinto, Chandran et al., 2013). Isolated hearts were minced using a sterile scalpel (Swann-Morton) before being added to a 15 ml tube containing 10 ml of Hank's Balanced Salt Solution (Life Technologies) and 14.5 mg (500 units/ml) of Collagenase II (Worthington). Tubes were placed in an orbital incubator at 37°C, moving at 190 RPM. A P1000 pipette (Gilson) was used to break-up the tissue suspension every 10 minutes during the 70 minute digestion within the incubator. Once digested the tissue suspension was passed through a 40 µm cell strainer into a 50 ml tube before being transferred back to a 15 ml tube. The tubes were then centrifuged at 350 g for 7 minutes at 4°C and the supernatant was discarded, leaving a pellet. The pellet was resuspended in 5 ml 1X red blood cell (RBC) lysis buffer (BioLegend) and was left on ice for 5 minutes. The tubes were then centrifuged again at 350 g for 7 minutes at 4°C and the supernatant was discarded, leaving a pellet. The cell pellet was resuspended in 500 µl 2% FBS/PBS.

2.5.2. Antibody staining

250 µl of the cell suspension was taken forward for antibody staining. 2.5 µl FcR blocking reagent (Miltenyi Biotech) was added to the suspension which was then left on ice for 15 minutes. Antibodies were then added at the dilutions shown in **Table 2.7** and the tubes were incubated at room temperature in the dark for 20 minutes. 750 µl 2% FBS/PBS was added to each tube and the samples were then centrifuged at 350 g for 7 minutes at 4°C to give a cell pellet. The pellet containing the stained cells was then resuspended in 500 µl 2% foetal bovine serum (FBS)/PBS and 5 µl 7AAD viability staining solution (eBioscience) was added to allow exclusion of dead cells. Flow cytometry quantification and FACS was performed on a FACSaria III machine (BD

Biosciences). The panel of antibodies was used to assess the populations of haematopoietic cells (CD45⁺), myeloid cells (CD45⁺ CD11b⁺), neutrophils (CD45⁺ CD11b⁺ Ly6G⁺), macrophages (CD45⁺ CD11b⁺ Ly6G⁻ F4/80⁺) and dendritic cells (CD45⁺ CD11b⁺ Ly6G⁻ F4/80⁻ CD11c⁺). Use of this panel of antibodies for flow cytometry experiments was an established procedure in the group. An appropriate gating strategy had previously been set up in pilot studies, which included isotype and fluorescence minus one (FMO) controls. The gating strategy is shown in **Figure 5.8** in chapter 5 of this thesis. Fluorescence compensation controls were used to correct for spectral overlap.

Table 2.7. Flow cytometry antibodies

Antibody	Company/Cat no.	Tissue expression/marker	Fluorophore	Diltution
CD45	BioLegend Inc (103124)	Haematopoietic cells	647	1 in 200
CD11b	BioLegend Inc (101206)	Myeloid cells	FITC	1 in 100
Ly6G	BioLegend Inc (127636)	Neutrophils	Brilliant violet 421	1 in 200
F4/80	BioLegend Inc (123110)	Macrophages	PE	1 in 100
CD11c	BioLegend Inc (117323)	Dendritic cells	APC/Cy7	1 in 100

2.5.3. Sample analysis

Flow cytometry data obtained from the FACSARIA III machine was exported to FlowJo software for full analysis.

2.6. Magnetic resonance imaging (MRI)

2.6.1. Cardiac cine-MRI scanning

I performed all MRI scanning and analysis in collaboration with Professor Carolyn Carr (DPAG, University of Oxford). Cardiac cine-MRI was performed post-MI at days 7, 14, 21 and 28 as previously described (Schneider, Cassidy et al., 2003). Mice were anesthetized with 4% isoflurane in O₂ in an induction chamber before being transferred into a purpose-built cradle and placed in the supine position. A nose cone connected to the cradle supplied 2% isoflurane in O₂ in order to maintain anaesthesia. Warm air was supplied to the cradle in order to maintain the temperature of the animals under anaesthesia. ECG electrodes were inserted into the forepaws and a respiration loop was taped across the chest. The cradle was lowered into a vertical-bore of an 11.7 T MR system (Magnex Scientific, Oxon, United Kingdom) with a 40 mm birdcage coil (Rapid Biomedical, Würzburg, Germany). The system was connected to a Bruker console running Paravision 2.1.1 (Bruker Medical, Ettlingen, Germany). The Paravision 2.1.1 software was set up to obtain a stack of contiguous 1 mm thick true short-axis ECG - gated cine-FLASH images. Images were acquired to cover the whole of the left ventricle (TE/TR 1.43/4.6 ms; 17.5° pulse; field of view 25.6 × 25.6 mm; matrix size 128 × 128 zero filled to 256 × 256 giving a voxel size of 100 × 100 × 1000 µm; 20 to 30 frames per cardiac cycle). Long-axis two-chamber and four-chamber cine images were also obtained.

2.6.2. MRI data analysis

MRI data was processed manually by image analysis with ImageJ (NIH Image, Bethesda, MD). All analysis was performed blind so as the vehicle/treated groups were unknown at the time of image processing. Measurements were calculated as previously described (Carr, Stuckey et al., 2008, Nahrendorf, Wiesmann et al., 2001). End-diastolic and end-

systolic volumes were measured for each slice and summed over the whole heart. Stroke volume was calculated by subtracting the end-systolic volume from the end-diastolic volume. Ejection fraction was calculated by dividing the end-diastolic volume by the stroke volume. End diastolic mass was calculated as the left-ventricular end-diastolic volume multiplied by the myocardial specific gravity (1.05 g/cm^3), end-systolic mass was also calculated in this was using the left-ventricular end-systolic volume. Cardiac output was calculated as stroke volume multiplied by heart rate. The relative infarct size was calculated from the average of the endocardial and epicardial circumferential lengths of the thinned, akinetic region of all slices, measured at diastole, and expressed as a percentage of the total myocardial surface.

2.7. Statistical analysis

All statistical analysis was performed using GraphPad Prism 6 software. Comparisons between two groups were made using an unpaired two-tailed T test, this included an F test to confirm the two groups had equal variances. A one-way ANOVA was used to make multiple comparisons between three experimental groups. In all cases a p-value of less than or equal to 0.05 was deemed significant.

3. Results I: Characterisation of the development of the cardiac lymphatic vasculature and identification of its cellular origins

3.1. Spatiotemporal development of the cardiac lymphatics in the embryo

It has been known that lymphatic vessels are present in the mammalian heart since they were first discovered by Rudbeck in the 17th century (Rudbeck, 1653). In recent years extensive research has been performed to characterise the development of the systemic lymphatic vasculature, however, the development of the cardiac lymphatic vessels had not been studied in detail prior to the work presented here. Therefore, the overall aims of the studies presented in sections 3.1. and 3.2. were to determine the time at which cardiac lymphatics begin to develop in the heart, to anatomically pinpoint where the cardiac lymphatic plexus first begins to form and observe how subsequent growth and expansion progresses through pre- and postnatal stages. Overall the data presented in these sections provide novel insights into the precise spatiotemporal development of cardiac lymphatic vessels.

3.1.1. Cardiac lymphatic vessels express LYVE-1, VEGFR-3 and PROX1

Lymphatic vessels are known to express a number of key functional proteins including LYVE-1 (Banerji et al., 1999), VEGFR-3 (Kaipainen et al., 1995) and PROX1 (Wigle & Oliver, 1999). PODOPLANIN is also known to be expressed by LECs (Hirakawa, Hong et al., 2003, Petrova, Makinen et al., 2002, Schacht et al., 2003), however, for these studies it was deemed unsuitable for whole-mount staining due to being expressed in the epicardium (Mahtab, Wijffels et al., 2008). Whole-mount immunofluorescent and 3,3'

Diaminobenzidine (DAB) staining was performed on E17.5 murine hearts in order to visualise the cardiac lymphatic network and to confirm cardiac LECs express the same key proteins present in the systemic lymphatic vasculature and other organs. E17.5 hearts were used as the cardiac lymphatic plexus is present on both the dorsal and ventral sides of the heart by this stage. Staining revealed an extensive network of lymphatic vessels on the epicardial surfaces of both dorsal and ventral sides of the heart, which were positive for LYVE-1, VEGFR-3 and PROX1 (**Figure 3.1a-g**). In addition to expression in lymphatic endothelium, LYVE-1 was also observed in cardiac resident macrophages (**Figure 3.1c**, arrows indicate macrophages), consistent with previous observations in other tissues (Carreira et al., 2001). VEGFR-3 was also expressed in the cardiac lymphatic endothelium, however, it was also detected at this stage in some angiogenic blood vessels, indicated by arrows in **Figure 3.1d**. This expression pattern of VEGFR-3 is consistent with previous studies outside of the heart (Hägerling et al., 2013, Kaipainen et al., 1995). PROX1 expression was observed within the nuclei of LECs and also at a lower level in cardiomyocytes (**Figure 3.1e-g**), again consistent with previous findings (Risebro et al., 2009). Although LYVE-1, VEGFR-3 and PROX1 label additional non-LEC cell types within the heart, each of these markers can clearly be used to visualise and identify cardiac lymphatic vessels. Consequently, LYVE-1, VEGFR-3 and PROX1 antibodies were used in combination, where possible, or independently to label the cardiac lymphatic vasculature.

Figure 3.1

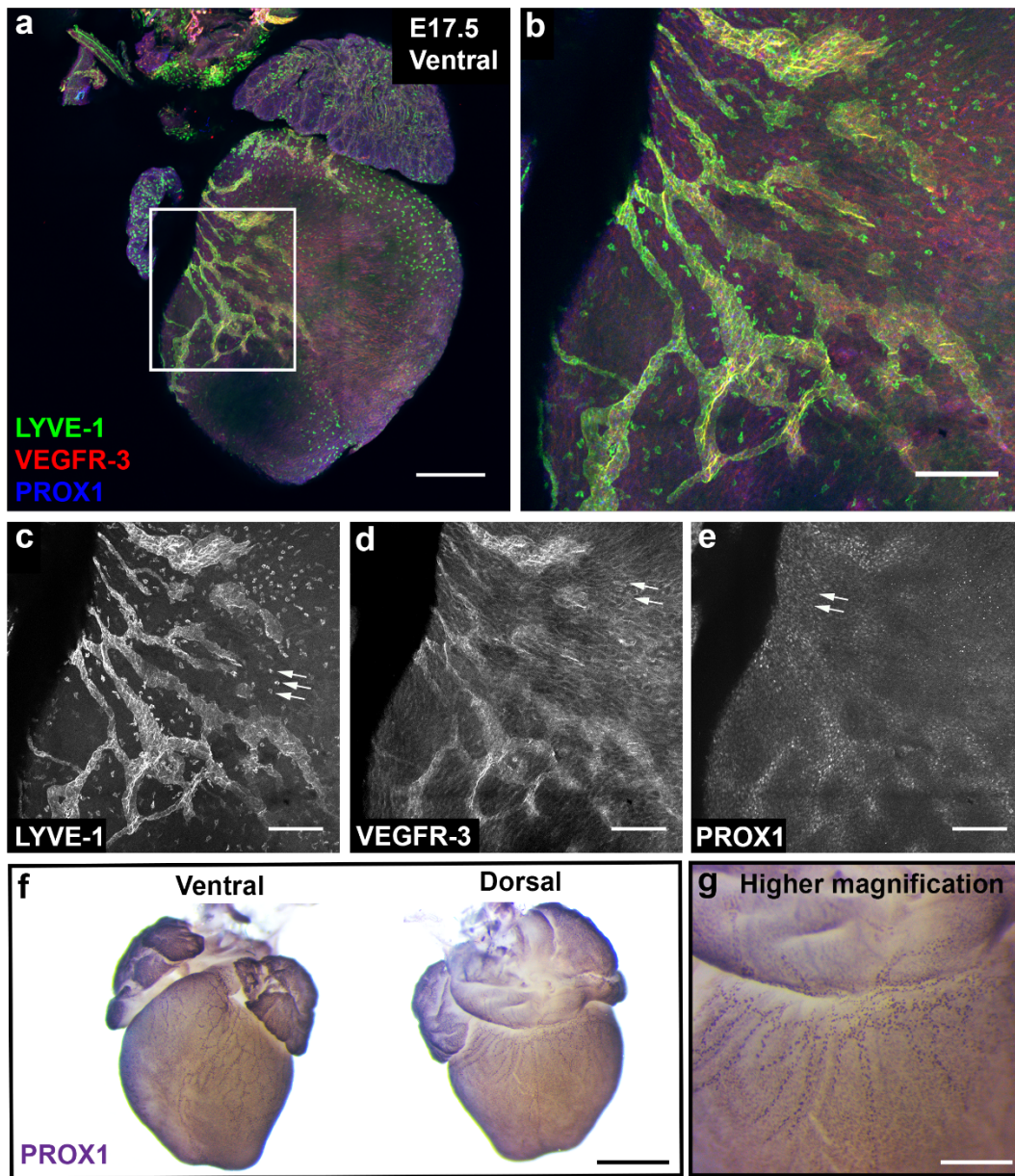


Figure 3.1: Cardiac lymphatic vessels express LYVE-1, VEGFR-3 and PROX1
Whole-mount immunofluorescent staining of E17.5 hearts for LYVE-1, VEGFR-3 and PROX1 (**a**), higher magnification in **b** and separate channels (**c**, **d** and **e**), revealed LYVE-1⁺ PROX1⁺ VEGFR-3⁺ lymphatic vessels on the surface of the heart. LYVE-1 also labelled cardiac resident macrophages (indicated by arrows in **c**). VEGFR-3 was expressed in angiogenic blood capillaries (indicated by arrows in **d**). PROX1 was also expressed in cardiomyocytes (indicated by arrows in **e**). PROX1 DAB staining revealed an extensive lymphatic network on both sides of the heart (**f**, **g**). Scale bars: **a** = 500 μ m, **b-e** = 200 μ m, **f** = 400 μ m, **g** = 200 μ m. n = 5.

3.1.2. Cardiac lymphatic endothelial cells bud from the cardinal vein region and migrate towards the outflow tract of the developing heart

E10.5 embryos were stained for ENDOMUCIN (EMCN), VEGFR-3 and PROX1. At this stage EMCN is expressed throughout the endothelium of blood vessels before later becoming restricted to veins and capillaries (Brachtendorf, Kuhn et al., 2001, Corada, Orsenigo et al., 2013, Kuhn, Brachtendorf et al., 2002). The stage E10.5 was selected as the emergence of LECs is ongoing at this point, having begun at E9.5 (Wigle & Oliver, 1999). Staining revealed the emergence of PROX1⁺ VEGFR-3⁺ LECs from the cardinal vein, intersomitic vessels (ISVs) and superficial venous plexus (sVP) regions (EMCN⁺) (**Figure 3.2a-e**). The PROX1⁺ VEGFR-3⁺ LECs (indicated by white arrows in **Figure 3.2b**) appeared to be migrating in the direction of the sinus venosus. At E10.5 VEGFR-3 is also expressed in angiogenic blood vessels and, therefore, is co-localized with EMCN in addition to PROX1. Whole-mount DAB staining on torsos isolated from E12.5 embryos revealed the migration of PROX1⁺ LECs from extra-cardiac tissue towards the outflow tract on the ventral surface of the heart (**Figure 3.2f-h**). Unfortunately it was not possible to perform whole-mount immunofluorescent staining on whole E12.5 embryos due to their size and thickness by this stage. These findings support those which have previously described the budding of LECs from the cardinal vein, ISV and sVP regions (Hägerling et al., 2013, Wigle & Oliver, 1999, Yang et al., 2012) and further demonstrate the LEC migration route into the heart at E12.5.

3.1.3. Cardiac lymphatic vessels develop from approximately E12.5 onwards in the mouse embryo

A time course analysis was performed to characterise the spatiotemporal development of cardiac lymphatic vessels. Hearts collected at E12.5, E15.5, E16.5 and E17.5 were stained for EMCN, VEGFR-3 and PROX1 (**Figure 3.3a-d**). VEGFR-3 staining was present

in lymphatic and blood vessel capillary endothelium from E12.5 to E17.5 with expression in blood vessel endothelium markedly reduced by E17.5. Staining at E12.5 revealed the infiltration of PROX1⁺ VEGFR-3⁺ LECs along the outflow tract region of the developing heart from extra-cardiac tissue (**Figure 3.3a**). From E15.5 to E17.5 the cardiac lymphatic network was observed to grow and extend down the heart from the base towards the apex. The developing cardiac lymphatics were VEGFR3⁺ PROX1⁺ and were closely aligned with EMCN⁺ coronary veins at later stages in development (**Figure 3.3b-d**).

Figure 3.2

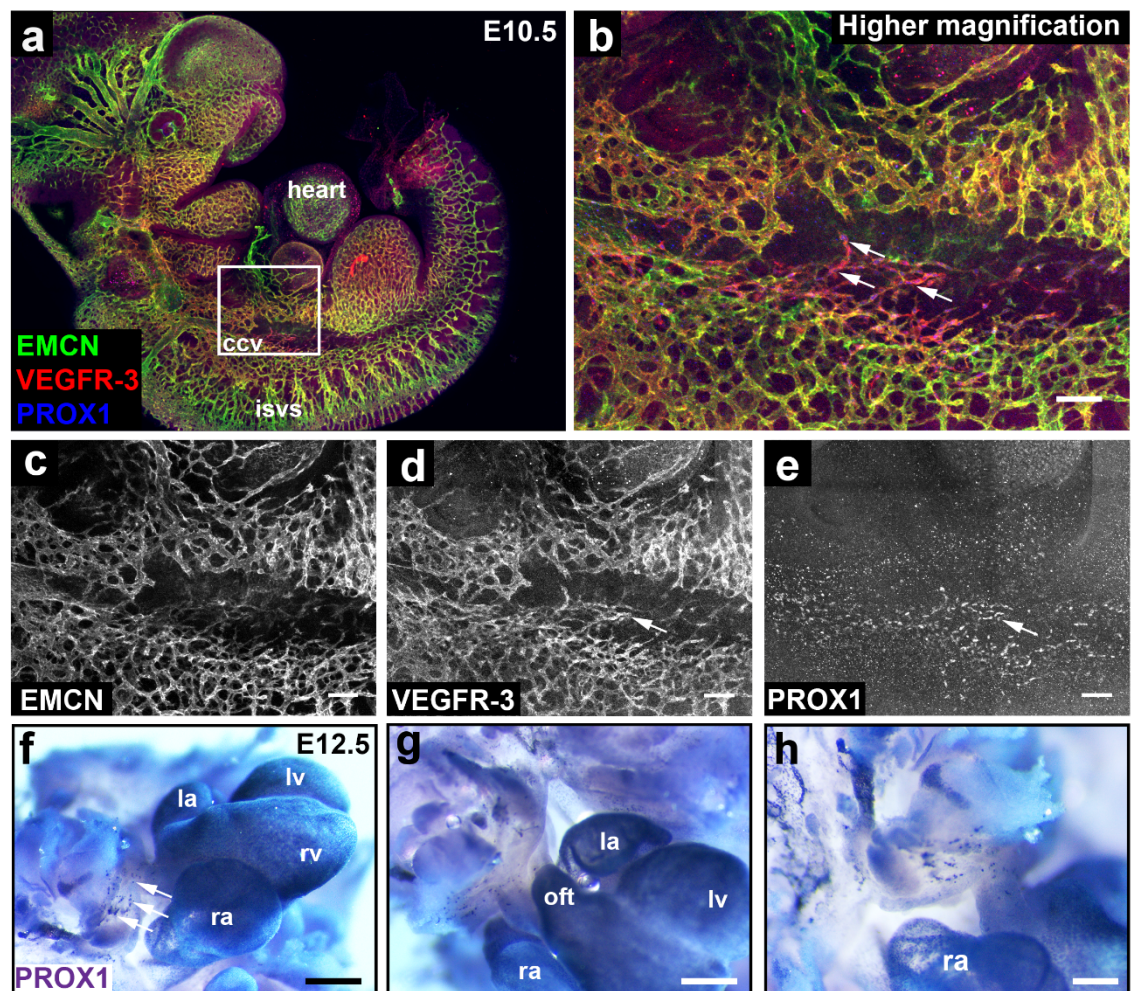


Figure 3.2: Cardiac LECs bud from the cardinal vein and migrate towards the outflow tract

Whole-mount immunofluorescent staining of E10.5 embryos for ENDOMUCIN (EMCN), VEGFR-3 and PROX1 (**a**), higher magnification in **b** and separate channels in **c**, **d** and **e**, revealed PROX1⁺ VEGFR-3⁺ cells budding from the cardinal vein (ccv)/intersomitic vessel (isvs) region. Example PROX1⁺ VEGFR-3⁺ cells are labelled with arrows in **b**, **d** and **e**. DAB staining on E12.5 torsos (**f-h**) revealed PROX1⁺ LECs migrating from extra-cardiac tissue into the heart, see arrows in **f**. Scale bars: **b-e** = 100 μ m, **f** = 600 μ m, **g** = 500 μ m, **h** = 300 μ m. E10.5 n = 3, E12.5 n = 3.

Figure 3.3

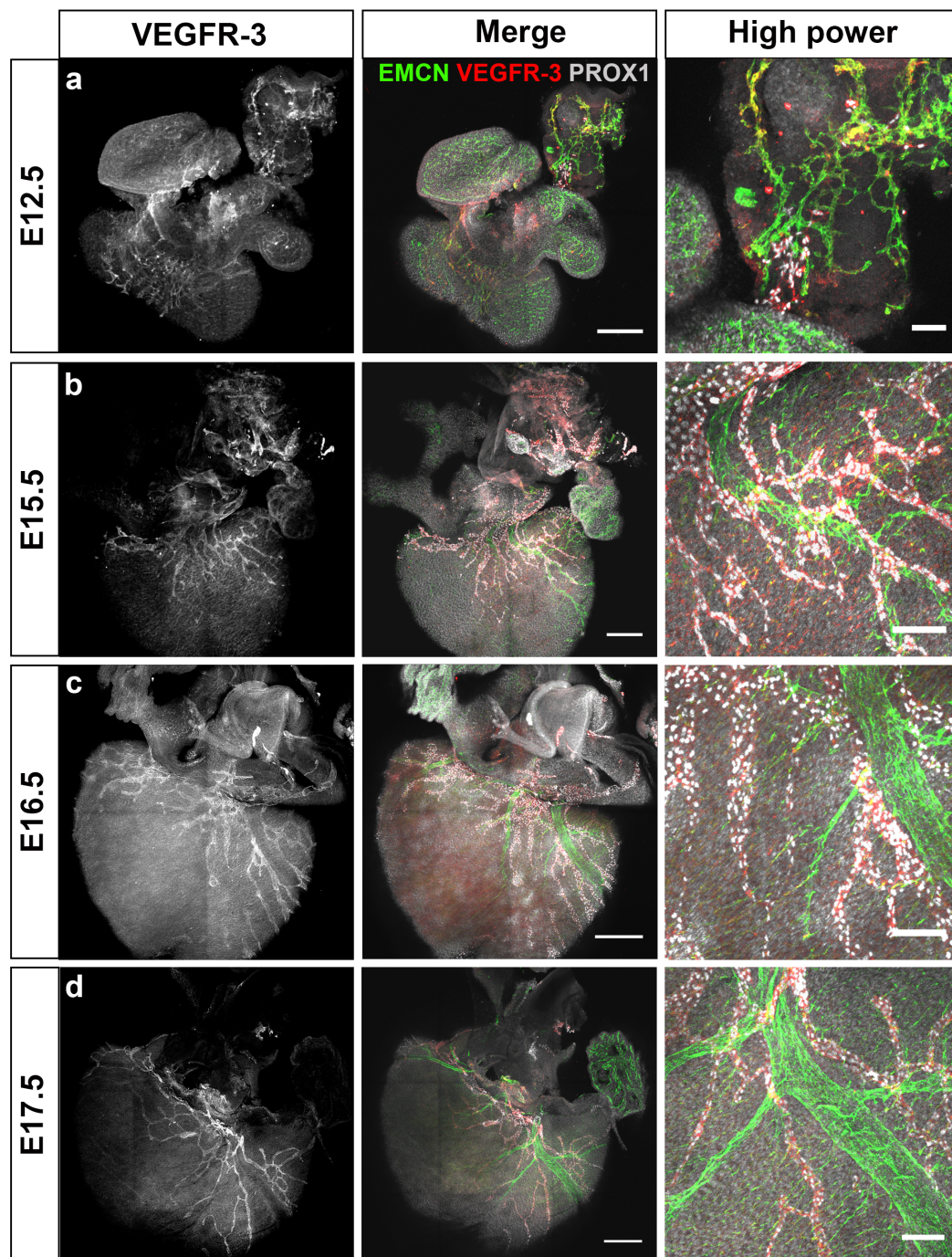


Figure 3.3: Cardiac lymphatic vessels develop from E12.5 onwards in the mouse embryo

Whole-mount immunofluorescent staining of E12.5 (a), E15.5 (b), E16.5 (c) and E17.5 (d) hearts for EMCN, VEGFR-3 and PROX1, imaged on the dorsal side, revealed expansion of the cardiac lymphatic network between E12.5 and E17.5 with growth occurring from the base towards the apex. Scale bars: **a** = 200 μ m, **b** = 300 μ m, **c**, **d** = 400 μ m. n = 3 for each time point.

3.1.4. Cardiac lymphatic endothelial cells do not sprout from coronary veins

Previous work performed in the Riley lab by Linda Klotz revealed that approximately 80% of cardiac LECs derive from Tie2⁺ venous endothelium. Therefore, whole-mount immunostainings were performed on developing hearts in order to investigate whether the endothelium of coronary veins provides a cellular contribution to the cardiac lymphatic vasculature. Hearts from E12.5 to E17.5 were stained for PROX1 and EMCN. The staining revealed the migration of PROX1⁺ LECs into the heart and the development of the coronary vasculature. PROX1⁺ LECs were observed to migrate in from extra-cardiac tissue in a process clearly independent of the coronary vasculature (**Figure 3.4a-l**). High power images (**Figure 3.4b, d, f, h, j, l**) revealed that at no stage in embryonic cardiac lymphatic development did PROX1⁺ cells appear to bud from the EMCN⁺ coronary endothelium (**Figure 3.4a-l**).

Figure 3.4

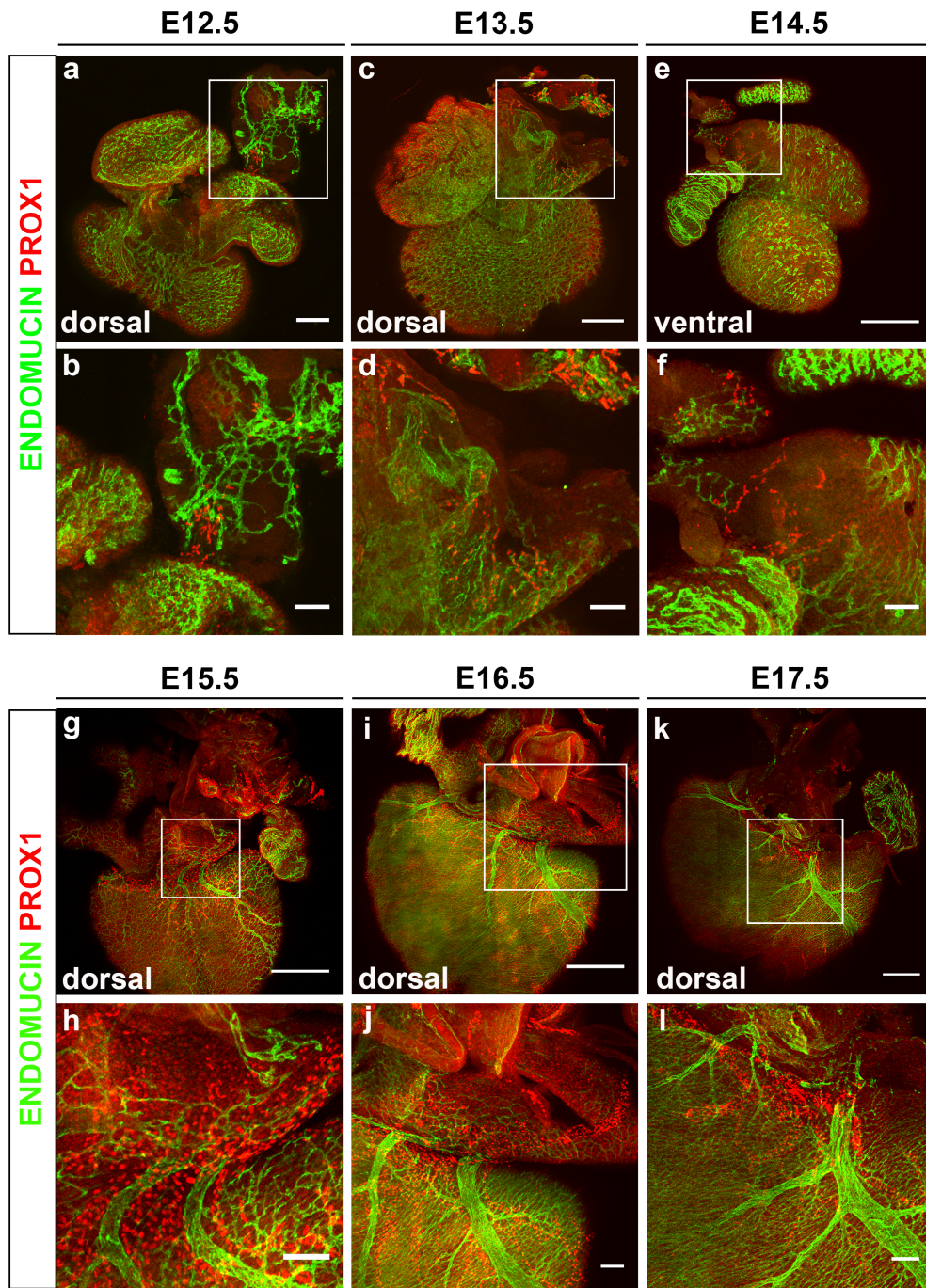


Figure 3.4: Cardiac lymphatic endothelial cells do not bud from coronary veins

Whole-mount immunofluorescent staining of E12.5 (**a**, higher magnification in **b**), E13.5 (**c**, higher magnification in **d**), E14.5 (**e**, higher magnification in **f**), E15.5 (**g**, higher magnification in **h**), E16.5 (**i**, higher magnification in **j**) and E17.5 (**k**, higher magnification in **l**) hearts for EMCN and PROX1, revealed growth of the cardiac lymphatics along coronary blood vessels, there was no evidence of PROX1⁺ LECs budding from coronary blood vessel endothelium. Scale bars: **a** = 200 μ m, **b**, **d**, **f**, **h**, **j**, **l** = 100 μ m, **c** = 300 μ m, **e**, **g**, **i** = 500 μ m, **k** = 400 μ m. n = 3 for each time point.

3.2. Postnatal growth of cardiac lymphatics

3.2.1. Cardiac lymphatic vessels continue to grow and mature postnatally

Hearts were collected at several stages between postnatal day (P) 0 and P7 and were subjected to whole-mount DAB staining for PROX1 or VEGFR-3 (**Figure 3.5** and **Figure 3.6**) in order to characterise the pattern of postnatal growth of cardiac lymphatics. Both PROX1 and VEGFR-3 were expressed by the postnatal cardiac lymphatic network. By P0 VEGFR-3 was expressed solely in the lymphatic endothelium and was no longer expressed in blood vessels. At P0, on the dorsal side of the heart (**Figures 3.5a-b, 3.6a**), the lymphatics were mainly located near the base of the heart, running alongside major coronary veins. On the ventral side at P0 (**Figures 3.5c, 3.6b**) the lymphatics were observed to extend further towards the apex than on the dorsal side at the same stage. Little expansion of the cardiac lymphatic network was observed between P1 and P3 (**Figures 3.5d-i, 3.6c-f**). Some growth occurred between P3 and P4 as a denser capillary network was visualised in regions on both the dorsal and ventral sides (**Figures 3.5j-l, 3.6g-h**). Growth of the network continued through to P6 (**Figures 3.5m-o, 3.6i-j**) and by P7 a substantially more extensive network had formed with an increased number of smaller, branched capillaries (**Figures 3.5p-r, 3.6k-l**).

Figure 3.5

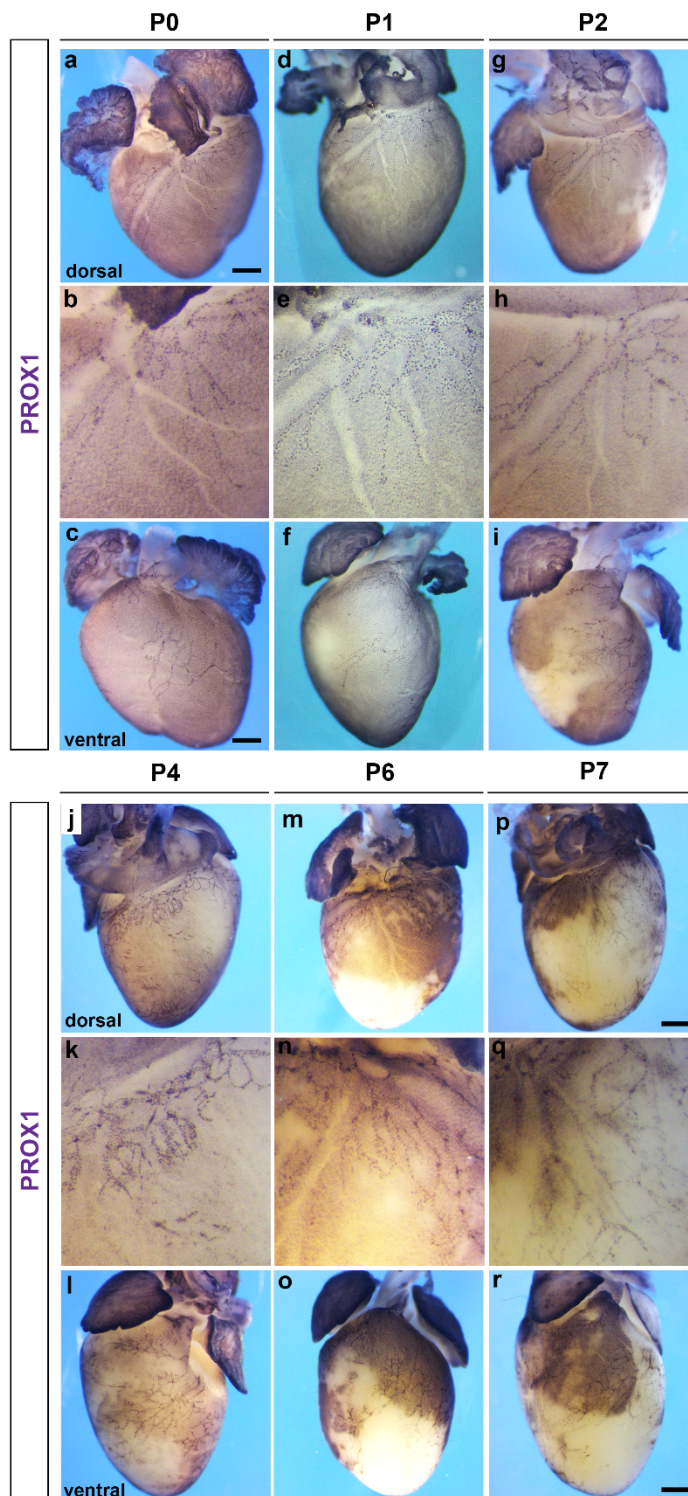


Figure 3.5: PROX1 staining on postnatal hearts revealed growth and expansion of the cardiac lymphatic network

Whole-mount DAB staining of P0 (dorsal side in **a**, high magnification dorsal in **b**, ventral side in **c**), P1 (**d**, **e**, **f**), P2 (**g**, **h**, **i**), P4 (**j**, **k**, **l**), P6 (**m**, **n**, **o**) and P7 (**p**, **q**, **r**) hearts for PROX1, revealed growth of the cardiac lymphatics through early postnatal stages. $n = 3$ to 5 for each time point. Scale bars **a**, **c** = $500\ \mu\text{m}$, **p**, **r** = $1\ \text{mm}$.

Figure 3.6

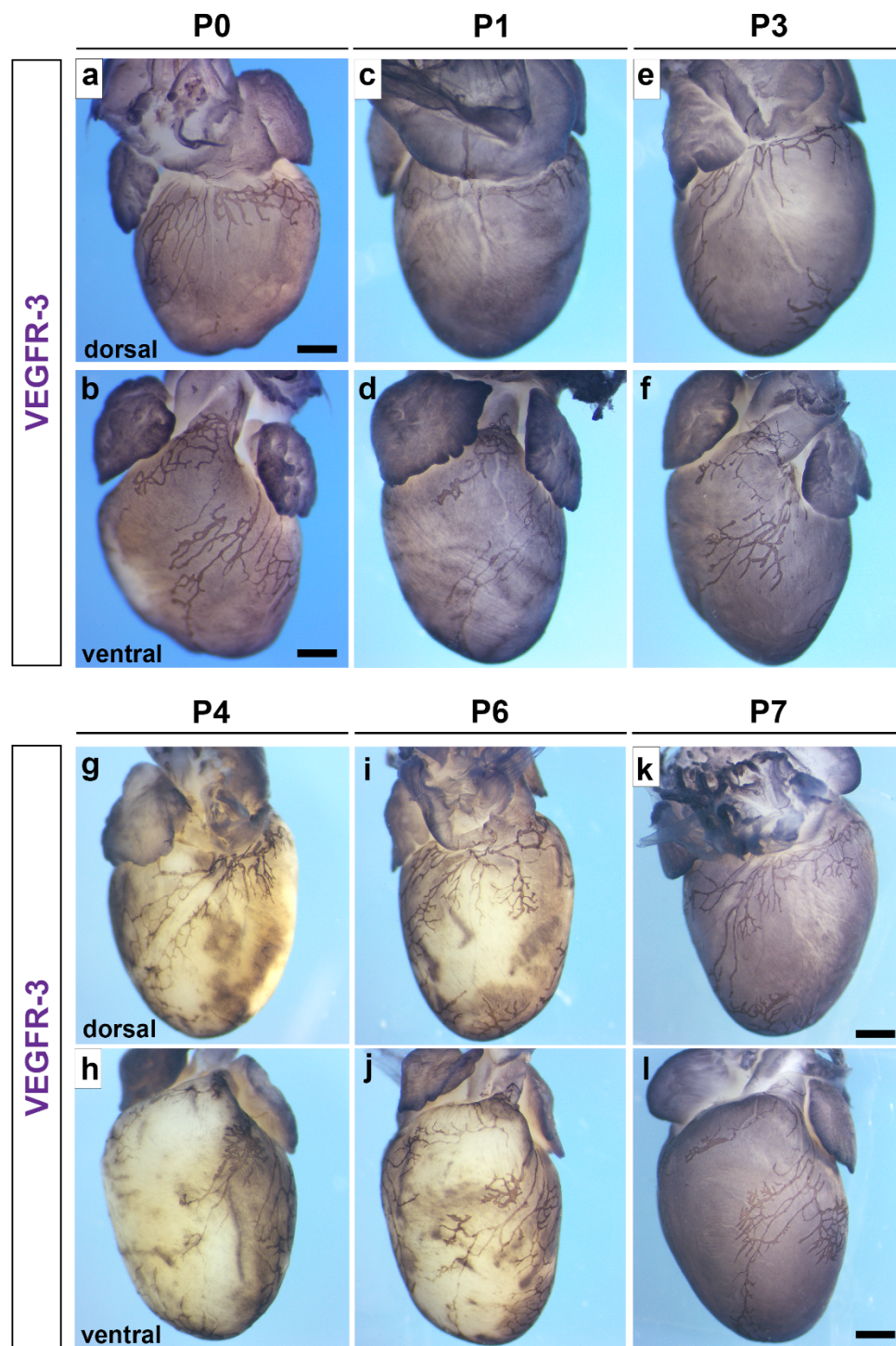


Figure 3.6: VEGFR-3 staining on postnatal hearts revealed growth and expansion of the cardiac lymphatic network

Whole-mount DAB staining of P0 (dorsal side in **a**, ventral side in **b**), P1 (**c**, **d**), P3 (**e**, **f**), P4 (**g**, **h**), P6 (**i**, **j**) and P7 (**k**, **l**) hearts for VEGFR-3, revealed growth of the cardiac lymphatics through early postnatal stages. $n = 3$ to 5 for each time point. Scale bars **a**, **b** = $500\ \mu\text{m}$, **k**, **l** = $1\ \text{mm}$.

3.2.2. ENDOMUCIN is expressed in the postnatal cardiac lymphatic endothelium and additional postnatal growth of the lymphatic network is not via sprouting from coronary veins

Whole-mount immunofluorescent staining was performed on hearts at P5. Initial antibody staining for PROX1 and EMCN revealed vessels which were co-labelled with PROX1 and EMCN (**Figure 3.7a**, arrows indicate co-labelled vessel). This finding was surprising as previous staining with PROX1 and EMCN antibodies on late embryonic hearts labelled only lymphatic (PROX1) or blood (EMCN) vessels, there was no evidence of co-labelling at embryonic stages. I, therefore, hypothesised that the PROX1⁺ EMCN⁺ vessels may have been venous endothelium in which PROX1 was switched on and from which novel postnatal PROX1⁺ LECs were derived i.e. LECs sprouting from coronary veins during additional postnatal growth of the lymphatics. In order to test this an erythrocyte antibody (TER119) was used to definitively determine whether the co-labelled vessels were veins expressing PROX1, or lymphatic vessels switching on EMCN postnatally. Staining with TER119 revealed, as expected, erythrocytes within EMCN⁺ blood vessels (**Figure 3.7b**, arrows indicate TER119⁺ erythrocytes in EMCN⁺ blood vessels). Co-staining with PROX1, EMCN and TER119 (**Figure 3.7c**, white box at higher magnification in **d**) revealed the PROX1⁺ EMCN⁺ vessels are not veins as they do not contain erythrocytes, indicating that they are in fact lymphatic vessels in which EMCN is expressed postnatally, thus disproving the hypothesis that PROX1 is switched on in venous endothelium postnatally.

Figure 3.7

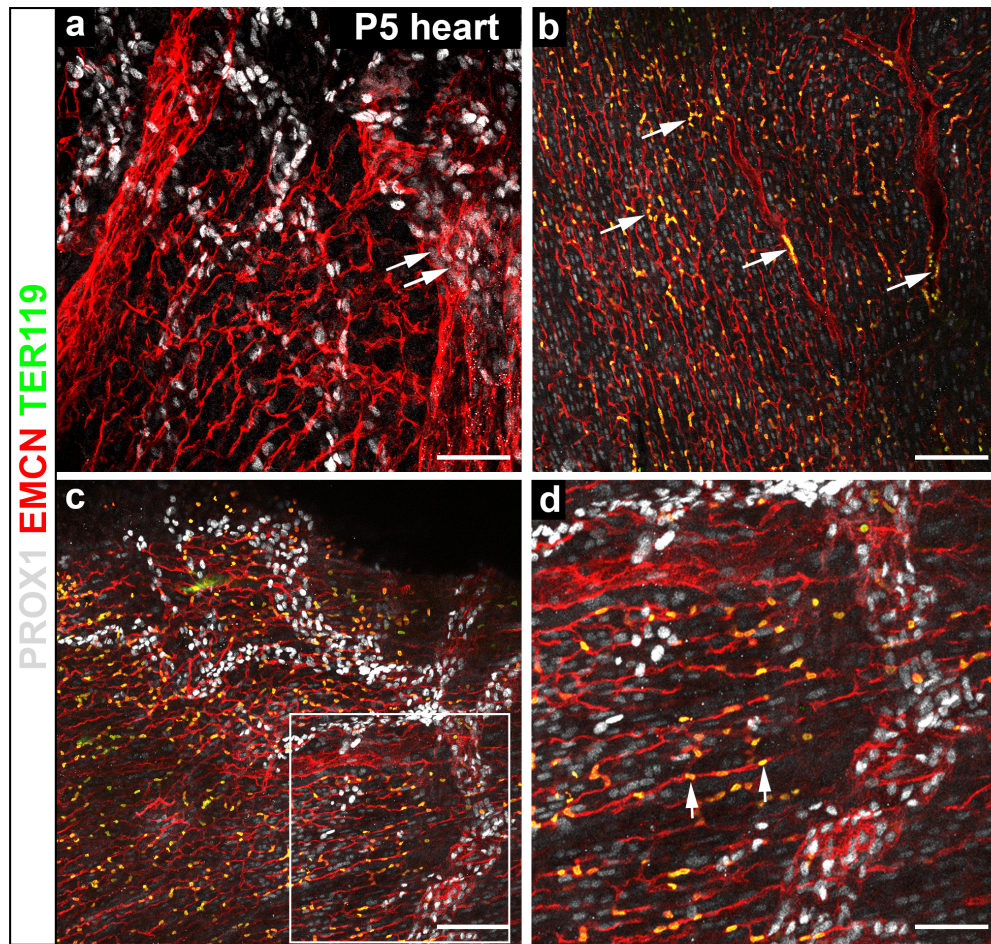


Figure 3.7: ENDOMUCIN is expressed in the postnatal cardiac lymphatic endothelium and additional postnatal growth of the lymphatic network is not via sprouting from coronary veins

Whole-mount immunofluorescent staining on P5 hearts for PROX1 (grey), EMCN (red) and erythrocytes labelled with TER119 (green) was performed. Staining for PROX1 and EMCN shown in **a** revealed vessels which expressed both EMCN and PROX1 (indicated with arrows in **a**). Staining for TER119, EMCN and PROX1 shown in **b** reveals the presence of TER119⁺ erythrocytes within EMCN⁺ blood vessels (indicated with arrows in **b**). Panels **c** and **d** demonstrate that TER119 is only co-localized with EMCN (indicated with arrows in **d**) and not with PROX1, therefore PROX1⁺ EMCN⁺ vessels are lymphatic vessels rather than blood vessels which express PROX1. Scale bars **a**, **d** = 50 μ m, **b**, **c**, = 100 μ m. n = 3 hearts analysed.

3.3. Further characterisation of the non-venous contribution to cardiac LECs

Previous work in the Riley lab performed by Linda Klotz revealed that not all cardiac LECs are derived from venous endothelium. Lineage tracing of venous endothelium using *Tie2-Cre* and *Pdgfb-CreER* consistently revealed areas of unlabelled cardiac lymphatic vasculature at E17.5, indicating there must be an alternative source for the unlabelled LECs (Klotz, Norman et al., 2015). Approximately 80% of LECs were found to be labelled by these endothelial Cre lines. In order to investigate the cellular sources which may give rise to the unlabelled LECs other potential sources were considered and subsequently excluded. Lineage tracing using *Mesp1-Cre* to label early pre-cardiac mesoderm (Saga, Miyagawa-Tomita et al., 1999), *Nkx2.5-Cre* to label late cardiac mesoderm (Moses, DeMayo et al., 2001), *Wt1-CreERT2* (Zhou, Ma et al., 2008) to label the epicardium and *Wnt1-Cre* to label cardiac neural crest (Danielian, Muccino et al., 1998) failed to fate map cardiac lymphatic endothelial cells. Subsequently the haemogenic endothelium was considered as a possible cellular source of LECs. Given its capacity as a specialised subset of endothelium which can give rise to haematopoietic stem and progenitor cells (HSPCs) it was hypothesised that the haemogenic endothelium may possess the ability to act as a multipotent progenitor which is also able to give rise to LECs. Therefore, the aim of the studies presented in this section was to perform lineage tracing using multiple Cre lines in order to determine whether there is a haematopoietic contribution to the cardiac lymphatic vasculature.

VAV1 is a GDP/GTP nucleotide-exchange factor for Rho/Rac and is known to be expressed in haematopoietic stem cells (HSCs) and their derivatives (Chen et al., 2009, de Boer et al., 2003), it has also been reported to be expressed in haemogenic

endothelium within the embryonic yolk sac, subaortic mesenchyme and intra-aortic haematopoietic clusters (Padron-Barthe, Temino et al., 2014). Therefore, the *Vav1-Cre* mouse line was employed for lineage tracing in order to determine whether a population of cardiac LECs do derive from the haemogenic endothelium. PDGFR β has also been reported to be expressed within haemogenic endothelium (Rolny, Nilsson et al., 2006), therefore, the *Pdgfr β -Cre* mouse line (Foo, Turner et al., 2006) was utilised as an additional Cre line to test the hypothesis that cells deriving from haemogenic endothelium contribute cardiac LECs.

3.3.1. *Vav1-Cre* does not label venous endothelium nor the systemic lymphatic vasculature and is, therefore, a distinct non-venous lineage which gives rise to cardiac LECs

3.3.1.1. *Vav1-Cre* does not label the systemic veins or lymphatics

In order to confirm any potential contribution to LECs from the *Vav1-Cre* lineage was non-venous derived, an experiment was performed to rule out labelling by *Vav1-Cre* of venous endothelium and the systemic lymphatic vasculature. *Vav1-Cre;R26R-tdTomato* embryos were collected at E10.0 and whole-mount immunofluorescent staining for PROX1 and EMCN revealed that *Vav1-Cre* did not label the common cardinal vein, nor the emerging LECs (**Figure 3.8a-d**). In addition, *Vav1-Cre;R26R-EYFP* embryos were harvested at E12.5 and cryo-sectioned transversally to reveal the jugular vein and jugular lymph sacs. Immunofluorescent staining demonstrated that *Vav1-EYFP*⁺ cells are not present in either the EMCN⁺ jugular vein or jugular lymph sacs (**Figure 3.8e-h**), thus confirming there is no contribution of *Vav1-Cre*⁺ cells to the blood vessel endothelium or the systemic lymphatic vasculature.

Figure 3.8

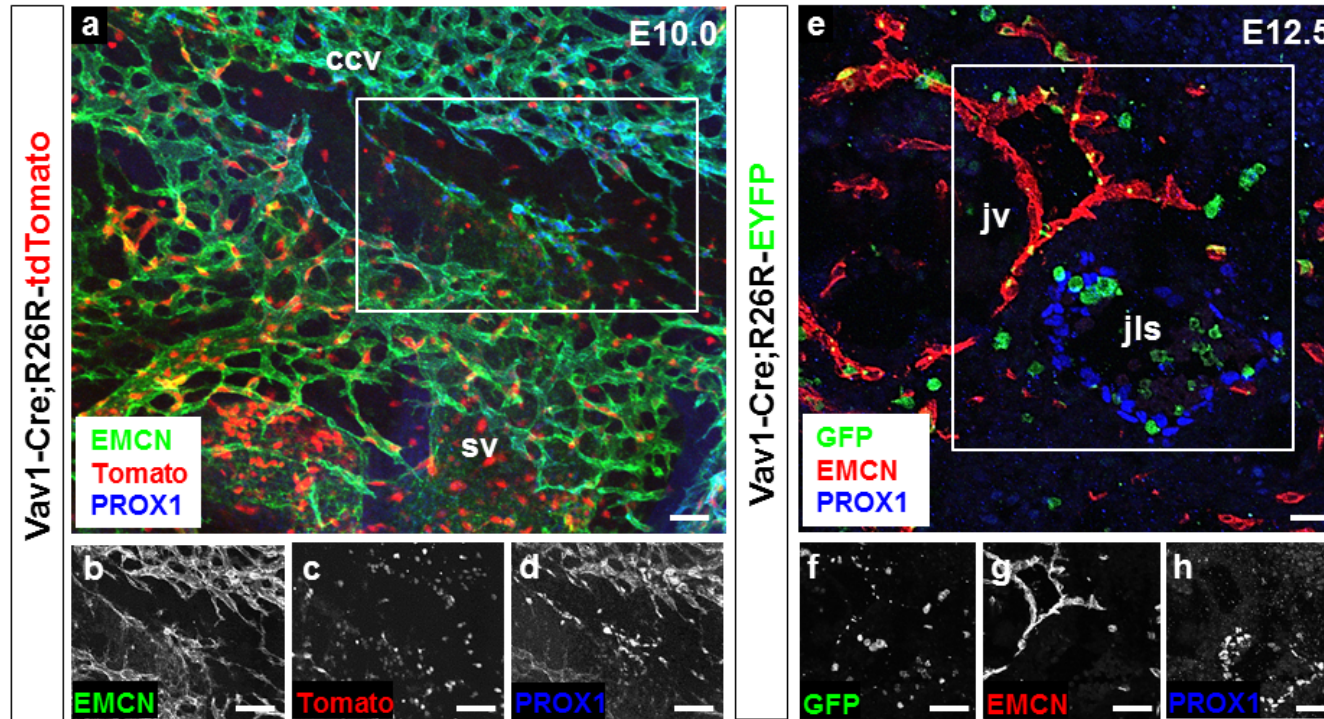


Figure 3.8: LECs which bud from the common cardinal vein or jugular lymph veins do not derive from a Vav1⁺ lineage
Whole E10.0 *Vav1-Cre;R26R-tdTomato* embryos were stained for EMCN (green) and PROX1 (blue), revealing the budding of PROX1⁺ LECs from the EMCN⁺ common cardinal vein (**a**). *Vav1-Cre;R26R-tdTomato* did not label any of the emerging LECs, nor the venous endothelium. White inset box in **a** highlights the emerging LECs and is enlarged in panels **b-d**. Cryosectioned E12.5 *Vav1-Cre;R26R-EYFP* embryos were also stained for EMCN (red) and PROX1 (blue), revealing no recombination nor labelling of LECs forming the jugular lymph sacs (**e**), highlighted by white box in **e**, enlarged in **f-h**. ccv, common cardinal vein; jls, jugular lymph sac; jv, jugular vein; sv, sinus venosus. Scale bars **a**, **e** = 50μm, **b-d**, **f-h** = 12.5 μm. n = 3 embryos analysed per time point.

3.3.1.2. The non-venous *Vav1-Cre* and *Pdgfr β -Cre* lineages contribute to cardiac LECs (Linda Klotz)

Lineage tracing using *Vav1-Cre* and *Pdgfr β -Cre* lines was initially performed by Linda Klotz to establish whether there is any lineage contribution from cells within the haemogenic endothelium to the cardiac lymphatic vasculature. Hearts from *Vav1-Cre;R26R-tdTomato* and *Pdgfr β -Cre;R26R-mTmG* embryos were collected at E17.5 and subjected to whole-mount immunofluorescent staining to label the cardiac lymphatics. E17.5 was the selected embryonic stage for analysis as there is an extensive lymphatic plexus on both sides of the heart by this point in development and whole-mount staining and mounting for confocal imaging can easily be performed on samples of this size. Confocal imaging of hearts from *Vav1-Cre;R26R-tdTomato* embryos revealed several regions of the cardiac lymphatic vasculature within the developing heart which were labelled by *Vav1-Cre;R26R-tdTomato* (**Figure 3.9a**); many examples of PROX1⁺ LYVE-1⁺ tdTomato⁺ LECs were observed throughout the lymphatic network (**Figure 3.9b, d**, PROX1⁺ LYVE-1⁺ tdTomato⁺ LECs indicated by white arrowheads in the far right panel of **3.9b**). Orthogonal views 1-4, displayed below **Figure 3.9a**, confirmed the co-localisation observed between PROX1, LYVE-1 and tdTomato. *Vav1-Cre;R26R-tdTomato* was also found to label LYVE-1⁺ cardiac resident macrophages, LYVE-1⁺ tdTomato⁺ macrophages are highlighted by white arrows in **Figure 3.9c**. *Pdgfr β -Cre;R26R-mTmG* hearts also revealed a contribution from this lineage to the cardiac lymphatic vasculature, demonstrated by extensive GFP⁺ labelling of the PROX1⁺ lymphatic network (**Figure 3.9e-h**). Within **Figure 3.9e** multiple regions are highlighted (regions 1-4) which are PROX1⁺ GFP⁺, orthogonal views confirming the lineage contribution in these example regions are displayed below **Figure 3.9e**. Quantification revealed that approximately 20% of LECs derived from the *Vav1*⁺ lineage and that approximately 30% derived from the *Pdgfr β* ⁺ lineage (Klotz et al., 2015).

Figure 3.9

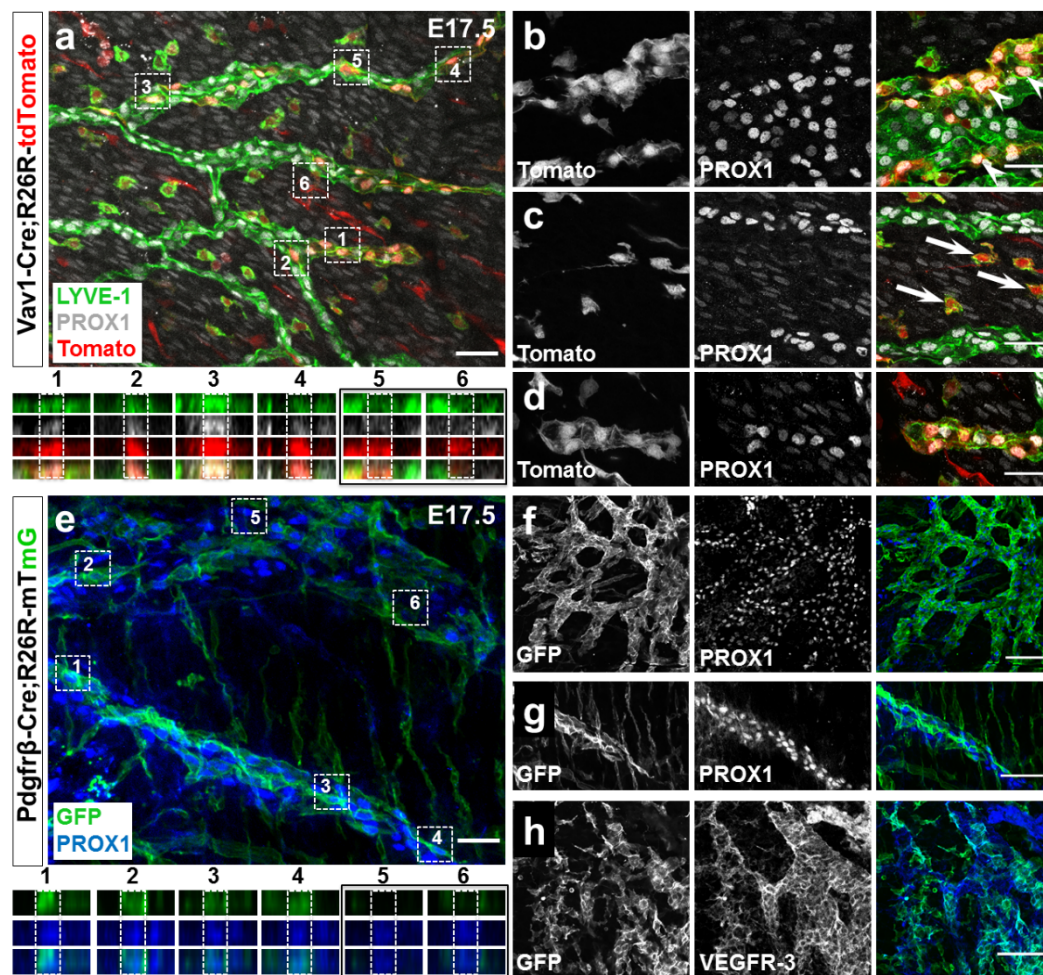


Figure 3.9: The non-venous *Vav1-Cre* and *Pdgfrβ-Cre* lineages contribute to LECs within the cardiac lymphatic vasculature (Linda Klotz)

Whole-mount immunofluorescent staining on E17.5 *Vav1-Cre;R26R-tdTomato* hearts for PROX1 (grey) and LYVE-1 (green) revealed a *Vav1*⁺ contribution to cardiac LECs (a, b, d). Orthogonal views (displayed below a) 1-4 highlight LECs in which recombination has taken place and 5-6 display cardiac macrophages which are LYVE-1⁺ PROX1⁻. Enlarged views from panel a are displayed in b-d. White arrowheads in b highlight *Vav1-Cre;R26R-tdTomato*⁺ PROX1⁺ LYVE-1⁺ LECs, white arrows in c indicate *Vav1-Cre;R26R-tdTomato*⁺ PROX1⁻ LYVE-1⁺ cardiac macrophages. Whole-mount immunofluorescent staining on E17.5 *Pdgfrβ-Cre;R26R-mTmG* hearts for PROX1 (blue) revealed a *Pdgfrβ*⁺ contribution to the cardiac lymphatic network (e-h). Orthogonal views (displayed below e) 1-4 highlight LECs in which recombination has taken place and 5-6 display regions of the cardiac lymphatic vasculature which are *Pdgfrβ*⁻. Scale bars a, e = 60 μm, b-d = 30 μm, f, h = 100 μm, g = 200 μm. *Vav1-Cre;R26R-tdTomato* n = 4 hearts analysed, *Pdgfrβ-Cre;R26R-mTmG* n = 3 hearts analysed.

3.3.2. The *Csf1r-CreER* lineage does not label the systemic lymphatic vasculature or venous endothelium and partially contributes to cardiac lymphatics

The constitutive *Vav1-Cre* and *Pdgfr β -Cre* lines, revealed a potential contribution from the haemogenic endothelium to the cardiac lymphatic vasculature, however, further investigation was required to confirm this contribution as neither the *Vav1-Cre* nor the *Pdgfr β -Cre* lines were reported to be entirely specific to the haemogenic endothelium. *Vav1-Cre* is known to be expressed throughout the entire haematopoietic system in addition to various lymphoid tissues (de Boer et al., 2003) and *Pdgfr β -Cre* is expressed in mural cells including pericytes and vascular smooth muscle cells (Foo et al., 2006). I, therefore, employed *Csf1r-CreER* as an additional, inducible Cre, for lineage tracing as the CSF1R protein has been reported to be expressed in within erythro-myeloid progenitors (EMPs) which arise from the yolk sac haemogenic endothelium (Gomez Perdiguero et al., 2015). Cre recombination in inducible CreER lines occurs when a pulse of tamoxifen is delivered, thus allowing a temporal recombination event which can occur during a specific time window. Inducible CreER lines are advantageous over constitutive Cre lines for lineage as the labelling can be induced at a specific stage.

3.3.2.1. The *Csf1r-CreER* lineage does not label the systemic lymphatic vasculature or venous endothelium

Initial lineage tracing using *Csf1r-CreER* was performed to rule out any contribution from this lineage to venous endothelium or systemic lymphatic vasculature. Pregnant females were pulsed with 4-OH tamoxifen at E7.5 in order to drive Cre recombination within EMPs arising from the haemogenic endothelium within *Csf1r-CreER;td-Tomato* embryos. Embryos were collected at E10.5 and subjected to whole-mount immunofluorescent staining to reveal the EMCN⁺ veins and PROX1⁺ LECs budding from

the cardinal vein (**Figure 3.10**). Imaging in the region of the cardinal vein revealed a small number of *Csf1r*-td-Tomato⁺ cells, however, no *Csf1r*-td-Tomato⁺ contribution to the EMCN⁺ venous endothelium nor the PROX1⁺ LECs was observed (**Figure 3.10a-d**); thus excluding labelling of these cell populations by *Csf1r-CreER*.

Figure 3.10

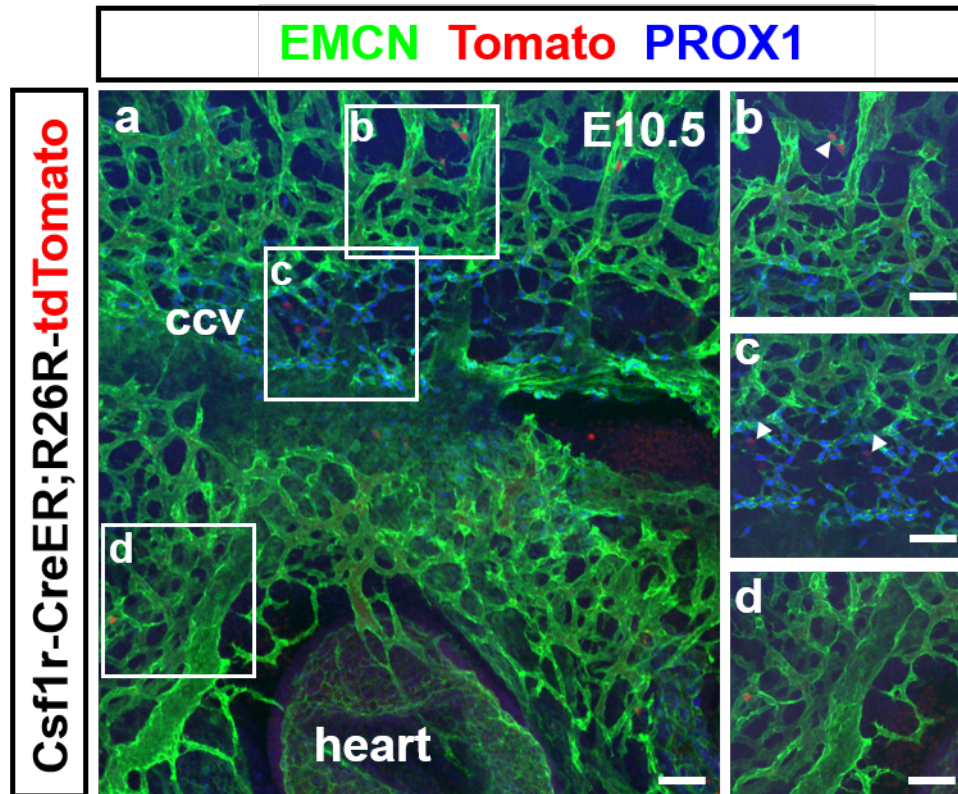


Figure 3.10: LECs which bud from the common cardinal vein do not derive from a *Csf1r*⁺ lineage

4-OH tamoxifen was injected to induce Cre recombination at E7.5. Whole E10.5 *Csf1r-CreER;R26R-tdTomato* embryos were stained for EMCN (green) and PROX1 (blue), revealing the budding of PROX1⁺ LECs from the EMCN⁺ common cardinal vein (a). *Csf1r-CreER;R26R-tdTomato* did not label any of the emerging LECs, nor the venous endothelium. White inset boxes in a, enlarged in b, c and d, highlight the presence of *Csf1r-CreER;R26R-tdTomato*⁺ macrophages indicated by white arrowheads (b,c) and PROX1⁺ *Csf1r-CreER;R26R-tdTomato*⁻ LECs emerging from the cardinal vein. ccv, common cardinal vein; jls, jugular lymph sac; jv, jugular vein; sv, sinus venosus. Scale bars a = 100µm, b-d = 50 µm. n = 3 embryos analysed.

3.3.2.2. The *Csf1r-CreER* lineage partially contributes to cardiac lymphatics

Csf1r-CreER;td-Tomato hearts were collected at E17.5 from embryos which had received a pulse of 4-OH tamoxifen at E7.5 in order to induce recombination within the haemogenic endothelium of the yolk sac. Immunofluorescent staining revealed LYVE-1⁺ PROX1⁺ cardiac lymphatics and a few rare examples of PROX1⁺ tdTomato⁺ LECs (**Figure 3.11a-c**, orthogonal views for PROX1⁺ tdTomato⁺ cells are displayed in panel **c** numbers 2-4), indicating a small contribution from the *Csf1r-CreER* labelled lineage to cardiac LECs. The low incidence of PROX1⁺ tdTomato⁺ LECs compared to the other Cre lines used to label the haemogenic endothelium possibly reflects the small number of cells targeted by temporal Cre recombination at E7.5 and a degree of labelling inefficiency. Indeed, in some hearts no contribution to the cardiac lymphatics from *Csf1r-CreER;td-Tomato* was observed, as demonstrated in **Figure 3.11d-f**, highlighting the rare incidence of contribution from this lineage when labelled at E7.5. In all hearts many tdTomato⁺ cells were observed, often in large clusters, on the surface (**Figure 3.11e-f**) which are likely to be tissue resident macrophages known to be derived from *Csf1r*⁺ EMPs (Gomez Perdiguero et al., 2015). Staining with EMCN, in addition to PROX1, revealed no contribution from the *Csf1r*⁺ haemogenic endothelium at E7.5 to the embryonic coronary endothelium (**Figure 3.11g-i**).

Figure 3.11

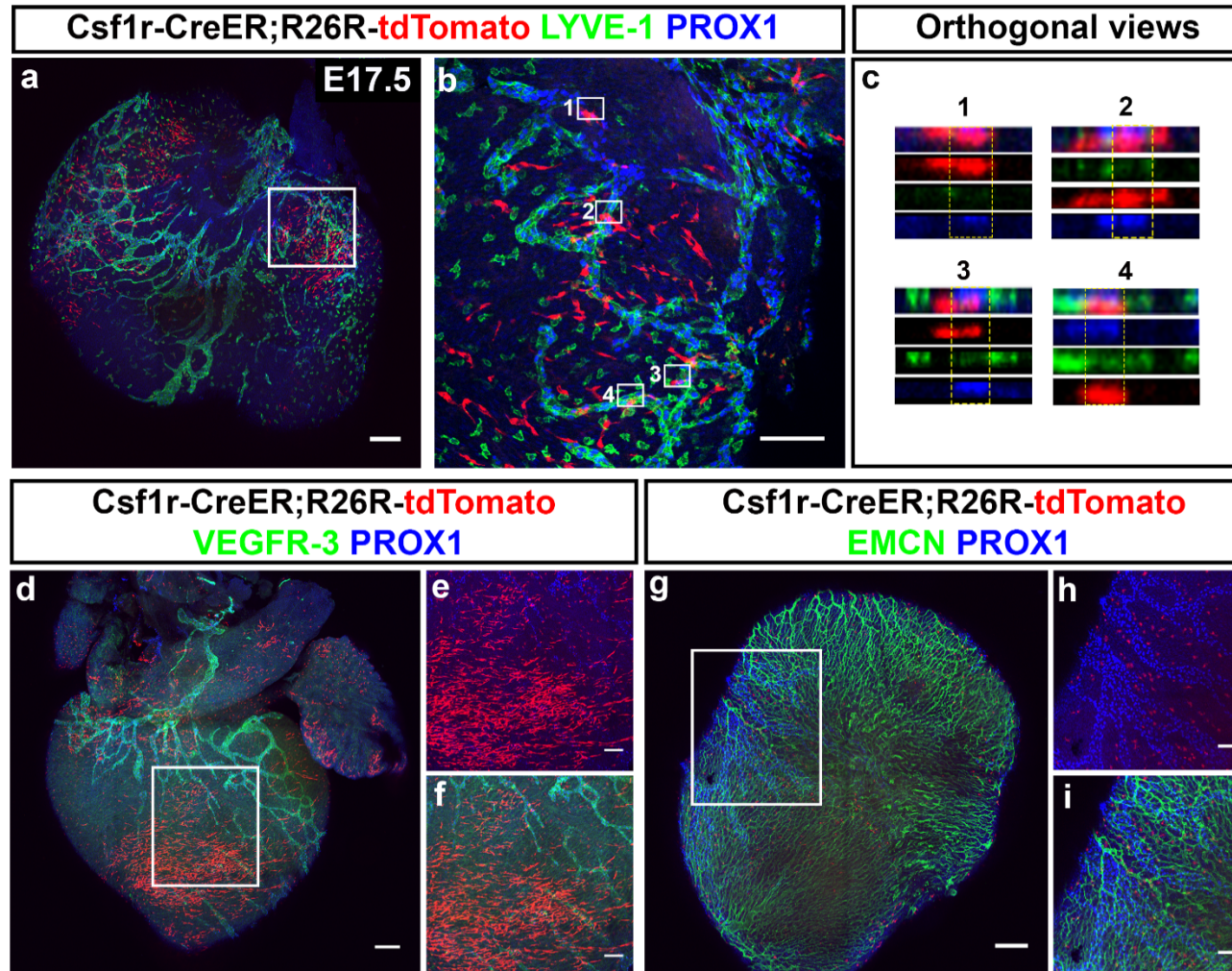


Figure 3.11: A small number of cardiac LECs may derive from a *Csf1r*⁺ lineage

4-OH tamoxifen was injected to induce Cre recombination at E7.5. Whole hearts from E17.5 *Csf1r-CreER;R26R-tdTomato* embryos were stained for LYVE-1 (green) and PROX1 (blue), revealing rare labelling of LECs by *Csf1r-CreER;R26R-tdTomato* (a, white box enlarged in b). Orthogonal views (c) are displayed for 4 example regions, a degree of co-localization was observed between PROX1 and tdTomato in views 2, 3, 4. Orthogonal view 1 displays an example of no co-localization.

E17.5 *Csf1r-CreER;R26R-tdTomato* hearts were stained for VEGFR-3 (green) and PROX1 (blue) (d, white inset box enlarged in e without VEGFR-3 and f with VEGFR-3). Hearts were also stained for EMCN (green) and PROX1 (blue) (g, white inset box enlarged in h without EMCN and i with EMCN). n = 5 hearts analysed.

3.4. Discussion

3.4.1. The development of the cardiac lymphatic vasculature

The work presented in this chapter has characterised the spatiotemporal development of the murine cardiac lymphatic vasculature in the embryo and early postnatal stages. Prior to this work several developmental anatomy studies, using techniques such as ink injections and immunohistochemistry, had shown that cardiac lymphatics are present within the mouse heart from E11.0 to E12.0 and grow down both sides of the heart from the base towards the apex (Flaht et al., 2012, Flaht-Zabost et al., 2014, Juszynski et al., 2008, Karunamuni et al., 2010). Much of the data presented here has utilised whole-mount immunofluorescent staining and confocal imaging which has allowed the detection of multiple proteins concomitantly. This, together with the collection of embryos at multiple time points, has enabled more detailed information on the development of the cardiac lymphatic vasculature to be acquired. This work established that cardiac LECs express LYVE-1, VEGFR-3 and PROX1, consistent with the systemic lymphatic vasculature and lymphatic beds within other organs. Antibody staining for these proteins revealed that cardiac lymphatic vessels begin to form in the heart from E12.5, appearing to enter along the outflow tract, having migrated from the cardinal vein region. Importantly they do not bud from blood vessels present within the heart, excluding an origin via the coronary veins. From E12.5 to E14.5 the cardiac lymphatics expand down the dorsal side of the heart and spread onto the ventral surface. At E15.5 a relatively extensive network is observed on both sides of the heart, which continues to extend towards the apex, and remodels through to postnatal stages. Analysis of postnatal hearts from P0 to P7 revealed that the lymphatic network continues to expand and grow extensively after birth.

3.4.2. Non-venous origins of cardiac LECs

Additionally, this chapter addresses the previous observation within the Riley group that a proportion of the cardiac lymphatic vasculature may derive from progenitors arising from the haemogenic endothelium, based on evidence that a proportion of cardiac LECs were not labelled by the venous endothelial *Tie2-Cre* and *Pdgfb-CreER* lines, and that some LECs were labelled by *Vav1-Cre* and *Pdgfr β -Cre*. It is important to note that in addition to being expressed in endothelial cells, Tie2 is also known to be expressed in the yolk sac from E6.5 in the mouse and has been detected with haematopoietic cells in the yolk sac at E7.5, E8.5 (Kisanuki, Hammer et al., 2001), and at E9.5 where it labels 88% of primitive haematopoietic progenitors (Tang et al., 2010). This leaves a small percentage of yolk sac progenitors which are not labelled by *Tie2-Cre*. The *Vav1-Cre*, *Pdgfr β -Cre* and *Csf1r-CreER* lines were employed in attempt to capture any non-venous derived, haematopoietic lineage contribution to cardiac LECs. **Table 3.1** summarizes the Cre lines used and the cell populations they target and label. *Pdgfr β -Cre* is not included in this table as recent evidence, discussed later in this section, has revealed that it does not target the yolk sac haemogenic endothelium.

Table 3.1. Summary of Cre lines used

		Cellular origin labelled				LEC contribution		References	Additional comments
Cre line	Recombination induced	Venous endothelium	Haemogenic endothelium	Primitive EMPs	HSCs and derivatives	Systemic	Cardiac		
Tie2-Cre	Constitutive	+	+	+	+	+	+	1-5	
Pdgfb-CreER	E9.5	+	–	–	–	+	+	5, 6	Labels non-venous progenitors when recombined at E8-8.5 (ref. 6)
Vav1-Cre	Constitutive	–	+	+	+	–	+	5, 7-9	
Csf1r-CreER	E7.5	–	+	+	–	–	+	4, 5	

EMPs = erythro-myeloid progenitors; HSCs = haematopoietic stem cells; LECs = lymphatic endothelial cells. References: (1) Srinivasan et al., 2007; (2) Kisanuki et al., 2001; (3) Tang et al., 2010; (4) Gomez Perdiguero et al., 2015; (5) Klotz et al., 2015; (6) Stanczuk et al., 2015; (7) Padron-Barthe et al., 2014; (8) Chen et al., 2009; (9) de Boer et al., 2003.

Work presented here established that *Vav1-Cre* does not label venous endothelium nor the systemic lymphatic vasculature, thus confirming the finding that the contribution to the cardiac lymphatics from this lineage is not from venous endothelium. A further inducible Cre line, *Csf1r-CreER* was employed to target EMPs which arise from the haemogenic endothelium. Once again labelling of the systemic lymphatic vasculature and venous endothelium was also ruled out with the *Csf1r-CreER* line and it was discovered that when induced with 4-OH tamoxifen at E7.5 *Csf1r-CreER* does, on rare occasions, contribute to PROX1⁺ LECs within the cardiac lymphatic vasculature, thus providing further evidence for a contribution from the haemogenic endothelium to cardiac LECs.

Pdgfr β -Cre has been shown to label a large proportion of the mesenteric lymphatic vasculature (Stanczuk et al., 2015), in addition to cardiac LECs. Since this work was performed a paper has been published which has revealed that *Pdgfr β -Cre* is not expressed in the yolk sac haemogenic endothelium or in yolk sac derived monocytes/macrophages, but does unexpectedly label the cardinal vein and jugular lymph sacs (Ulvmar, Martinez-Corral et al., 2016). In light of this finding it must be concluded that the labelling of a proportion of the cardiac lymphatic vasculature with *Pdgfr β -Cre* does not necessarily provide evidence for a non-venous lineage contribution. However, it remains possible that *Pdgfr β -Cre* may label other areas of haemogenic endothelium such as the AGM region but further experiments would be required to investigate this. Despite these findings the labelling observed with the *Vav1-Cre* and *Csf1r-CreER* lines does still suggest that there is a non-venous contribution to the cardiac lymphatic vasculature.

The *Vav1-Cre* line labels the haemogenic endothelium in addition to HSCs and their derivatives (de Boer et al., 2003, Padron-Barthe et al., 2014) and when induced at E7.0-8.0 *Csf1r-CreER* labels EMPs (Gomez Perdiguero et al., 2015). It is possible, therefore, that the contribution from these two lines is in fact from early yolk-sac derived monocytes/macrophages which arise from haemogenic endothelium. Interestingly prior work has demonstrated that macrophages do not provide a lineage contribution to the dermal lymphatic vasculature, however, they are intimately associated with LECs and do play a role in the regulation of vessel calibre as PU.1 macrophage deficient embryos presented with hyperplastic lymphatic vessels (Gordon, Rao et al., 2010). Further work is required using monocyte/macrophage Cre lines to determine whether the non-venous lineage contributions in the heart may arise specifically from these cell types.

Recently several other studies have revealed non-venous contributions to lymphatic vasculature. In the zebrafish a niche of specialised angioblasts within the cardinal vein have been found to give rise to paracordal cells (PACs), which have been described as the building blocks of the lymphatic system (Nicenboim, Malkinson et al., 2015). In the mouse non-venous contributions have been recorded for the skin and gut mesentery in addition to the heart. Lineage tracing revealed that 33% of the dermal lymphatic vasculature does not derive from venous endothelium labelled by *Tie2-Cre* and additional lymphatic endothelial cells form by a process termed lymphvasculogenesis, although the precise cellular source remains to be determined (Martinez-Corral et al., 2015). Lineage tracing has also revealed that a proportion of the mesenteric lymphatic vasculature derives from *ckit*⁺ haemogenic endothelium (Stanczuk et al., 2015) and another study demonstrated that the mesenteric lymphatics are heterogeneous in

origin and derive from a venous population within the subcardinal vein and a non-venous population which is dependent on arteriogenesis (Mahadevan et al., 2014).

3.5. Conclusions

The work presented in this chapter has demonstrated that LECs bud from the cardinal vein region from E10.5 and migrate towards the heart where they enter from E12.5 onwards and begin to form cardiac lymphatic vessels. An extensive time course analysis with multiple markers has revealed that the cardiac lymphatics continue to expand down both sides of the heart and grow and remodel through to postnatal stages. Furthermore, this analysis revealed that cardiac LECs do not bud from coronary veins.

Previous evidence collected by the Riley group suggested that not all cardiac LECs are venous-derived and that a small proportion may derived from the haemogenic endothelium. The data presented here shows that *Vav1-Cre*, a line used to label the haemogenic endothelium, in addition to HSCs and their derivatives, does not label venous endothelium nor systemic lymphatic vasculature, despite labelling LECs within the heart. This suggests that a proportion of heart-based lymphatics do derive from an alternative source to the systemic lymphatic vasculature. Lineage tracing using the haemogenic endothelium/EMP *Csf1r-CreER* line, also revealed rare labelling of LECs in the heart, again indicating that a small proportion of cardiac LECs do derive from a non-venous source. Together these data demonstrate that the previous dogma that all LECs are venous-derived does not hold true for the cardiac lymphatic vasculature. Furthermore, other groups have also recently presented examples of non-venous derived LECs in other organ-based lymphatic networks, supporting these findings. By improving our understanding of the cellular origins from which lymphatic vessels

derive during embryonic development we are better placed to investigate the mechanisms by which lymphatic vessels expand in response to disease in the adult.

4. Results II: Characterisation of the cardiac lymphatic vasculature in conditional PROX1 mutants

PROX1 is a key transcription factor which is essential for the specification and maintenance of LECs which form systemic lymphatic vessels (Hong et al., 2002, Wigle & Oliver, 1999). In order to determine whether PROX1 is required for the development of cardiac LECs which derive from venous endothelium, or those which may derive from the haemogenic endothelium, PROX1 was deleted from each of these compartments using the Cre-lox system. PROX1 was targeted by crossing *Tie2-Cre* (Koni et al., 2001) and *Vav1-Cre* (de Boer et al., 2003) mouse lines with a *Prox1^{fllox}* (Iwano et al., 2012) conditional mouse line. The *Prox1^{fllox}* allele allows conditional knockout and simultaneous knock-in of EGFP (schematic shown in **Figure 4.1**) thus labelling *Tie2*⁺ and *Vav1*⁺ cells in which PROX1 has been targeted. For these experiments *Tie2-Cre;Prox1^{fl/+}* and *Vav1-Cre;Prox1^{fl/+}* heterozygous animals were used as controls due to the GFP labelling of the lymphatic network induced by the deletion of one copy of PROX1.

Previous work has demonstrated that severe *Tie2-Cre;Prox1^{fl/fl}* mutants have defective systemic lymphatic vasculature (Srinivasan et al., 2007, Vondenhoff, van de Pavert et al., 2009). A range of phenotypes were observed due to variability with Cre deletion. Severe mutants, however, were found to completely lack systemic lymphatics but still retained a few PROX1⁺ LECs which were attributed to incomplete Cre deletion. The cardiac lymphatics of *Tie2-Cre;Prox1^{fl/fl}* mutants were not investigated in these studies.

Figure 4.1

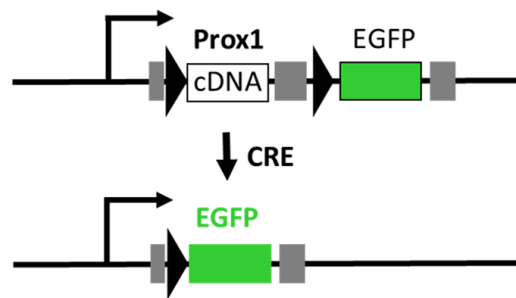


Figure 4.1 *Prox1^{lox}* construct

Cre drives deletion of PROX1 via floxed excision of exon 1 and 2, resulting in EGFP expression in targeted cells.

In order to generate *Tie2-Cre;Prox1^{fl/fl}* and *Vav1-Cre;Prox1^{fl/fl}* embryos for analysis a breeding strategy was set up as illustrated for *Tie2-Cre;Prox1^{fl/fl}* in **Figure 4.2**.

Figure 4.2

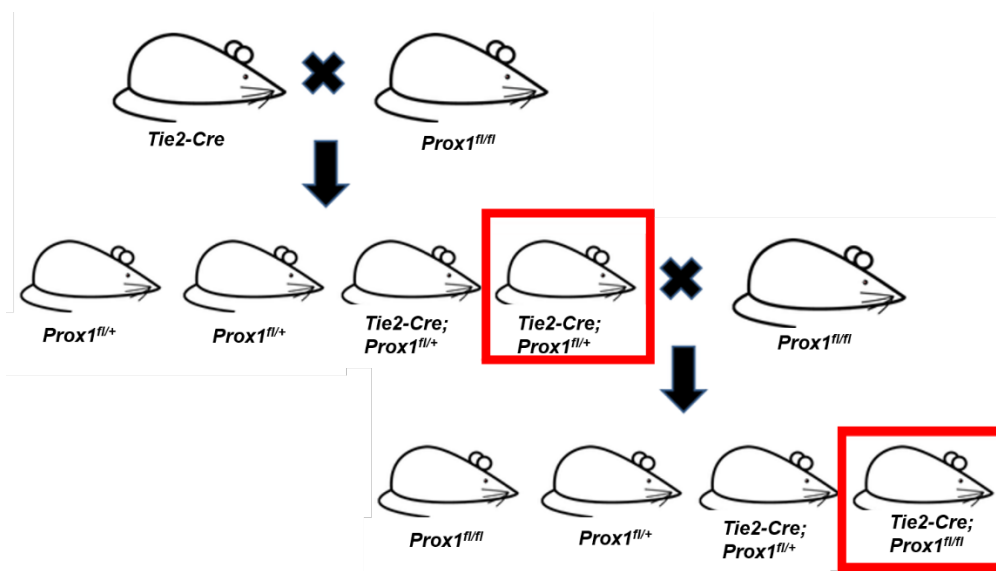


Figure 4.2: Breeding strategy to generate *Tie2-Cre;Prox1^{fl/fl}* embryos

Two generations of breeding were required to generate *Tie2-Cre;Prox1^{fl/fl}* embryos. Firstly a *Tie2-Cre* male was crossed with a *Prox1^{fl/fl}* female. Resulting litters were genotyped and *Tie2-Cre;Prox1^{fl/+}* male studs were used in timed matings with *Prox1^{fl/fl}* females in order to generate *Tie2-Cre;Prox1^{fl/fl}* embryos and *Prox1^{fl/fl}*, *Prox1^{fl/+}*, *Tie2-Cre;Prox1^{fl/+}* littermate controls. The same breeding strategy was used to generate *Vav1-Cre;Prox1^{fl/fl}* embryos except females carried the Cre.

4.1. Characterisation of the cardiac lymphatic vasculature in *Tie2-Cre;Prox1^{fl/fl}* conditional mutants

4.1.1. Survival of *Tie2-Cre;Prox1^{fl/fl}* animals

Hearts from a *Tie2-Cre;Prox1^{fl/+}* x *Prox1^{fl/fl}* cross were collected at E14.5, E17.5, E18.5 and P7 for experimental analysis. Each litter collected was genotyped. Genotypes were recorded in order to determine if *Tie2-Cre;Prox1^{fl/fl}* mutants survive at normal Mendelian ratios. The expected percentage for each genotype would be 25% *Prox1^{fl/fl}*, 25% *Prox1^{fl/+}*, 25% *Tie2-Cre;Prox1^{fl/+}* and 25% *Tie2-Cre;Prox1^{fl/fl}*. Across the various stages collected the percentages for each genotype were 12% *Prox1^{fl/fl}*, 14% *Prox1^{fl/+}*, 40% *Tie2-Cre;Prox1^{fl/+}* and 34% *Tie2-Cre;Prox1^{fl/fl}* (full breakdown for each stage is displayed in **Table 4.3**). The percentages of *Tie2-Cre;Prox1^{fl/+}* and *Tie2-Cre;Prox1^{fl/fl}* animals were higher than expected. Conversely the percentages of *Prox1^{fl/+}* and *Prox1^{fl/fl}* mice were lower than expected. This is possibly due to the *Tie2-Cre* being a transgene (Koni et al., 2001) with unknown copy number integration resulting in over 50% of the offspring inheriting the transgene. The observation that 34% of all animals from this cross were *Tie2-Cre;Prox1^{fl/fl}* indicated that this genotype is not embryonic lethal, however, animals which survive may not have undergone full Cre recombination in all *Tie2⁺* cells.

Table 4.3. Survival of *Tie2-Cre;Prox1^{fl/fl}* animals

Genotype	% E14.5	%E17.5 and E18.5	% P7	% all stages (n=106)
<i>Tie2-Cre;Prox1^{fl/fl}</i>	38	34	20	34
<i>Tie2-Cre;Prox1^{fl/+}</i>	14	45	60	40
<i>Prox1^{fl/fl}</i>	10	12	20	12
<i>Prox1^{fl/+}</i>	38	9	0	14

Table 4.3: Survival of *Tie2-Cre;Prox1^{fl/fl}* animals

The table shows the percentage of each genotype collected at each developmental stage: E14.5, E17.5, E18.5 and P7. The percentage of each genotype totalled across all stages is also shown.

4.1.2. E14.5 and E17.5 *Tie2-Cre;Prox1^{fl/fl}* mutants display gross vascular abnormalities

Embryos were collected at E14.5 and E17.5 in order to determine the effect of PROX1 deletion in the Tie2 lineage. At E14.5 some *Tie2-Cre;Prox1^{fl/fl}* mutants presented with severe oedema along the dorsal side (**Figure 4.4b-c**) which was not observed in littermate controls (**Figure 4.4a**). Furthermore, some superficial haemorrhaging was observed in E14.5 *Tie2-Cre;Prox1^{fl/fl}* embryos, particularly around the facial region (**Figure 4.4b**). These small areas of haemorrhaging may be defective areas of the developing lymphatic vasculature which are blood filled. By E17.5 mutant embryos no longer presented with oedema, however, a gross vascular phenotype was observed when compared to littermate controls (**Figure 4.4d**) consisting of blood filled nodules and a hyperplastic network of blood filled capillaries (**Figure 4.4e**). Mutant hearts also displayed superficial blood filled nodules and capillaries (**Figure 4.4f**).

Figure 4.4

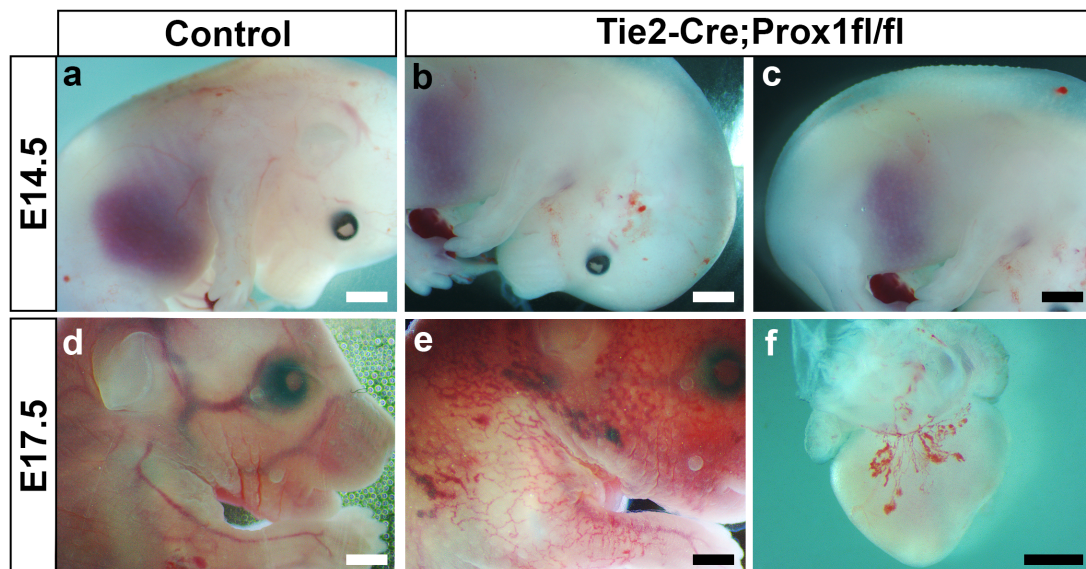


Figure 4.4: E14.5 and E17.5 *Tie2-Cre;Prox1^{fl/fl}* embryos display gross abnormalities

An example E14.5 control embryo is shown in **a**. Two views of an example E14.5 mutant embryo are shown in **b**, **c** in which severe oedema was present along the dorsal side. E17.5 mutant embryos displayed blood filled nodules and a hyperplastic network of blood filled vessels (**e**) which were not present in controls (**d**). Mutant hearts also displayed a blood filled vessels and nodules. E14.5 controls n = 10, mutants n = 8. E17.5 controls n = 17, mutants n = 27. Scale bars **a-e** = 100 μ m, **f** = 400 μ m.

4.1.3. The cardiac lymphatics of *Tie2-Cre;Prox1^{fl/fl}* embryos are either mildly or severely disrupted dependant on Cre recombination levels

Whole-mount immunofluorescent staining was performed to determine whether *Tie2-Cre;Prox1^{fl/fl}* mutants had defective cardiac lymphatic vasculature. E14.5 hearts were collected in order to assess whether there was an impact on the initial sprouting and infiltration of lymphatic vessels into the heart. Hearts were stained for GFP, LYVE-1 and DAPI. GFP staining of *Tie2-Cre;Prox1^{fl/+}* hearts revealed a GFP⁺ network of lymphatic vessels sprouting from the sinus venosus region on the dorsal side of the heart indicating one copy of PROX1 had successfully been deleted in contributing Tie2⁺ cells (**Figure 4.5a, c**). These sprouting vessels were only partially positive for LYVE-1 (**Figure 4.5b**) indicating that LYVE-1 is possibly expressed subsequently to the initial lymphatic network formation in the heart. E14.5 *Tie2-Cre;Prox1^{fl/fl}* hearts did not display any sprouting GFP⁺ or LYVE-1⁺ lymphatic vessels suggesting either a complete loss of cardiac lymphatics or a delay in the initial sprouting of lymphatics within the heart. PECAM-1 (Platelet endothelial cell adhesion molecule 1, also known as CD31, labels blood vessel endothelium (Delisser, Baldwin et al., 1997)) staining was also performed and confirmed that there was no disruption to the coronary vasculature in *Tie2-Cre;Prox1^{fl/fl}* hearts when compared to *Tie2-Cre;Prox1^{fl/+}* controls (**Figure 4.5g-h**).

Figure 4.5

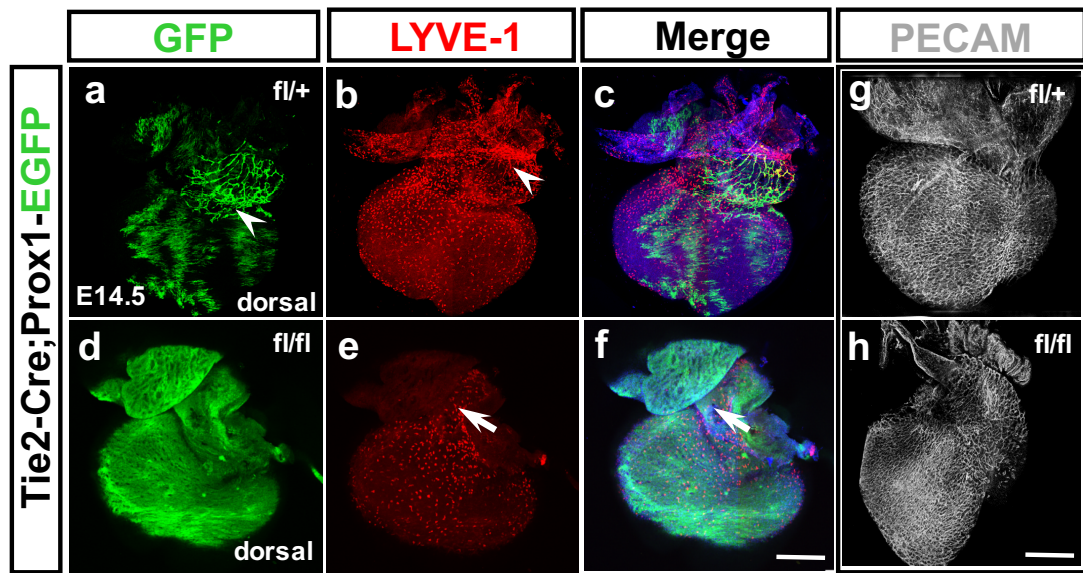


Figure 4.5: E14.5 *Tie2-Cre;Prox1^{fl/fl}* mutants and controls whole-mount immunofluorescent staining

Staining for GFP (green), LYVE-1 (red) and DAPI (blue) was performed. Staining of *Tie2-Cre;Prox1^{fl/+}* hearts revealed a GFP⁺ emergent lymphatic plexus on the dorsal side of the heart (**a, c**) which was partially LYVE-1⁺ (**b, c**) arrowhead in **b** indicates the LYVE-1⁺ region of the lymphatic plexus. Staining of *Tie2-Cre;Prox1^{fl/fl}* mutant hearts revealed a complete absence of a lymphatic plexus (**d-f**), arrows in **e-f** indicate the expected area where a lymphatic plexus should be present at this stage. Hearts were also stained for PECAM (grey) which revealed the presence of normal coronary blood vessel endothelium in both controls (**g**) and mutants (**h**). Scale bars **f, h**=500 μ m. Heterozygotes n = 3, mutants n = 6.

In order to further characterise the phenotype of cardiac lymphatic vessels in *Tie2-Cre;Prox1^{fl/fl}* mutants embryos were also collected at E17.5, a stage in development when there is an extensive cardiac lymphatic network present. Hearts underwent whole-mount staining for GFP, LYVE-1 and PROX1. PROX1 was included in the staining panel in order to confirm PROX1 was deleted appropriately when targeted with *Tie2-Cre*, to account for the mosaicism of recombination often seen with Cre lines (Heffner, Herbert Pratt et al., 2012). *Tie2-Cre;Prox1^{fl/+}* hearts displayed an extensive LYVE-1⁺ PROX1⁺ network which was also GFP⁺ (**Figure 4.6d-f**). However, this network was not as extensive on the ventral side of the hearts when compared to *Prox1^{fl/fl}*

controls (**Figure 4.6a-c**). Staining of *Tie2-Cre;Prox1^{fl/fl}* hearts (**Figure 4.6g-l**) revealed a gradient of phenotypes, reflective of the level of Cre recombination and PROX1 deletion. Some mutant hearts in which PROX1 was fully deleted in the Tie2 lineage displayed no LYVE-1⁺ lymphatic vessels on either the dorsal or ventral sides of the heart (**Figure 4.6g-i**), suggesting that PROX1 is necessary for development of cardiac LECs and indicating that any LECs which derive from an alternate source are unable to form a cardiac lymphatic network without the presence of venous derived LECs. Due to varying levels of Cre efficiency and mosaicism other mutant hearts retained some PROX1 expression in LECs (**Figure 4.6j-l**). Here only a small truncated cardiac lymphatic network was present on the dorsal side of the heart which retained PROX1 expression (**Figure 4.6j, l**).

4.1.4. Partial PROX1 deletion results in a hypoplastic network indicating a novel role for PROX1 in remodelling of the lymphatic network

The cardiac lymphatic networks of *Tie2-Cre;Prox1^{fl/fl}* mutant hearts which retained partial PROX1 expression were analysed. Compared with controls these hearts showed hypoplasia and a truncated network, present only on the dorsal side of the heart (see **Figure 4.7a-c** for controls, **g-i** for mutants). Comparable regions from each heart were selected (example regions are shown in **Figure 4.7d-f, j-l**) and analysed, using Angiotool software (Zudaire et al., 2011), to quantify the defects in the lymphatic network observed in mutants. Quantification revealed that the lymphatic vessels in *Tie2-Cre;Prox1^{fl/fl}* mutant hearts were on average shorter in length ($p \leq 0.001$, $n=4$), with an increased number of end points ($p \leq 0.05$, $n=4$) and were significantly thinner ($p \leq 0.001$, $n=4$) relative to *Tie2-Cre;Prox1^{fl/+}* controls (**Figure 4.7m-o**).

Figure 4.6

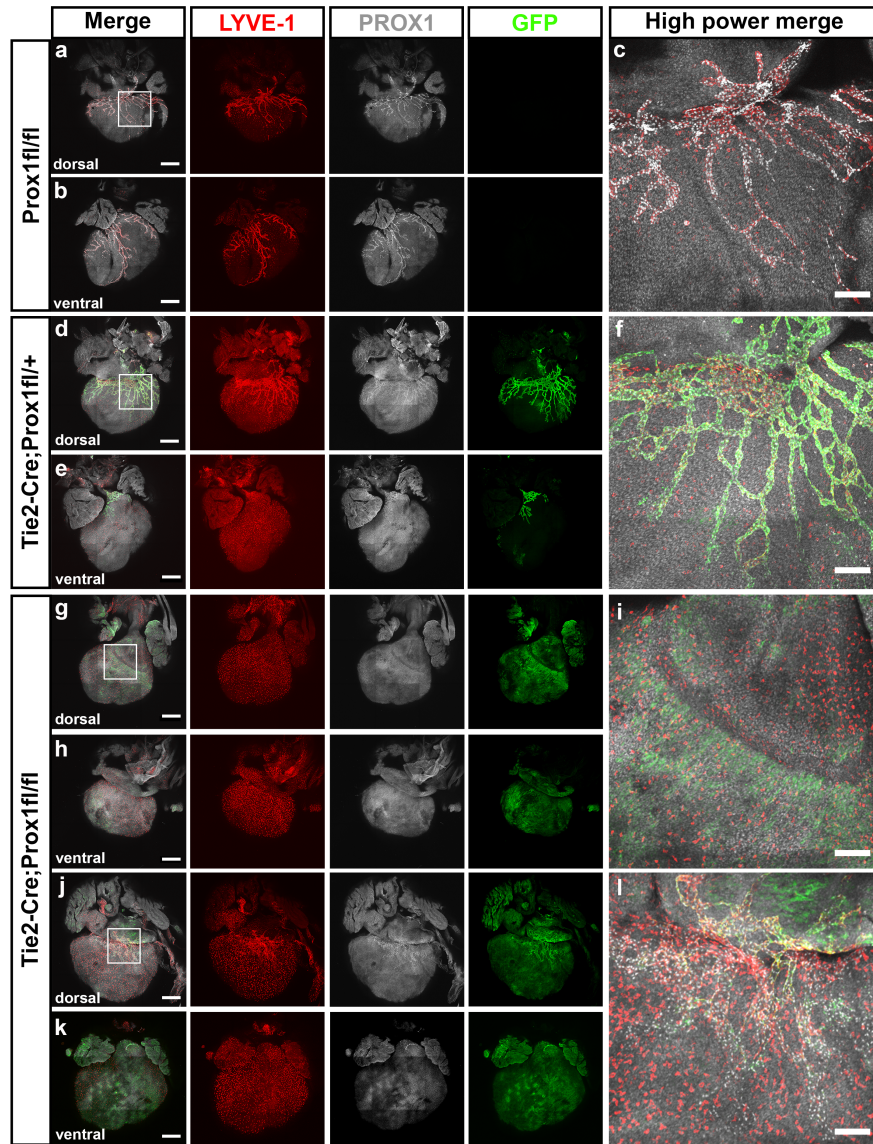


Figure 4.6: E17.5 *Tie2-Cre;Prox1^{fl/fl}* mutants and controls whole-mount immunofluorescent staining

Staining for GFP (green), LYVE-1 (red) and PROX1 (grey) was performed. *Prox1^{fl/fl}* hearts displayed an extensive LYVE-1⁺ PROX1⁺ GFP⁻ network on both sides (**a-c**). Staining of *Tie2-Cre;Prox1^{fl/+}* hearts revealed an extensive lymphatic network on the dorsal side which was GFP⁺, LYVE-1⁺ and PROX1⁺ (**d, e**, white box in **d** displayed at high power in **f**). Fewer lymphatic vessels were present on the ventral side of *Tie2-Cre;Prox1^{fl/+}* hearts (**e**). *Tie2-Cre;Prox1^{fl/fl}* mutant hearts (**g-l**) displayed a range of phenotypes which corresponded to the level of Cre recombination and PROX1 deletion as indicated by GFP⁺ labelling. Some mutant hearts in which PROX1 was fully deleted in the Tie2 lineage displayed no LYVE-1⁺ lymphatic vessels on either the dorsal or ventral sides of the heart (**g-i** white box in **g** displayed at high power in **i**). Due to varying levels of Cre efficiency and mosaicism other mutant hearts retained some PROX1 expression in LECs (**j-l** white box in **j** displayed at high power in **l**). Here only a small truncated cardiac lymphatic network was present on the dorsal side of the heart which retained PROX1 expression (**g, i**). Scale bars **a, b, d, e, g, h, j, k** = 500 μ m, **c, f, i, l** = 150 μ m. Controls n = 4, heterozygotes n = 3, mutants n = 6.

Figure 4.7

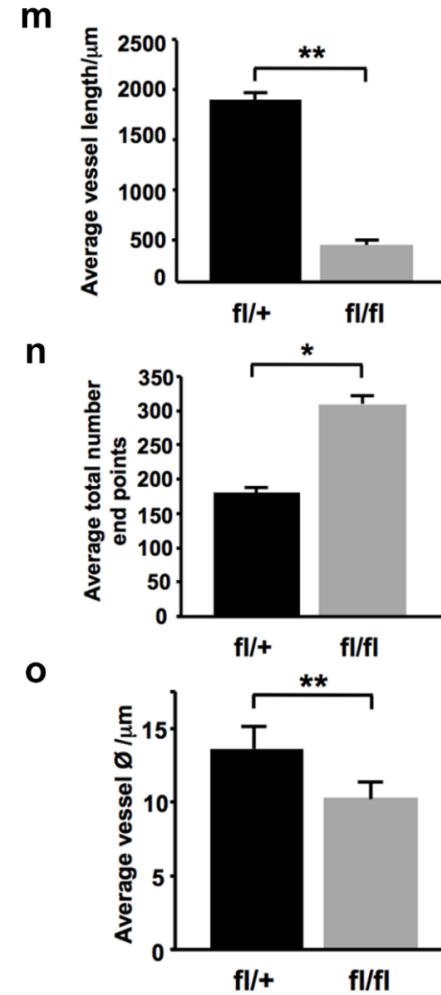
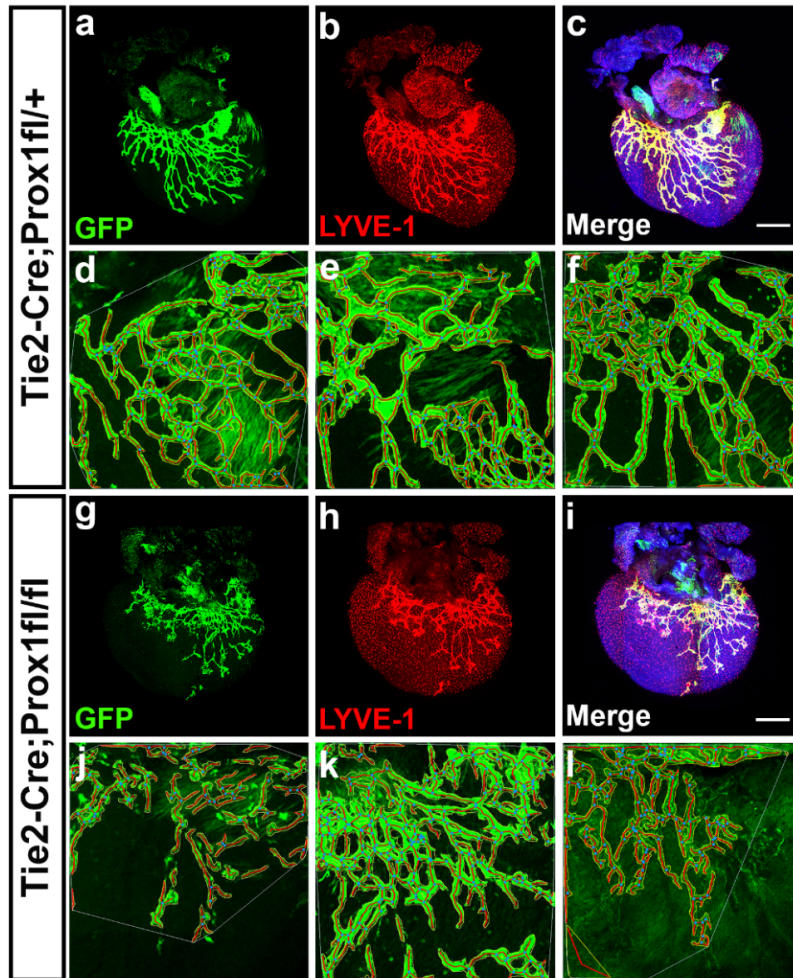


Figure 4.7: Quantitative analysis of the disrupted lymphatic network in E17.5 *Tie2-Cre;Prox1^{fl/fl}* mutant hearts which retain some PROX1 expression

Tie2-Cre;Prox1^{fl/fl} hearts (g-i) which retained partial PROX1 expression displayed a truncated cardiac lymphatic network present only on the dorsal side, when compared to controls (a-c). Quantification of comparable areas of the lymphatic plexus in controls (d-f) and mutants (j-l) revealed that the lymphatic vessels in mutant hearts were on average shorter in length (m), with an increased number of end points (n) and were significantly thinner (o). All graphs show mean $\pm$ S.E.M. * $p \leq 0.05$, ** $p \leq 0.001$, Student's t-test. Scale bars c, i = 500 μ m. n = 4 hearts analysed per genotype.

4.1.5. E17.5 *Tie2-Cre;Prox1^{fl/fl}* mutants display disrupted dermal lymphatics

Dorsal skin samples were collected from E17.5 embryos in order to determine the consistency between the phenotype of *Tie2-Cre;Prox1^{fl/fl}* cardiac lymphatics and the lymphatic network in another organ system. A proportion of the lymphatic vasculature in the skin is known to be derived from venous endothelium and recently it has been demonstrated that an alternative source also contributes to dermal LECs (Martinez-Corral et al., 2015). Therefore, it was hypothesised that the dermal lymphatics of *Tie2-Cre;Prox1^{fl/fl}* mutant embryos would display a similar phenotype to the mutant cardiac lymphatics. Whole-mount staining of dorsal skin samples revealed an extensive LYVE-1⁺ network in *Prox1^{fl/+}* controls (**Figure 4.8a-c**) which also appeared normal and expressed GFP in *Tie2-Cre;Prox1^{fl/+}* embryos (**Figure 4.8d-f**). In some cases *Tie2-Cre;Prox1^{fl/fl}* embryos displayed an extensive, intact dermal lymphatic vascular network (**Figure 4.8g-h**). However, this was likely due to incomplete PROX1 deletion as other mutant dorsal skin samples revealed a disorganised network of thin, broken vessels (**Figure 4.8j-l**), similar to those observed in hearts where a significant level of PROX1 deletion had occurred. These data indicate that the disrupted lymphatic phenotype observed in *Tie2-Cre;Prox1^{fl/fl}* embryonic hearts is conserved in other lymphatic organ beds such as the dermal lymphatic vasculature.

Figure 4.8

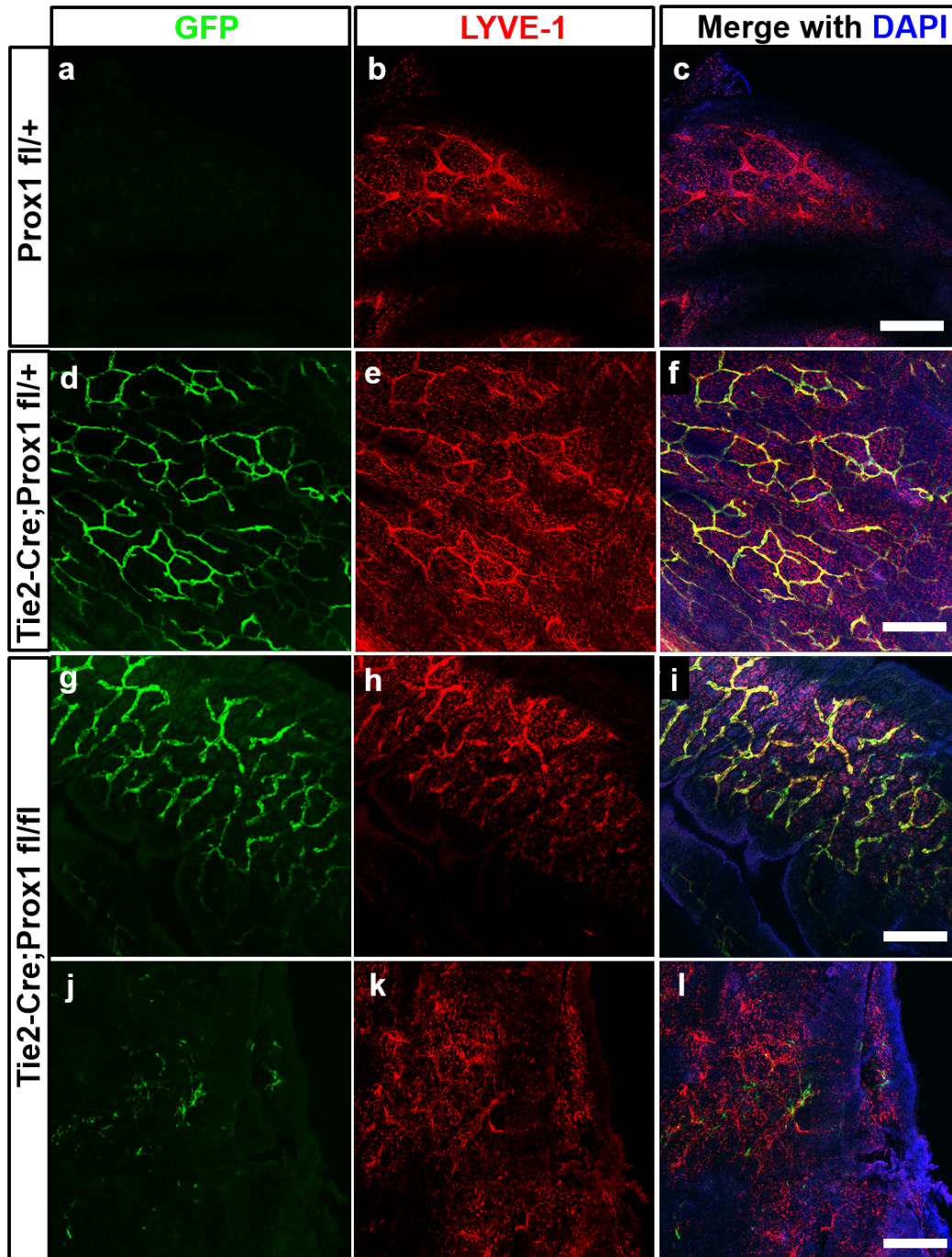


Figure 4.8: Whole-mount immunofluorescent staining of dorsal skin from E17.5 control and *Tie2-Cre;Prox1^{fl/fl}* mutant embryos

Staining for GFP (green), LYVE-1 (red) and DAPI (blue) was performed. Staining of *Prox1^{fl/+}* controls revealed an extensive LYVE-1⁺ dermal lymphatic network (**a-c**) which was also GFP⁺ in *Tie2-Cre;Prox1^{fl/+}* samples (**b-d**). Staining of *Tie2-Cre;Prox1^{fl/fl}* mutant skin samples displayed a range of phenotypes from a well formed lymphatic network (**g-i**) to a severely disrupted, disorganised network (**j-l**). Scale bars **c, f, i, l**=500 μ m. n = 3 samples analysed per genotype.

4.1.6. Severe E18.5 *Tie2-Cre;Prox1^{fl/fl}* mutants display abnormal cardiac morphology

In order to assess the gross cardiac anatomy arising from targeting PROX1 in the *Tie2*-derived LECs, E18.5 *Tie2-Cre;Prox1^{fl/fl}* and *Tie2-Cre;Prox1^{fl/+}* hearts were collected, sectioned and subjected to haematoxylin and eosin (H&E) staining (**Figure 4.9**). Histology revealed *Tie2-Cre;Prox1^{fl/fl}* mutant hearts were markedly smaller than controls, with smaller chambers and thickening of the ventricular compact layer. Furthermore, mutants appeared dysmorphic along the axis from apex to base with a rounded appearance at the apex. Endocardial cushions, however, appeared to have developed normally.

Figure 4.9

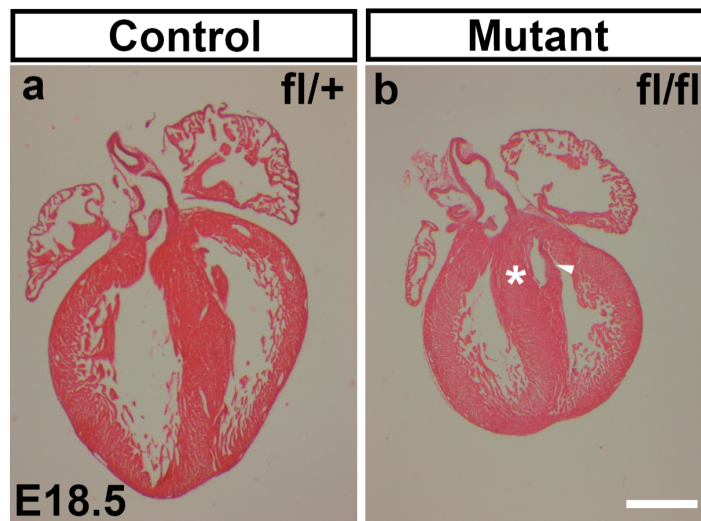


Figure 4.9: Histology revealed E18.5 *Tie2-Cre;Prox1^{fl/fl}* mutant hearts are dysmorphic

Haematoxylin and eosin staining is shown for sections of E18.5 control (**a**) and mutant hearts (**b**). Compared to controls mutant hearts were significantly smaller and dysmorphic. Ventricular septation (white asterix) and valve leaflet formation (white arrowhead) appeared to have developed normally. $n = 3$ hearts analysed per genotype. Scale bar = 1 mm.

4.1.7. Postnatal *Tie2-Cre;Prox1^{fl/fl}* mutants appear to be recovered, or Cre deletion may have been incomplete

To assess whether the cardiac lymphatic phenotype seen in milder mutants at E17.5 persisted into early postnatal stages, hearts were collected at 7 days after birth (P7). At this stage both *Prox1^{fl/fl}* and *Tie2-Cre;Prox1^{fl/+}* control hearts and those of *Tie2-Cre;Prox1^{fl/fl}* mutants displayed an equivalent VEGFR-3⁺ lymphatic network on both sides of the heart (**Figure 4.10a-f**). This suggests that the hypoplastic and truncated network present in E17.5 mutants with partial PROX1 knockdown may be recoverable, or animals which survive to postnatal stages may have incomplete deletion of PROX1 in the Tie2 lineage.

Figure 4.10

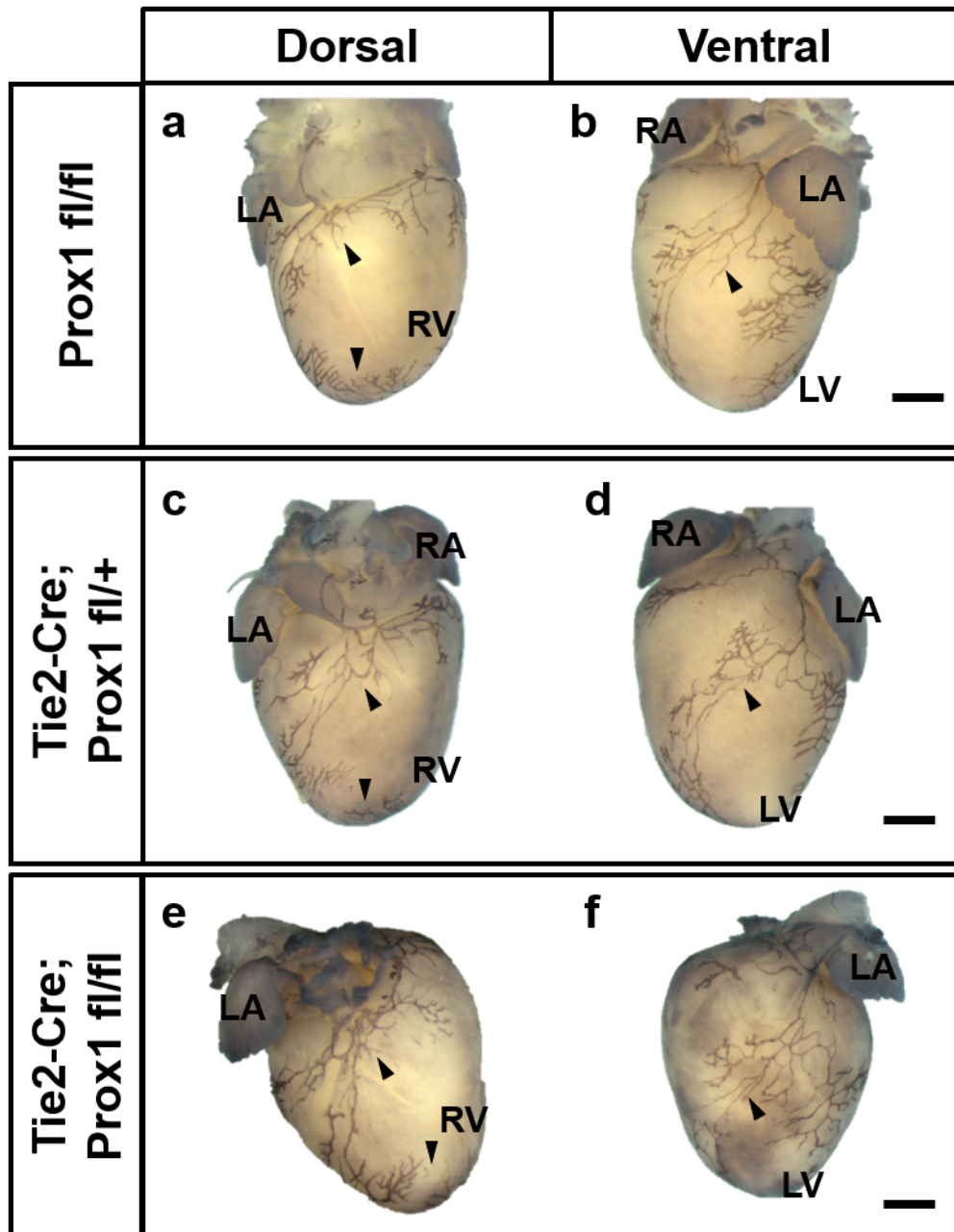


Figure 4.10: Postnatal assessment of *Tie2-Cre;Prox1^{fl/fl}* mutants

DAB staining for VEGFR-3 was used to visualise the cardiac lymphatic network in P7 hearts (indicated by black arrowheads). At this stage *Prox1^{fl/fl}* and *Tie2-Cre;Prox1^{fl/+}* control hearts displayed an extensive VEGFR-3⁺ lymphatic network (**a-d**). *Tie2-Cre;Prox1^{fl/fl}* mutants displayed an extensive network similar to that observed in controls (**e, f**). LA = left atrium, RA = right atrium, LV = left ventricle, RV = right ventricle. Scale bars **b, d, f** = 500 μ m. n = 3 hearts analysed per genotype.

4.2. Characterisation of the cardiac lymphatic vasculature in *Vav1-Cre;Prox1^{fl/fl}* conditional mutants

4.2.1. Survival of *Vav1-Cre;Prox1^{fl/fl}* animals

As performed previously for *Tie2-Cre;Prox1^{fl/fl}* animals, Mendelian ratios were calculated for a *Vav1-Cre;Prox1^{fl/+}* x *Prox1^{fl/fl}* cross where samples were collected for genotyping at E14.5, E17.5, E18.5 and P7. Again, the expected percentage for each genotype would be 25% *Prox1^{fl/fl}*, 25% *Prox1^{fl/+}*, 25% *Vav1-Cre;Prox1^{fl/+}* and 25% *Vav1-Cre;Prox1^{fl/fl}*. Across the various stages collected the percentages for each genotype were 33% *Prox1^{fl/fl}*, 14.5% *Prox1^{fl/+}*, 22% *Vav1-Cre;Prox1^{fl/+}* and 30.5% *Vav1-Cre;Prox1^{fl/fl}* (full breakdown for each stage is displayed in **Table 4.11**). The ratio of *Vav1-Cre;Prox1^{fl/fl}*, *Vav1-Cre;Prox1^{fl/+}* and *Prox1^{fl/fl}* genotypes were close to the expected 25%, however, there were approximately 10% less *Prox1^{fl/+}* animals than expected. The *Vav1-Cre* line is a “knock-in” rather than a transgene so this discrepancy in the expected embryo ratios cannot be explained by the integration of unknown copy numbers of the genome, therefore, this difference may be due to the fact that in practise the expected Mendelian ratios are not always perfectly met. The observation that 30.5% of all animals were *Vav1-Cre;Prox1^{fl/fl}* demonstrates that deletion of PROX1 in the *Vav1* lineage is not embryonic lethal.

Table 4.11. Survival of *Vav1-Cre;Prox1^{fl/fl}* animals

Genotype	% E14.5	%E17.5 and E18.5	% P7	% all stages (n=69)
<i>Vav1-Cre;Prox1^{fl/fl}</i>	50	27	40	30.5
<i>Vav1-Cre;Prox1^{fl/+}</i>	12.5	25	0	22
<i>Prox1^{fl/fl}</i>	37.5	34	20	33
<i>Prox1^{fl/+}</i>	0	14	40	14.5

Table 4.11: Survival of *Vav1-Cre;Prox1^{fl/fl}* animals

The table shows the percentage of each genotype collected at each developmental stage: E14.5, E17.5, E18.5 and P7. The percentage of each genotype totalled across all stages is also shown.

4.2.2. E14.5 and 17.5 *Vav1-Cre;Prox1^{fl/fl}* mutants appear grossly normal

In order to determine whether PROX1 is required in the *Vav1*⁺ lineage for the development of cardiac lymphatic network, and to confirm that *Vav1-Cre*⁺ cells contribute to cardiac LECs, *Vav1-Cre;Prox1^{fl/fl}* embryos were collected at E14.5 and E17.5. Upon dissection both E14.5 and E17.5 control (**Figure 4.12a, c**) and *Vav1-Cre;Prox1^{fl/fl}* mutant embryos (**Figure 4.12b, d**) appeared grossly normal.

Figure 4.12

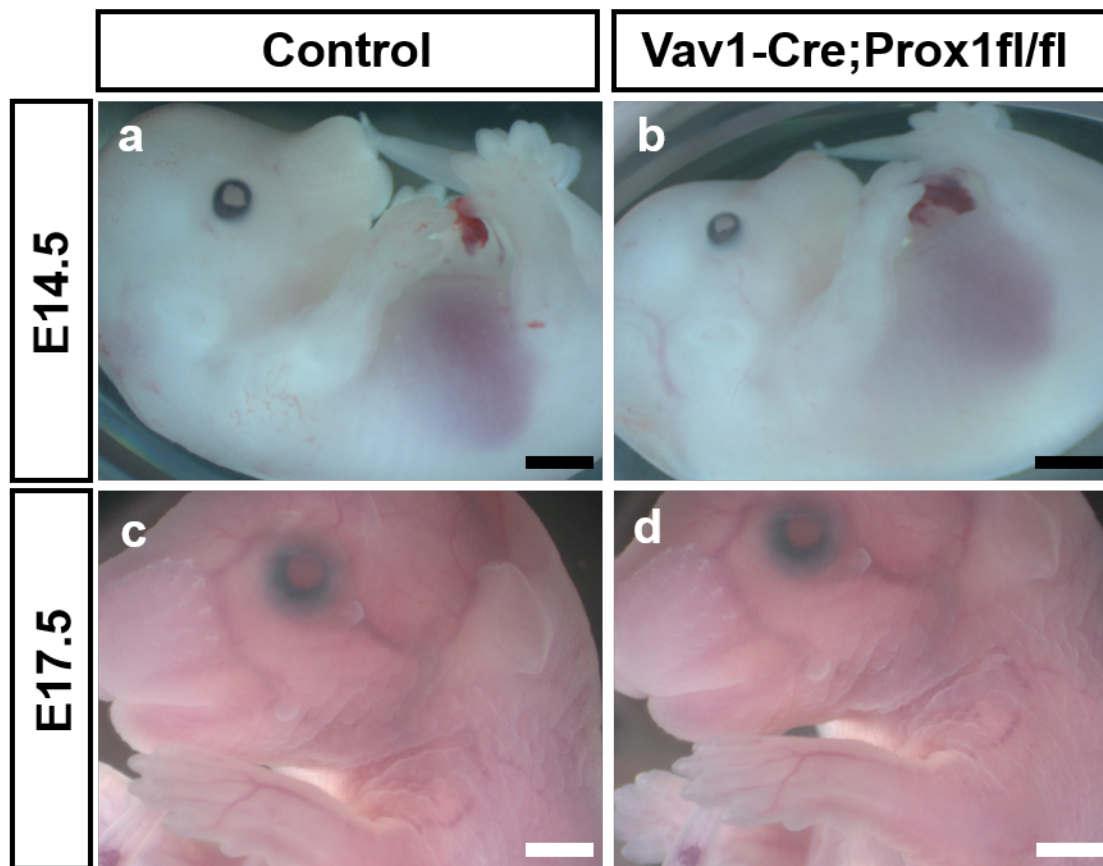


Figure 4.12: E14.5 and E17.5 *Vav1-Cre;Prox1^{fl/fl}* embryos do not display gross abnormalities

Control and mutant embryos are shown at E14.5 (control **a**, mutant **b**) and E17.5 (control **c**, mutant **d**). No gross differences between controls and mutants were observed at either E14.5 or E17.5. E14.5 controls n = 3, mutants n = 4. E17.5 controls n = 27, mutants n = 15. All scale bars = 100 μ m.

4.2.3. The cardiac lymphatics of *Vav1-Cre;Prox1^{fl/fl}* embryos appear normal

Whole-mount immunofluorescent staining was performed on E14.5 *Vav1-Cre;Prox1^{fl/fl}* mutant hearts and controls to detect GFP, LYVE-1 and PROX1 in order to assess whether the emergence of the cardiac lymphatic network is disturbed by loss of LECs from the *Vav1* lineage. *Vav1-Cre;Prox1^{fl/+}* hearts revealed an emergent lymphatic plexus sprouting from the base on the dorsal side which was partially GFP⁺ (**Figure 4.13a, c**) and LYVE-1⁺ (**Figure 4.13b, c**), thus confirming some of the emergent LECs derived from the *Vav1-Cre* lineage. *Vav1-Cre;Prox1^{fl/fl}* mutant hearts were also found to have GFP⁺ LYVE-1⁺ developing lymphatics which were present in a well-organised network (**Figure 4.13d-f**). This suggests that loss of LECs from the *Vav1* lineage does not impair the initial formation of the cardiac lymphatic network. Staining for PECAM-1 on *Vav1-Cre;Prox1^{fl/fl}* hearts also revealed the presence of normally formed coronary endothelium (**Figure 4.13g-h**).

Figure 4.13

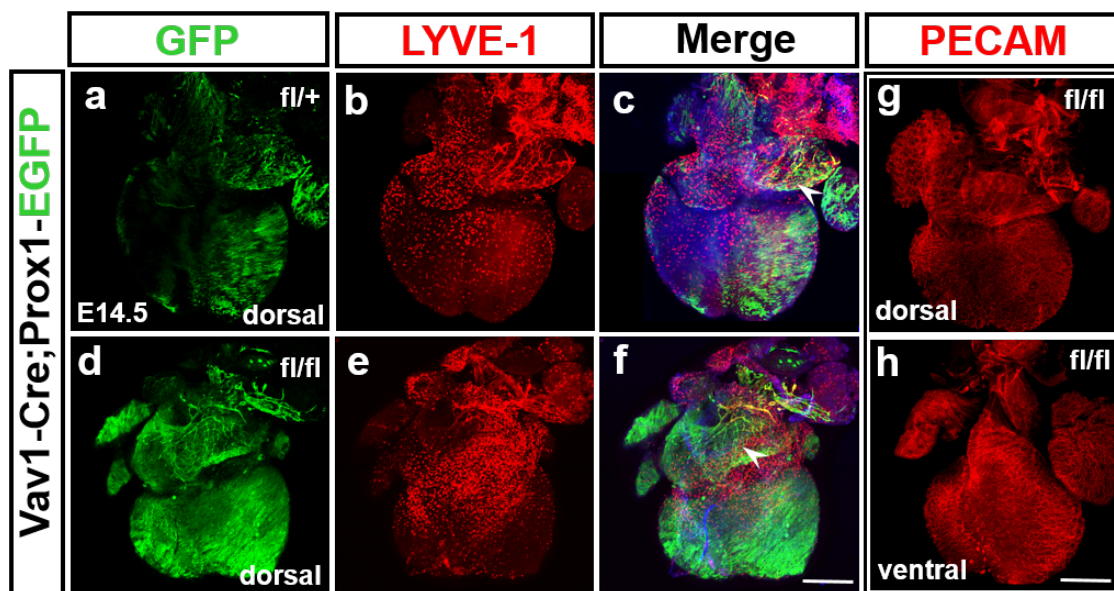


Figure 4.13: E14.5 *Vav1-Cre;Prox1^{fl/fl}* mutants and controls whole-mount immunofluorescent staining

Staining for GFP (green), LYVE-1 (red) and DAPI (blue) was performed. Staining of *Vav1-Cre;Prox1^{fl/+}* hearts revealed a partially GFP⁺, LYVE-1⁺ emergent lymphatic plexus on the dorsal side of the heart (**a-c**, arrowhead in **c** indicates the developing plexus). Staining of *Vav1-Cre;Prox1^{fl/fl}* mutant hearts also revealed an emergent lymphatic plexus which was partially GFP⁺ and LYVE-1⁺ (**d-f**, arrowhead in **f** indicates the developing plexus). *Vav1-Cre;Prox1^{fl/fl}* hearts were also stained for PECAM, **g-h**, which revealed the presence of normal coronary blood vessel endothelium. Scale bars **f**, **h**=500 μ m. n = 3 hearts analysed per genotype.

Vav1-Cre;Prox1^{fl/+} and *Vav1-Cre;Prox1^{fl/fl}* hearts were also collected at E17.5 in order to assess whether PROX1 deletion in the *Vav1-Cre* lineage has any impact on later network growth. Staining for LYVE-1, PROX1 and GFP revealed a grossly normally formed network on both the dorsal and ventral sides of *Vav1-Cre;Prox1^{fl/+}* control hearts (**Figure 4.14a, b**). High resolution imaging revealed GFP⁺ cells present within the lymphatic endothelium (**Figure 4.14g-i**) thus confirming the lineage contribution from *Vav1-Cre* to the GFP⁺ LECs in which one copy PROX1 had been deleted. *Vav1-Cre;Prox1^{fl/fl}* hearts displayed well-formed, extensive LYVE-1⁺ PROX1⁺ networks on both sides of the heart (**Figure 4.14c-f**), similar to those observed in controls.

Vav1-Cre;Prox1^{fl/fl} hearts also displayed GFP⁺ cells within the lymphatic endothelium, where GFP⁺ was present PROX1 loss was observed (**Figure 4.14j-l**, GFP presence and PROX1 loss is indicated by arrowheads in **j, k**). These results confirm a minor *Vav1*⁺ lineage contribution to cardiac LECs but demonstrate that the loss of LECs which potentially derive from the haemogenic endothelium does not impair the formation of the lymphatic network in the heart. Unexpectedly non-specific GFP labelling was also observed in some regions of myocardium at both E14.5 and E17.5 in *Vav1-Cre;Prox1^{fl/fl}* and *Vav1-Cre;Prox1^{fl/fl}* hearts. (**Figure 4.13a, d, Figure 4.14a-f**). 'Off-target' Cre activity has previously been described for the *Vav1-Cre* line (Heffner et al., 2012), therefore, the likely explanation for GFP expression observed in the myocardium is Cre deletion of PROX1 which is expressed in cardiomyocytes.

Figure 4.14

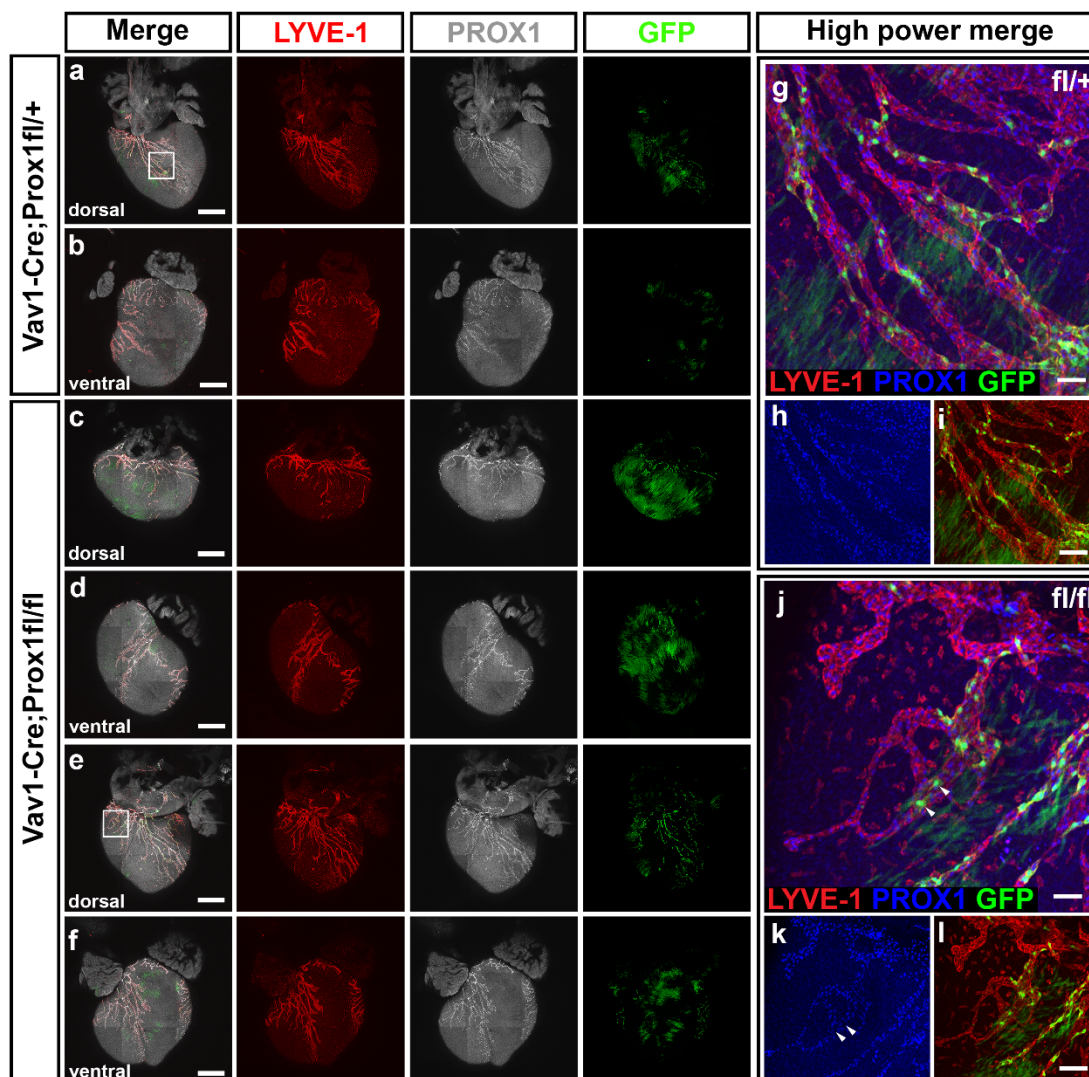


Figure 4.14: E17.5 *Vav1-Cre;Prox1^{fl/fl}* mutants and controls whole-mount immunofluorescent staining

Staining for GFP (green), LYVE-1 (red) and PROX1 (grey/blue) was performed. *Vav1-Cre;Prox1^{fl/+}* hearts displayed an extensive lymphatic network on the dorsal and ventral sides which was partially GFP⁺ and fully LYVE-1⁺ and PROX1⁺ (**a, b**, white box in **a** displayed at high power in **g-i**). *Vav1-Cre;Prox1^{fl/fl}* mutant hearts were also found to have an organised, extensive LYVE-1⁺, PROX1⁺ network, partially positive for GFP (**c-f** white box in **e** displayed at high power in **j-l**). Scale bars **a-f** = 500 μ m, **g, j** = 50 μ m, **i, l** = 100 μ m. Heterozygotes n = 4, mutants n = 6.

4.2.4. Postnatal *Vav1-Cre;Prox1^{fl/fl}* hearts appear phenotypically normal

Vav1-Cre;Prox1^{fl/fl} and control hearts were collected at P7 to investigate whether there was any impact of PROX1 deletion from the *Vav1-Cre* lineage on the postnatal growth and development of the cardiac lymphatics. Whole-mount DAB staining for VEGFR-3 revealed an extensive lymphatic network on both the dorsal and ventral sides of *Prox1^{fl/fl}* (**Figure 4.15a-b**), *Vav1-Cre;Prox1^{fl/+}* (**Figure 4.15c-d**) control and *Vav1-Cre;Prox1^{fl/fl}* (**Figure 4.15e-f**) mutant hearts, indicating that deletion of PROX1 in the *Vav1-Cre* lineage does not significantly affect the postnatal growth and expansion of the cardiac lymphatic network.

Figure 4.15

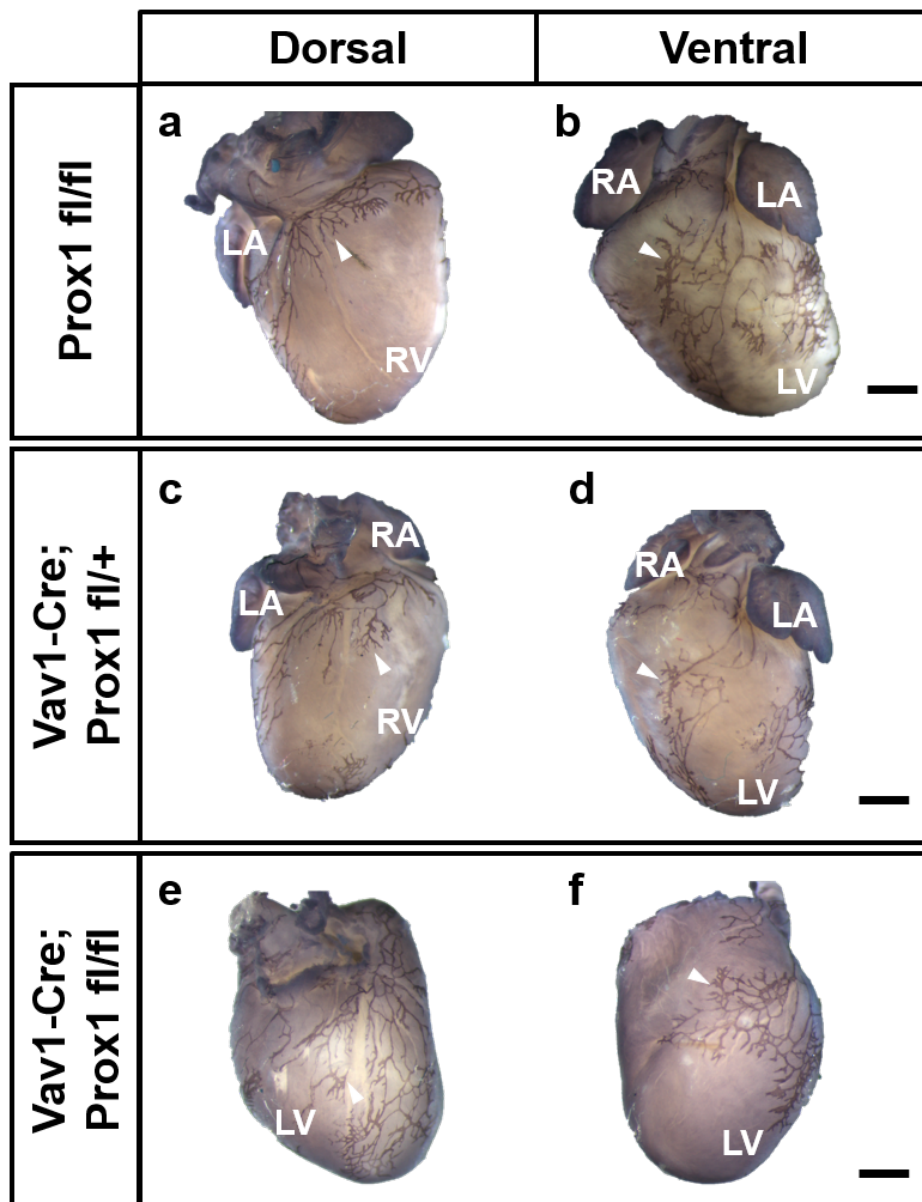


Figure 4.15: Postnatal assessment of *Vav1-Cre;Prox1^{fl/fl}* mutants

DAB staining for VEGFR-3 on P7 hearts. At this stage *Prox1^{fl/fl}* and *Vav1-Cre;Prox1^{fl/+}* control hearts displayed an extensive VEGFR-3⁺ lymphatic network (**a-d**, lymphatics indicated by white arrowheads). *Vav1-Cre;Prox1^{fl/fl}* mutants displayed an extensive network similar to that observed in controls (**e, f**). LA = left atrium, RA = right atrium, LV = left ventricle, RV = right ventricle. Scale bars **b, d, f** = 500 μ m. n = 3 hearts analysed per genotype.

4.3. Discussion

4.3.1. Deletion of PROX1 in the *Tie2-Cre* lineage

The work presented in this chapter has characterised the phenotype of *Tie2-Cre;Prox1^{fl/fl}* mutants. Upon dissection *Tie2-Cre;Prox1^{fl/fl}* mutant embryos displayed oedema at E14.5 and blood filled nodules and capillaries at E17.5. It has previously been demonstrated that deletion of PROX1 causes LECs to dedifferentiate into blood endothelial cells (BECs) (Johnson, Dillard et al., 2008). It is, therefore, possible that the blood filled capillaries observed may have resulted from LECs which dedifferentiated into BECs, leading to a hyperplastic network of blood vasculature. Previous phenotypes of blood-filled lymphatic vessels have also been observed in mutants where there is a defective separation of the venous and lymphatic systems (Abtahian et al., 2003, Uhrin, Zaujec et al., 2010). Further work is required to determine whether the blood-filled vessels observed here are a hyperplastic blood capillary network or blood-filled lymphatic vessels resulting from insufficient separation of the venous and lymphatic systems.

There were some mutant hearts in which PROX1 was not fully deleted in the *Tie2-Cre* lineage due to inefficient Cre deletion or mosaicism as previously reported for many Cre lines (Heffner et al., 2012) and for *Tie2-Cre;Prox1^{fl/fl}* mutants (Srinivasan et al., 2007). *Tie2-Cre;Prox1^{fl/fl}* hearts analysed at E17.5 revealed a gradient of phenotype severity that correlated with the extent of deletion of PROX1. Where antibody staining confirmed complete or almost complete loss of PROX1 protein in the lymphatic network there was severe disruption with few or no vessels observed. These results indicate that PROX1 is required for cardiac LEC fate specification as has been confirmed previously in the development of the systemic lymphatic vessels (Wigle, Chowdhury et al., 1999). Severe disruption of the network with loss of PROX1 in the *Tie2* lineage also confirmed

previous observations made in the Riley lab that approximately 80% of cardiac LECs are derived from a venous endothelial (Tie2⁺) lineage (Klotz et al., 2015). Furthermore, the disruption observed also suggests that cells from alternate sources were insufficient to give rise to a lymphatic network as no compensation for loss of the Tie2⁺ contribution was detected. Severe *Tie2-Cre;Prox1^{fl/fl}* mutant hearts also displayed a gross phenotype of being dysmorphic and significantly smaller than controls. During postnatal stages hearts were recovered from both heterozygous and homozygous mutants at P7 where there was no obvious disruption to the VEGFR-3⁺ lymphatic network; this may have been due to network remodelling in early postnatal stages or more likely incomplete deletion of PROX1 by *Tie2-Cre*.

Some milder E17.5 *Tie2-Cre;Prox1^{fl/fl}* mutants displayed a truncated, hypoplastic plexus of cardiac lymphatics, demonstrated by the pattern of staining and also by quantification of vessel network parameters. The hypoplastic phenotype resulting from the partial loss of PROX1 protein suggested a novel dose-dependent role for PROX1 in patterning of lymphatic vessels given the increased number of end points, reduced vessel diameter and shorter length of cardiac lymphatics observed with incomplete PROX1 deletion. Interestingly a PROX1 dosage effect has previously been reported in lymphovenous valve formation within the jugular and subclavian veins where two copies of PROX1 are required. *Prox1* heterozygous animals were found to completely lack lymphovenous valves (Srinivasan & Oliver, 2011).

Tie2-Cre;Prox1^{fl/+} heterozygous mutants also displayed a mild phenotype where the lymphatic network appeared to be less extensive on the ventral side of the heart when compared to controls. This may indicate that there is some impairment in the formation of the network with the loss of a single copy of PROX1 which is supported by a previous

report in which lymphatic vessels were mispatterned in *Tie2-Cre;Prox1^{fl/+}* animals (Harvey, Srinivasan et al., 2005). Further experiments were performed on *Tie2-Cre;Prox1^{fl/+}* heterozygous adult animals which are presented in chapter 6 of this thesis.

4.3.2. Deletion of PROX1 in the *Vav1-Cre* lineage

Vav1-Cre;Prox1^{fl/fl} mutants were also characterised in order to assess whether there is an effect of deletion of PROX1 in non-venous derived LECs on the formation of the cardiac lymphatic network. A proportion of LECs were labelled with GFP in E14.5 and E17.5 *Vav1-Cre;Prox1^{fl/fl}* hearts, showing that PROX1 has been deleted in *Vav1*⁺ cells which would have contributed to cardiac LECs. This confirmed previous lineage tracing data which demonstrated that cells from the *Vav1*⁺ lineage contribute to approximately 20% of cardiac lymphatic vessels. Despite the loss of some *Vav1*⁺ LECs within the cardiac lymphatic network these mutant hearts did not display an obvious phenotype with regards to disruption of the lymphatic vasculature at E14.5, E17.5 or P7. This is consistent with the data that cells from the *Tie2*⁺ lineage are the main progenitor source for cardiac LECs and also demonstrates that LECs derived from the *Vav1*⁺ lineage are not essential for the formation of the cardiac lymphatics. In addition to specific targeting of LECs by *Vav1-Cre* some examples of off-target GFP expression were observed in the heart muscle in cells which appeared to be deletion of PROX1 in cardiomyocytes. This observation once again highlights the problem of mosaicism and off-target effects often observed with Cre lines as previously described (Heffner et al., 2012).

The data presented here has confirmed a lineage contribution from a non-venous source to cardiac LECs and has also demonstrated that LECs from this lineage are not

required for the formation of an extensive cardiac lymphatic network in the embryo. What remains to be answered is why do LECs derive from multiple origins, how do they integrate into the network and what is their functional importance.

4.4. Conclusions

As discussed in chapter 3 it has been demonstrated that a small proportion of cardiac LECs are derived from non-venous origins, including from the Vav1⁺ lineage. In this chapter genetic targeting of PROX1 in both venous endothelium (Tie2⁺) and cells derived from the Vav1⁺ lineage confirmed that the majority of cardiac lymphatic vessels are derived from a venous endothelial source and a smaller proportion are derived from a Vav1⁺, non-venous cellular source. In addition these studies confirmed that as in the systemic vasculature, PROX1 is required to specify LEC fate in cardiac lymphatic vasculature. Targeting of cardiac lymphatics derived from venous endothelium resulted in severe disruption of the lymphatic network for which LECs derived from alternate sources, such as the Vav1⁺ lineage, appeared unable to compensate. Furthermore, some E17.5 *Tie2-Cre;Prox1^{fl/fl}* mutant hearts in which incomplete Cre deletion had occurred displayed a milder phenotype with a disrupted and hypoplastic cardiac lymphatic network, suggesting a novel role for PROX1 in remodelling of the network, which has not previously been appreciated in other lymphatic vessel beds during development.

5. Results III: VEGF-C modulates post-myocardial infarction lymphangiogenesis and inflammation

Lymphangiogenesis has previously been observed in humans (Ishikawa et al., 2007, Kholova et al., 2011) and rats (Sun et al., 2012) following MI and more recently studies performed by Linda Klotz in the Riley lab have investigated this response in mice. Klotz demonstrated by whole-mount immunostaining that significant lymphangiogenesis occurs by day 7 post-MI, which is coupled with an increase in the mRNA expression levels of *Vegfr3*, *Lyve1* and *Prox1*. Furthermore, the newly formed lymphatics were found to persist in the infarcted region until at least 35 days following injury (Klotz et al., 2015). In this chapter I have further investigated the response and role of the cardiac lymphatic vessels following MI using a mouse permanent LAD ligation model. Experiments focused on the role of cardiac lymphatic vessels in the post-MI inflammatory response and examined whether therapeutic targeting of this response could be beneficial for cardiac function.

5.1. A proportion of macrophages are potentially cleared by cardiac lymphatic vessels post-MI

The post-MI inflammatory process has been the subject of many studies with the hope that modulation of inflammation may result in improved healing and reduced scarring. There has been some investigation into the fate of the large numbers of immune cells which infiltrate the heart following injury. In one study day 3 post-MI hearts were transplanted heterotopically into donor animals and the fate of labelled CD45 leucocytes was assessed 24 hours later. This revealed that at this time point a large proportion of leucocytes die locally but some are trafficked to other sites including the

spleen and liver (Leuschner, Rauch et al., 2012). Another study found that tissue resident macrophages mostly die in the heart by 24 hours post-MI. There was also some evidence to suggest that some tissue resident macrophages may actively depart to the spleen and bone marrow, although the mechanism for this remains unclear (Heidt, Courties et al., 2014). Whilst these studies provide insights into the fate of leucocytes and macrophages during the relatively early stages of the post-MI inflammatory process they do not address later time points such as at day 7 post-MI by which point extensive lymphangiogenesis has occurred (Klotz et al., 2015). Leucocytes (Johnson, Clasper et al., 2006), including macrophages (Lawrance et al., 2016), neutrophils (Rigby et al., 2015) and dendritic cells (Randolph et al., 2005) enter lymphatic vessels in other settings of inflammation (Lawrance et al., 2016, Rigby et al., 2015). Therefore, I hypothesised that a proportion of post-MI macrophages may exit the heart, via the cardiac lymphatics and drain to the mediastinal lymph nodes. In order to test this hypothesis immunofluorescent staining and imaging methods were established to visualise the presence of macrophages within cardiac lymphatic vessels. Days 4 and 7 post-MI were selected for analysis as there is a large population of macrophages within the heart at these time points which are constantly undergoing a process of recruitment to the heart followed by apoptosis and egression (Leuschner et al., 2012, Nahrendorf et al., 2007).

5.1.1. Visualisation of macrophages within cardiac lymphatic vessels

Initially immunofluorescent staining was performed on transverse cryosections from intact, day 4 and day 7 post-MI hearts. Staining on intact (uninjured) hearts revealed the presence of LYVE-1⁺ lymphatic vessels within the sub-epicardial region and dispersed tissue resident CD68⁺ macrophages (**Figure 5.1a-b**). By day 4 post-MI an increased number of F4/80⁺ macrophages were detected within the infarcted region

(**Figure 5.1c-d**), this is in agreement with previous studies (Nahrendorf et al., 2007). An increased number of LYVE-1⁺ lymphatic vessels were also observed at day 4 post-MI (**Figure 5.1c-d**). These newly formed vessels were observed to sprout deeper within the myocardial region when compared to the sparser sub-epicardial plexus observed in intact controls (**Figure 5.1a-d**, white arrowheads in **c** mark newly formed lymphatics deeper within the myocardium). By day 7 post-MI macrophages were observed in close proximity to cardiac lymphatic vessels (**Figure 5.1e-f**) and in some cases appeared to be positioned for trafficking into the lymphatics as indicated by white arrowheads in **Figure 5.1e-f**.

Whilst informative the data collected from staining on cryosections is limited as it only reveals a relatively small snapshot of the sub-epicardial cardiac lymphatic plexus which does not give substantial information about the extent of post-MI lymphangiogenesis or clear evidence as to whether macrophages do actually enter the lymphatic vessels. In order to address these issues a whole-mount immunofluorescent staining protocol was developed to allow better visualisation of the sub-epicardial lymphatic network. The development of this method allowed whole post-MI adult hearts to be stained and imaged using light sheet microscopy, or as small biopsies using high resolution confocal microscopy.

Whole day 4 post-MI hearts underwent immunofluorescent staining for LYVE-1 and CD68, followed by light sheet imaging. This revealed the extensive LYVE-1 sub-epicardial lymphatic network (**Figure 5.2a-b, d-e**) and CD68⁺ macrophages across the epicardial surface (**Figure 5.2a, c, d, f**). As previously reported (Klotz et al., 2015), lymphangiogenesis was observed close to the infarcted region (**Figure 5.2d-e**, higher power in **g-h**). Within this lymphangiogenic region, close to the infarct, CD68⁺

macrophages were observed at the tips of lymphatic capillaries (**Figure 5.2g-i**) which may suggest entrance of macrophages into the lymphatic vessels for clearance.

Despite providing an informative snapshot of the post-MI lymphatic network and macrophages present on the surface of the heart, the light sheet images do not provide clear evidence as to whether macrophages are trafficked within cardiac lymphatic vessels. Therefore, large pieces of stained heart were imaged using confocal microscopy in order to provide higher resolution Z-stack images. Orthogonal views on Z-stacks from day 4 post-MI hearts stained for CD68 and LYVE-1 revealed a large number of macrophages within the myocardium and a small number of macrophages within the lymphatic endothelium, as confirmed by orthogonal views showing CD68⁺ macrophages between LYVE-1⁺ lymphatic endothelium (**Figure 5.3a-c**). In other regions CD68⁺ macrophages were observed lining up at lymphangiogenic tips in an apparent vessel conformation (**Figure 5.3d-e**). In some cases co-localisation between CD68 and LYVE-1 was observed within the same plane of the vessel suggesting there may be a lineage contribution from macrophages to LECs (**Figure 5.3d-f**). At day 7 post-MI *Csf1r-CreER;R26R-tdtomato* hearts were collected in which post-MI macrophages were labelled by a single dose of 4-OH tamoxifen at day 3 post-MI. Induction of Cre activity at this point produced fluorescent labelling of macrophages and dendritic cells present in the heart at this time point after MI (Qian et al., 2011). Additional staining for LYVE-1 and confocal imaging on these hearts also revealed a small number of macrophages within lymphatic vessels (**Figure 5.3g-h**), again confirmed by orthogonal views which display CD68⁺ cells sandwiched by LYVE-1 endothelium (**Figure 5.3h-i**).

Together these data indicate that some macrophages are indeed transported away from the infarcted myocardium by cardiac lymphatic vessels as observed by imaging at days

4 and 7 post-MI. Furthermore, a small number of macrophages may potentially transdifferentiate into LECs at lymphangiogenic tips, as suggested by co-localization between CD68 and LYVE-1 at these tips and the apparent lining up of macrophages in a vessel like formation leading towards the tip.

Figure 5.1

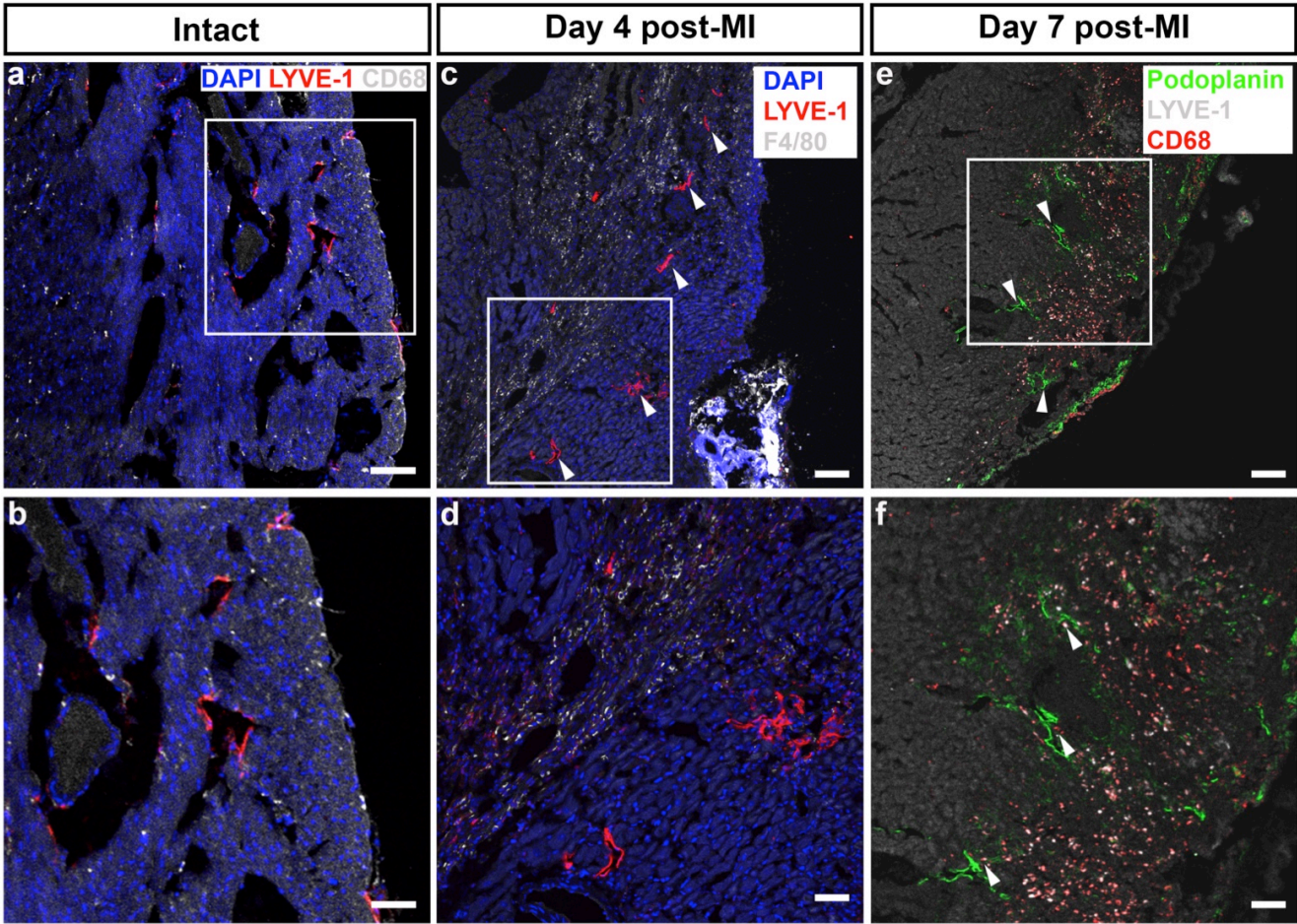


Figure 5.1: Post-MI macrophages are situated proximal to newly formed lymphatic vessels

Immunofluorescent staining was performed on cryosections from intact (uninjured), day 4 post-MI and 7 day post-MI hearts to detect macrophages (CD68 or F4/80) and lymphatic vessels (LYVE-1 or PODOPLANIN). Intact hearts displayed a number of LYVE-1⁺ lymphatic vessels and dispersed tissue resident CD68⁺ macrophages (a, white box enlarged in b). An increased number of macrophages (F4/80⁺) and lymphatic vessels (LYVE-1⁺) were observed at day 4 post-MI (c, white box enlarged in d). By day 7 post-MI macrophages were detected proximal to cardiac lymphatic vessels, as indicated by white arrowheads in e (white box enlarged in f). Scale bars a, c, e = 100 μ m, b, d, f = 50 μ m.

Figure 5.2

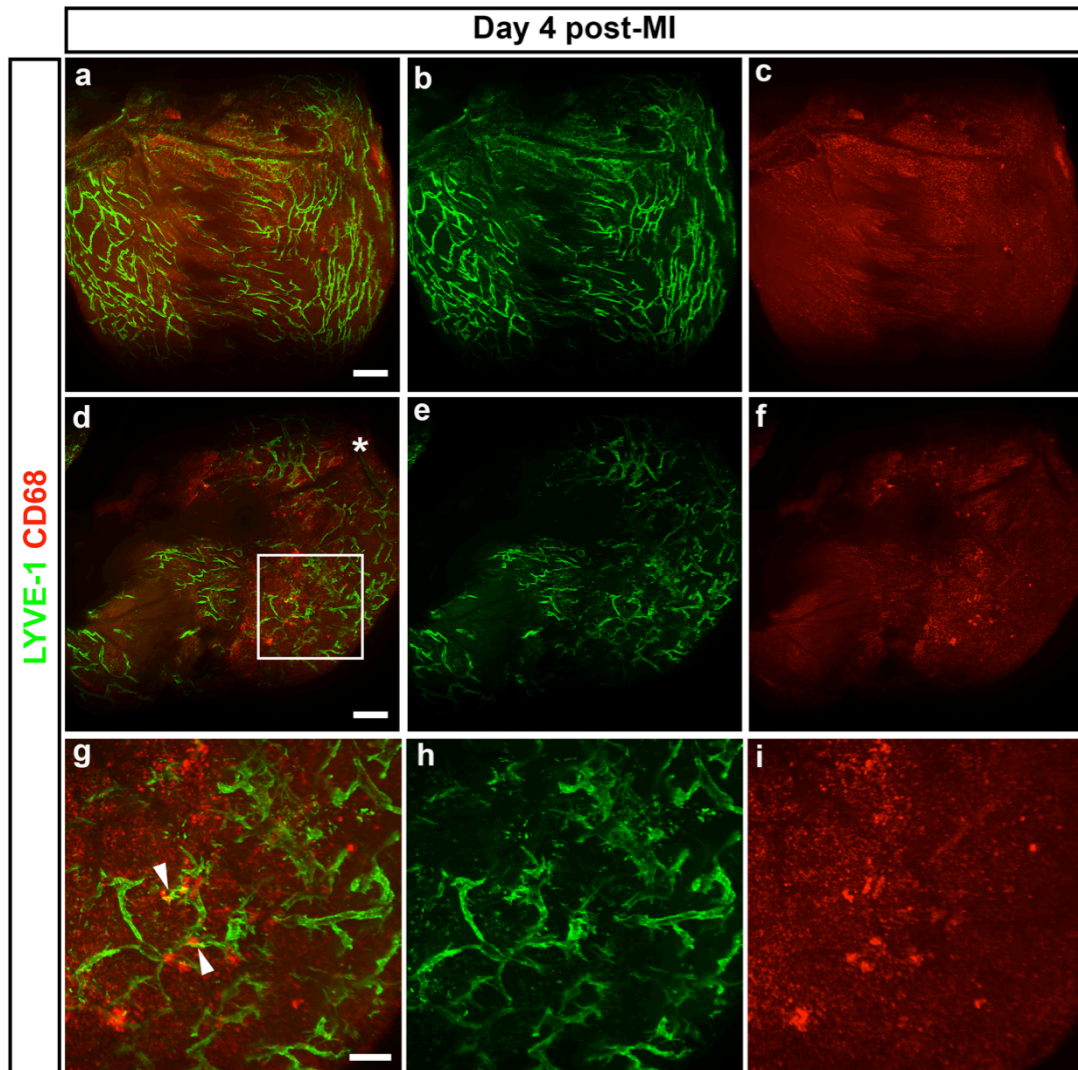


Figure 5.2: Whole-mount immunofluorescent staining and light sheet imaging reveals the sub-epicardial lymphatic plexus and post-MI epicardial macrophages

Immunofluorescent staining for LYVE-1 (green) and CD68 (red) was performed on whole adult hearts at day 4 post-MI. Light sheet imaging of the whole heart revealed the LYVE-1⁺ sub-epicardial cardiac lymphatic plexus (**a-b, d-e**) and macrophages (**a, c, d, f**) on the surface of the heart. Close to the infarcted region (point of LAD ligation marked by * in **d**) macrophages were observed at the tips of lymphatic vessels (white box in **d** enlarged in **g**, white arrowheads indicate macrophages at tips, separate channels displayed in **h** and **i**). Scale bars **a, d** = 500 μ m, **g** = 200 μ m.

Figure 5.3

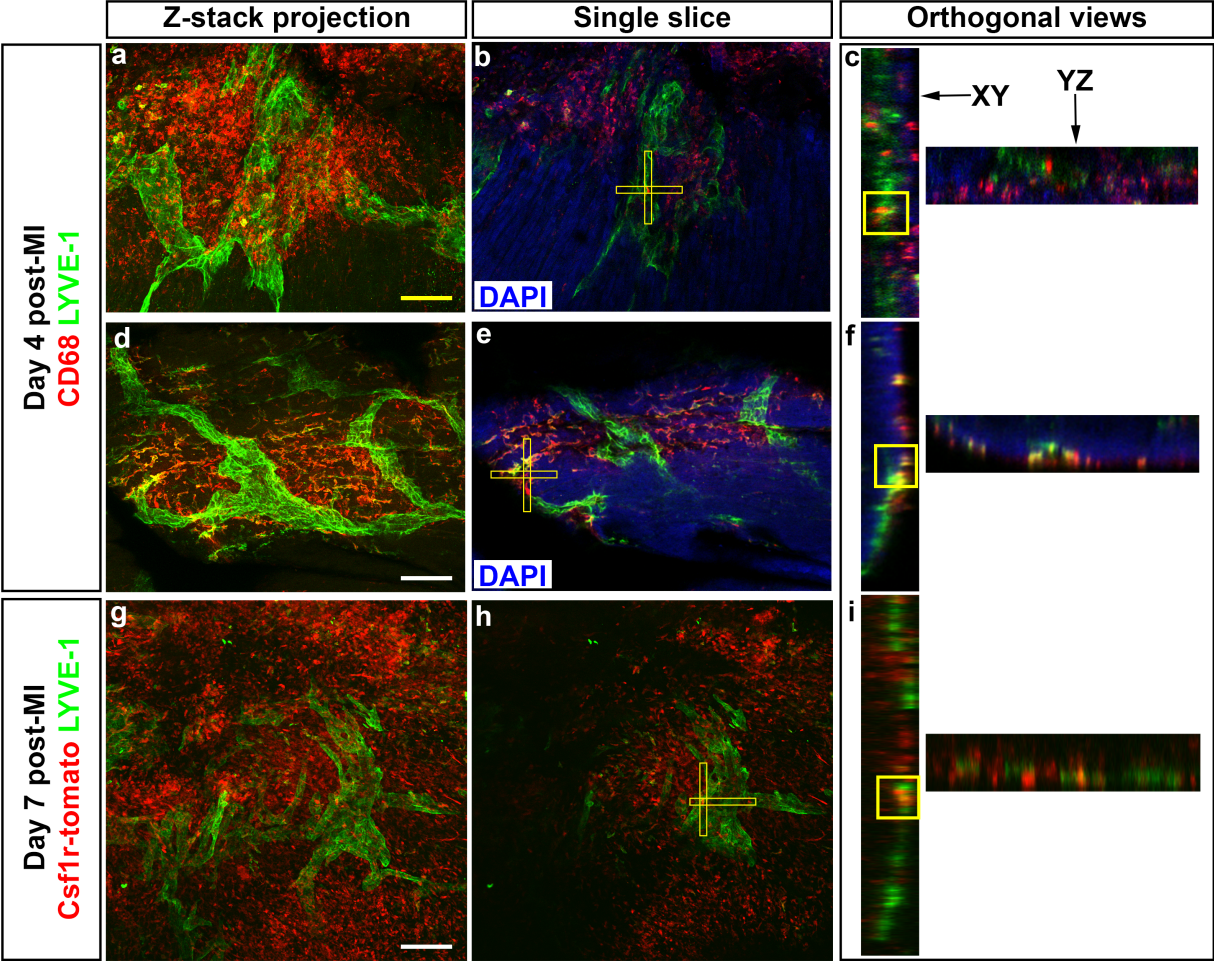


Figure 5.3: Macrophages appear to be trafficked within lymphatic vessels post-MI

Immunofluorescent staining for LYVE-1 (green) and CD68 (red) was performed on whole adult hearts at day 4 post-MI (a-f). Confocal imaging of heart biopsies revealed a large number of CD68⁺ macrophages. A small number of these macrophages were located within the LYVE-1⁺ lymphatic endothelium (orthogonal views c, f), suggesting they are trafficked away from the site of inflammation by the cardiac lymphatics. This observation was also made at day 7 post-MI (g-h) in *Csf1r-tomato* hearts where macrophages are tomato⁺ and LYVE-1 antibody staining (green) labels the lymphatic endothelium. Scale bars a, d, g = 100 μ m.

5.2. VEGF-C is expressed endogenously by the epicardium and macrophages post-MI

VEGF-C is a lymphatic growth factor which, in addition to being essential for embryonic lymphatic vessel development (Karkkainen et al., 2004), is upregulated in states of inflammation (Baluk et al., 2005, Paavonen, Mandelin et al., 2002, Ristimäki, Narko et al., 1998, Zhang, Lu et al., 2007) and is able to trigger lymphangiogenesis which occurs in a many diseases where there is an increase demand on lymphatic vessels (Baluk et al., 2005, D'Alessio et al., 2014, Kaiserling, Krober et al., 2003, Kerjaschki et al., 2004, Kunstfeld, Hirakawa et al., 2004, Zhang et al., 2007). During cardiac development VEGF-C has been shown to be secreted by the outflow tract, epicardium and valves (Chen, Poduri et al., 2014a, Chen, Sharma et al., 2014b) and is secreted by mesenchymal cells close to developing lymph sacs elsewhere in the embryo (Kukk et al., 1996). In the adult, VEGF-C is known to be secreted by vascular smooth muscle cells and lymph nodes in the steady state and is additionally secreted by macrophages during inflammation (Granata, Frattini et al., 2010, Kataru et al., 2009). Therefore, I hypothesised that VEGF-C is secreted endogenously by the heart to drive post-MI lymphangiogenesis and may be secreted by macrophages, as observed in other disease states, and/or by the epicardium by reactivation of embryonic mechanisms.

Immunofluorescent staining was performed on cryosections from wild type, uninjured (intact) hearts which revealed a complete absence of VEGF-C, including within the PODOPLANIN⁺ epicardium and CD68⁺ cardiac resident macrophages (**Figure 5.4a-c**). At day 4 post-MI VEGF-C expression was observed within the activated epicardium (**Figure 5.4d-e**) where it was found to co-localize with PODOPLANIN expression (**Figure 5.4g**). VEGF-C was also observed to be expressed by a subset of CD68⁺

macrophages within the infarct core (**Figure 5.4f**) but was not observed in all macrophages present within the heart at day 4 post-MI.

Figure 5.4

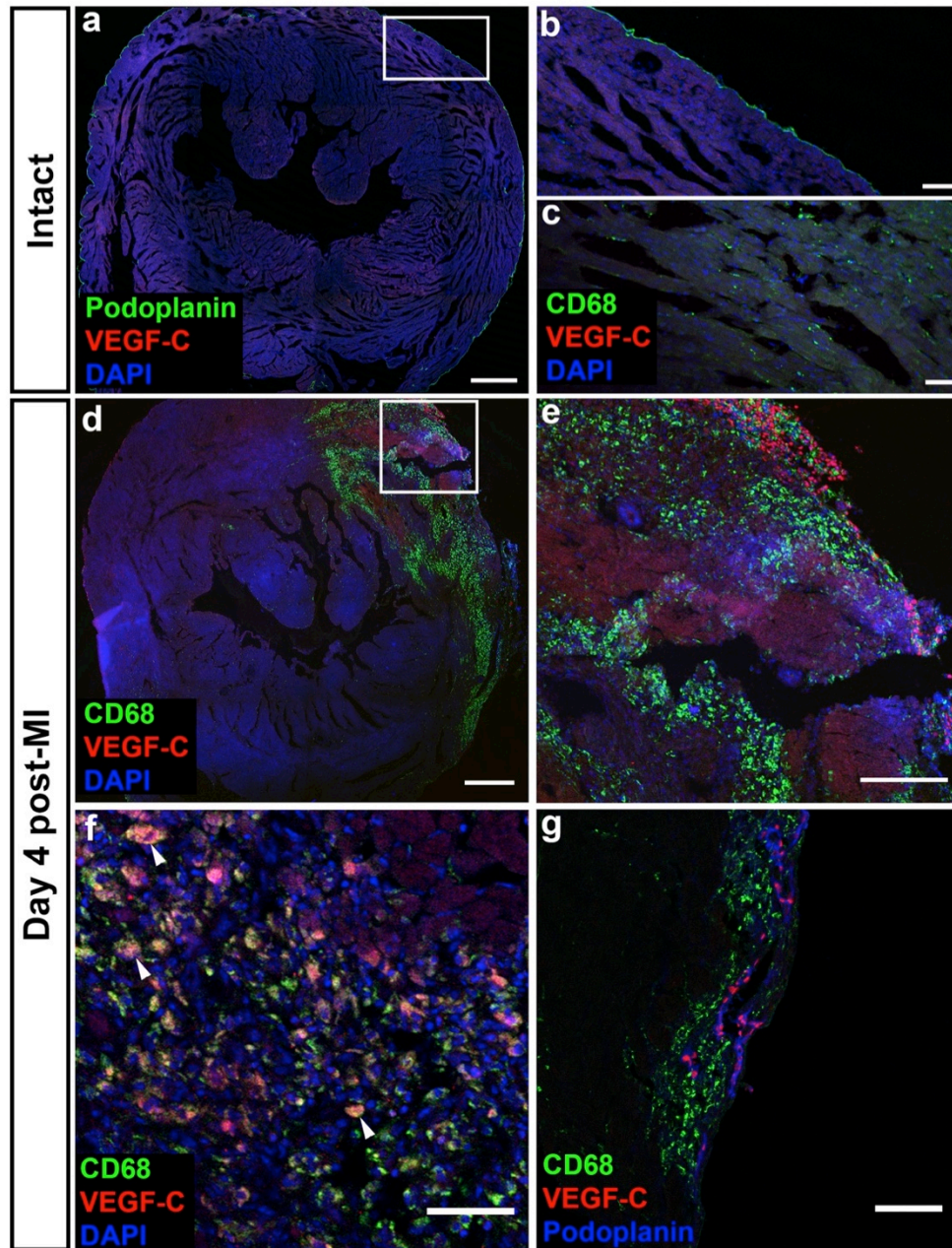


Figure 5.4: VEGF-C is secreted from the epicardium and a subpopulation of macrophages by day 4 post-MI

Immunofluorescent staining on uninjured intact hearts revealed the PODOPLANIN⁺ epicardium which displayed a complete absence of VEGF-C (**a**, white box in **a** enlarged in **b**). CD68⁺ tissue resident macrophages (green) were also detected within the myocardium but did not express VEGF-C (**c**). At day 4 post-MI VEGF-C expression was observed within the activated epicardium (**d**, white box enlarged in **e**). A large number of macrophages were also observed (**d**), a subpopulation of which were found to express VEGF-C (examples indicated by white arrowheads in **f**). Expression of VEGF-C within the epicardium was found to co-localize with PODOPLANIN expression (**g**). Scale bars **a**, **d** = 500 μ m, **b**, **f**, **g** = 100 μ m, **c** = 50 μ m, **e** = 200 μ m.

5.3 Post-MI recombinant VEGF-C (C156S) administration enhances lymphangiogenesis

Several studies have previously reported that exogenous administration of VEGF-C is able to trigger additional lymphangiogenesis in animal models (Anisimov, Alitalo et al., 2009, Lahtenvuo, Honkonen et al., 2011, Rissanen, Markkanen et al., 2003) which may aid the clearance of inflammation and resolution of oedema via the lymphatic vasculature (Cui et al., 2015, Kataru et al., 2009, Polzer, Baeten et al., 2008). I hypothesised that administration of exogenous VEGF-C following MI could stimulate additional lymphangiogenesis, enhancing the response of the lymphatic vessels which already occurs following MI. Several methods of VEGF-C administration have been used previously including adenoviral delivery (D'Alessio et al., 2014, Saaristo, Tammela et al., 2004, Yoon, Murayama et al., 2003) and administration of recombinant protein (Cui et al., 2015, Huang et al., 2016, Szuba, Skobe et al., 2002). Due to its commercial availability and ease of delivery recombinant VEGF-C with a C156S mutation was selected. The C156S mutation prevents binding of VEGF-C to VEGFR-2, restricting binding to the VEGFR-3 receptor, thus limiting additional effects on blood vessel angiogenesis (Veikkola, Jussila et al., 2001). Intraperitoneal (I.P.) injection was selected as the delivery route due to its efficacy in a previous study where daily 100ng/g VEGF-C injections for 7 days were found to improve outcomes in a model of polycystic kidney disease (Huang et al., 2016).

5.3.1. Multiple doses of VEGF-C (C156S) within 7 days post-MI leads to enhanced lymphangiogenesis

Hearts from *Vegfr3^{LacZ/+}* animals (Dumont, 1998) were used for whole-mount X-gal staining in order to visualise the sub-epicardial cardiac lymphatic network. Staining on hearts with no MI revealed the presence of an extensive cardiac lymphatic network which did not display any particularly dense areas of lymphatic capillaries nor many lymphangiogenic tips (**Figure 5.5a**). In contrast to uninjured hearts, vehicle treated hearts at day 7 post-MI displayed significant lymphangiogenesis and a dense network of sprouting lymphatic capillaries around the infarcted region (**Figure 5.5b**, asterisk marks suture and arrowheads indicate lymphangiogenic vessels), presumably triggered by endogenous VEGF-C and possibly also VEGF-D secretion. Hearts from animals treated with five 100 ng/g doses of VEGF-C (C156S) at days 0, 2, 3, 4 and 6 post-MI displayed an even denser network of lymphatic capillaries with lymphangiogenic tips (**Figure 5.5c**, asterisk marks suture and arrowheads indicate lymphangiogenic vessels) when compared to vehicle treated controls (**Figure 5.5b**), demonstrating that additional post-MI administration of VEGF-C (C156S) enhances lymphangiogenesis.

Figure 5.5

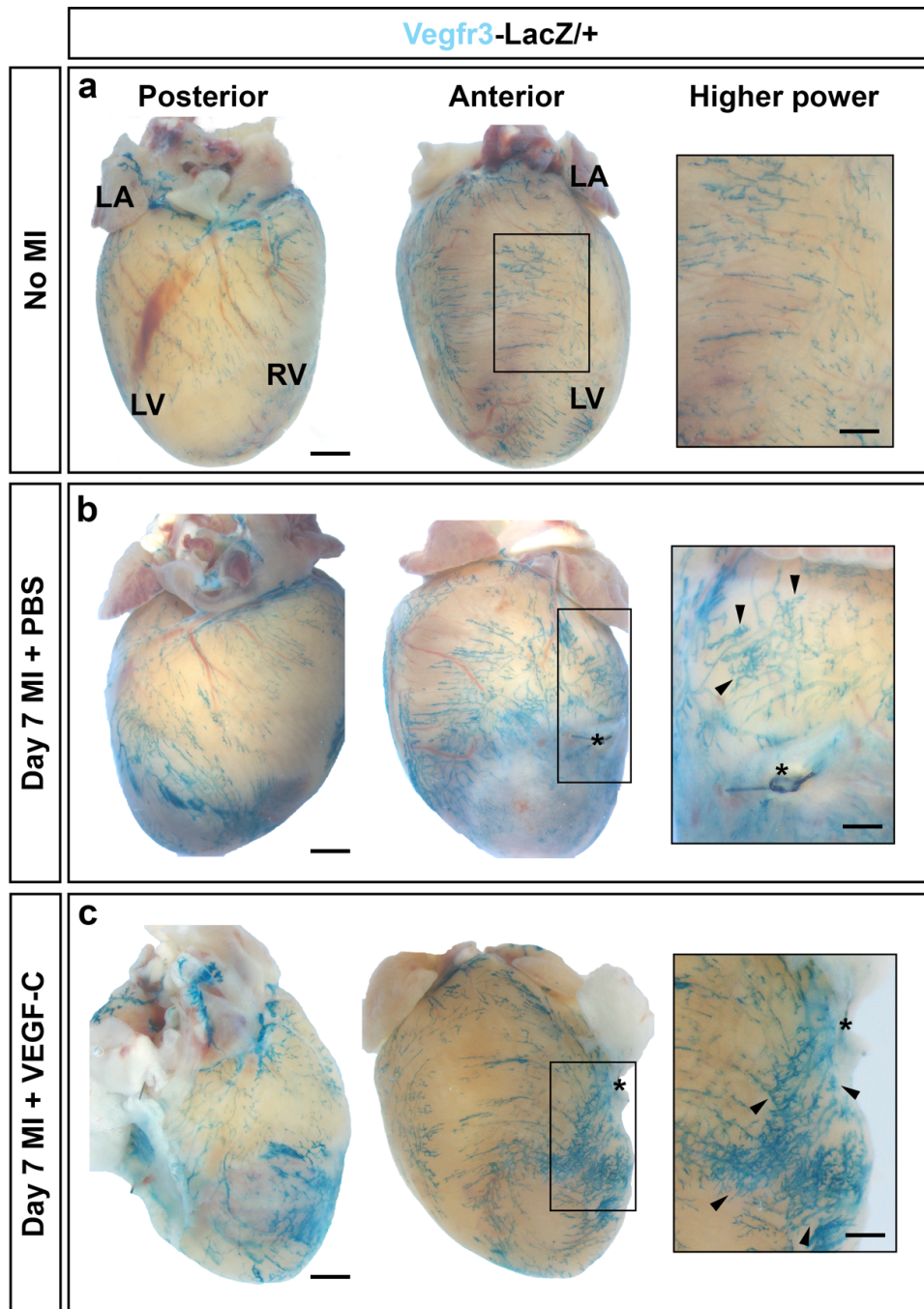


Figure 5.5: Post-MI administration of VEGF-C (C156S) enhances lymphangiogenesis

X-gal staining on adult mouse hearts was used to detect *Vegfr3-LacZ/+* expression within the sub-epicardial lymphatic capillary network. An extensive network is present in uninjured hearts (a) which undergoes significant lymphangiogenesis by day 7 post-MI (b). Post-MI treatment with four doses of VEGF-C (C156S) was found to enhance lymphangiogenesis, resulting in a denser lymphatic capillary network (c). Asterisk marks suture. Arrowheads indicate lymphangiogenic capillaries. Low power scale bars = 1 mm, high power scale bars = 250 μ m. n = 4-5 hearts per group.

5.4 Cardiac magnetic resonance imaging (MRI) revealed post-MI improvements in cardiac function following treatment with VEGF-C (C156S)

In order to determine whether the enhanced lymphangiogenesis induced by VEGF-C (C156S) could lead to improvements in post-MI healing and cardiac function a longitudinal cardiac MRI study was performed. Following MI animals were given either PBS or VEGF-C (C156S) I.P. injections at days 0, 2, 3, 4, and 6 post-MI. Cardiac MRI imaging was performed at days 7, 14, 21, and 28 to assess the effect of treatment on post-MI healing and function. Left ventricular ejection fraction (LVEF) was found to be significantly improved in the VEGF-C (C156S) treated group at days 14 and 21 post-MI (**Figure 5.6a**). LVEFs % $\pm$ S.E.M.: day 14 vehicle 43 ± 5 , day 14 VEGF-C 57 ± 4 , $p=0.029$; day 21 vehicle 45 ± 5 , day 21 VEGF-C 60 ± 5 , $p=0.045$; $n=8$ in each group. Representative short axis stills for vehicle and VEGF-C (C156S) treated groups are displayed in **Figure 5.6b**. No statistically significant improvement was observed with VEGF-C (C156S) treatment at day 7 post-MI ($p=0.200$). At day 28 there was a trend ($p=0.084$) towards increased LVEF with a mean $\pm$ S.E.M. LVEF % in the vehicle group of 45 ± 4 % and 56 ± 4 % in the VEGF-C (C156S) treated group. In addition to LVEF other left ventricular parameters were measured, including end diastolic volume, end systolic volume, stroke volume, cardiac output, left ventricular mass, absolute infarct size, and relative infarct size (**Table 5.7**). The measurements for end systolic volume at day 14 post-MI were significantly improved within the treated group compared to controls, with a mean of 21.5 ± 4.1 μ l in the VEGF-C (C156S) group versus 43.5 ± 10 μ l in the vehicle group ($p=0.046$). No significant differences were observed in absolute and relative infarct sizes across all time points in the VEGF-C (C156S) treated group when compared to vehicle controls (**Table 5.7**). Collectively this data suggests that VEGF-C (C156S)

therapy does have beneficial impact on cardiac function although further investigations, possibly with a larger group of animals, are necessary to determine whether this effect persists.

Figure 5.6

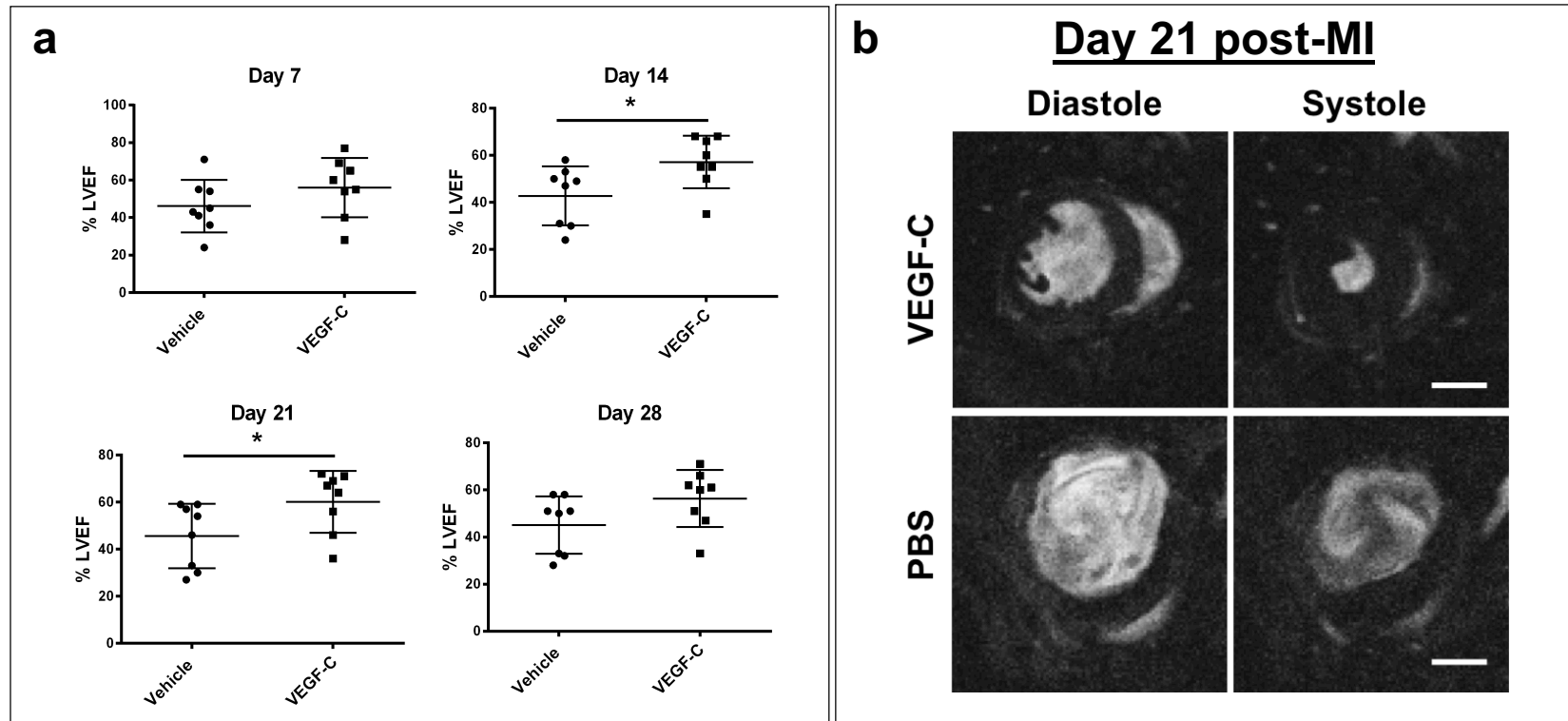


Figure 5.6: Post-MI VEGF-C (C156S) treatment leads to improved left ventricular ejection fraction at days 14 and 21 post-MI
 Animals were subjected to a four I.P. injections of VEGF-C (C156S) or PBS at days 0, 2, 4 and 6 post-MI. Cardiac Cine MRI was performed at days 7, 14, 21 and 28 post-MI in order to assess cardiac function, data presented in panel **a**. A significant increase in left ventricular ejection fraction (LVEF) was observed at days 14 and 21 with VEGF-C treatment. Both groups $n = 8$. Data presented as mean $\pm$ standard error of mean. An unpaired, two-tailed t-test was used to assess statistically significant changes. * $p \leq 0.05$. Actual p-values: day 7 = 0.200, day 14 = 0.029, day 21 = 0.045, day 28 = 0.084. Representative short axis stills for VEGF-C and vehicle groups at day 21 post-MI are displayed in **b**. Scale bars = 2mm.

Table 5.7. Functional cardiac parameters from MRI analyses of animals treated with VEGF-C (C156S) or PBS following MI

	7 days post-surgery		14 days post-surgery		21 days post-surgery		28 days post-surgery	
	<i>VEGF-C</i> (n=8)	<i>PBS</i> (n=8)	<i>VEGF-C</i> (n=8)	<i>PBS</i> (n=8)	<i>VEGF-C</i> (n=8)	<i>PBS</i> (n=8)	<i>VEGF-C</i> (n=8)	<i>PBS</i> (n=8)
Body weight [g]	19.0±0.6	19.8±0.5	19.6±0.7	20.5±0.5	19.8±0.5	20.4±0.4	20.9±0.7	21.3±0.6
Heart rate [bpm]	515±10	499±19	511±6	496±19	510±9	494±10	469±52	496±26
Left Ventricle								
End diastolic volume [μl]	49.6±4.4	68.9±11.1	48.3±4.0	70.9±10.9	56.6±5.4	72.1±11.5	60.9±7.4	73.3±13.7
End systolic volume [μl]	23.1±4.8	40.3±9.7	21.5±4.1*	43.5±10.0*	24.0±5.3	42.1±10.2	27.9±7.6	42.3±12.1
Stroke volume [μl]	26.5±2.3	28.6±2.3	26.8±1.5	27.3±1.5	32.6±1.6	30.0±2.7	32.9±2.0	31.0±3.2
Ejection fraction [%]	56±6	46±5	57±4*	43±5*	60±5*	45±5*	56±6	45±6
Cardiac output [ml/min]	13.5±0.9	14.3±1.4	13.7±0.6	13.6±1.0	16.6±0.8	14.7±1.2	15.4±2.2	15.5±2.1
Left ventricular mass [mg]	80±5	96±8	80±4	90±7	88±6	90±4	94±9	95±6
Absolute infarct size [mm ²]	13.6±5.6	36.4±11.4	14.2±5.8	35.0±10.8	14.1±5.3	34.5±11.0	13.5±7	28.9±12.0
Relative infarct size [%]	10±4	21±5	11±4	20±5	10±3	21±6	9±4	18±7

Data presented as mean ± standard error of mean. Asterices indicate significant differences between VEGF-C (C156S) and PBS treated:
 *p≤0.05; repeated measures t-test two-tailed distribution two-sample equal variance.

5.5. Post-MI recombinant VEGF-C (C156S) administration modulates clearance of post-MI inflammatory cells

Having established that VEGF-C (C156S) therapy has a beneficial impact on cardiac function at days 14 and 21 post-MI further experiments were performed to investigate how this occurs. In various other disease models VEGF-C therapy has been observed to promote immune cell clearance, resulting in improved clinical outcomes (Cui et al., 2015, D'Alessio et al., 2014, Huang et al., 2016, Huggenberger et al., 2011). Furthermore, it has been suggested that a prolonged inflammatory response post-MI can lead to poorer outcomes including LV dilation and heart failure (Frangogiannis, 2014). I, therefore, hypothesised that VEGF-C (C156S) therapy would exert a beneficial effect through increasing LEC permeability and/or increased uptake of leucocytes into the expanded lymphatic network, thereby enhancing clearance of inflammatory cells from the post-MI heart.

VEGF-C has previously been shown to increase the permeability of human intestinal LECs by promoting the internalisation of vascular endothelial cadherin (CDH5) (Tacconi, Correale et al., 2015). CDH5 is a key adherin which provides cell-to-cell contact and maintenance of the integrity of the endothelial barrier through the formation of an intracellular protein network with catenin proteins (Dejana, Orsenigo et al., 2008). Internalisation or blockade of CDH5 destabilises endothelial cell contacts and increases the permeability of the vessels to leucocytes (Corada, Mariotti et al., 1999, Weis, Cui et al., 2004). I, therefore, hypothesised that VEGF-C (C156S) therapy may have an early effect on increasing the permeability of the cardiac lymphatics which would lead to an increase in the clearance of post-MI inflammation and interstitial fluid before an extensive lymphangiogenic response, which occurs by day 7 post-MI, had been

established. In order to test whether there is an effect on the clearance of leucocytes 100ng/g VEGF-C (C156S) injections were administered at day 0 or days 0, 2 and 3 post-MI with collection of hearts at days 1 and 4 respectively for flow cytometry analysis of immune cell numbers.

The other possibility, based on previous findings in other models (Baluk et al., 2005, D'Alessio et al., 2014, Huggenberger et al., 2011, Kataru et al., 2009), was that the increased density of the lymphatic network which occurs by day 7 post-MI would allow improved clearance of innate immune cells from the heart and drainage to regional lymph nodes, thus reducing the potential for persistent inflammation, and excessive fibrosis and scarring. In order to investigate this, flow cytometry and qRT-PCR experiments were performed at day 7 post-MI to assess the numbers of the innate immune cells and their cytokine profile within the post-MI heart. For these experiments treated animals received four 100ng/g doses of VEGF-C (C156S) treatment at days 0, 2, 4 and 6 days post-MI. This was the same dosing regime which resulted in enhanced lymphangiogenesis observed at day 7 post-MI (Section 5.3.1) and improvements in cardiac function at days 14 and 21 post-MI (Section 5.4).

5.5.1. Early VEGF-C (C156S) treatment does not modulate post-MI inflammation within the heart at days 1 and 4 post-MI

Flow cytometry was performed on whole digested hearts at days 1 and 4 post-MI in order to assess the numbers of total leucocytes (CD45⁺) and myeloid cells (CD45⁺ CD11b⁺), including neutrophils (CD45⁺ CD11b⁺ Ly6G⁺), macrophages (CD45⁺ CD11b⁺ Ly6G⁻ F4/80⁺) and dendritic cells (CD45⁺ CD11b⁺ Ly6G⁻ F4/80⁻ CD11c⁺) present. Gates were set up as shown in **Figure 5.8**. Hearts collected at day 1 post-MI had received either a single dose of VEGF-C (C156S) at day 0 (VEGF-C group) or PBS (vehicle group).

Hearts collected at day 4 post-MI had received either three doses of VEGF-C (C156S) at days 0, 2 and 3 (VEGF-C group) or vehicle. At both days 1 and 4 post-MI (**Figures 5.9** and **5.10**) no significant differences were observed between the control and VEGF-C groups in any of the cell populations suggesting at these time points there is no impact of VEGF-C (C156S) treatment on the clearance of innate immune cells from the infarcted heart at these early time points before extensive lymphangiogenesis has occurred. This suggests that there is no substantial early effect of VEGF-C (C156S) treatment on the permeability of the cardiac lymphatics or if there is such an effect it does not result in increased clearance of leucocytes from the heart at days 1 and 4 post-MI.

Figure 5.8

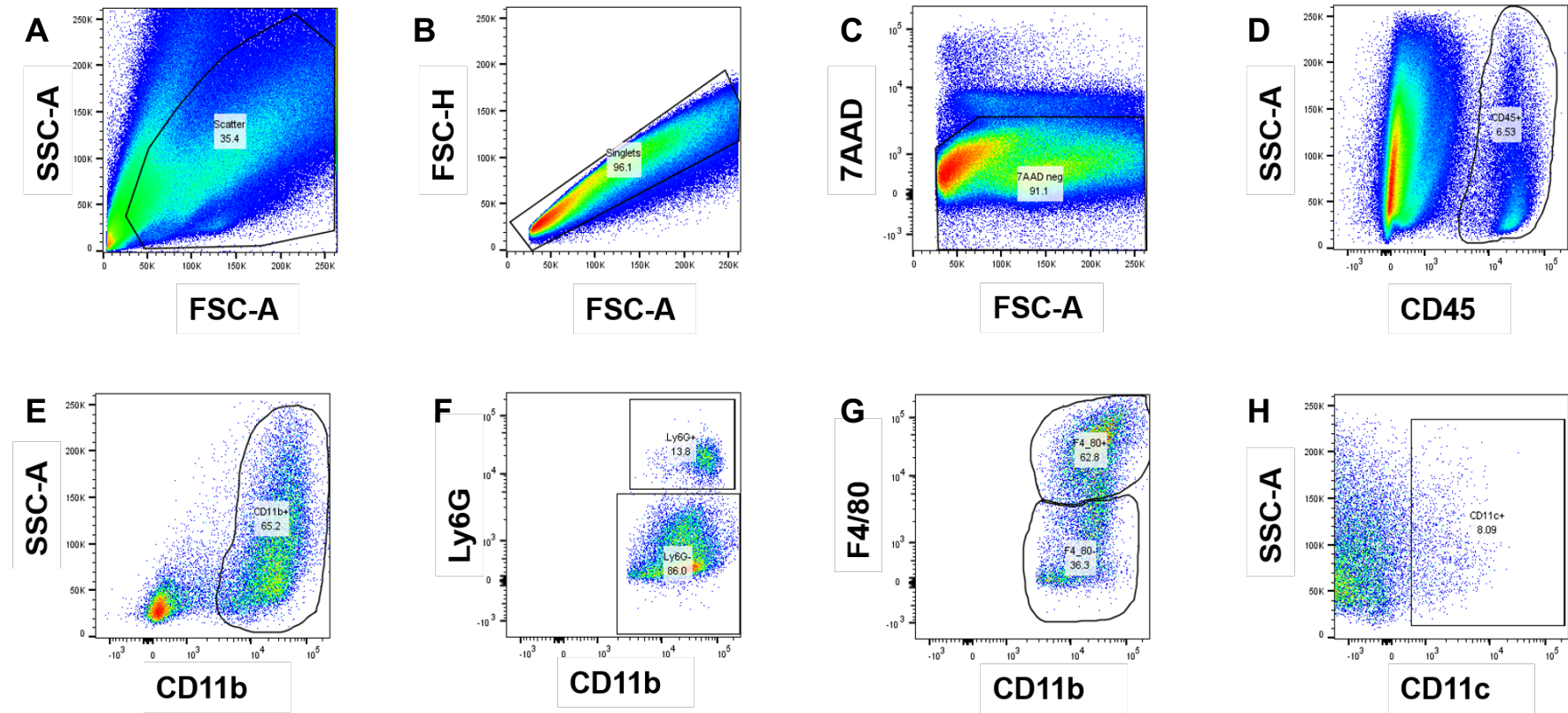


Figure 5.8: Gating strategy for flow cytometry experiments

The gates shown here were set up to measure cell populations using the following markers: 7-Aminoactinomycin D (7AAD -live/dead population), CD45 (leucocytes), CD11b (myeloid cells), Ly6G (neutrophils), F4/80 (macrophages) and CD11c (dendritic cells). Populations were expressed as percentages of scatter/singlets/live cells from digested post-MI cardiac tissue. This set-up enabled comparison of cell populations between experimental groups. SSC= side scatter, FSC= forward scatter.

Figure 5.9

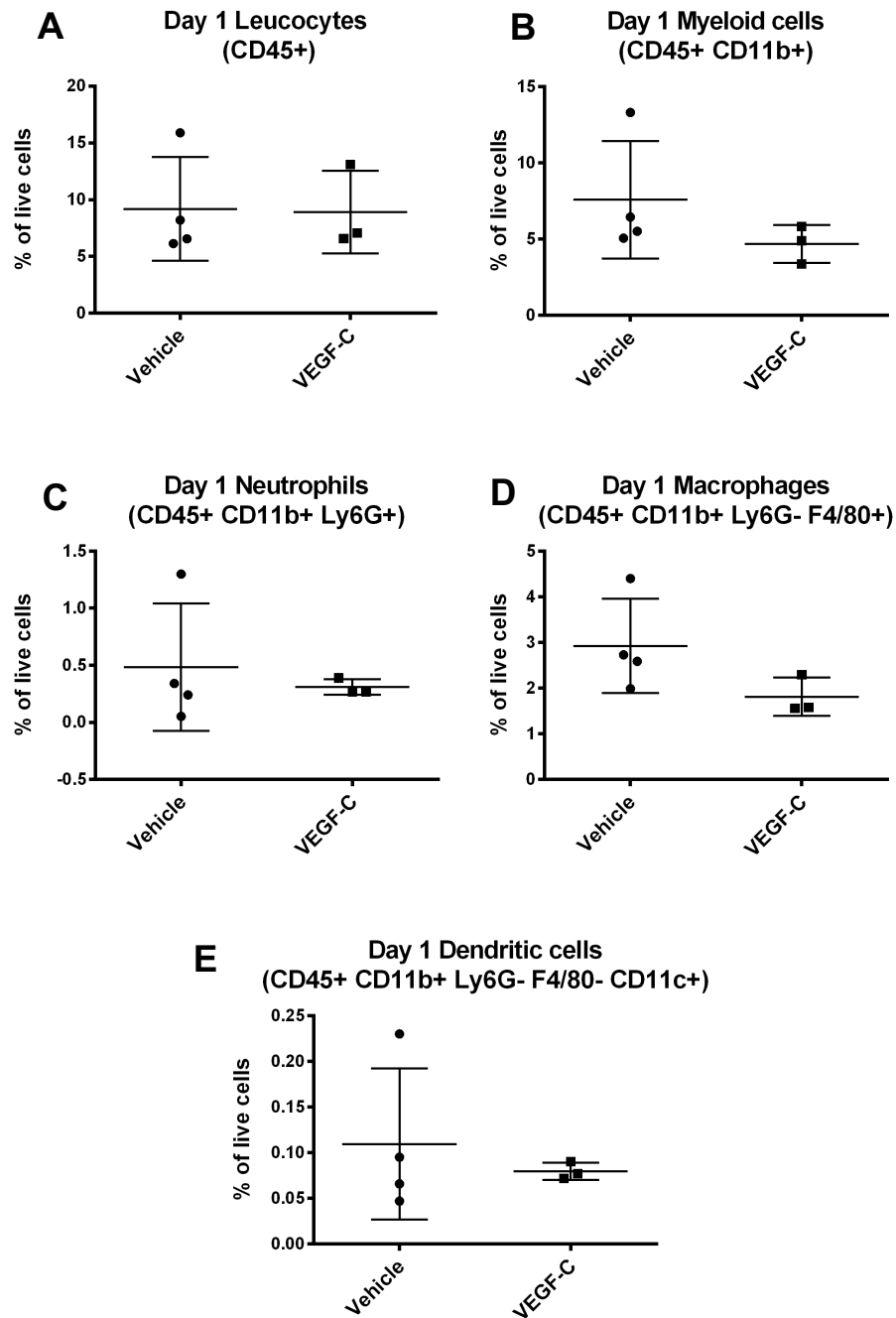


Figure 5.9: Flow cytometry for immune cells within cardiac tissue at day 1 post-MI with and without VEGF-C (C156S) treatment

Flow cytometry was performed on digested hearts at day 1 post-MI which had received a single I.P. injection of PBS (vehicle) or VEGF-C (C156S) at day 0. No significant changes were observed in the % of live cells which were leucocytes (A), myeloid cells (B), neutrophils (C), macrophages (D) and dendritic cells (E) between groups. Vehicle group n = 4, VEGF-C group n = 3. Data presented as mean $\pm$ standard error of mean. An unpaired, two-tailed t-test was used to assess statistical significance. p-values: A=0.932, B=0.275, C=0.624, D=0.144 and E=0.570.

Figure 5.10

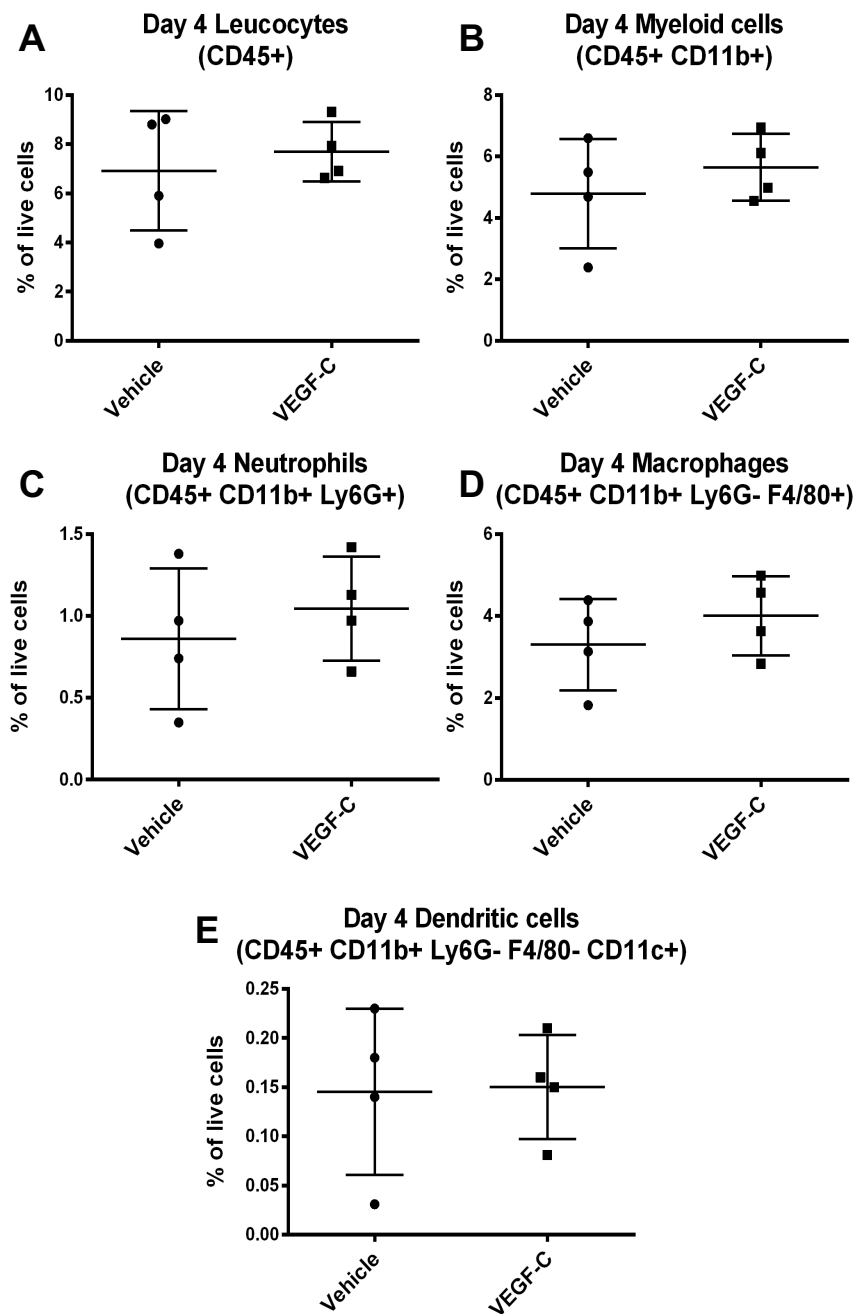


Figure 5.10: Flow cytometry for immune cells within cardiac tissue at day 4 post-MI with and without VEGF-C (C156S) treatment

Flow cytometry was performed on digested hearts at day 4 post-MI which had received I.P. injections of PBS (vehicle) or VEGF-C (C156S) at days 0, 2 and 3. No significant changes were observed in the % of live cells which were leucocytes (A), myeloid cells (B), neutrophils (C), macrophages (D) and dendritic cells (E) between groups. Vehicle group n = 4, VEGF-C group n = 4. Data presented as mean ± standard error of mean. An unpaired, two-tailed t-test was used to assess statistical significance. p-values: A=0.588, B=0.442, C=0.515, D=0.376, E=0.924.

5.5.2. Multiple doses of VEGF-C (C156S) treatment leads to a reduction in macrophages within the heart at day 7 post-MI

Flow cytometry was performed on whole digested hearts at day 7 post-MI, which had received either four doses of VEGF-C (C156S) at days 0, 2, 4 and 6 (VEGF-C group) or vehicle. A significant reduction in the percentages of total myeloid cells (% mean $\pm$ S.E.M. vehicle: 4.730 ± 0.5257 %, VEGF-C: 3.117 ± 0.4401 %, $p=0.040$) and macrophages (% mean $\pm$ S.E.M. vehicle: 3.497 ± 0.4169 %, VEGF-C: 2.238 ± 0.3390 %, $p=0.041$) were observed with VEGF-C (C156S) treatment. No statistically significant changes were observed between groups in the percentages of total-leucocytes, neutrophils and dendritic cells (**Figure 5.11**). Together these data demonstrate that fewer myeloid cells, including macrophages, remain within the post-MI heart following multiple doses VEGF-C (C156S) treatment. This suggests that more of these cells are cleared via the expanded lymphatic capillary network that is present by day 7 post-MI in the treated group.

Figure 5.11

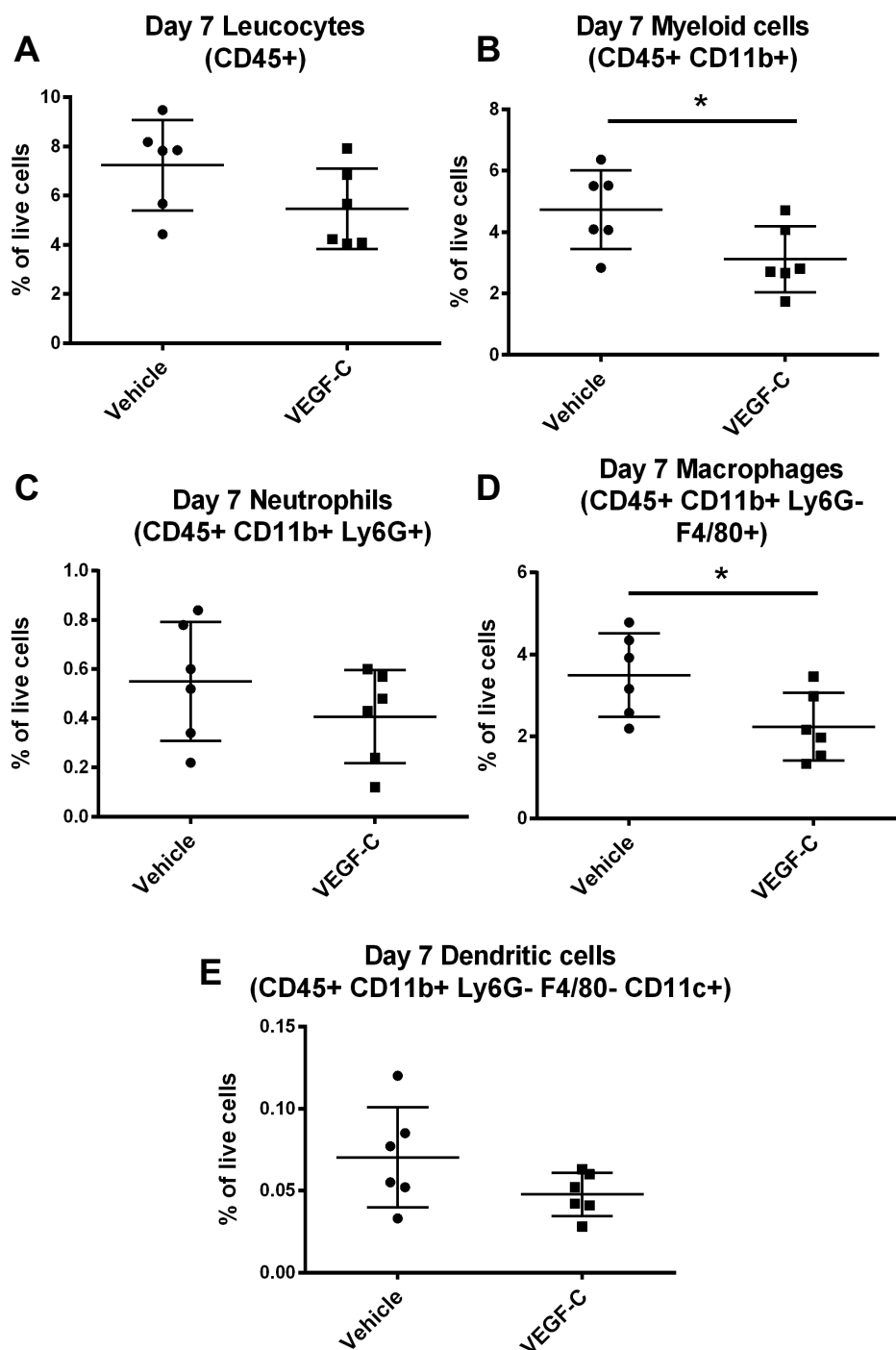


Figure 5.11: Flow cytometry for immune cells within cardiac tissue at day 7 post-MI with and without VEGF-C (C156S) treatment

Flow cytometry was performed on digested hearts at day 7 post-MI which had received I.P. injections of PBS (vehicle) or VEGF-C (C156S) at days 0, 2, 4 and 6. A significant reduction in the % of live cells which were myeloid cells (B) and macrophages (D) was observed with VEGF-C (C156S) treatment. Vehicle group $n = 6$, VEGF-C group $n = 6$. Data presented as mean $\pm$ standard error of mean. An unpaired, two-tailed t-test was used to assess statistical significance, * $p \leq 0.05$. p-values: A=0.108, B=0.040, C=0.280, D=0.041, E=0.127.

5.5.3. A single dose of VEGF-C (C156S) treatment has no significant impact on the clearance of immune cells by day 7 post-MI

The data presented in **Figure 5.11** revealed that multiple doses of VEGF-C (C156S) post-MI are sufficient to enhance the clearance of macrophages from the heart by day 7 post-MI. However, it was still unknown as to whether a single dose of VEGF-C (C156S) would be sufficient to enhance immune cell clearance, and whether injection at a particular time point would be a critical factor in stimulating enhanced lymphangiogenesis by day 7 post-MI. In order to investigate this further flow cytometry experiments were performed on hearts collected at day 7 post-MI which had received a single dose of VEGF-C (C156S) at day 0 or day 4 post-MI, or no treatment (untreated group).

Flow cytometry was performed at day 7 post-MI on whole digested hearts to quantify the numbers of leucocytes, myeloid cells, neutrophils, macrophages and dendritic cells present. Overall no significant differences were observed between the percentages of innate immune cells remaining in the heart between any of the groups at day 7 post-MI (**Figure 5.12**). These data suggest that a single dose of VEGF-C (C156S) at day 0 or day 4 is not sufficient to enhance lymphangiogenesis and immune cell clearance. This demonstrates that continued stimulation with VEGF-C (C156S) is required for adequate lymphangiogenesis to take place, which in turn leads to optimal clearance of macrophages.

Figure 5.12

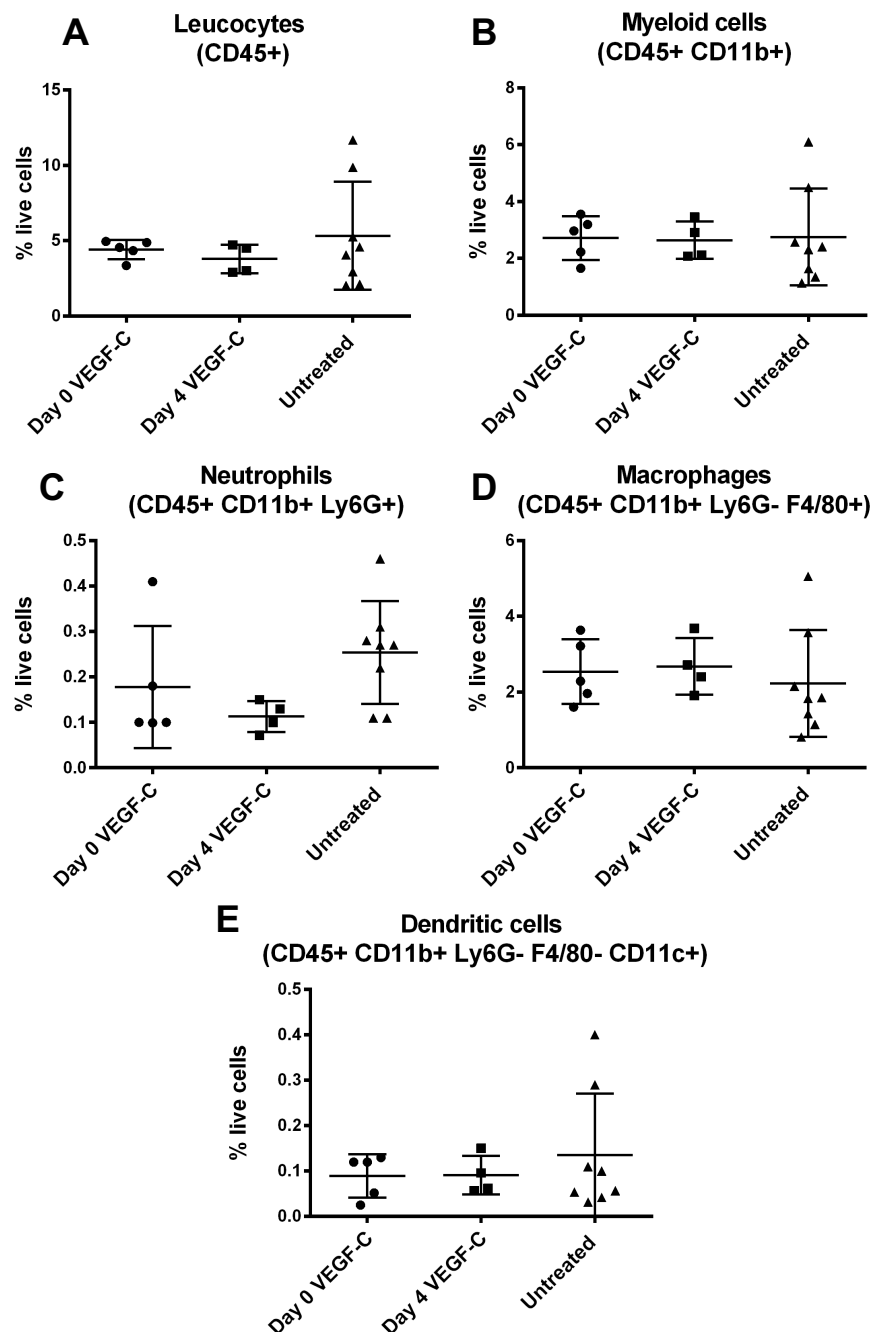


Figure 5.12: A single dose of VEGF-C (C156S) at day 0 or day 4 post-MI is not sufficient to promote enhanced clearance of immune cells by day 7 post-MI
 Animals were subjected to a single dose of VEGF-C (C156S) at day 0 or day 4 post-MI, or were given no treatment. Flow cytometry was performed on whole digested hearts at day 7 post-MI revealed no significant changes in the percentage of live cells which were leucocytes, myeloid cells, neutrophils, macrophages and dendritic cells. Day 0 VEGF-C group n = 5, day 4 VEGF-C group n = 5, untreated group n = 8. Data presented as mean $\pm$ standard deviation. A one-way ANOVA was used to assess any statistical changes between groups.

5.5.4. A single injection of VEGF-C (C156S) at day 0 is not sufficient to enhance post-MI lymphangiogenesis

A single dose of VEGF-C (C156S) is not sufficient to enhance the clearance of immune cells from the heart by day 7 post-MI, thus I hypothesised that this dose would also not be sufficient to enhance lymphangiogenesis. In order to investigate this control hearts (MI, no treatment) and hearts which were treated with a single dose of VEGF-C at day 0 post-MI were collected at day 7 post-MI. Immunofluorescent staining for VEGFR-3 or PODOPLANIN and light sheet imaging was performed to visualise the cardiac lymphatic network. A similar lymphangiogenic network was present in both control and VEGF-C (C156S) treated hearts (**Figure 5.13a-d**) which appeared of similar density, with comparable numbers of lymphangiogenic tips (marked by white arrowheads in **Figure 5.13b, d**). The lymphatic network in hearts treated with a single dose VEGF-C (C156S) did not appear to undergo increased lymphangiogenesis as previously observed in *Vegfr3^{LacZ/+}* animals at day 7 post-MI following four doses of VEGF-C (C156S). These findings confirm that a single 100ng/g dose of VEGF-C (C156S) at day 0 post-MI is not sufficient to enhance lymphangiogenesis, which supports the hypothesis that increasing the density of lymphatic capillaries leads to increased clearance of immune cells from the heart.

Figure 5.13

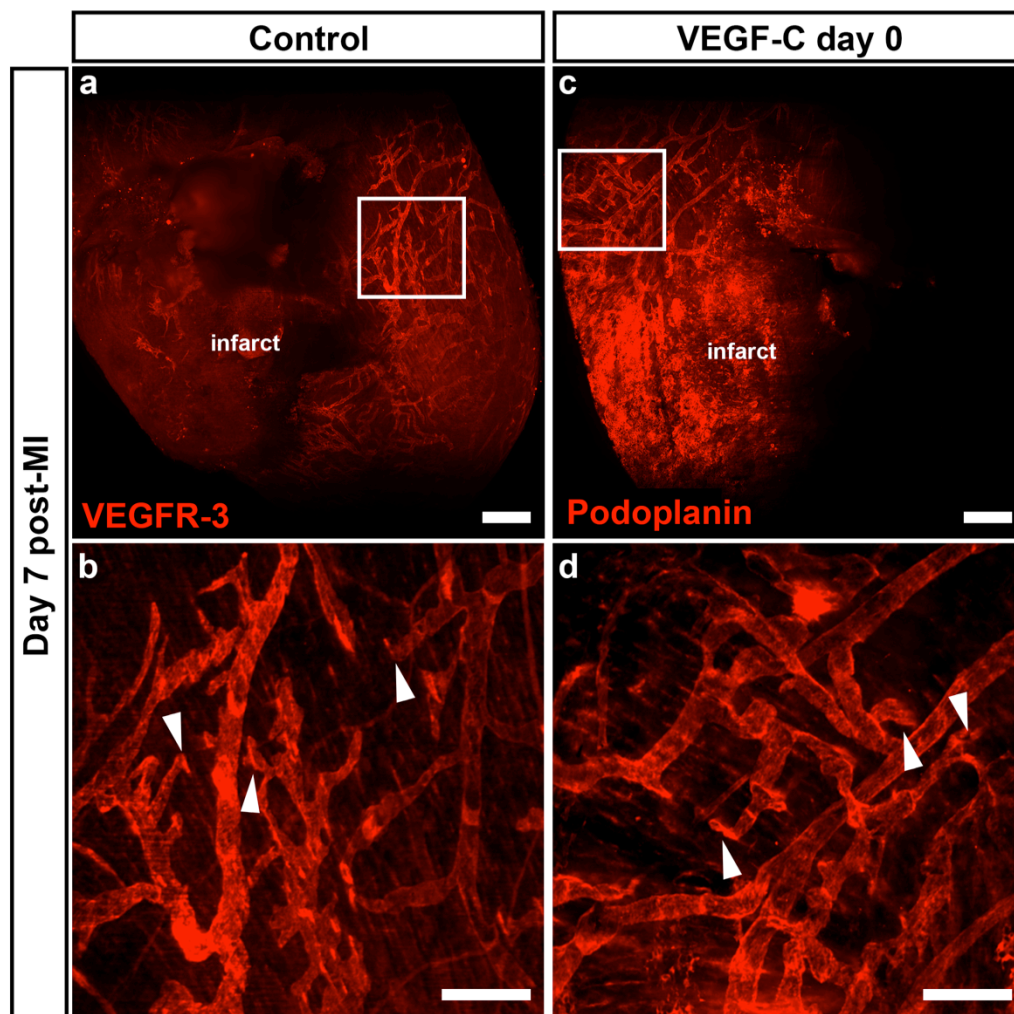


Figure 5.13: A single dose of VEGF-C (C156S) is not sufficient to enhance post-MI lymphangiogenesis

Whole-mount immunofluorescent staining and light sheet imaging was performed on hearts at day 7 post-MI. VEGFR-3 staining on control hearts revealed the superficial cardiac lymphatic plexus with lymphangiogenic capillary tips (**a**, white box enlarged in **b**, white arrowheads indicate example lymphangiogenic capillary tips). Hearts which had received a single dose of VEGF-C (C156S) at day 0 post-MI displayed a similar lymphangiogenic PODOPLANIN⁺ capillary network (**c**, white box enlarged in **d**, white arrowheads indicate example lymphangiogenic tips) which did not appear to be significantly denser than the network observed in controls. Scale bars **a**, **c** = 500 μ m, **b**, **d** = 200 μ m. n = 3-5 hearts per group.

5.5.5. Inflammatory profile of macrophages remaining in the heart following post-MI VEGF-C (C156S) treatment

Having established that a reduced number of macrophages are present in the heart at day 7 post-MI following multiple doses of VEGF-C (C156S) treatment (Section 5.5.2), work was undertaken to determine the molecular phenotype of the remaining macrophage population. In particular, whether this was mainly composed of inflammatory (M1) macrophages, reparative (M2) macrophages or a combination of both.

qRT-PCR for M1/M2 markers and macrophage expressed cytokines was performed on FACS-isolated CD45⁺ CD11b⁺ Ly6G⁻ F4/80⁺ macrophages (gating strategy shown in **Figure 5.8**) at day 7 post-MI from whole-digested hearts which had received either four doses of VEGF-C (C156S) at days 0, 2, 4 and 6 post-MI or no treatment (control). A list of the genes analysed are included in **Table 5.14**. The post-MI roles of inflammatory and reparative macrophages and the key cytokines they release were previously introduced in section 1.4.2. I hypothesised that increased clearance of inflammatory macrophages in treated hearts would result in a higher proportion of reparative macrophages remaining within the heart that would express higher levels of M2 markers and pro-repair cytokines: *Cd206*, *Chi3l3*, *Il10* and *Tgfb1* (Gallego-Colon et al., 2015, Murray & Wynn, 2011, Shiraishi et al., 2016). Conversely, if there were increased clearance of reparative macrophages, due to preference for uptake of this cell type by lymphatic vessels, this could result an increased number of inflammatory macrophages remaining within the heart with higher mRNA expression levels of *Cd80*, *Cd86*, *Il6*, *Il1b* and *Tnf* (de Couto, Liu et al., 2015, Murray & Wynn, 2011, Pinto et al., 2014). The alternative hypothesis to both of these situations would be that both inflammatory and

reparative macrophages are able to be cleared, meaning there would be little change in the ratio of inflammatory:reparative gene expression.

Table 5.14. Macrophage markers and cytokines used for qRT-PCR profiling

Gene	Property
<i>Cd80</i>	M1 macrophage marker
<i>Cd86</i>	M1 macrophage marker
<i>Cd206</i>	M2 macrophage marker
<i>Chi3l3</i>	M2 macrophage marker
<i>Il1b</i>	pro-inflammatory cytokine
<i>Il6</i>	pro-inflammatory cytokine
<i>Il10</i>	anti-inflammatory cytokine
<i>Tgfb1</i>	pro-fibrotic cytokine
<i>Tnf</i>	pro-inflammatory cytokine

The qRT-PCR data revealed similar expression levels of *Il6*, *Il1b* and *Il10* in macrophages from both the control and VEGF-C (C156S) treated groups (**Figure 5.15**). Cycle threshold (CT) values obtained for *Tnf*, *Tgfb1*, *Cd80*, *Cd86*, *Cd206* and *Chi3l3* were greater than 30 indicating these genes are expressed only at very low levels, preventing quantification by qRT-PCR. No change the expression levels of the pro-inflammatory cytokines *Il6* and *Ilb* was observed between groups, nor a change between the expression levels of the anti-inflammatory cytokine *Il10*. This could indicate that there are similar proportions of inflammatory/reparative macrophages which are cleared via the lymphatic vessels. As such, the effect of VEGF-C (C156S) induction on lymphangiogenesis is likely to reduce the overall macrophage load in the heart. However, it is important to consider the limitations of this data set given the very low expression levels of many of the cytokines and M1/M2 markers.

Figure 5.15

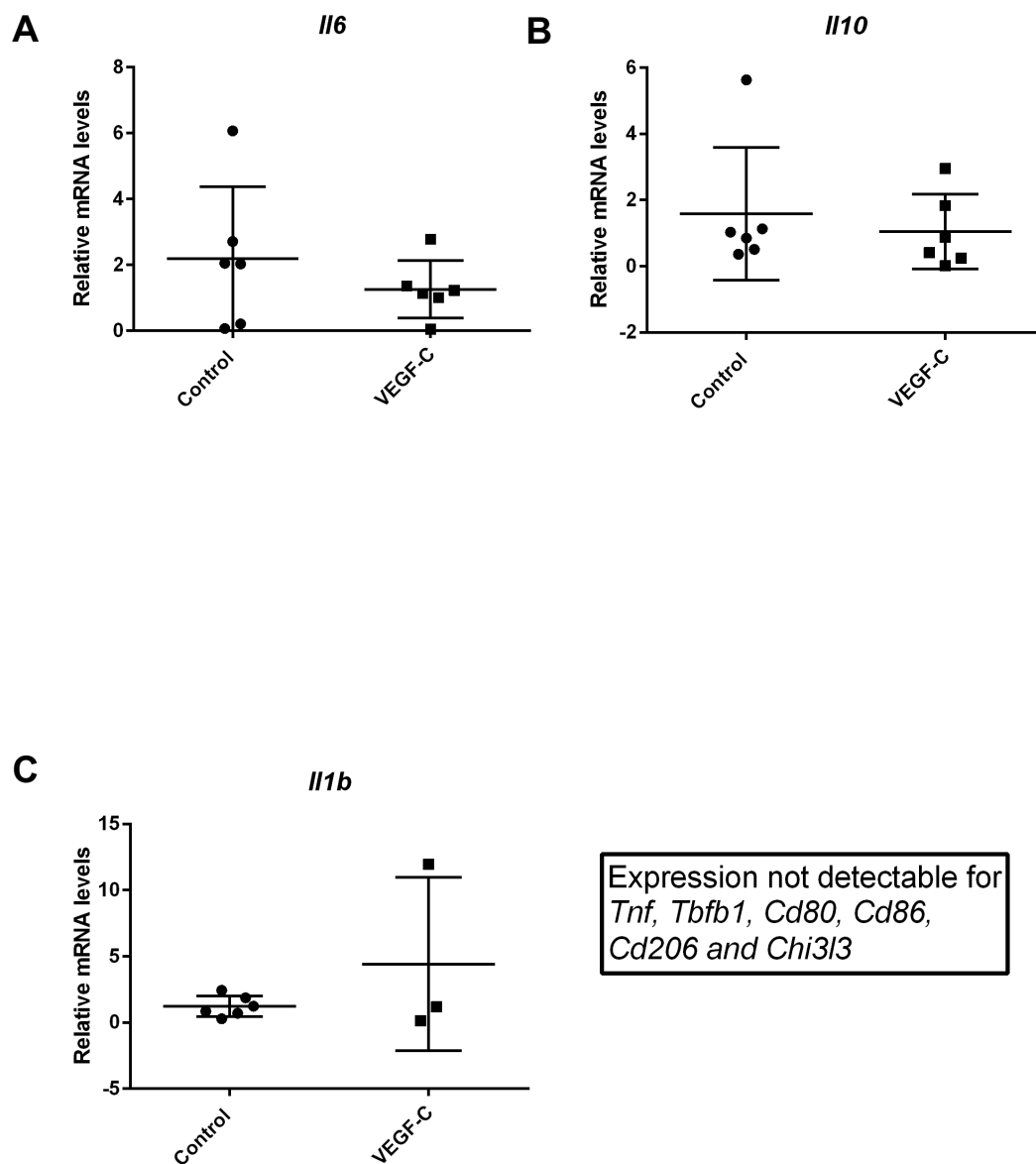


Figure 5.15: qRT-PCR analysis for key inflammation and fibrosis genes in day 7 post-MI FACS-isolate macrophages with and without VEGF-C (C156S) treatment

qRT-PCR was performed on cDNA from sorted cardiac macrophages collected by FACS at day 7 post-MI. Animals received either no treatment (control) or I.P. injections of VEGF-C (C156S) at days 0, 2, 4 and 6 post-MI. A panel of inflammation, fibrosis and M1/M2 genes were tested, pro-inflammatory: *Il6*, *Il1b*, *Tnf*, anti-inflammatory/pro-resolution: *Il10*, *Tgfb1*, M1: *Cd80*, *Cd86*, M2: *Cd206*, *Chi3l3*. No statistically significant changes were observed in the mRNA expression of *Il6* (A), *Il10* (B) and *Il1b* (C) between groups. mRNA expression levels of *Tnf*, *Tgfb1*, *Cd80*, *Cd86*, *Cd206* and *Chi3l3* were too low to detect (CT values > 30). Control group n = 6, VEGF-C group n = 6 (some data points not shown due to undetectable expression). Data presented shows all data points with mean $\pm$ standard deviation. An unpaired, two-tailed t-test was used to assess statistical significance. p-values: A=0.356, B=0.584 and C=0.245.

5.5.6. Inflammatory profile of the infarcted myocardium following post-MI VEGF-C (C156S) treatment

Due to the low levels of expression of many of the M1/M2 markers and cytokine genes in the FACS-isolated macrophages, an alternative approach was taken to investigate whether there may be a preference towards clearance of inflammatory versus reparative macrophages. Infarcts were dissected from hearts at day 7 post-MI that had received either four doses of VEGF-C (C156S) or no treatment. qRT-PCR was performed to detect levels of the M1 markers/pro-inflammatory cytokines (*Cd80*, *Cd86*, *Il6*, *Il1b*, and *Tnf*) and the M2 markers/anti-inflammatory/pro-fibrosis cytokines (*Cd206*, *Chi3l3*, *Il10* and *Tgfb1*). Five collagen genes were included in the panel, including *Col1a1*, *Col1a2*, *Col6a1*, *Col4a1* and *Col3a1*. These genes were included to assess the hypothesis that a reduction in the number of reparative macrophages within the heart would lead to a reduction in the levels of *Tgfb1* within the infarct, and, therefore, a reduction in the level of collagen production by fibroblasts.

qRT-PCR performed on infarcts isolated at day 7 post-MI revealed no statistically significant changes in the relative mRNA levels of the M1 marker genes *Cd80* and *Cd86*, nor the M2 marker genes *Cd206* and *Chi3l3* within the infarcts of control and VEGF-C (C156S) treated groups (**Figure 5.16**). Furthermore, no statistically significant changes were observed between the relative mRNA levels of *Il1b*, *Il6*, *Il10*, *Tgfb1* or *Tnf* between VEGF-C (C156S) animals and controls (**Figure 5.17**). mRNA expression levels of the collagen genes *Col1a1*, *Col1a2*, *Col6a1*, *Col4a1* and *Col3a1* were also similar when comparing between untreated and VEGF-C (C156S) treated samples (**Figure 5.18**). Together these data indicate that at day 7 post-MI, despite increasing the clearance of macrophages from the heart, VEGF-C (C156S) treatment has no significant impact on the cytokine profile or collagen gene expression within the infarcted tissue.

Figure 5.16

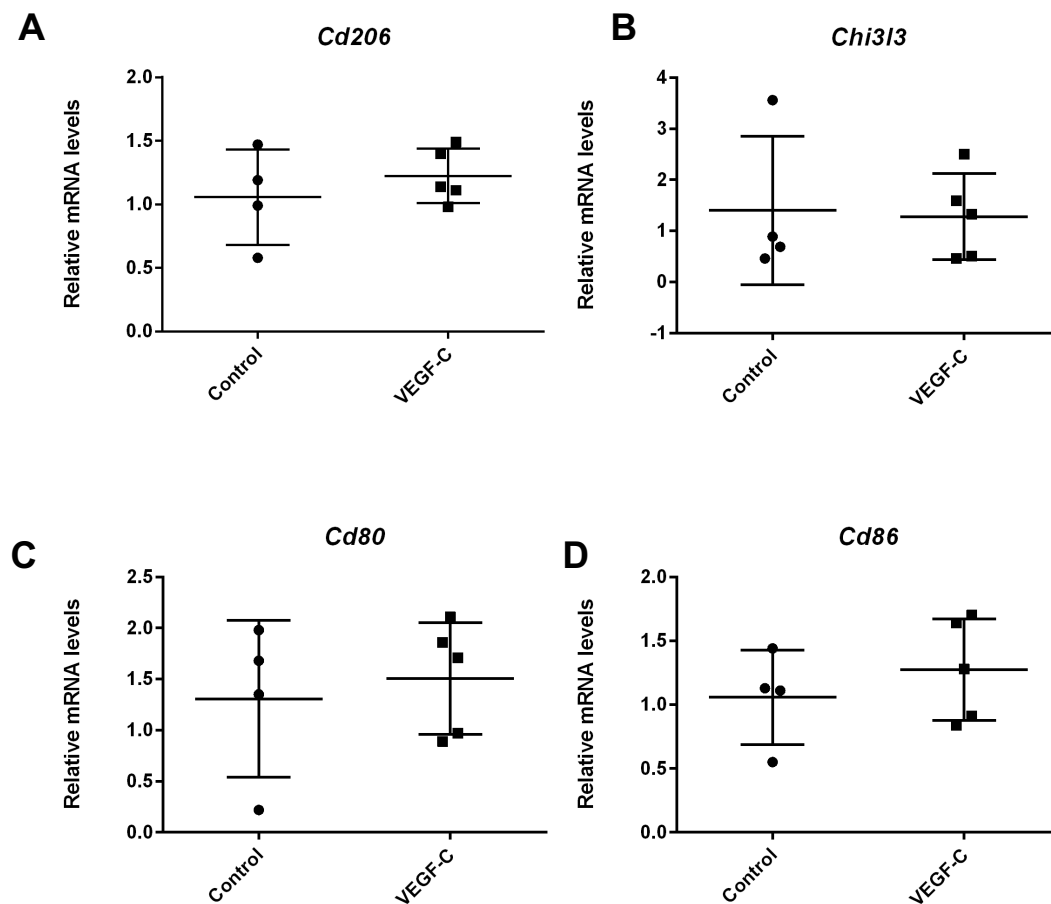


Figure 5.16: Post MI VEGF-C (C156S) treatment does not significantly affect the expression of M1 and M2 macrophage markers within the infarcted region at day 7 post-MI

qRT-PCR was performed on isolated infarcted tissue at day 7 post-MI from animals that had received either no treatment (control group) or VEGF-C (C156S) (VEGF-C group) via I.P injection at days 0, 2, 4, 6 post-MI. No statistically significant changes were observed in the expression of M2 markers *Cd206* (A) *Chi3l3* (B) nor M1 markers *Cd80* (C) *Cd86* (D) between groups. Control group n = 4, VEGF-C group n = 5. Data presented as mean $\pm$ standard error of mean. An unpaired, two-tailed t-test was used to assess statistical significance. p-values: A=0.425, B=0.878, C=0.661 and D=0.432.

Figure 5.17

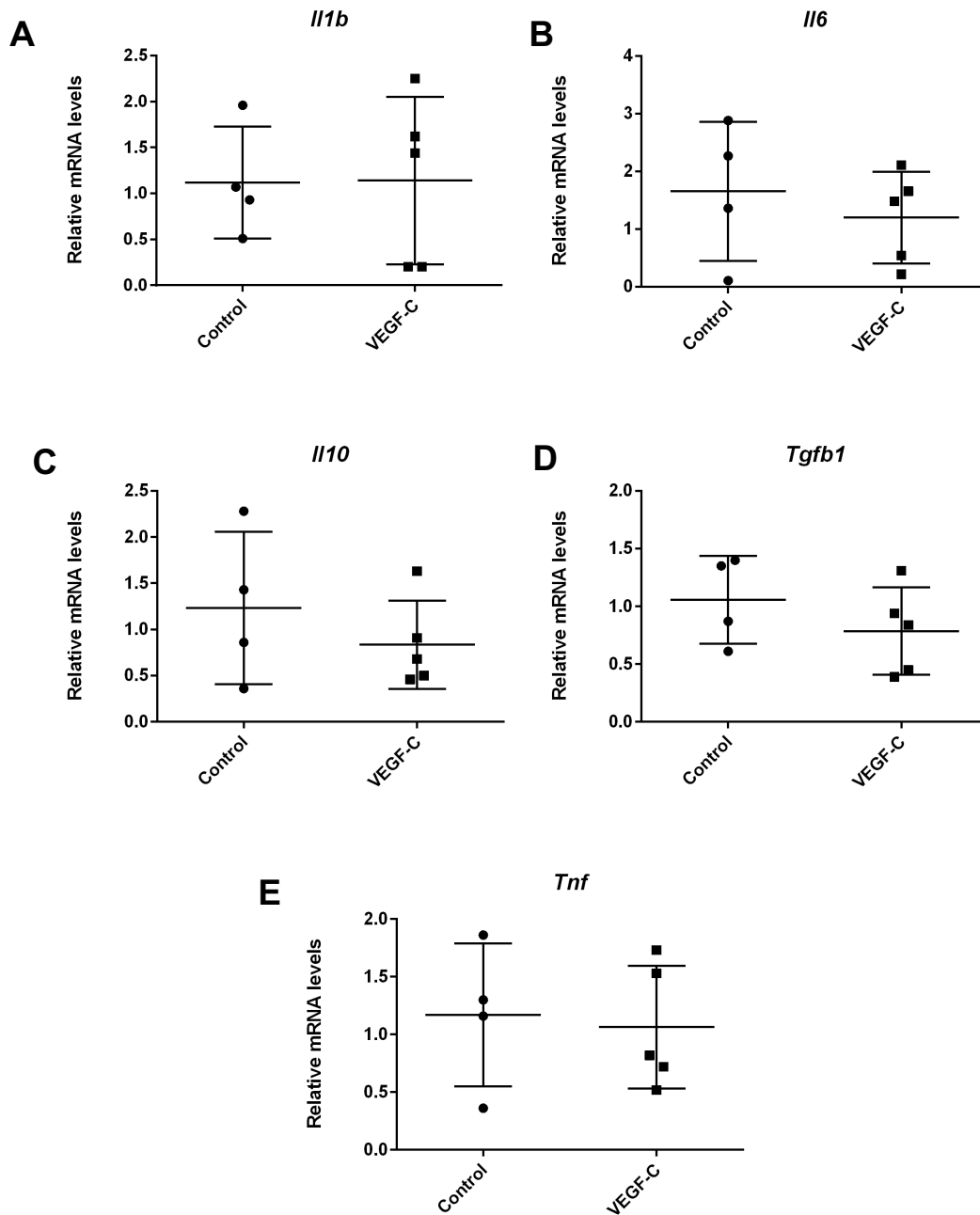


Figure 5.17: Post MI VEGF-C (C156S) treatment does not significantly affect the inflammatory profile within the infarcted region at day 7 post-MI

qRT-PCR was performed on isolated infarcted tissue at day 7 post-MI from animals that had received either no treatment (control group) or VEGF-C (C156S) (VEGF-C group) via I.P injection at days 0, 2, 4, 6 post-MI. No statistically significant changes were observed in the expression of pro-inflammatory markers (*Il1b*, *Il6*, *Tnf*) nor anti-inflammatory/pro-fibrotic markers (*Il10*, *Tgfb1*) between groups. Control group n = 4, VEGF-C group n = 5. Data presented as mean $\pm$ standard error of mean. An unpaired, two-tailed t-test was used to assess statistical significance. p-values: A=0.965, B=0.517, C=0.393, D=0.322 and E=0.790.

Figure 5.18

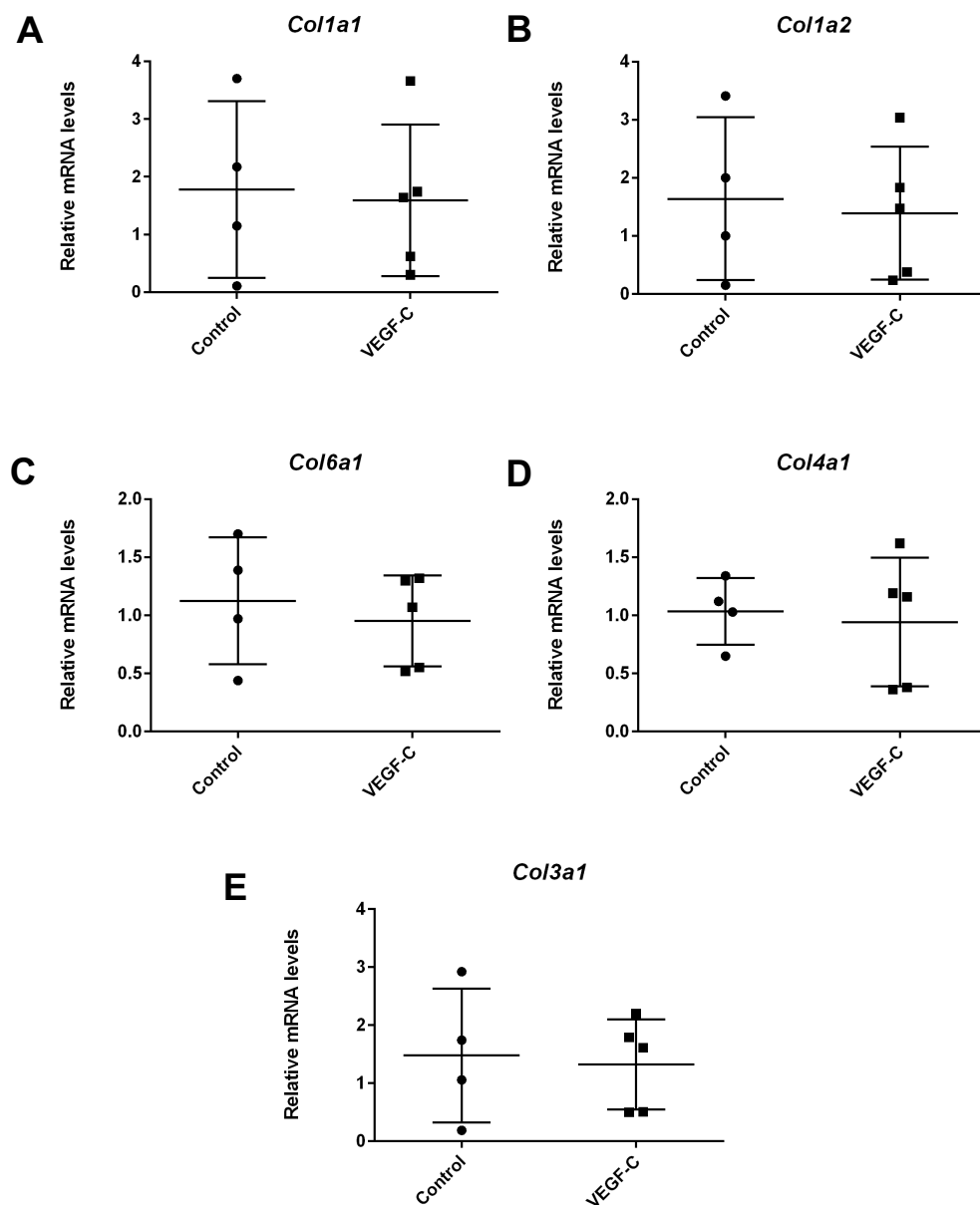


Figure 5.18: Post MI VEGF-C (C156S) treatment does not affect the expression of collagen genes within the infarct at day 7 post-MI

qRT-PCR was performed on isolated infarcted tissue at day 7 post-MI from animals that had received either no treatment (control group) or VEGF-C (C156S) (VEGF-C group) via I.P injection at days 0, 2, 4, 6 post-MI. No significant changes were observed in the expression of the collagen genes *Col1a1*, *Col1a2*, *Col6a1*, *Col4a1* or *Col3a1* between groups. Control group n = 4, VEGF-C group n = 5. Data presented as mean $\pm$ standard error of mean. An unpaired, two-tailed t-test was used to assess statistical significance. p-values: A=0.846, B=0.778, C=0.596, D=0.771 and E=0.815.

5.6. Discussion

5.6.1. Visualisation of macrophages within cardiac lymphatic vessels

Previous studies have demonstrated that leucocytes can be cleared by the lymphatic vasculature during inflammation (Johnson et al., 2006, Lawrance et al., 2016, Randolph et al., 2005, Rigby et al., 2015). In this chapter I revealed that a small number of CD68⁺ macrophages are present within the LYVE-1⁺ lymphatic endothelium, suggesting that macrophages are taken up by cardiac lymphatics. However, it was not possible to unequivocally identify the lumen of the cardiac lymphatics vessels which are only open when an accumulation of interstitial fluid is present. The images presented, coupled with the previous literature documenting macrophage entry into lymphatic vessels, strongly suggests that cardiac lymphatic vessels do traffic macrophages in the heart post-MI. To definitely prove this live intravital imaging would need to be performed *in vivo* in order to directly visualise the trafficking of macrophages and other leucocytes in the cardiac lymphatic vessels, this is discussed further in chapter 8. Another alternative would be to detect trafficked macrophages in the mediastinal lymph nodes, to which the cardiac lymphatics drain, this is also discussed in more detail in chapter 8.

A second observation from the images at day 4 post-MI was the presence of CD68⁺ LYVE-1⁺ cells which appeared to be macrophages lining up in a tube like structure to form the tip of a new lymphatic vessel. There have been previous reports of macrophage to LEC transdifferentiation in the context of inflammation-induced lymphangiogenesis (Hall et al., 2012, Kataru et al., 2009, Kerjaschki et al., 2006, Kerjaschki et al., 2004, Maruyama et al., 2005, Zumsteg et al., 2009), which supports the possibility that macrophages may provide a lineage contribution during the post-MI expansion of the lymphatic network in the heart. In order to confirm this observation further work would be required, including lineage tracing, this is discussed further in chapter 8.

5.6.2. Expression of VEGF-C within the epicardium and macrophages in the post-MI heart

Prior work performed by Linda Klotz in the Riley group had revealed that the cardiac lymphatics undergo extensive lymphangiogenesis in response to MI, however, the driver of this network expansion had not been identified. Here, I have demonstrated that VEGF-C, a known key lymphangiogenic protein, is secreted from the activated epicardium and a subset of macrophages following MI. Secretion of VEGF-C from the epicardium likely reflects a recapitulation of foetal gene programs, as VEGF-C is secreted from the epicardium during cardiac development (Chen et al., 2014a, Chen et al., 2014b) and the epicardium is known to be reactivated upon injury (Huang, Thatcher et al., 2012, Limana, Bertolami et al., 2010, Mercer, Odelberg et al., 2013, van Wijk, Gunst et al., 2012, Wang, Karra et al., 2013, Zhou, Honor et al., 2011). VEGF-C is also secreted from macrophages during states of inflammation elsewhere in the body (Granata et al., 2010, Kataru et al., 2009) and my data reveals that this mechanism is conserved in the heart. The observation that only a subpopulation of macrophages secrete VEGF-C may reflect the differential expression by subsets of macrophages; higher levels of VEGF expression have previously been observed in Ly6C^{lo} /M2 monocytes/macrophages than in Ly6C^{hi} /M1 monocytes/macrophages (Nahrendorf et al., 2007).

5.6.3. Effect of post-MI VEGF-C (C156S) administration on lymphangiogenesis and cardiac function

I have demonstrated that administration of multiple doses of VEGF-C (C156S) post-MI resulted in a denser, more extensive lymphatic network around the infarcted region. This finding is in agreement with several studies in other models which found that delivery of adenoviral or recombinant VEGF-C resulted in increased lymphangiogenesis and lymphatic function (Anisimov et al., 2009, Cui et al., 2015, D'Alessio et al., 2014,

Lahtenvuo et al., 2011, Rissanen et al., 2003), and that a blockade of the VEGFR-3 signalling pathway attenuates lymphangiogenesis and lymphatic draining (D'Alessio et al., 2014). Furthermore my findings are supported by a recent study based on an ischaemia reperfusion model in the rat heart. Here it was reported that intra-ventricular delivery of VEGF-C (C156S) resulted in an increased number of epicardial lymphatic capillaries at 3 weeks post-injury and later an increase in the frequency of epicardial pre-collectors at 8 weeks post-injury (Henri et al., 2016).

The cardiac MRI data presented in this chapter revealed that multiple injections of VEGF-C (C156S) during the first week following MI resulted in improved LVEF at days 14 and 21 post-MI. However, at day 28 post-MI, the end point for this experiment, the improvement was no longer statistically significant. The loss of significance at day 28 in this study may simply reflect the high levels of variability within the relatively small experiments groups of eight animals and it is, therefore, difficult to ascertain from this result whether or not VEGF-C (C156S) treatment has a long term effect on cardiac function. Henri et al. have also investigated the effect of VEGF-C (C156S) treatment on post-MI cardiac function in the rat and reported contradictory results using two experimental methods (Henri et al., 2016). They reported at 6 weeks post-MI that echo revealed no improvement in fractional shortening in the treated group when compared to controls, however, at 8 weeks haemodynamic assessment by left ventricular catheterisation revealed a significant improvement in the diastolic function in the treated group. To conclusively determine whether or not post-MI VEGF-C (C156S) treatment does have a lasting effect on cardiac function, additional longitudinal MRI studies with larger experiments groups and a longer time scale would be required.

5.6.4. Assessment of inflammation in post-MI VEGF-C (C156S) treated animals

I hypothesised that the improvement in cardiac function observed at days 14 and 21 post-MI in the VEGF-C (C156S) treated group may be due to improved clearance of inflammatory cells. Increased clearance of inflammatory cells via the lymphatics could prevent the persistence of inflammation and the associated increase adverse remodelling, fibrosis and poor functional outcomes. Another explanation for improved cardiac function is that additional lymphangiogenesis may be beneficial for the clearance of tissue fluid which otherwise accumulates causing myocardial oedema and cardiac dysfunction (Mehlhorn, Geissler et al., 2001). The latter hypothesis has already been investigated in the work published by Henri et al., where they demonstrated that treatment with VEGF-C (C156S) resulted in decreased water content in the heart at 8 weeks post-injury (Henri et al., 2016). For that reason I focused on the possibility that post-MI VEGF-C (C156S) treatment could result in enhanced immune cell clearance. This could happen by increasing the permeability of LECs and/or through increased leucocyte uptake into denser the lymphatic network.

In order to test whether there was an early pre-lymphangiogenesis effect of VEGF-C (C156S) therapy on immune cell clearance via increased LEC permeability post-MI animals were treated at day 0 or days 0, 2 and 3 and collected at days 1 and 4, respectively. The timing of treatments and collection of heart was designed to occur before significant lymphangiogenesis had occurred. At both days 1 and 4 post-MI no significant effects on the percentages of innate immune cells remaining within the hearts were detected. There are several explanations for the observation that no significant improvement in immune cell clearance occurs by these time points. The VEGF-C (C156S) treatment regime may not be sufficient to increase the permeability of the cardiac lymphatics. To investigate this further immunofluorescent staining and

confocal imaging should be performed to assess CDH5 expression in the lymphatic endothelium of post-MI hearts following VEGF-C (C156S) treatment; a reduction in expression or internalisation of the protein would suggest that LEC permeability is increased. If the early VEGF-C (C156S) treatment is sufficient to increase LEC permeability the lack of increased immune cell clearance may be explained by the fact that days 1 and 4 represent early time points in the post-MI inflammatory process. At these early time points neutrophils and inflammatory macrophages are actively performing crucial roles in the clearance of necrotic cells and debris so it is possible that any signalling required for them to exit the heart is not yet active.

At day 7 post-MI, significantly reduced numbers of myeloid cells, including macrophages, were observed in the heart following VEGF-C (C156S) treatment at days 0, 2, 4 and 6. These data suggested that by this time the denser, more extensive lymphatic network formed following VEGF-C (C156S) treatment is sufficient to allow increased clearance of macrophages from the injured heart. This finding is also supported by Henri et al. (2016) who reported a reduced number of CD68⁺ macrophages within the left ventricle of the rat by 3 weeks post-MI with VEGF-C (C156S) treatment. Furthermore, studies in other disease models have also reported an increased clearance of macrophages via the lymphatic vessels with VEGF-C treatment (Cui et al., 2015, D'Alessio et al., 2014).

I also performed experiments to determine whether a single injection of VEGF-C (C156S) treatment at day 0 or day 4 would be sufficient to enhance post-MI lymphangiogenesis and increase clearance of immune cells from the heart by day 7 post-MI. However, I found that a single 100 ng/g injection of VEGF-C (C156S) at day 0 or 4 did not lead to significantly reduced numbers of innate immune cells within the

heart by day 7 post-MI, suggesting that an enhanced lymphangiogenic response had not been induced; this was confirmed by immunofluorescent staining.

Together these data reveal that multiple doses of VEGF-C (C156S) therapy are required to promote optimal lymphangiogenesis by day 7 post-MI and this event is coupled with an increased clearance of macrophages from the heart. Recent studies, as discussed in section 1.4.2., have indicated that reducing the number of pro-inflammatory macrophages in the heart following MI and increasing the number of reparative macrophages, or the factors they release, may be beneficial for cardiac healing. However, persistence of reparative macrophages may also result in excessive fibrosis, leading to cardiac stiffness and diastolic dysfunction (Hulsmans et al., 2016). Therefore, an overall reduction in the number of macrophages in the heart, as I have observed with VEGF-C (C156S) treatment, may prevent the persistence of pro-inflammatory cytokines and pro-fibrotic factors, which in excess can have a negative impact on the healing process.

I performed qRT-PCR on FACS-isolated F4/80⁺ macrophages from vehicle and VEGF-C (C156S) treated hearts at day 7 post-MI to characterise how the expression profiles of the macrophages which remain in the heart following VEGF-C (C156S) treatment are effected in their polarisation. The results of this experiment revealed similar levels of *Il6*, *Il10* and *Il1b* mRNA expression in macrophages from control and VEGF-C (C156S) treated animals, suggesting that the cytokine profile of macrophages remains similar with treatment. mRNA expression levels of *Tnf*, *Tgfb1*, *Cd80*, *Cd86*, *Cd206* and *Chi3l3* were not quantifiable by qRT-PCR as only CT values above 30 were obtained, indicating that expression levels of these genes are very low. One possible explanation for this may be that only small quantities of RNA (concentration ranged between 2 and 130 ng/ μ l)

were obtained from the sorted macrophages which subsequently required amplification for cDNA synthesis using the Quantitect Whole Transcriptome kit (Qiagen). Interestingly a recent study using a model of inflammatory bowel disease revealed that VEGF-C not only modulates inflammation via increased clearance of immune cells by the lymphatics, but also acts directly, via the STAT signalling pathway, to modulate the polarisation of macrophages. VEGF-C was found to induce a hybrid phenotype in colonic macrophages, where both high levels of M1 and M2 genes were expressed (D'Alessio et al., 2014). These M1/M2 hybrid macrophages have been reported to play an important role in the resolution of inflammation (Bystrom, Evans et al., 2008, Stables, Shah et al., 2011). Further investigations would be required to determine whether such an effect also occurs in the heart.

Due to the technical difficulties experienced with profiling gene expression in sorted macrophages, whole infarcts were also collected for qRT-PCR at day 7 post-MI following multiple doses of VEGF-C (C156S) or no treatment. No statistically significant changes were observed in mRNA expression levels of M1/M2 markers, cytokines nor collagen genes. It is surprising that there is no change in the mRNA expression inflammatory cytokines nor the collagen genes given that there are fewer macrophages present within the heart which would suggest the overall levels of macrophage expressed cytokines would be reduced. One explanation for this is that changes in the mRNA expression levels of cytokines and collagen genes may be difficult to dissect out due to the large, heterogeneous population of cell types and ECM within the infarct. Another possibility is that a change in the factors macrophages are secreting may happen later than day 7 post-MI which is supported by the recently published finding that VEGF-C (C156S) treatment completely prevents cardiac fibrosis by 8 weeks post-MI as assessed by sirius red staining for collagen on sections (Henri et al., 2016). With multiple doses

of VEGF-C (C156S) treatment I observed an overall reduced number of macrophages in the heart at day 7 post-MI which was coupled with improved left-ventricular ejection fraction at days 14 and 21 post-MI. Together these data suggest that reducing the overall number of macrophages in the heart by day 7 post-MI with VEGF-C (C156S) treatment is beneficial, at least in the short-medium term, for cardiac healing.

5.7. Conclusions

The work presented in this chapter has further characterised the endogenous lymphangiogenic response of the cardiac lymphatic vessels that occurs following MI and has investigated the effect of VEGF-C (C156S) treatment on this response. It has been shown here that the activated epicardium and a subset of macrophages are able to act as sources of endogenous VEGF-C post-MI which stimulates lymphangiogenesis following injury. Furthermore, administration of multiple doses of recombinant VEGF-C (C156S) was found to enhance the post-MI lymphangiogenic response. The presence of a more extensive, denser lymphatic network resulted in increased clearance of myeloid cells including macrophages by 7 days post-MI, thereby reducing the overall macrophage load in the heart, which may prevent the build-up of inflammatory cells and associated excessive fibrosis and scarring. Cardiac LVEF was also observed to improve at days 14 and 21 with VEGF-C (C156S) treatment. However, further MRI studies are required to establish whether this is long-lasting effect.

6. Results IV: Genetic perturbation of the cardiac lymphatics to assess their post-myocardial infarction roles

In chapter 5 I showed that administration of VEGF-C (C156S) following MI results in enhanced lymphangiogenesis and an improvement in cardiac function. At day 7 post-MI reduced numbers of myeloid cells, including macrophages, remained in the heart following VEGF-C (C156S) treatment, suggesting improved clearance via the lymphatic vasculature. To further test the hypothesis that innate immune cells are cleared by cardiac lymphatic vessels following MI, two additional mouse models, *LYVE-1*^{-/-} (Gale et al., 2007) and *Tie2-Cre;Prox1*^{fl/+}, were employed in which the lymphatic vasculature is genetically perturbed. LYVE-1 is a receptor for hyaluronan which is expressed on the surface of LECs. Whilst it is redundant for lymphatic vessel development (Gale et al., 2007), it has recently been demonstrated that LYVE-1 is functionally required for the transmigration of innate immune cells including macrophages across the inflamed lymphatic endothelium (Lawrance et al., 2016), (Bhattacharjee & Jackson, unpublished data). Therefore, the *LYVE-1*^{-/-} mouse model provides a useful tool to assess the impact of blocking innate immune cell clearance via the lymphatic vasculature on the cardiac healing process. *Tie2-Cre;Prox1*^{fl/+} mice are haploinsufficient for PROX1 in the 80% of cardiac LECs which derive from a venous source (Klotz et al., 2015). PROX1 is a master regulator of lymphatic development as it is required for the specification and maintenance of LEC fate (Hong et al., 2002, Wigle & Oliver, 1999). *Tie2-Cre;Prox1*^{fl/+} mice are known to have defective adult lymphatic vasculature elsewhere in the body (Harvey et al., 2005) and, therefore, may be susceptible to impaired lymphangiogenesis and immune cell clearance following cardiac injury. Together, these two models impose

distinct impairments on the lymphatic vasculature, which allow the lymphangiogenic response and immune cell clearance post-MI to be further investigated.

6.1. Effects of *LYVE-1*^{-/-} on cardiac macrophages and post-MI lymphangiogenesis

The initial characterisation of *LYVE-1*^{-/-} mice revealed a structurally normal lymphatic network and normal circulating levels of leucocytes (Gale et al., 2007). LYVE-1 is also expressed in yolk-sac derived macrophages which are present within the heart (Pinto et al., 2012, Stevens, Gise et al., 2016). These LYVE-1⁺ resident macrophages have been described to have a gene signature similar to TIE2-expressing monocytes that play important roles in angiogenesis (Gordon et al., 2010). The effect of LYVE-1 deletion on the presence of tissue resident macrophages has not previously been established.

6.1.1. *LYVE-1*^{-/-} mice display tissue resident cardiac macrophages

In wild type, cryosectioned adult hearts, staining for LYVE-1 and CD68 revealed the presence of many CD68⁺ LYVE-1⁺ tissue resident macrophages throughout the myocardium (**Figure 6.1a, c and e**). The presence of CD68⁺ macrophages was retained in *LYVE-1*^{-/-} hearts (**Figure 6.1b, d and f**). These data demonstrate that the deletion of LYVE-1 does not affect the presence of tissue resident macrophages in the heart. This, therefore, excludes a confounding effect of deleting LYVE-1 in tissue resident macrophages when using *LYVE-1*^{-/-} animals to assess the post-MI clearance of immune cells by the lymphatics.

Figure 6.1

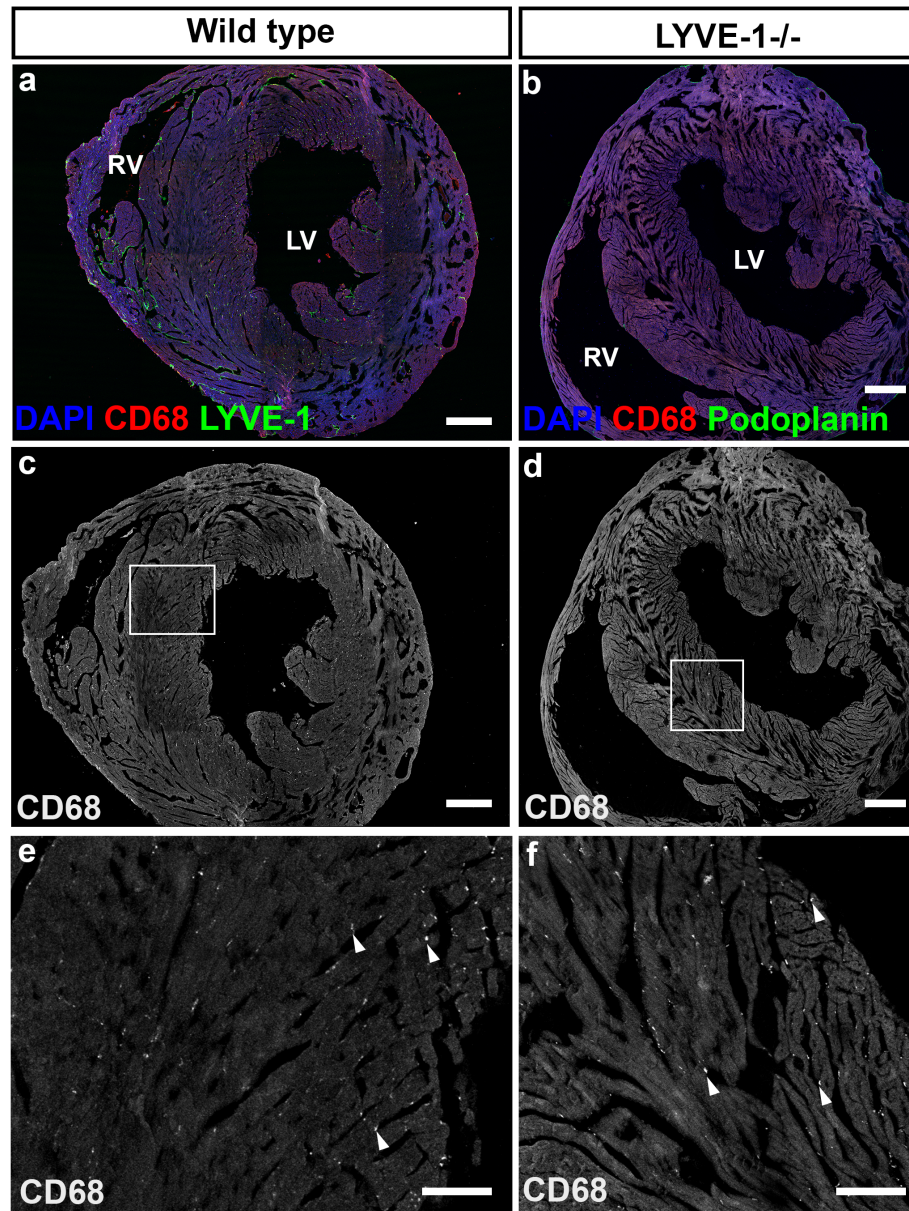


Figure 6.1: *LYVE-1*^{-/-} animals possess CD68⁺ resident cardiac macrophages
Immunofluorescent staining was performed on transverse cryosectioned adult hearts from wild type and *LYVE-1*^{-/-} animals. Wild type samples were stained for DAPI (nuclei, blue), CD68 (macrophages, red) and LYVE-1 (lymphatic endothelium and macrophages); *LYVE-1*⁺ CD68⁺ tissue resident macrophages were observed throughout the myocardium (**a**), CD68 only shown in **c**, high power area in white box shown in **e**. *LYVE-1*^{-/-} samples were stained for DAPI, CD68 and PODOPLANIN (lymphatic vessels, epicardium), revealing the presence of tissue resident CD68⁺ cardiac macrophages (**b**), CD68 only shown in **d**, high power area in white box shown in **f**. White arrowheads in **e** and **f** highlight CD68⁺ macrophages. Scale bars **a-d** = 500 μ m, **e, f** = 100 μ m. RV = right ventricle, LV = left ventricle.

6.1.2. *LYVE-1*^{-/-} mice display a normal post-MI lymphangiogenic response

Lymphatic vessels in *LYVE-1*^{-/-} mice have previously been shown to undergo normal lymphangiogenesis during subcutaneous tumour growth (Gale et al., 2007). In order to determine whether a normal lymphangiogenic response also occurs post-MI in these animals, whole-mount immunofluorescent staining and light sheet imaging were performed on hearts at day 7 post-MI. VEGFR-3 antibody staining revealed lymphangiogenesis in wild type (control) injured hearts at this time point which included the presence of sprouting lymphangiogenic capillaries (**Figure 6.2a-c**, white arrowheads in **b** and **c** mark example lymphangiogenic tips). A similar lymphangiogenic phenotype was also observed post-MI in *LYVE-1*^{-/-} hearts (**Figure 6.2d-f**, white arrowheads in **e** and **f** mark example lymphangiogenic tips) demonstrating that despite *LYVE-1* deletion a normal lymphangiogenic response is retained. Ideally, quantification of various network parameters would have been performed using Angiotool software, as previously employed in chapter 4. However, the whole-mount staining protocol used on these samples, whilst optimised, often resulted in patchy areas of staining due to incomplete penetration of antibody into the tissue. This prevented the comparison of multiple areas from multiple hearts using Angiotool.

Figure 6.2

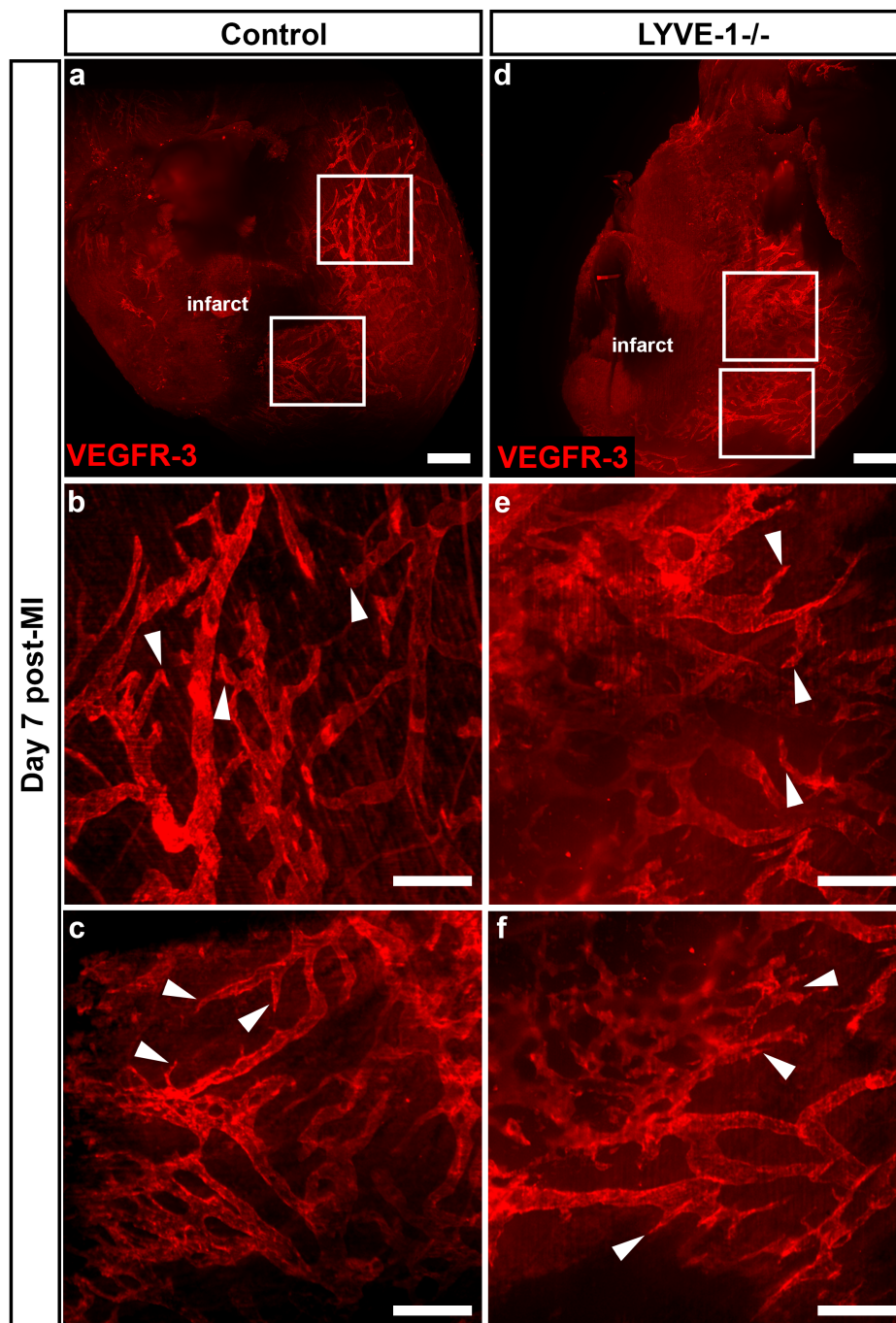


Figure 6.2: *LYVE-1*^{-/-} animals display normal post-MI lymphangiogenesis
 Whole-mount immunofluorescent staining and light sheet imaging was performed on hearts at day 7 post-MI. VEGFR-3 staining on control hearts revealed the superficial cardiac lymphatic plexus with lymphangiogenic capillary tips (**a**, white boxes enlarged in **b** and **c**, white arrowheads indicate example lymphangiogenic capillary tips). *LYVE-1*^{-/-} hearts displayed a similar lymphangiogenic VEGFR-3⁺ capillary network (**d**, white boxes enlarged in **d** and **e**, white arrowheads indicate example lymphangiogenic tips) to controls. Scale bars **a**, **d** = 500 μ m, **b**, **c**, **e**, **f** = 200 μ m. n = 4-5 hearts per group.

6.2. *LYVE-1*^{-/-} mice display an accumulation of macrophages and neutrophils post-MI

In chapter 5 I showed that administration of VEGF-C (C156S) enhanced post-MI lymphangiogenesis, leading to a reduced percentage of myeloid cells including macrophages within the heart at day 7 post-injury. This suggested that an increase in the density of the cardiac lymphatic network provided additional routes for innate immune cells to exit the heart following MI. LYVE-1 is involved in the transmigration of innate immune cells including macrophages across inflamed lymphatic endothelium (Lawrance et al., 2016), (Bhattacharjee & Jackson, unpublished data). Therefore, the *LYVE-1*^{-/-} mouse represents a potentially useful tool to study impaired clearance of immune cells post-MI. I hypothesised that MI in *LYVE-1*^{-/-} animals would result in a build-up of innate immune cells within the heart, due to their reduced ability to enter the lymphatic vessels for clearance.

6.2.1. *LYVE-1*^{-/-} mice display an accumulation of innate immune cells at day 7 post-MI

Flow cytometry was performed on whole digested hearts at day 7 post-MI from control (C57BL/6) and *LYVE-1*^{-/-} animals to assess the numbers of leucocytes (CD45⁺), myeloid cells (CD45⁺ CD11b⁺), neutrophils (CD45⁺ CD11b⁺ Ly6G⁺), macrophages (CD45⁺ CD11b⁺ Ly6G⁻ F4/80⁺) and dendritic cells (CD45⁺ CD11b⁺ Ly6G⁻ F4/80⁻ CD11c⁺) present. Gates were set up as previously shown in **Figure 5.8**. A significant increase in the percentages of myeloid cells (% mean $\pm$ S.E.M. control: 2.750 ± 0.6037 %, *LYVE-1*^{-/-}: 5.608 ± 1.239 %, $p=0.044$), including macrophages (control: 2.230 ± 0.4979 %, *LYVE-1*^{-/-}: 4.417 ± 0.8771 %, $p=0.040$) and neutrophils (control: 0.2538 ± 0.0378 %, *LYVE-1*^{-/-}: 0.8917 ± 0.3154 %, $p=0.038$), were observed in *LYVE-1*^{-/-} hearts when compared to controls. No

significant changes were observed in the percentages of the overall leucocyte population and dendritic cells (**Figure 6.3**). These data demonstrate that there is retention of myeloid cells including macrophages and neutrophils within the *LYVE-1*^{-/-} heart following MI, suggesting that in this model there is a failure of these cells to clear via the cardiac lymphatic vessels.

Figure 6.3

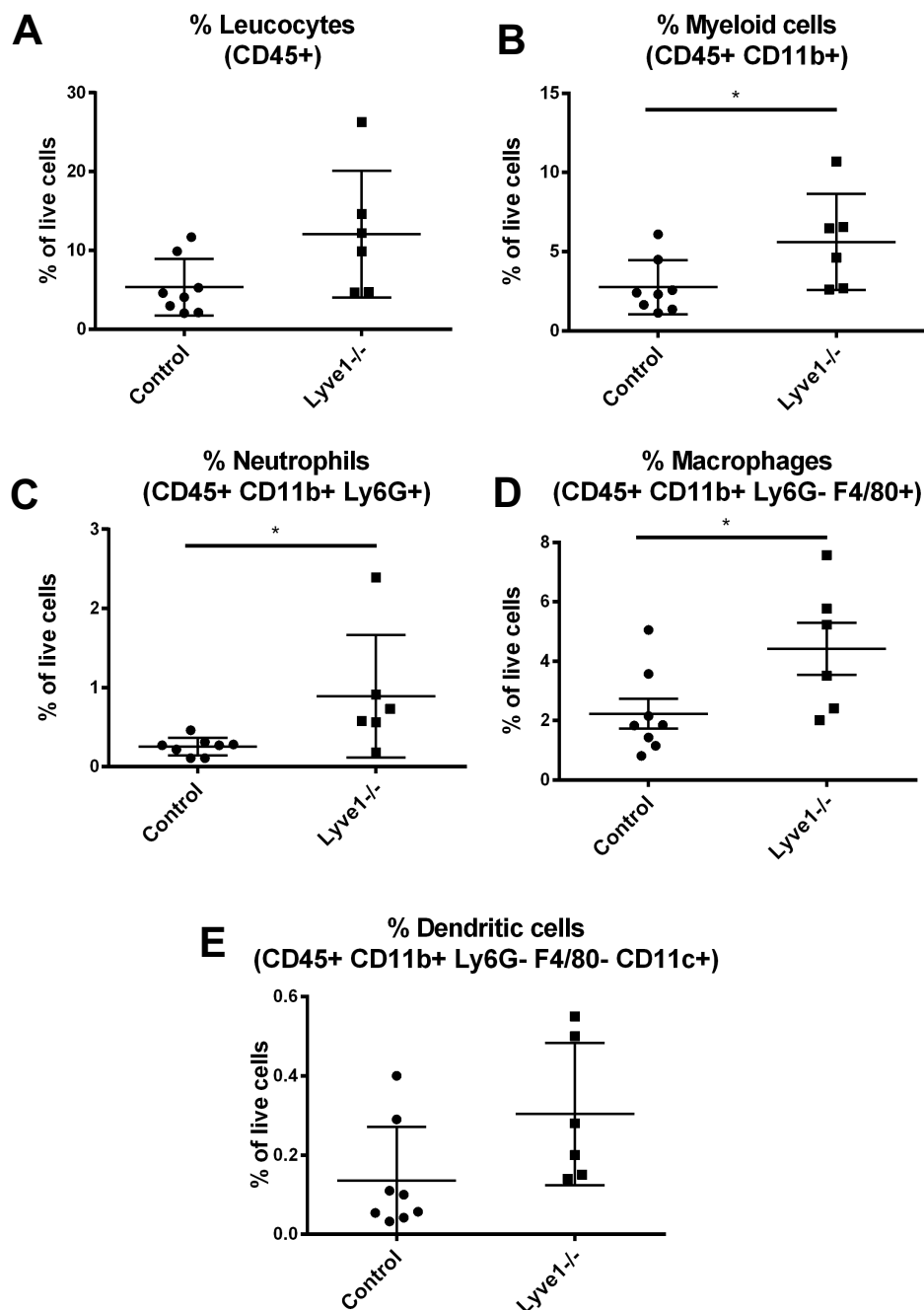


Figure 6.3: Flow cytometry for immune cells within cardiac tissue at day 7 post-MI in control and *LYVE-1*^{-/-} animals

Flow cytometry was performed on digested hearts at day 7 post-MI from control (C57Bl/6) or *LYVE-1*^{-/-} animals. A significant increase in the % of live cells which were myeloid cells, macrophages and neutrophils was observed in the *LYVE-1*^{-/-} hearts. Control group n = 8, *LYVE-1*^{-/-} group n = 6. Data presented as mean ± standard error of mean. An unpaired, two-tailed t-test was used to assess statistical significance, *p ≤ 0.05. p-values: A=0.055, B=0.044, C=0.038, D=0.040 and E=0.069.

6.2.2. Inflammatory profile of macrophages remaining in the heart following MI in *LYVE-1*^{-/-} animals

FACS-isolated CD45⁺ CD11b⁺ Ly6G⁻ F4/80⁺ macrophages (gating strategy shown in **Figure 5.8**) were collected at day 7 post-MI from whole-digested hearts from C57BL/6 (control) and *LYVE-1*^{-/-} animals. qRT-PCR was performed for M1/M2 markers and macrophage expressed cytokines in order to characterise the polarisation of the macrophages that accumulate post-MI in *LYVE-1*^{-/-} hearts, the same panel of genes were used as in chapter 5 (**Table 5.14**).

The qRT-PCR data revealed similar expression levels of *Il6*, *Il1b* and *Il10* in macrophages from both the control and *LYVE-1*^{-/-} groups (**Figure 6.4**). CT values obtained for *Tnf*, *Tgfb1*, *Cd80*, *Cd86*, *Cd206* and *Chi3l3* were greater than 30 indicating that these genes are expressed only at very low levels, preventing quantification by qRT-PCR. Although *LYVE-1*^{-/-} animals display an increased number of macrophages in the heart at day 7 post-MI the data presented here indicates that these macrophages have similar levels of *Il6*, *Il1b* and *Il10* to wild type animals. This suggests the macrophages that accumulate in *LYVE-1*^{-/-} hearts at day 7 post-MI maintain a similar mixture of macrophage polarisations to those present in lower numbers in post-MI C57BL/6 hearts.

Figure 6.4

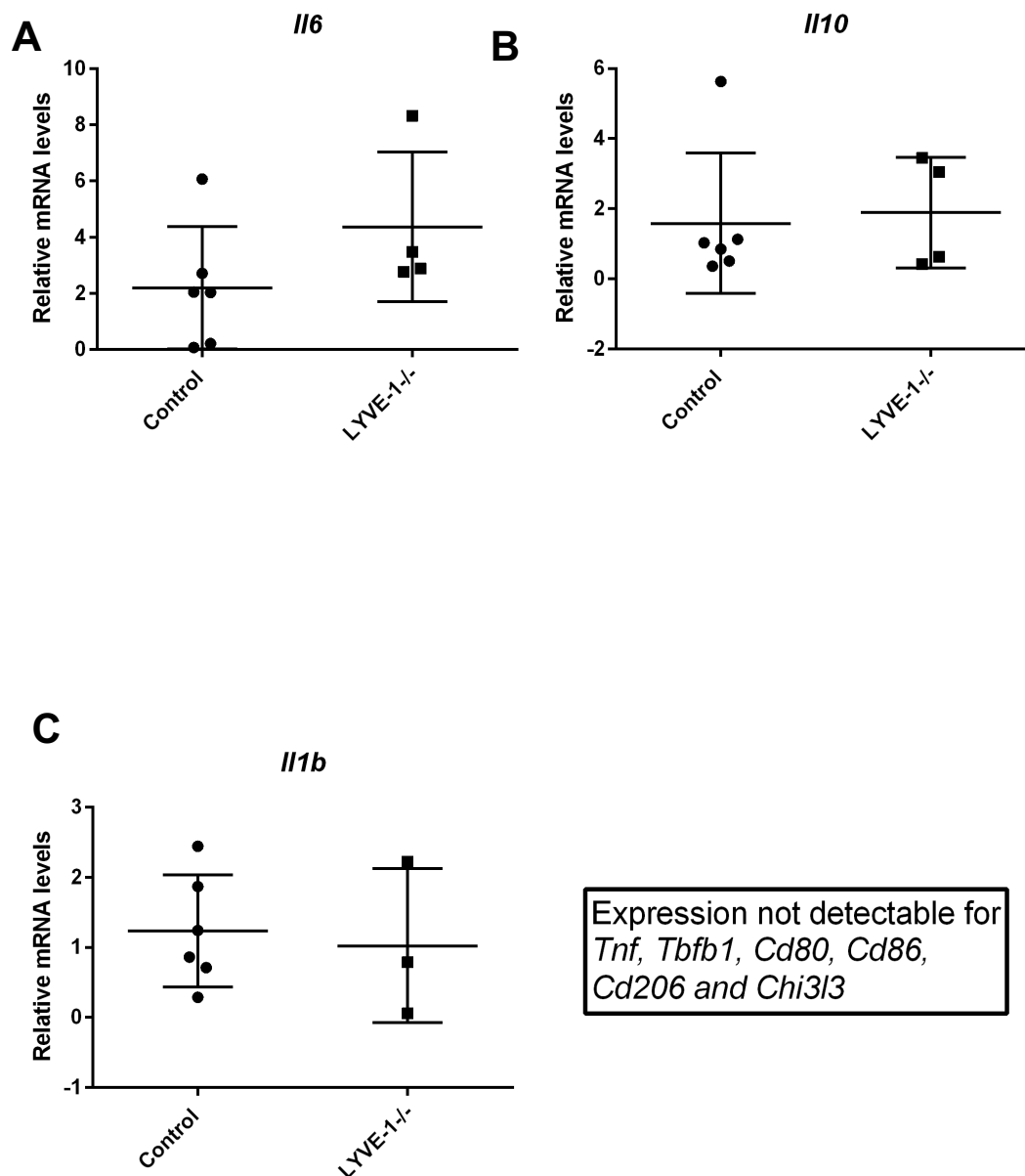


Figure 6.4: qRT-PCR analysis for key inflammation and fibrosis genes in day 7 post-MI FACS sorted macrophages from control and *LYVE-1*^{-/-} animals

qRT-PCR was performed on cDNA from sorted cardiac macrophages collected by FACS at day 7 post-MI from control and *LYVE-1*^{-/-} animals. A panel of inflammation, fibrosis and M1/M2 genes were tested, pro-inflammatory: *Il6*, *Il1b*, *Tnf*, anti-inflammatory/pro-fibrosis: *Il10*, *Tgfb1* M1: *Cd80*, *Cd86*, M2: *Cd206*, *Chi3l3*. No statistically significant changes were observed in the mRNA expression of *Il6*, *Il10* and *Il1b* between groups. mRNA expression levels of *Tnf*, *Tgfb1*, *Cd80*, *Cd86*, *Cd206* and *Chi3l3* were too low to detect (CT values > 30). Control group n = 6, *LYVE-1*^{-/-} group n = 4. Data presented shows all data points with mean ± standard deviation. An unpaired, two-tailed t-test was used to assess statistical significance. p-values: A=0.193, B=0.806 and C=0.747.

6.2.3. Inflammatory profile of the infarcted myocardium in *LYVE-1*^{-/-} animals

In order to further assess the effect of the accumulation of macrophages due to *LYVE-1* deletion, infarcts were collected at day 7 post-MI from control and mutant animals. I hypothesised that an increase in the number of macrophages remaining in the heart would lead to overall increased levels of macrophage released cytokines and the upregulation of collagen production by fibroblasts and myofibroblasts. RNA was extracted in order to investigate the mRNA expression levels of M1 and M2 markers, macrophage released cytokines and collagens within the infarct. qRT-PCR revealed no statistically significant changes in the M1 marker genes *Cd80* and *Cd86*, nor the M2 marker genes *Cd206* and *Chi3l3* (**Figure 6.5**). mRNA expression levels of the pro-inflammatory cytokines *Il6*, *Il1b*, and *Tnf* were also not significantly altered between groups (**Figure 6.6**). Expression levels of the anti-inflammatory *Il10* and the pro-fibrosis cytokine *Tgfb1* were similar in both groups (**Figure 6.6**). A trend towards increased expression of *Col1a1* was observed in the *LYVE-1*^{-/-} group, however, this was not statistically significant ($p=0.0573$) (**Figure 6.7**). Expression levels of *Col1a2*, *Col6a1*, *Col4a1* and *Col3a1* remained similar in both groups (**Figure 6.7**). Together, these data indicate that the increased number of macrophages present in *LYVE-1*^{-/-} animals at day 7 post-MI does not result in an overall increase in macrophage produced cytokines or significantly upregulated collagen production at this time point. The observation that the expression of M1/M2 markers remain similar also suggests that polarisations of macrophages that accumulate in *LYVE-1*^{-/-} animals is similar to the mixture of macrophages present in wild types at day 7 post-MI.

Figure 6.5

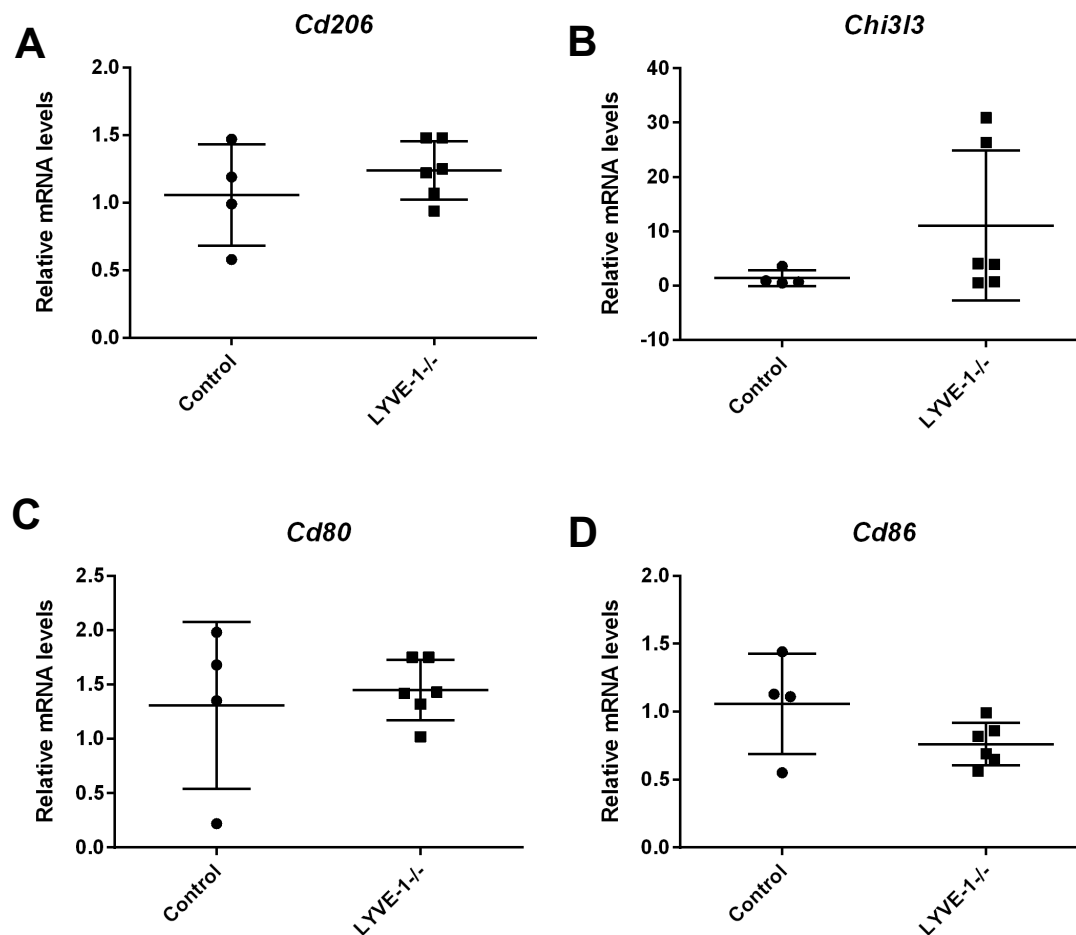


Figure 6.5: Deletion of LYVE-1 does not significantly affect the expression M1 and M2 markers within the infarcted region at day 7 post-MI

qRT-PCR was performed on isolated infarcted tissue at day 7 post-MI from control or LYVE-1^{-/-} animals. No statistically significant changes were observed in the expression of M1 markers (*Cd80*, *Cd86*) nor M2 markers (*Cd206*, *Chi3l3*) between groups. Control group n = 4, LYVE-1^{-/-} group n = 6. Data presented as mean $\pm$ standard error of mean. An unpaired, two-tailed t-test was used to assess statistical significance. p-values: A=0.352, B=0.207, C=0.690 and D=0.114.

Figure 6.6

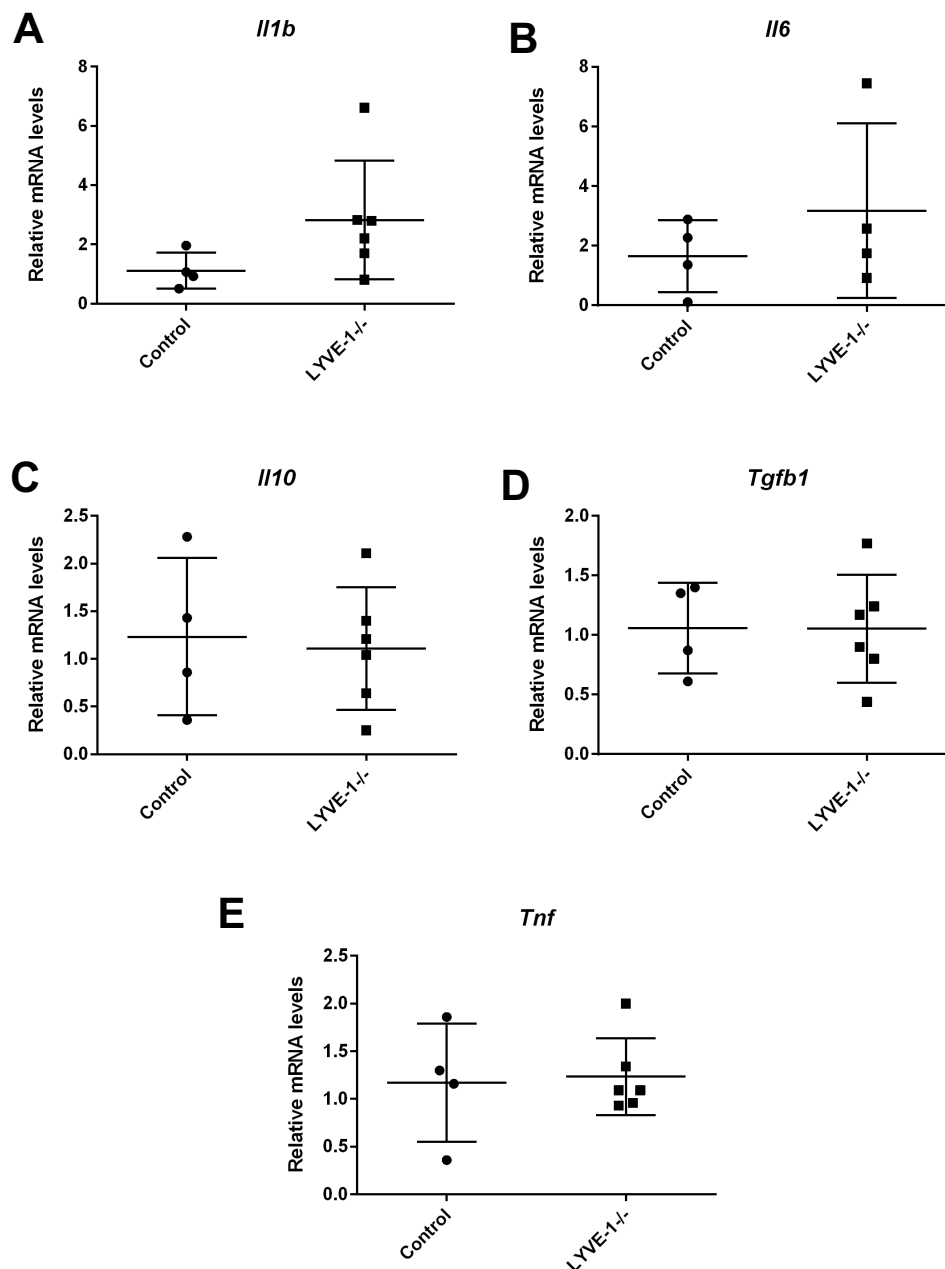


Figure 6.6: Deletion of LYVE-1 does not significantly affect the inflammatory profile within the infarcted region at day 7 post-MI

qRT-PCR was performed on isolated infarcted tissue at day 7 post-MI from control or LYVE-1^{-/-} animals. No statistically significant changes were observed in the expression of pro-inflammatory markers (*Il1b*, *Il6*, *Tnf*) nor anti-inflammatory markers (*Il10*, *Tgfb1*) between groups. Control group n = 4, LYVE-1^{-/-} group n = 6. Data presented as mean ± standard error of mean. An unpaired, two-tailed t-test was used to assess statistical significance. p-values: A=0.144, B=0.374, C=0.795, D=0.988 and E=0.844.

Figure 6.7

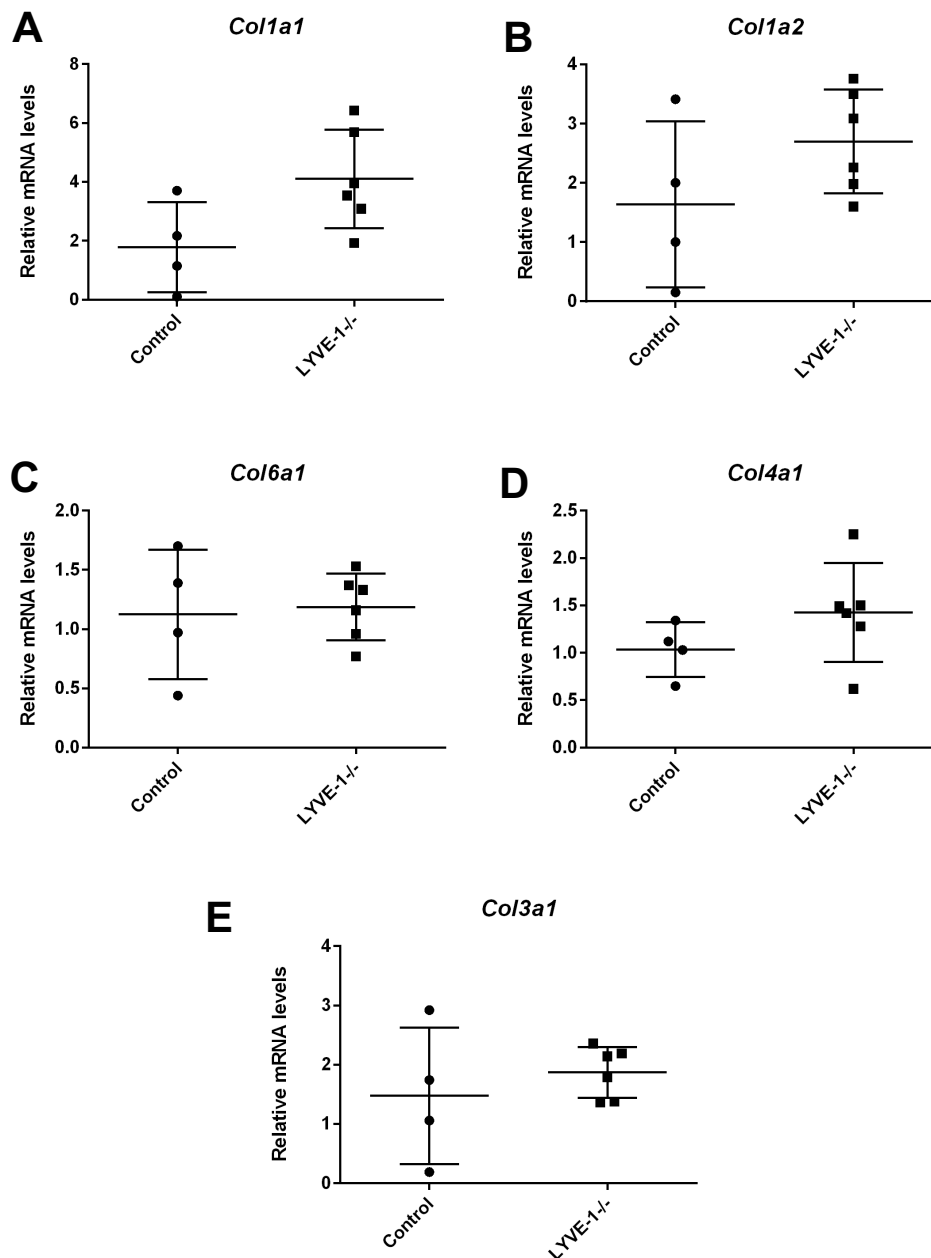


Figure 6.7: Deletion of LYVE-1 does not affect the expression of collagen genes within the infarct at day 7 post-MI

qRT-PCR was performed on isolated infarcted tissue at day 7 post-MI from control or LYVE-1^{-/-} animals. No statistically significant changes were observed in the expression of the collagen genes *Col1a1*, *Col1a2*, *Col6a1*, *Col4a1* or *Col3a1* between groups. Control group n = 4, LYVE-1^{-/-} group n = 6. Data presented as mean ± standard error of mean. An unpaired, two-tailed t-test was used to assess statistical significance. p-values: A=0.057, B=0.175, C=0.818, D=0.213 and E=0.460.

6.3. Assessment of post-MI responses in *Tie2-Cre;Prox1^{fl/+}* mice

Work presented in chapter 4 described the phenotype of the cardiac lymphatic vessels in *Tie2-Cre;Prox1^{fl/fl}* mutants and *Tie2-Cre;Prox1^{fl/+}* heterozygotes. At E17.5 *Tie2-Cre;Prox1^{fl/+}* embryos presented a mild phenotype of delayed lymphatic vessel growth on the ventral side of the heart. Furthermore, adult *Tie2-Cre;Prox1^{fl/+}* animals have been shown to exhibit mispatterned and ruptured lymphatic vessels (Harvey et al., 2005). I, therefore, hypothesised that *Tie2-Cre;Prox1^{fl/+}* adult animals would display impaired lymphangiogenesis in response to MI, resulting in a build-up of innate immune cells due to insufficient clearance.

6.3.1. Key lymphatic genes are downregulated following MI in *Tie2-Cre;Prox1^{fl/+}* mice

qRT-PCR was performed on hearts from control (*Prox1^{fl/+}*) and heterozygous (*Tie2-Cre;Prox1^{fl/+}*) animals at days 4 and 7 post-MI in order to assess the mRNA expression levels of the key lymphatic genes *Vegfr3*, *Prox1*, and *Lyve1* that are upregulated during post-MI lymphangiogenesis (Klotz et al., 2015). At day 4 post-MI mRNA expression levels of both *Lyve1* and *Prox1* were significantly reduced in heterozygous animals when compared to controls (**Figure 6.8C, E**). *Vegfr3* expression was not significantly changed at this time point (**Figure 6.8A**). Expression levels of *Vegfr3* and *Prox1* at day 7 post-MI were significantly reduced (**Figure 6.8B, F**), however, no change in *Lyve1* expression at this time point was observed (**Figure 6.8D**). Together these data suggest lymphangiogenesis in *Tie2-Cre;Prox1^{fl/+}* animals is impaired, as expression levels of the key lymphatic genes in response to cardiac injury are reduced. Furthermore, these data confirm partial knockdown of *Prox1* in the heterozygous animals.

Figure 6.8

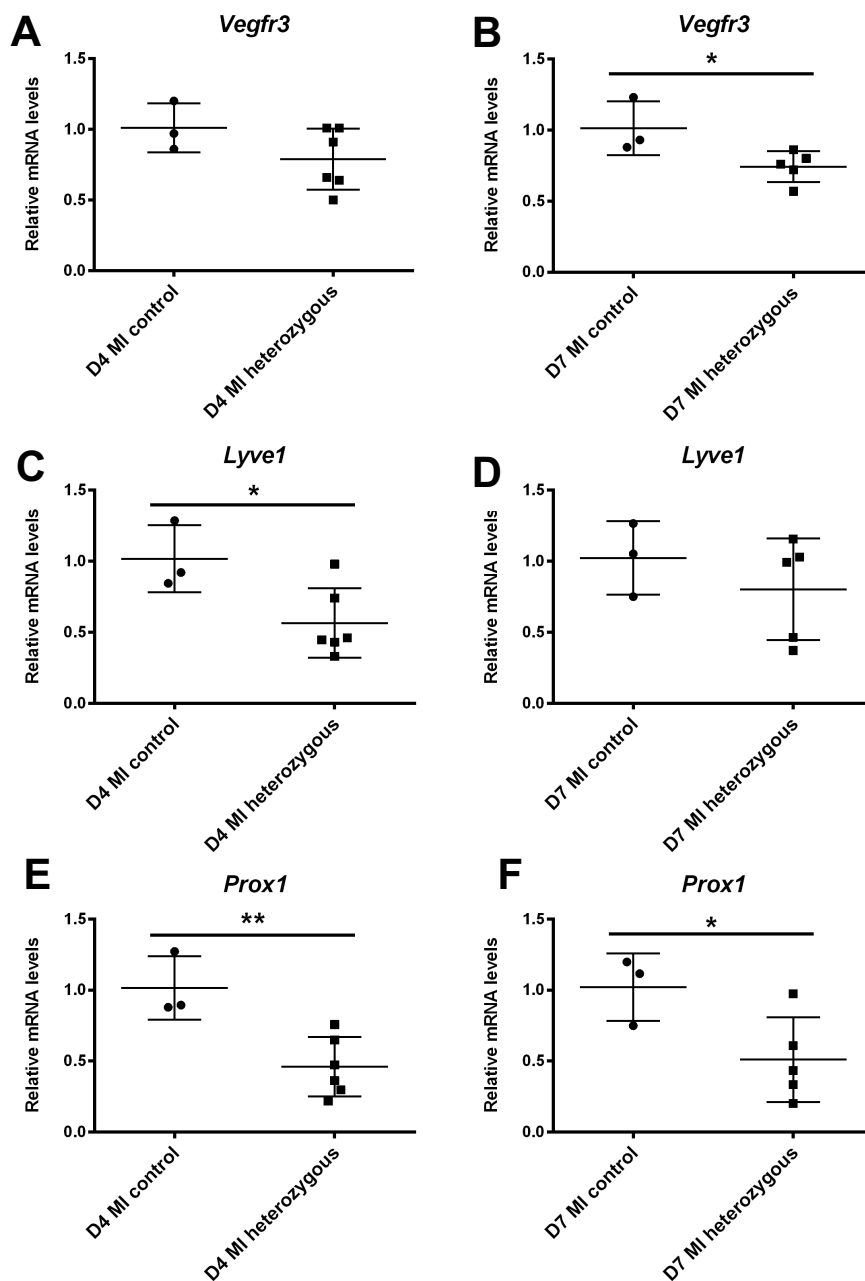


Figure 6.8: *Tie2-Cre;Prox1^{fl/+}* animals display a reduction in key lymphatic genes at days 4 and 7 post-MI

qRT-PCR was performed on cardiac tissue at days 4 and 7 post-MI from control (*Prox1^{fl/+}*) and heterozygous (*Tie2-Cre;Prox1^{fl/+}*) animals. At day 4 post-MI no significant change was observed in *Vegfr3* mRNA levels, but mRNA levels of *Lyve1* and *Prox1* were reduced in heterozygous animals. At day 7 post-MI no significant change was observed in *Lyve1* mRNA levels, but mRNA levels of *Vegfr3* and *Prox1* were significantly reduced in heterozygous animals. Control group day 4 n = 3, heterozygous group day 4 n = 6, Control group day 7 n = 3, heterozygous group day 7 n = 5. Data presented as mean ± standard error of mean. An unpaired, two-tailed t-test was used to assess statistical significance, **p ≤ 0.01, *p ≤ 0.05. p-values: A=0.171, B=0.039, C=0.034, D=0.393, E=0.008 and F=0.047.

6.3.2. Assessment of inflammation and fibrosis in *Tie2-Cre;Prox1^{fl/+}* mice post-MI

I hypothesised that impaired lymphangiogenesis in *Tie2-Cre;Prox1^{fl/+}* animals would result in increased levels of inflammation within the heart due to reduced clearance of innate immune cells via the lymphatic vasculature. In order to investigate this qRT-PCR was performed at days 4 and 7 post-MI in control (*Prox1^{fl/+}*) and heterozygous (*Tie2-Cre;Prox1^{fl/+}*) animals to assess the mRNA expression levels of the pro-inflammatory cytokines *Il6* and *Tnfα*, the anti-inflammatory cytokine *Il10*, and collagen (*Col1a1*). No significant changes were observed between controls and heterozygotes in the mRNA expression levels of any of the tested genes at days 4 or 7 post-MI (**Figure 6.9**). This data indicates that the expression of macrophage released cytokines and production of collagen is not increased during the first week post-MI in *Tie2-Cre;Prox1^{fl/+}* animals.

Figure 6.9

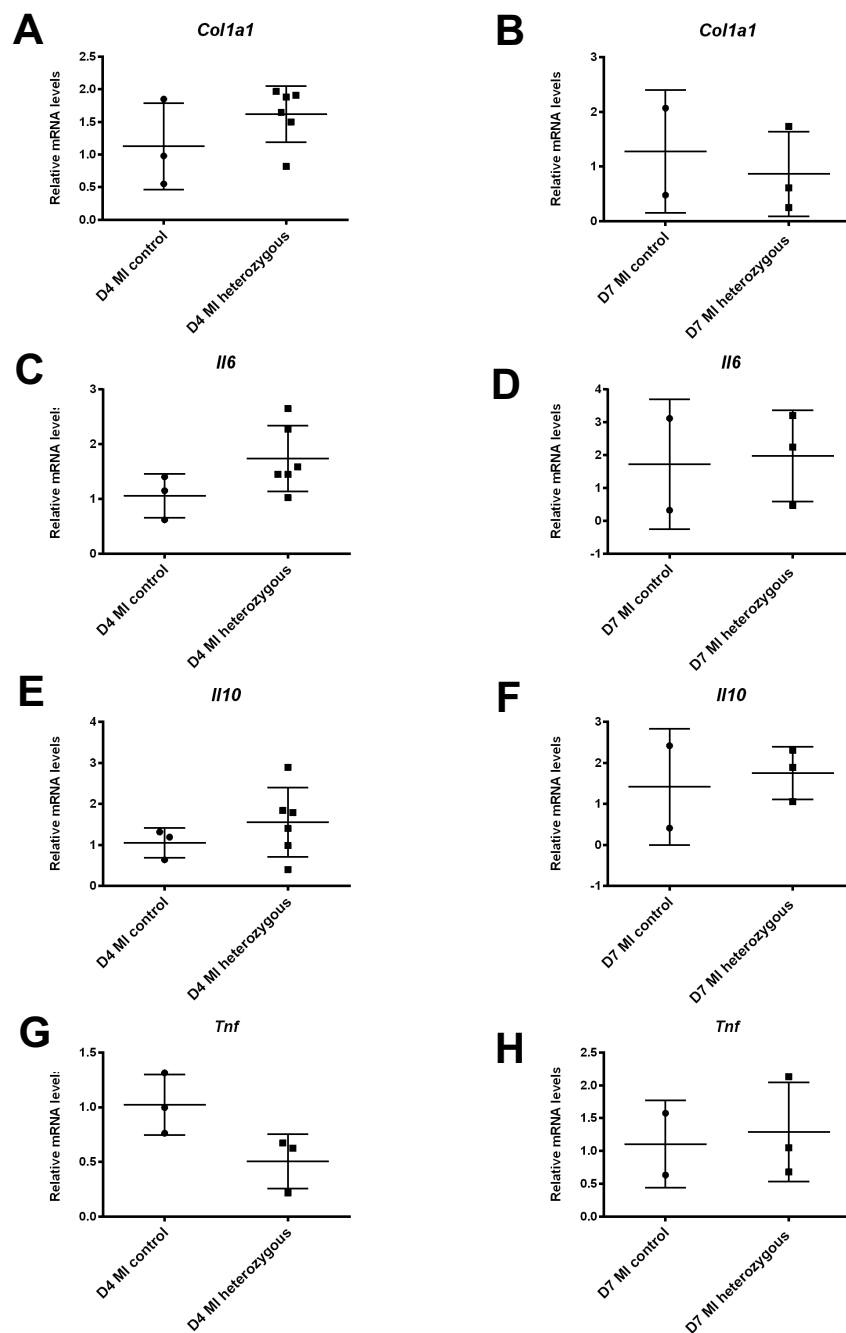


Figure 6.9: *Tie2-Cre;Prox1^{fl/+}* animals do not display any significant changes in inflammation/fibrosis genes at days 4 and 7 post-MI

qRT-PCR was performed on cardiac tissue at days 4 and 7 post-MI from control (*Prox1^{fl/+}*) and heterozygous (*Tie2-Cre;Prox1^{fl/+}*) animals. A panel of inflammation and fibrosis genes were tested, pro-inflammatory: *Il6*, *Tnf*, anti-inflammatory/pro-fibrosis: *Il10*, *Col1a1*. At both days 4 and 7 post-MI no statistically significant changes were observed between groups. Control group day 4 n = 3, Heterozygous group day 4 n = 6, Control group day 7 n = 2, Heterozygous group day 7 n = 3. Data presented as mean $\pm$ standard error of mean. An unpaired, two-tailed t-test was used to assess statistical significance. p-values: A=0.211, B=0.652, C=0.125, D=0.873, E=0.369, F=0.729, G=0.073 and H=0.802.

6.4. Discussion

6.4.1. *LYVE-1*^{-/-} as a model to study the clearance of post-MI inflammation via the lymphatic vasculature

Given that LYVE-1 is required for the transmigration of macrophages (Lawrance et al., 2016), (Bhattacharjee & Jackson, unpublished data) into the lymphatic vasculature during inflammation, the *LYVE-1*^{-/-} mouse model provides a useful tool to study the role of the lymphatic vasculature in the clearance of post-MI inflammatory cells. I performed characterisation of *LYVE-1*^{-/-} animals prior to MI studies. This revealed that cardiac tissue resident macrophages are present in these animals and a normal post-MI lymphangiogenic response is retained. These findings confirm that the only known effect of LYVE-1 deletion would be on the transmigration of innate immune cells into the lymphatic endothelium and not on the response of tissue resident macrophages to MI, nor the extent of inherent lymphangiogenesis.

6.4.2. Assessment of post-MI inflammation in *LYVE-1*^{-/-} animals

Flow cytometry performed at day 7 post-MI revealed an accumulation of myeloid cells including macrophages and neutrophils within the myocardium of *LYVE-1*^{-/-} animals. Interestingly this contrasts with the effect of VEGF-C (C156S) treatment which resulted in a reduced number of macrophages remaining within the heart (data presented in chapter 5). Together, these results indicate that the cardiac lymphatics do play an important role in the clearance of myeloid cells including macrophages and neutrophils via a LYVE-1 dependant mechanism. Increasing the extent of lymphangiogenesis resulted in reduced numbers of innate immune cells within the heart. Conversely blocking migration of these cells by deleting LYVE-1 resulted in increased numbers remaining in the heart. Whilst these data are suggestive of lymphatic vessels playing an important role in the clearance of inflammation, further work needs to be performed to

confirm this. This should include collection of the mediastinal lymph nodes, to which the cardiac lymphatics drain, in order to assess any changes in the numbers of innate immune cells that have been trafficked there.

qRT-PCR was performed on isolated macrophages from control and *LYVE-1*^{-/-} hearts at day 7 post-MI in order to characterise the gene expression profile of the macrophages which build-up post-MI in the hearts of *LYVE-1*^{-/-} animals. mRNA expression levels of *Il6*, *Il1b* and *Il10* were similar in macrophages from control and *LYVE-1*^{-/-} hearts which may suggest that the ratio of inflammatory:reparative macrophages is similar in both groups. qRT-PCR was also performed for *Tnf*, *Tgfb1*, *Cd80*, *Cd86*, *Cd206* and *Chi3l3*. However, these genes were all expressed at levels that were too low to quantify. An alternative experiment to assess the ratio of M1:M2 macrophages which build-up in *LYVE-1*^{-/-} hearts would be to perform quantification by flow cytometry using an M1/M2 discriminatory marker such as CD206 (Lorchner, Poling et al., 2015). This experiment will be discussed further in the future work section of this thesis.

qRT-PCR on whole infarcts from control and *LYVE-1*^{-/-} animals at day 7 post-MI revealed no significant changes in the mRNA expression levels of M1/M2 markers, macrophage released inflammatory cytokines, nor collagen genes. There was a trend, however, towards increased *Col1a1* expression in the *LYVE-1*^{-/-} group (p=0.0573). This trend may be explained by the overall increase in the number of macrophages present in the heart at day 7 post-MI in the *LYVE-1*^{-/-} group, which may lead to an overall increase in levels of TGFβ release and stimulation of collagen production by fibroblasts and myofibroblasts. The observation that no significant changes in any of the inflammation/fibrosis genes assessed were detected, despite the significantly increased number of macrophages present within the *LYVE-1*^{-/-} hearts at this time point,

may be because it is difficult to detect small changes in gene expression when the infarct tissue from which the RNA was isolated contains such a heterogeneous population of cells and ECM. Another alternative could be that any changes in cytokine or collagen gene expression occur at a later or earlier time point than day 7 post-MI.

Whilst at day 7 post-MI *LYVE-1*^{-/-} animals appeared to have similar expression levels of cytokine and collagen genes, the finding that macrophages and neutrophils accumulate in *LYVE-1*^{-/-} hearts post-injury could result in negative outcomes. High levels of neutrophils post-MI in humans have been associated with increased levels of adverse remodelling and a higher incidence of LV cardiac rupture (van den Borne, Cleutjens et al., 2009). In mouse models inhibition of ECM degrading MMPs, which are produced by neutrophils, has been found to be beneficial for cardiac healing by increasing angiogenesis, reducing collagen accumulation and attenuating LV dilation (Ducharme, Frantz et al., 2000, Lindsey, Escobar et al., 2006). Given these findings it is likely that the persistence of neutrophils, due to impaired clearance by cardiac lymphatics in *LYVE-1*^{-/-} animals, could result in increased scarring, fibrosis, and poor functional outcomes. Furthermore, the accumulation of macrophages post-MI in *LYVE-1*^{-/-} animals may contribute to these adverse effects as well. Blocking the infiltration of pro-inflammatory macrophages, which have phagocytic and proteolytic properties, has been found to improve post-MI cardiac healing (Kaikita et al., 2004, Majmudar et al., 2013). Moreover, the persistence of reparative macrophages may result in excessive fibrosis, adverse remodelling and poor functional outcomes (Hulsmans et al., 2016). Further work, which is outlined in chapter 8, is required to determine whether the impaired clearance of neutrophils and macrophages via the cardiac lymphatics does have a negative impact on cardiac healing.

6.4.3. *Tie2-Cre;Prox1^{fl/+}* animals as a model to investigate the role of post-MI lymphangiogenesis in the clearance of inflammation

I hypothesised that *Tie2-Cre;Prox1^{fl/+}* haploinsufficient animals would provide a useful model to study the requirement of post-MI lymphangiogenesis for the clearance of inflammation. Preliminary data presented here demonstrated that the expression of the key lymphangiogenic genes *Prox1*, *Vegfr3* and *Lyve1* is reduced following MI in *Tie2-Cre;Prox1^{fl/+}* animals when compared to controls. These data suggest that this model does provide a useful tool to determine the requirement for lymphangiogenesis in the clearance of post-MI inflammation and the subsequent healing and scarring process. However, any impairment in lymphangiogenesis should be confirmed by whole-mount antibody staining as discussed further in the future work section in chapter 8.

I also performed qRT-PCR to assess the expression of various inflammation and fibrosis genes at days 4 and 7 post-MI, however, no significant changes were observed in the *Tie2-Cre;Prox1^{fl/+}* group. In other experiments where I detected changes in the number of macrophages within the heart, measured by flow cytometry, these were not translated into changes in inflammation and fibrosis genes at the same time point. Therefore, although *Tie2-Cre;Prox1^{fl/+}* animals do not show any changes in the expression of inflammation and fibrosis genes, there may still be an effect on the numbers of innate immune cells cleared by the cardiac lymphatics. The observation that inhibition of lymphangiogenesis in an inflammatory bowel disease model resulted in reduced clearance of macrophages via the lymphatic vasculature supports this possibility (D'Alessio et al., 2014). Due to the number of animals available for surgery and time constraints a flow cytometry study on *Tie2-Cre;Prox1^{fl/+}* animals to assess the number of immune cells within the heart has not been included in this thesis, however,

animal breeding is currently ongoing to enable this study to be carried out as outlined in chapter 8.

6.5. Conclusions

Two genetic mouse models which will be useful in assessing the roles of post-MI lymphangiogenesis and immune cell clearance via the cardiac lymphatics have been characterised. It has been demonstrated that the *LYVE-1*^{-/-} mouse retains tissue resident cardiac macrophages and a normal lymphangiogenic response and can, therefore, be used as a model to study the effects of blocking clearance of innate immune cells, including macrophages, via the cardiac lymphatics. MI experiments using this model have revealed that myeloid cells, including macrophages and neutrophils, accumulate within the infarcted *LYVE-1*^{-/-} heart. This indicates that the cardiac lymphatics do play an important role in the clearance of inflammation. In other studies the accumulation of neutrophils and macrophages post-MI has been associated with increased fibrosis, scarring and adverse remodelling. Therefore, further work needs performed to determine whether impaired lymphatic clearance of inflammation in the *LYVE-1*^{-/-} model does result in increased post-MI scarring and poorer functional outcomes. It has also been shown here that the *Tie2-Cre;Prox1*^{fl/+} haploinsufficiency model displays an impaired post-MI lymphangiogenic response, paving the way for further experiments which will allow the requirement for lymphangiogenesis for the clearance of cardiac inflammation to be further investigated.

7. General discussion

The following aims as set out in chapter 1 of this thesis have been achieved:

- 1) Characterise the spatiotemporal development of cardiac lymphatic vessels during embryonic development and early postnatal stages – *chapter 3, sections 3.1 and 3.2.*
- 2) Perform lineage tracing to further investigate whether a proportion of cardiac LECs are derived from a non-venous origin – *chapter 3, section 3.3.*
- 3) Utilise Cre-lox genetic models to determine whether PROX1 is required in cardiac LECs which derive from venous and non-venous origins – *chapter 4*
- 4) Further characterise the response of the cardiac lymphatics to myocardial injury and investigate the effect of recombinant VEGF-C therapy on the resolution of post-MI inflammation and healing – *chapter 5*
- 5) Employ models in which lymphangiogenesis or clearance of immune cells by lymphatic vessels is genetically perturbed to further determine the importance of cardiac lymphatics in the clearance of post-MI inflammation – *chapter 6*

7.1. Spatiotemporal characterisation of the development of cardiac lymphatic vessels

The work presented in sections 3.1 and 3.2 of this thesis provides a detailed characterisation of the development of the cardiac lymphatic vessels, therefore completing what was set out in aim 1. I undertook this work to gain further scientific insight into the development of cardiac lymphatic vessels, a process which has not previously been well-characterised. Understanding the mechanisms by which cardiac lymphatics form is of significant importance as it is these processes that may become

disrupted in congenital diseases and are reactivated during expansion of the lymphatic network in pathological conditions. Prior to my work it had previously been established that the systemic lymphatic vasculature begins to form in the mouse embryo from around E9-9.5, with the formation of a primitive plexus extending throughout the embryo by E14.5 (Hägerling et al., 2013, Wigle & Oliver, 1999, Yang et al., 2012). Multiple studies, reviewed in (Brade, Pane et al., 2013, Laugwitz, Moretti et al., 2008), had also provided key insights into the processes by which the heart develops and had identified the cellular sources from which it derives. Briefly, the heart begins to develop from cardiogenic mesoderm at E7.5 through the formation of the cardiac crescent, which consists of the first and second heart fields. By E8.0 a beating, linear heart tube is formed. Additional progenitors then migrate into the heart tube before it loops at E8.5. At E10.5 neural crest and proepicardial cells also contribute to the heart, by which point it is four chambered. This is followed by chamber maturation, forming a fully septated heart by E14.5.

I performed a detailed analysis of the spatiotemporal development of cardiac lymphatics at multiple embryonic time points which revealed that the LECs originating from the cardinal vein/ISV/sVP region first enter the heart around E12.5 via migration along the sinus venosus region. A primitive plexus of cardiac lymphatics that run closely alongside coronary veins is formed by E13.5-14.5 which then branches and expands from the base towards the apex by the process of lymphangiogenesis through to postnatal stages. Together, these findings provide novel insights into the key time points and patterning of cardiac lymphatic formation, a diagrammatic summary of my findings is shown in **Figure 7.1**. Ideally *in vivo* live imaging would be performed to follow the dynamic movements of LECs as they form the cardiac lymphatic network. However, live imaging requires successful culture of mouse embryos which is currently

limited to stages around mid-gestation and cannot be maintained to capture later events such as the formation of the cardiac lymphatic vessels.

Figure 7.1

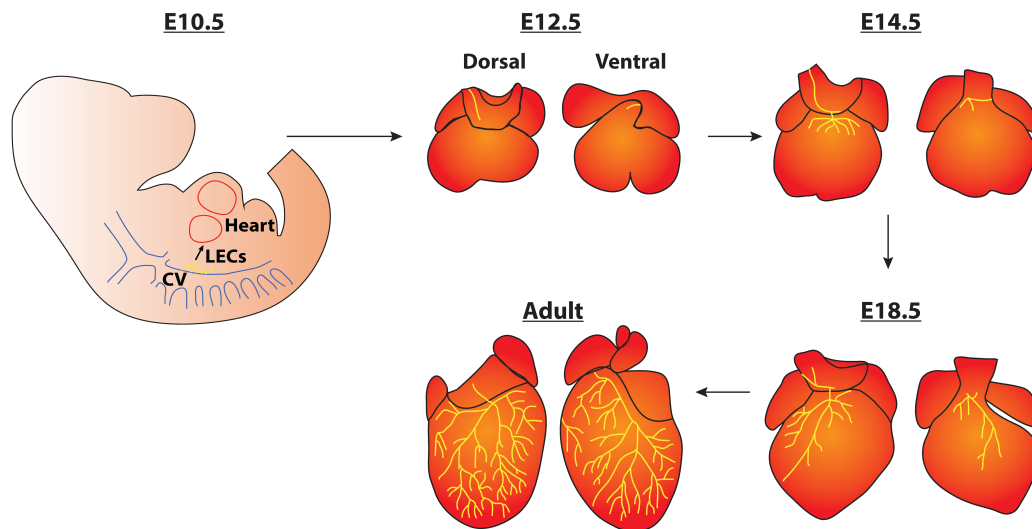


Figure 7.1. The cardiac lymphatic vasculature begins to develop from E12.5 in the mouse embryo and forms an extensive network covering the heart by postnatal stages

From E10.0-11.5 lymphatic endothelial cells begin to bud from the cardinal vein, intersomitic vessels, and surface venous plexus regions and migrate to the heart. The first lymphatic endothelial cells enter the heart along the sinus venosus and by E12.5 primitive lymphatic capillaries can be observed on the dorsal and ventral sides of the heart. The cardiac lymphatic network continues to grow and expand on both sides of the heart from the base towards the apex. This process continues through to postnatal stages, ultimately forming an extensive lymphatic network which covers the epicardial surface of the heart. This figure was included in a review article (Norman & Riley, 2016).

7.2. A proportion of cardiac lymphatic endothelial cells derive from non-venous origins

Prior to the work performed for this thesis, preliminary work in the Riley group had suggested that a proportion of cardiac LECs are non-venous in origin which contradicted previous claims that all lymphatic vessels derive from veins (Srinivasan et

al., 2007). I therefore performed lineage tracing as outlined in aim 2 to further investigate whether a proportion of cardiac LECs do derive from non-venous cellular sources. The results of these experiments are presented in chapter 3 and confirmed that a minor population of cardiac LECs are non-venous in origin. These findings were particularly exciting as they provided some of the first evidence in the field that not all LECs arise from veins. My novel results were based on lineage tracing using R26R-tdTomato/EYFP reporters and *Vav1-Cre*, and *Csf1r-CreER* lines, both of which are expressed within haemogenic endothelium/erythro-myeloid progenitor populations. For both lines immunofluorescent staining and imaging ruled out a lineage contribution to the systemic lymphatic vasculature and venous endothelium. The *Vav1-Cre* line was found to label approximately 20% of LECs within the heart, whereas only rare labelling of a handful of LECs was observed with the *Csf1r-CreER* when induced with 4-OH tamoxifen administered at E7.5. It is interesting that the *Vav1-Cre* line labels a much larger proportion of cardiac LECs than the *Csf1r-CreER* line. This is most likely due to the *Csf1r-CreER* being inducible rather than constitutive. In the inactivated state the CreER fusion protein is sequestered in the cytosol by a heat shock 90 protein (Hsp90). When 4-OH tamoxifen is delivered it binds to the oestrogen receptor (ER), releasing the protein from Hsp90. The CreER fusion protein then translocates to the nucleus and recombination can occur at the *loxP* sites of the R26R-based reporters to generate fluorescent labelling. Consequently inducible (4-OH tamoxifen) labelling is relatively inefficient and only targets a proportion of progenitor cells which are present at E7.5. It is also possible that the additional cells labelled by the *Vav1-Cre* line are progenitors within the haemogenic endothelium which arise later than E7.5 as this specialised cell population persists in the embryo until around E10.5 in the AGM region (Gritz & Hirschi, 2016); furthermore they could also arise from HSCs within the foetal liver and bone marrow at later stages.

Over the past 18 months other groups have also published observations of non-venous derived LECs in other organ-based lymphatic beds in the skin and gut mesentery (Mahadevan et al., 2014, Martinez-Corral et al., 2015, Stanczuk et al., 2015). These studies also employed a lineage tracing approach which revealed that a proportion of LECs in the gut mesentery are derived from the haemogenic endothelium, however, the non-venous origins of dermal LECs remain to be discovered. The finding that there is a contribution of non-venous LECs to some organ-based lymphatic beds, including the heart, which does not occur in the formation of the systemic lymphatic vasculature, prompts the questions of why this cellular heterogeneity occurs and how LECs from multiple origins integrate into the wider organ-based lymphatic network. In the heart non-venous LECs appear to be dispersed randomly with no particular clustering of these cells in specific regions of the vasculature. Rather than coming together from multiple regions all LECs appear to migrate into the heart by tracking along the sinus venosus region to form a network. It is, therefore, likely that the non-venous LECs are already present within the population of cells which enter the heart at this site. This is in contrast to the observation in the skin that non-venous LECs clustered in close proximity to the tips of sprouting vessels and then integrated into the network in a process described as “lymphvasculogenesis” (Martinez-Corral et al., 2015). Precisely how non-venous derived LECs integrate with venous LECs (derived from the cardinal vein/ISV/sVP region) to enter the heart remains to be established. One approach to further investigate this would be to collect *Vav1-Cre;R26R-tdTomato* and *Csf1r-CreER;R26R-tdTomato* embryos at E12.5 in order to detect the location of non-venous LECs at the initial stages where lymphatics begin to form in the heart. Immunofluorescent staining for lymphatic markers and light sheet imaging would be performed on isolated torsos (see **Figure 3.2f-h**) in which non-venous LECs are labelled.

All of the lineage tracing studies performed to gain insight into the origins of LECs have made use of multiple Cre lines. The Cre-lox system is an extremely useful tool for the labelling of distinct cell populations and fate mapping, however, it is not without confounding issues. Recent investigations by the JAX laboratory for various Cre lines have revealed multiple problems which include Cre activity in off-target tissues, parent-of-origin effects and variable activity between littermates (Heffner et al., 2012). The observation that Cre activity can occur in off-target cell populations means that conclusions drawn from lineage tracing data must be made with caution. Inducible CreER lines offer the advantage of being activated only at the specific time point when tamoxifen is administered, thus allowing early embryonic recombination events in unwanted cell types to be avoided. The work presented here which supports the non-venous origin of LECs has employed the constitutive *Vav1-Cre* line and the inducible *Csf1r-CreER* line. By using multiple lines, including an inducible line, and analysing a large number of samples the conclusions from these lineage tracing studies are made with as much confidence as is possible given the tools which are currently available. Further work could be performed to confirm my conclusions by combining the Dre-rox and Cre-lox systems to perform dual labelling on non-venous and venous derived LECs in parallel. The Dre-rox system is similar to Cre-lox in that it can provide highly efficient site-specific recombination (Anastassiadis, Fu et al., 2009). Importantly for dual labelling, Cre cannot induce recombination at *rox* sites and conversely Dre cannot drive recombination at *loxP* sites. This means that two cell populations can be traced concomitantly (Zhang, Pu et al., 2016). However, the commercial availability of Dre and rox lines is limited meaning that lines to perform additional lineage tracing would first have to be generated in-house.

7.3. PROX1 is required for the formation of the cardiac lymphatic vasculature

PROX1 has been termed the “master regulator” in the development of the systemic lymphatic vasculature in mammals as it is essential for specification and maintenance of LEC fate (Hong et al., 2002, Wigle & Oliver, 1999). In zebrafish, however, *prox1a* is not essential for the formation of lymphatics (van Impel, Zhao et al., 2014). Aim 3 of this thesis was to determine whether PROX1 is also required for the development of murine cardiac lymphatic vessels and to determine its role in LECs derived from both venous and non-venous origins. I generated mutants in which PROX1 was deleted in the venous (*Tie2-Cre*) or non-venous (*Vav1-Cre*) compartments.

Tie2-Cre;Prox1^{fl/fl} embryonic hearts displayed severely disrupted lymphatics which in some cases were completely absent. This demonstrates that LECs derived from a non-venous source are not sufficient to compensate for the loss of LECs from a venous origin and shows that PROX1 is required for the formation of cardiac lymphatics that derive from veins. Furthermore, mild *Tie2-Cre;Prox1^{fl/fl}* mutants, in which PROX1 was partially deleted, displayed a truncated lymphatic network which was also mispatterned. This indicates a novel role for PROX1 in remodelling of the lymphatic plexus.

Vav1-Cre;Prox1^{fl/fl} animals displayed a grossly normal lymphatic network in the heart although specific examples of where LECs from the *Vav1* lineage had been deleted and lost from the network were detected revealing that PROX1 is also essential for the specification of LECs from this origin. Whilst I have established here that non-venous derived LECs are not essential for the formation of the embryonic cardiac lymphatic network, the question of the purpose of these cells and whether they have a distinct

functional roles remains. In order to investigate this further experiments would need to be performed in adult *Vav1-Cre;Prox1^{fl/fl}* animals to determine whether the deletion of these cells has any effect on the function of the cardiac lymphatics in homeostasis and pathological settings.

7.4. Limitations of the murine MI model

To gain further understanding of the role and response of cardiac lymphatic vessels to MI a mouse model of permanent occlusion of the LAD was employed to generate the data included in chapters 5 and 6 of this thesis. Whilst this model provides a useful tool to study the cardiac injury which occurs in humans it is important to note that it does have certain limitations. Occlusion of the LAD represents the clinical event in terms of the ischemic injury that occurs upon blockage of a coronary artery, but it does not model the subsequent angioplasty to restore perfusion which usually occurs when the patient is admitted to hospital. Arguably to better model the events associated with reperfusion a surgical ischemia reperfusion model would have been used for these studies, however, this technique has not yet been established in the Riley group. It is also important to consider that MI surgeries were performed on young, healthy mice which does not accurately represent the situation in humans where MI usually occurs due to the rupture of an inflamed atherosclerotic plaque. Recent evidence has revealed that atherosclerosis induces chronic monocytosis which impacts on the inflammatory environment during post-MI healing (Nahrendorf, Pittet et al., 2010). It has also been demonstrated that atherosclerotic *ApoE^{-/-}* mice have impaired infarct healing when compared with controls (Panizzi, Swirski et al., 2010). Ideally to better model the human condition, all MI studies should be performed in atherosclerotic mice, however, in practise this would be challenging as all transgenic lines used for surgery would have to be crossed onto the *ApoE^{-/-}* background.

The surgical MI model can result in significant variability between animals independent of surgical expertise and attempts to ligate at equivalent points down the length of the LAD. Variations in infarct size may have implications on some of the data presented in this thesis. It is likely that animals with larger infarcts experience a larger inflammatory response and possibly greater levels of lymphangiogenesis. Therefore, variations in the extent of the lymphatic response and degree of inflammation within experimental groups may mask the detection of changes which could be significant were all animals to have equivalent infarct sizes. In order to overcome this issue it would be preferential to use large cohorts of animals for all studies, however, in practice the number of animals used in studies was limited by the number of genetically modified mice available, the number of surgery hours required, and UK Home Office regulations. Another option to improve the MI protocol would be to employ methods to assess the extent of injury shortly after surgery in order to exclude any infarcts which are significantly smaller or larger than average. This could be performed by carrying out an echocardiogram of the heart or by collecting blood plasma as early as 8 hours after injury to measure levels of cardiac troponin T which have been reported to correlate with infarct size (Metzler, Hammerer-Lercher et al., 2002, Vietta, Andrades et al., 2013).

7.5. Clearance of post-MI macrophages by cardiac lymphatics

As introduced in chapter 1, many studies have revealed that lymphatic vessels are essential for the clearance of inflammatory cells and fluid that accumulate in inflamed tissue. In chapters 5 and 6 of this thesis aims 4 and 5 were addressed in order to gain further insight into the significance of the lymphangiogenic process that occurs post-MI and how modulation of this response affects the clearance of inflammation and cardiac healing. Prior to my work many papers studying the inflammatory response to MI had been published but none had investigated the role of lymphatic vessels in this process.

Whilst innate immune cells including macrophages (Bellingan et al., 1996, Bellingan et al., 2002, Lawrance et al., 2016), neutrophils (Beauvillain et al., 2011, Rigby et al., 2015), and dendritic cells (Randolph et al., 2005) are known to be transported in lymphatic vessels evidence as to whether these cells are cleared by this route post-MI was lacking. I therefore performed immunofluorescent staining on post-MI hearts in order to establish whether macrophages are present within the lymphatics of the inflamed heart. The staining indicated that CD68⁺ macrophages are indeed taken up by the cardiac lymphatics, thereby providing the first direct visual evidence of this process. Ideally live intravital imaging would be performed to observe the transport of immune cells within cardiac lymphatics, this is discussed further in chapter 8. Another approach to further investigate the trafficking of macrophages from the heart would be to collect the mediastinal lymph nodes to which the cardiac lymphatics drain.

7.6. VEGF-C (C156S) therapy modulates post-MI inflammation and healing

Cardiac injury triggers a significant lymphangiogenic response as demonstrated previously in the Riley lab by Klotz (Klotz et al., 2015) and by my data in chapter 5. Furthermore, others have established that the growth factor protein VEGF-C drives lymphangiogenesis during embryonic development and in states of inflammation where expansion of the lymphatic network occurs. It has also been demonstrated that addition of adenoviral or recombinant VEGF-C can enhance lymphangiogenesis, leading to improved clearance of inflammation and better clinical outcomes (Cui et al., 2015, D'Alessio et al., 2014, Huang et al., 2016, Huggenberger et al., 2011). In chapter 5 I have shown that both macrophages and the activated epicardium secrete VEGF-C in response to cardiac injury, driving expansion of the lymphatic network. I also revealed

that administration of several doses of recombinant VEGF-C (C156S) protein, rather than a single dose, during the first week after MI could significantly enhance lymphangiogenesis. Dosing with multiple VEGF-C (C156S) injections lead to improvements in left-ventricular ejection fraction at days 14 and 21 post-MI, however, further work, as outlined in chapter 8 needs to be performed to determine whether this effect persists. Should there be a long term improvement in cardiac function this would suggest that recombinant VEGF-C (C156S) may be useful to treat patients. However, given the short half-life of VEGF-C, which is less than 15 minutes (Veikkola et al., 2001), and the need for multiple doses, large amounts of protein would be required to achieve a therapeutic effect which would be associated with a high cost. It is, therefore, important to consider alternative strategies for enhancing the lymphangiogenic response. There is a need for further drug discovery research in this field as no synthetic pro-lymphangiogenic drugs currently exist.

Whilst pro-lymphangiogenic therapy may provide a promising way of augmenting the inflammatory response to promote improved healing conditions it must be considered that alone it may not be sufficient to permanently improve cardiac function. During MI there is a large area of necrotic tissue meaning that multiple cell types including cardiomyocytes and blood vessels need to be replaced. Therefore, it is possible that optimal repair of the damaged heart may require a combination of therapeutic approaches, including pro-lymphangiogenic drugs, which combined may be capable of reducing scarring and aiding in the regeneration process to replace the damaged tissue with new cells and blood vessels as discussed in section 1.4.1.

Investigations presented in chapter 5 were also performed to gain a better understanding of how VEGF-C (C156S) induced enhanced lymphangiogenesis impacts

on post-MI inflammation within the heart. The key finding from this work was that multiple doses of VEGF-C (C156S) post-MI leads to a reduction in the numbers of myeloid cells, including macrophages remaining within the heart by day 7 post-MI. However, at days 1 and 4 post-MI there was no impact of VEGF-C (C156S) treatment on the number of innate immune cells remaining within the myocardium. This indicates that the increased clearance of inflammation observed by day 7 post-MI is likely due to increased uptake of cells by the denser lymphatic network present by day 7 post-MI in VEGF-C (C156S) treated animals. Further experiments should be performed to determine whether numbers of immune cells, including macrophages, collected in the draining mediastinal lymph nodes correlate with the reduction observed in the heart. Increased apoptosis of leucocytes in the heart of VEGF-C (C156S) treated animals should also be ruled out. These experiments are described in more detail in chapter 8.

Several studies have previously reported that reducing the number of inflammatory (M1) macrophages and increasing the number of reparative (M2) macrophages and the factors they release is beneficial for post-MI healing (Burchfield et al., 2008, Kaikita et al., 2004, Majmudar et al., 2013, Santini et al., 2007, Shiraishi et al., 2016). However, a prolonged presence of reparative macrophages in the infarcted region can exacerbate the fibrotic response, resulting in compromised function (Hulsmans et al., 2016). I investigated whether there is a preference for the cardiac lymphatics to clear inflammatory or reparative macrophages when stimulated with VEGF-C (C156S) treatment. I performed qRT-PCR at day 7 post-MI to characterise gene expression profiles of sorted cardiac macrophages remaining within the heart and of the pro-inflammatory cytokines and collagen genes expressed in the infarct. This data did not reveal any statistically significant changes with VEGF-C (C156S) treatment which suggests that there is no change in the ratio of inflammatory/reparative markers of

macrophages remaining in the heart. In order to investigate this further, another flow cytometry study should be performed where CD206 is included in the panel to allow M1 and M2 numbers to be directly quantified (Lorchner et al., 2015). However, it is important to consider that the M1/M2 classification is an oversimplification with macrophages *in vivo* being much more plastic with graded responses acting across the inflammation and repair phases. It is surprising that no changes in the pro-inflammatory cytokines and collagen genes expressed in the infarcted tissue were detected, as an overall reduction in the number of macrophages present was expected to lead to a reduction in the levels of cytokines expressed and induced by macrophages. It may be that such an effect does occur at a later time point, or the dense and heterogeneous infarct tissue makes it difficult to dissect out subtle gene expression changes.

Together, the data I have collected reveals that multiple doses of VEGF-C (C156S) treatment post-MI results in enhanced lymphangiogenesis by day 7 post-MI, at which time point a reduced number of macrophages in the heart is also observed. This suggests that the expanded lymphatic network is capable of clearing an increased number of macrophages from the infarcted heart. The timely clearance of macrophages to reduce the load in the heart may create an improved inflammatory environment for cardiac healing and a reduction of the adverse effects associated with macrophage persistence. Both inflammatory, and reparative macrophages, if present in the post-MI heart at high levels for too long, can have a negative impact on cardiac healing and functional outcomes. Excessive levels of inflammatory cytokines can have deleterious effects due to the excessive break down of cardiac tissue, whilst the prolonged presence of reparative macrophages may result in excessive fibrosis and increased scarring. I also observed improvements in cardiac function at days 14 and 21 post-MI with VEGF-C

(C156S) which supports the theory that an improved clearance of macrophages from the heart is beneficial for cardiac healing. A diagrammatic summary of these results is shown in **Figure 7.2**.

Figure 7.2

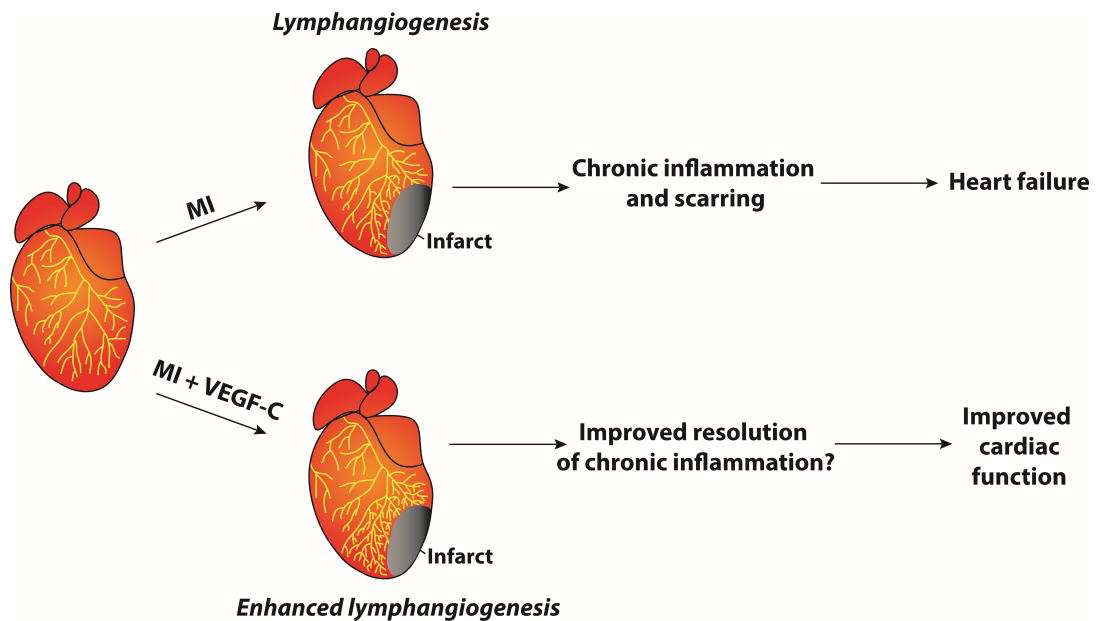


Figure 7.2. Treatment with recombinant VEGF-C (C156S) following MI in a murine model leads to enhanced lymphangiogenesis and improved cardiac function

Treatment with VEGF-C (C156S) post-MI induces an enhanced lymphangiogenic response which may improve the resolution of inflammation, reducing scarring. Results from cardiac MRI revealed that treatment with recombinant VEGF-C improves cardiac function at days 14 and 21 post-MI. This figure was included in a review article (Norman & Riley, 2016).

7.7. Genetic perturbation of the cardiac lymphatics to assess the requirement for post-MI lymphangiogenesis and immune cell clearance

Two genetic models were employed to further investigate the requirement for the cardiac lymphatics during in the post-MI inflammatory and healing processes. LYVE-1

is known to be required for the transmigration of innate immune cells including macrophages across the inflamed lymphatic endothelium (Lawrance et al., 2016), (Bhattacharjee & Jackson, unpublished data). The previously published *LYVE-1*^{-/-} mouse model (Gale et al., 2007), therefore, provides a useful tool to block the clearance of innate immune cells from the post-MI heart via lymphatic vessels and to study the downstream effects of this. Myeloid cells, including macrophages and neutrophils failed to clear from the hearts of post-MI *LYVE-1*^{-/-} animals. This observation is in contrast to the effect of VEGF-C (C156S) therapy which resulted in a reduction in the number of macrophages within the heart post-MI. This is illustrated in a diagrammatic summary shown in **Figure 7.3**. My data is also in agreement with the results of studies where suppression of lymphangiogenesis in inflammatory models has been found to result in the persistence of inflammation and poorer outcomes (Baluk et al., 2005, Guo et al., 2009, Wirzenius et al., 2007). Together these findings strongly indicate that cardiac lymphatic vessels do play an important role in the clearance of immune cells from the infarcted tissue. Further experiments, as discussed in more detail in chapter 8, are required to determine whether the failed clearance of inflammatory cells in *LYVE-1*^{-/-} hearts has a downstream impact on fibrosis, scarring and cardiac function.

Tie2-Cre;Prox1^{fl/+} animals were also employed for MI studies which are presented in chapter 6. I hypothesised that these mice, which are haploinsufficient for *Prox1*, would display an impaired lymphangiogenic response to MI, thus allowing the impact of suboptimal lymphangiogenesis on cardiac healing to be investigated. I performed qRT-PCR on *Tie2-Cre;Prox1*^{fl/+} hearts which revealed that a significant downregulation of key lymphangiogenic genes does indeed occur. This result provides the basis for further use of this model in MI studies to assess the impact of impaired post-MI lymphangiogenesis on the clearance of inflammation.

Figure 7.3

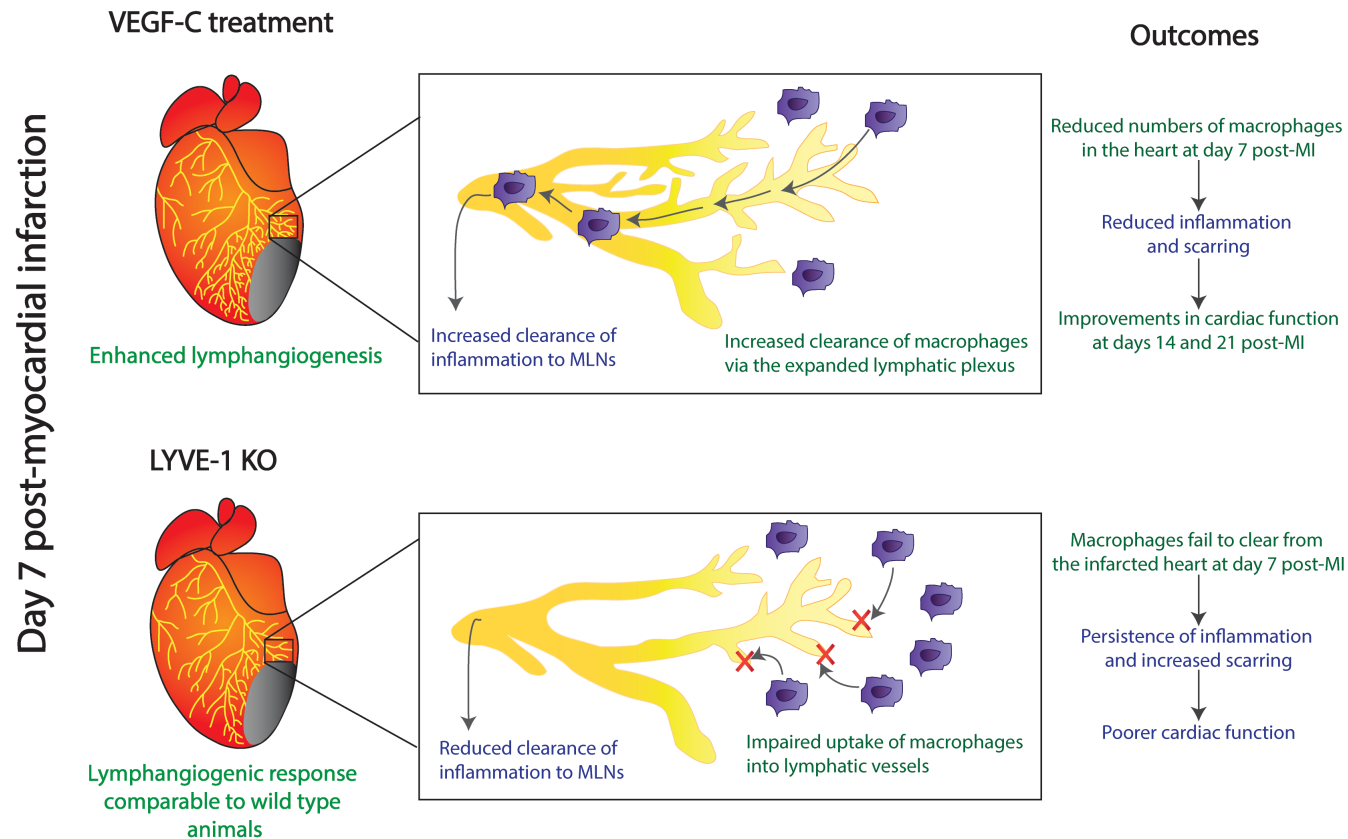


Figure 7.3. Summary of the post-MI effects of VEGF-C treatment or LYVE-1 deletion

Treatment with multiple doses of VEGF-C (C156S) post-MI induces an enhanced lymphangiogenic response which leads to increased clearance of macrophages via the expanded lymphatic network. LYVE-1 is involved in the transport of macrophages into lymphatic vessels during inflammation. LYVE-1 knockout (KO) mice display a normal lymphangiogenic response but the impaired uptake of macrophages into the lymphatics results in the accumulation of these cells within the heart by day 7 post-MI. Green text denotes conclusions which can be drawn from the data presented in this thesis. Blue text denotes hypotheses which require further investigation.

7.8. Conclusions

The work presented in this thesis has provided novel insights into the development of cardiac lymphatic vessels and their response to MI. A detailed analysis of embryos at multiple time points revealed that the cardiac lymphatics begin to form in the heart in the sinus venosus region around E12.5, with LECs appearing to track towards the heart from the cardinal vein/ISV region. The LECs then migrate down both sides of the heart from the base towards the apex and expand to form a branched network which runs closely alongside coronary veins. The network increases in density and continues to undergo remodelling through to postnatal stages. Lineage tracing demonstrated that a proportion of LECs are labelled by the non-venous *Vav1-Cre* and *Csf1r-CreER* lines, which target early progenitor populations in the yolk sac blood islands and haemogenic endothelium. My findings, together with those recently made by others in the skin and gut, have provided evidence countering the previous dogma that all lymphatic vessels are derived exclusively from veins. This highlights discrepancies between the mechanisms by which organ based and systemic lymphatic beds develop.

Investigations in adult mice revealed that the cardiac lymphatic vessels undergo extensive lymphangiogenesis in response to MI, which is driven by VEGF-C secreted from the epicardium and a subpopulation of macrophages. Administration of several doses of recombinant VEGF-C (C156S) protein post-MI was sufficient to enhance this lymphangiogenic response, leading to a denser lymphatic capillary network. This VEGF-C (C156S) treatment regime also resulted in increased clearance of macrophages from the heart by day 7 post-MI and improvements in cardiac function at days 14 and 21. It was also revealed that when uptake of myeloid cells into the lymphatics is blocked post-MI in *LYVE-1*^{-/-} animals macrophages and neutrophils accumulate in the heart. These data highlight the importance of the cardiac lymphatics as a route for clearance

of inflammatory cells from the infarcted tissue and indicate that they may be a useful therapeutic target to modulate post-MI inflammation and healing.

8. Future work

Following on from the findings of this thesis the main aims of future work will be to further investigate the following points:

1. Are bone marrow derived cells able to contribute to expansion of the cardiac lymphatic network post-MI?
2. Further evidence of macrophage trafficking by cardiac lymphatic vessels post-MI
3. Do cardiac lymphatics discriminate between macrophage subtypes during the clearance of post-MI inflammation?
4. Does post-MI VEGF-C (C156S) therapy lead to maintained, long-term improvements in cardiac function?
5. Do *LYVE-1*^{-/-} animals display impaired post-MI healing and poorer cardiac outcomes?
6. Use of the *Tie2-Cre;Prox1*^{fl/+} model to further investigate the requirement for lymphangiogenesis in the resolution of post-MI inflammation

8.1. Are bone marrow-derived macrophages able to contribute to the expansion of the cardiac lymphatic network post-MI?

I have established here that in the adult mouse the cardiac lymphatic vasculature undergoes significant expansion during the first 7 days following MI in a process which is driven by the release of VEGF-C from the activated epicardium and macrophages. Whilst lymphangiogenesis is the main accepted mechanism by which growth of the lymphatic network occurs during pathological conditions there have been some reports of a direct lineage contribution from macrophages which are able to transdifferentiate

into LECs (Hall et al., 2012, Kataru et al., 2009, Kerjaschki et al., 2006, Kerjaschki et al., 2004, Maruyama et al., 2005, Zumsteg et al., 2009). My observation that cells from the embryonic yolk sac and haemogenic endothelium contribute to cardiac LECs during development also suggests that it may be possible that bone marrow-derived cells (BMCs), including macrophages, could contribute to new lymphatics upon injury. Furthermore, in section 5.1.1. I observed co-localization between CD68⁺ macrophages and lymphangiogenic tips at day 4 post-MI, again indicating that there may be a lineage contribution from BMCs to the expanded lymphatic network. In order to investigate this finding in more detail we will make use of the *Csf1r-CreER* mouse line which, when induced with tamoxifen in the adult, targets bone marrow-derived macrophages and dendritic cells (Qian et al., 2011). We will cross this Cre line to the *R26R-tdTomato* reporter line and then perform MI and induce Cre activity to label macrophages at day 4 after injury. Hearts will be collected at day 7 post-MI, as newly formed lymphatics have previously been observed to be formed by this time point, and will then be subjected to whole-mount immunofluorescent staining for PROX1 and VEGFR-3 to label the lymphatics. Confocal imaging of large biopsies of these stained hearts will allow us to identify infiltration of macrophages to the infarcted region and determine whether there are any newly formed LECs which are also tdTomato⁺. This will enable us to establish whether there is a contribution from infiltrating macrophages to the cardiac lymphatics post-MI in addition to the apparent lymphangiogenesis previously described.

8.2. Further evidence of macrophage trafficking by cardiac lymphatic vessels post-MI

In inflammatory conditions leucocytes, including macrophages, have been observed to be cleared via lymphatic vessels (Beauvillain et al., 2011, Bellingan et al., 1996, Bellingan et al., 2002, Lawrance et al., 2016, Randolph et al., 2005, Rigby et al., 2015). In chapter 5 I presented data which strongly suggests that macrophages are transported into the cardiac lymphatics post-MI. This finding is based on immunofluorescent staining and imaging performed on whole hearts and sections, however, to definitively prove that macrophages are trafficked away from the heart by lymphatics further experiments are required.

We will develop a protocol in order to perform live imaging of immune cell trafficking within lymphatic vessels of the heart. Although live imaging of the heart is challenging, due to the motion caused by the heart beating, one previous study has successfully performed live imaging of immune cells in infarcted mouse hearts (Jung, Kim et al., 2013). Jung and co-workers made a small incision in the intercostal muscles and used a suction-assisted endoscope that exerted pressure on the heart to stabilize its motion. The endoscope was integrated into a confocal microscope which allowed live imaging of immune cells in the same mouse at multiple time points after MI. We will adopt this set-up to image the trafficking of macrophages within cardiac lymphatics following injury. We will cross *CD68-GFP* animals (Iqbal, McNeill et al., 2014), with the *PROX1-tdTomato* line (Truman, Bentley et al., 2012) to generate *CD68-GFP;PROX1-tdTomato* animals in which macrophages will be labelled green and lymphatic vessels will be labelled red. MI surgery will be performed on these animals, followed by live imaging at days 1, 4 and 7 post-MI to assess the role of cardiac lymphatics in trafficking

macrophages during the initial innate immune response. Once this technique has been established further experiments with VEGF-C (C156S) treatment and/or LYVE-1 blocking antibodies could be performed to observe the *in vivo* effect of enhancing or blocking the clearance of immune cells from the post-MI heart.

We will also perform experiments on post-MI mediastinal lymph nodes (MLNs) to which the cardiac lymphatics drain. This will allow detection of macrophages and other innate immune cells that have been trafficked from the post-MI. I have already performed a pilot experiment in which I have successfully isolated the MLNs from uninjured and day 7 post-MI mice, and performed immunofluorescent staining (**Figure 8.1**). This confirmed the samples collected are MLNs as they express the lymphatic markers LYVE-1 and PODOPLANIN, and contain CD68 and F4/80⁺ lymph node resident macrophages. These MLNs will be collected in several post-MI experiments in order to further investigate the inflammatory cells which are drained here from the infarcted heart. However, due to the presence of lymph node resident macrophages in uninjured animals a method needs to be established to distinguish these macrophages from those which have trafficked from the heart following injury. CD169 is only expressed within resident macrophages in the bone marrow, lymph nodes, liver, and spleen. Importantly it has not been previously detected within tissue resident or infiltrating macrophages in the heart (Gray & Cyster, 2012). Staining for CD169, CD68 and F4/80 will be performed on cryosections from hearts at days 4 and 7 post-MI to confirm that CD169 is not expressed within resident or infiltrating cardiac macrophages. If expression of CD169 in cardiac macrophages is excluded further staining will be performed on post-MI MLNs for CD169, CD68 and F4/80. Using confocal imaging we will be able to establish whether there are any CD169⁻ CD68⁺ F4/80⁺ cells present within the post-MI MLNs which should represent any macrophages trafficked from the site of injury.

We will perform MI surgery on *Myh6-CreER;R26R-tdTomato* (Sohal, Nghiem et al., 2001) animals which will be induced with tamoxifen prior to surgery in order to fluorescently label cardiomyocytes. MLNs will be harvested at days 4 and 7 post-MI and subjected to immunofluorescent staining for CD68 and subsequent confocal imaging. This will allow any CD68⁺ tdTomato⁺ macrophages which have phagocytosed cardiomyocytes and have migrated to the MLNs to be detected. Staining will also be performed for CD169 to distinguish between MLN resident macrophages and those which have been trafficked from the heart.

Figure 8.1

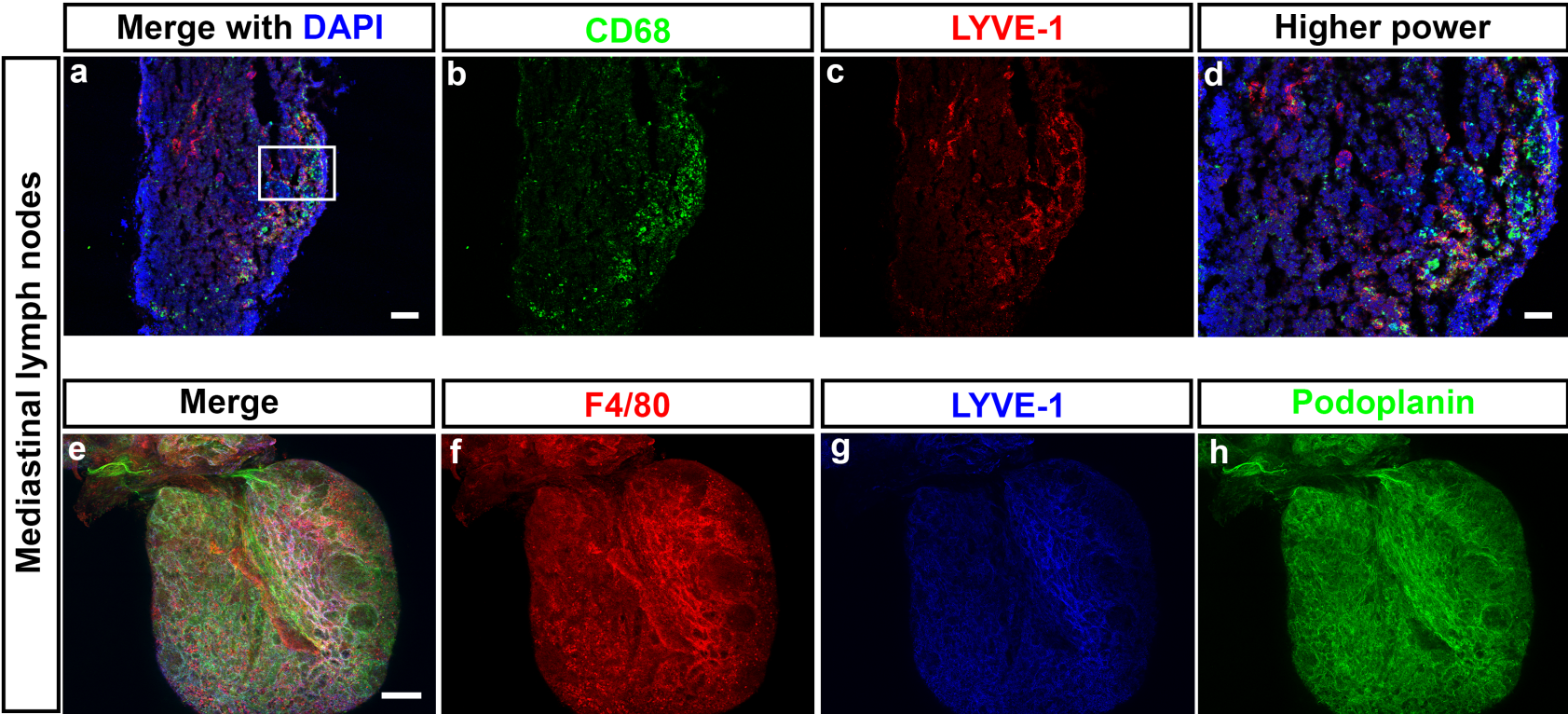


Figure 8.1. Isolation and characterisation of mediastinal lymph nodes

MLNs were isolated from adult mice and subjected to immunofluorescent staining in order to confirm their identity. Panel **a** shows staining for DAPI (nuclei), CD68 (macrophages) and LYVE-1 (lymphatic endothelium) on cyrosections from uninjured animals, separate channels shown in **b**, **c** and higher power is displayed in **d**. Whole-mount MLNs from day 7 post-MI animals were also stained for F4/80 (macrophages), LYVE-1 and PODOPLANIN (lymphatic endothelium) as displayed in **e**, separate channels shown in **f**, **g** and **h**. Scale bars: **a** = 100 μ m, **d** = 50 μ m, **e** = 300 μ m.

8.3. Do cardiac lymphatics discriminate between macrophage polarisations during the clearance of post-MI inflammation?

In chapters 5 and 6 I presented data which revealed that VEGF-C (C156S) therapy enhances lymphangiogenesis, resulting in improved clearance of myeloid cells, including macrophages from the heart by day 7 post-MI. Conversely, MI in *LYVE-1*^{-/-} animals, where uptake of myeloid cells into the lymphatics is impaired resulted in a build-up of neutrophils and macrophages. Both inflammatory and reparative macrophages perform essential roles during the cardiac healing process, however the prolonged presence of these cells may increase adverse remodelling and excessive fibrosis, which is detrimental to cardiac function. We, therefore, propose that timely clearance of both inflammatory and reparative from macrophages from the heart is beneficial for healing to prevent the persistence of inflammation or excessive fibrosis. In order to investigate whether there is preference for the clearance of inflammatory versus reparative macrophages by the cardiac lymphatics I performed qRT-PCR profiling to investigate the cytokine expression profile of the macrophages retained within day 7 post-MI hearts in wild type, VEGF-C (C156S) treated and *LYVE-1*^{-/-} animals. This did not reveal any significant changes in gene expression, suggesting that the clearance of macrophages from the heart by the lymphatics is not dependent on their inflammatory status. However, further work needs to be performed to confirm this. MI will be performed on C57BL/6 and *LYVE-1*^{-/-} animals; half of the C57BL/6 animals will receive four doses of VEGF-C (C156S) as described earlier in this thesis, thus giving three experimental groups. Hearts and MLNs will be collected and digested and stained for flow cytometry. In addition to the panel of antibodies used previously in chapters 5 and 6 to measure numbers of leucocytes, myeloid cells, macrophages, neutrophils, and dendritic cells, antibodies for CD206, and CD169 will be included. CD169 will allow MLN

resident macrophages to be distinguished from those which have trafficked there. CD206 will enable the numbers of M1 and M2 macrophages remaining within the heart and also the numbers of those trafficked to the MLNs to be quantified. This will allow us to determine whether there is a preference for the clearance of a particular subtype of macrophages by the cardiac lymphatics post-MI.

8.4. Does post-MI VEGF-C (C156S) therapy lead to maintained improvements in cardiac function?

In chapter 5 of this thesis I presented the results of a 28-day longitudinal MRI study which was performed on animals treated with four doses of VEGF-C (C156S) during the first week post-MI. This study revealed significant improvements in left-ventricular ejection fraction, a key readout of cardiac function, at days 14 and 21 post-MI in the treated group. However, at day 28 post-MI this significance was lost, although there was still a trend towards improvement with treatment. An additional study with the same experimental set-up and an increased number of animals will be performed where MRI scanning is performed at 6 weeks post-MI. These hearts will also be collected for subsequent histology and Masson's trichrome staining to assess scarring. Our Home Office licencing states that 6 weeks is latest time point at which we are permitted to perform experiments on post-MI animals. Furthermore, by 6 weeks the remodelling and scar maturation processes which occur post-MI are largely complete (Blankestijn et al., 2001). Therefore, assessment at 6 weeks should provide evidence as to whether this VEGF-C (C156S) therapeutic regime does or does not have a long-term beneficial impact on cardiac function.

8.5. Do *LYVE-1*^{-/-} animals display persistent inflammation, impaired post-MI healing and poorer cardiac function?

In chapter 6 I revealed that there is a build-up of neutrophils and macrophages within the hearts of *LYVE-1*^{-/-} mice at day 7 post-MI. We speculate that this is due to impaired clearance of innate immune cells from the heart. Therefore, MLNs from *LYVE-1*^{-/-} injured animals will contain either a reduced or completely diminished number of trafficked macrophages. To investigate this we will collect MLNs from *LYVE-1*^{-/-} and control animals also at day 7 post-MI and perform immunofluorescent staining on cryosections for CD68, F4/80 and CD169 to identify lymph node resident and trafficked macrophages.

To determine whether the inflammatory cells accumulated by day 7 post-MI in *LYVE-1*^{-/-} animals persist we will perform additional flow cytometry experiments to quantify immune cell numbers at days 14 and 21 post-injury. Normally by 2-3 weeks after injury, during the scar maturation stage, macrophages are no longer present in the infarcted heart in large numbers (Nahrendorf et al., 2007). We hypothesise that in *LYVE-1*^{-/-} hearts there will be increased numbers of innate immune cells, including macrophages, at days 14 and 21 post-MI due to impaired clearance by the cardiac lymphatics. We will also perform FACS to collect F4/80⁺ macrophages from control and *LYVE-1*^{-/-} hearts at these stages and will analyse the cytokine expression profile of the accumulated cells by qRT-PCR.

We will investigate whether the accumulation of inflammatory cells within *LYVE-1*^{-/-} hearts results in excessive cardiac remodelling, fibrosis and scarring, leading to impaired cardiac function. MI surgery will be performed on *LYVE-1*^{-/-} and C57BL/6

animals which will then undergo repeat MRI scanning at days 7, 14, 21, 28 and 6 weeks post-injury in order to measure a variety of cardiac parameters including left-ventricular ejection fraction. Hearts will also be collected from animals at the end of the study for histology and Masson's trichrome staining to assess scarring.

8.6. Further investigations into the role of post-MI lymphangiogenesis using the *Tie2-Cre;Prox1^{fl/+}* model

Data presented in chapter 6 demonstrated that *Tie2-Cre;Prox1^{fl/+}* animals may provide a useful tool to determine the requirement for lymphangiogenesis in the clearance of post-MI inflammation and the subsequent healing and scarring process. However, this initial observation, which was solely based on reduced expression of the key lymphangiogenic genes *Prox1*, *Vegfr3* and *Lyve1*, requires further investigation. We will, therefore, perform whole-mount immunofluorescent staining for LYVE-1 and VEGFR-3 and light sheet imaging on hearts from *Tie2-Cre;Prox1^{fl/+}* animals at day 7 post-MI. The labelled lymphatic network will then be imaged using light sheet microscopy in order to assess whether lymphangiogenesis is indeed impaired as we hypothesise based on the gene expression data. Hearts and MLNs from *Tie2-Cre;Prox1^{fl/+}* animals will also be collected at day 7 post-MI for flow cytometry to assess whether there is an effect of this genetic perturbation on clearance of innate immune cells via the cardiac lymphatics. Should post-MI lymphangiogenesis be disrupted in the *Tie2-Cre;Prox1^{fl/+}* animals this may result in impaired clearance of immune cells and the persistence of inflammation. Flow cytometry will be performed using the same panel of antibodies as in this thesis to measure numbers of pan-leucocytes, myeloid cells, macrophages, neutrophils and dendritic cells. If clearance of inflammation via the cardiac lymphatics is impaired, we would expect to observe an increase in the number of innate immune cells remaining

within the heart and a reduction in the number which arrive at the MLNs. This may result in the persistence of inflammation which has the potential to increase scarring and fibrosis, resulting in poorer cardiac outcomes. We will, therefore, also perform a longitudinal MRI study with repeat scanning at days 7, 14, 21, 28 and 6 weeks post-injury in order to measure a variety of cardiac parameters including left-ventricular ejection fraction. Hearts will also be collected from animals at the end of the study for histology and Masson's trichrome staining to assess scarring.

By employing both *LYVE-1*^{-/-} and *Tie2-Cre;Prox1*^{fl/+} animals for MI studies we will be able to compromise lymphatic function in two separate ways; by blocking immune cell uptake and impairing lymphangiogenesis. We hypothesise that MI in these models will result in the persistence of inflammatory cells, impaired cardiac healing and poor functional outcomes which would elevate the cardiac lymphatics as an important anti-inflammatory target post-MI.

9. Final remarks

The hypotheses developed at the outset of this project were:

- 1) A proportion of the cardiac lymphatic vasculature is non-venous in origin
- 2) PROX1 is essential for the development of cardiac lymphatic vessels
- 3) Administration of the pro-lymphangiogenic growth factor VEGF-C will promote enhanced lymphangiogenesis and aid the clearance of inflammation post-MI
- 4) Impaired lymphangiogenesis or disrupted uptake of post-MI immune cells by cardiac lymphatic vessels will result in impaired clearance of immune cells and a poor outcome post-MI

The data presented in this thesis has provided validation for the above hypotheses. From this work I have contributed new findings to the field of lymphatic development by describing how and when the cardiac lymphatics form and by revealing that cardiac LECs are heterogeneous in origin, deriving from veins and non-venous progenitors. I have also demonstrated that the cardiac lymphatics play an important role in the clearance of post-MI inflammatory cells and that therapeutic modulation of this response may provide an improved inflammatory environment, conferring benefits to the cardiac healing process. My data, which has been obtained from a murine model, provides findings which act a basis for extrapolation to humans. The recent development of a model to generate LECs *in vitro* from human pluripotent stem cells (Lee, Park et al., 2015) provides a platform to perform human based drug screening for novel regulators of lymphangiogenesis. This may lead to the identification of new therapies to modulate inflammation and cardiac healing in post-MI patients via targeting of the cardiac lymphatics.

Appendix I – Achievements

Meetings attended

Oral presentations:

- 19th International Vascular Biology Meeting, Boston, October 2016
- BHF Oxbridge Centres for Regenerative Medicine meeting, Royal Society London, October 2015
- BSCR Cell therapy for cardiovascular disease in the 21st century: a reflective and prospective update, Glasgow, June 2015
- London Heart Development meeting, May 2013

Poster presentations:

- GRS Lymphatics, Ventura CA, March 2016
- GRC Lymphatics, Ventura CA, March 2016
- Weinstein Cardiovascular Development and Regeneration Conference, Madrid, April 2014

Prizes

Runner-up prize, Sherrington Talks 2016

- Prize awarded for my presentation which was at a day of talks given by final year DPhil students in the Department of Physiology, Anatomy and Genetics

Best young investigator talk, BSCR Glasgow 2015

Peter Beaconsfield Prize, University of Oxford Medical Sciences division, 2015

- Runner-up prize awarded for an essay written on my research and how it has the potential to be translated for clinical therapies. This competition was open to all DPhil candidates across five university departments.

Poster prize, DPAG poster day 2014

- 1st prize awarded at a graduate student poster day in the Department of Physiology, Anatomy and Genetics

Publications

Published papers:

Klotz, L.*, Norman, S.*, Vieira, J. M.* et al. (2015). "Cardiac lymphatics are heterogeneous in origin and respond to injury." Nature **522**(7554): 62-67.

* denotes joint-first authorship. See appendix II for abstract.

Iqbal, A. J., McNeill E., Kapellos T.S., Regan-Komito D., Norman S et al. (2014). "Human CD68 promoter GFP transgenic mice allow analysis of monocyte to macrophage differentiation in vivo." Blood **124**(15): E33-E44. See appendix III for abstract.

Papers in preparation:

Sophie Norman, Thomas Cahill, Joaquim Miguel Vieira, Mala Rohling, Louise A. Johnson, David G. Jackson and Paul R. Riley. "Developmental programming of the cardiac lymphatics towards immunomodulation and improved heart repair." See appendix IV for abstract.

Published reviews:

Norman, S. and P. R. Riley (2016). "Anatomy and development of the cardiac lymphatic vasculature: Its role in injury and disease." Clinical Anatomy **29**(3): 305-315. See appendix V for abstract.

Appendix II

Joint first authorship on a paper published in *Nature* in 2015. I performed experiments and analysis of data.

ARTICLE

doi:10.1038/nature14483

Cardiac lymphatics are heterogeneous in origin and respond to injury

Linda Klotz^{1*}, Sophie Norman^{2*}, Joaquim Miguel Vieira^{2*}, Megan Masters², Mala Rohling², Karina N. Dubé¹, Sveva Bollini³, Fumio Matsuzaki⁴, Carolyn A. Carr² & Paul R. Riley²

The lymphatic vasculature is a blind-ended network crucial for tissue-fluid homeostasis, immune surveillance and lipid absorption from the gut. Recent evidence has proposed an entirely venous-derived mammalian lymphatic system. By contrast, here we show that cardiac lymphatic vessels in mice have a heterogeneous cellular origin, whereby formation of at least part of the cardiac lymphatic network is independent of sprouting from veins. Multiple Cre-*lox*-based lineage tracing revealed a potential contribution from the putative haemogenic endothelium during development, and discrete lymphatic endothelial progenitor populations were confirmed by conditional knockout of *Prox1* in *Tie2*⁺ and *Vav1*⁺ compartments. In the adult heart, myocardial infarction promoted a significant lymphangiogenic response, which was augmented by treatment with VEGF-C, resulting in improved cardiac function. These data prompt the re-evaluation of a century-long debate on the origin of lymphatic vessels and suggest that lymphangiogenesis may represent a therapeutic target to promote cardiac repair following injury.

In 1902, Florence Sabin proposed that the primary lymph sacs originate from the embryonic veins and then give rise to the entire lymphatic vasculature by sprouting and remodelling¹. An alternative model of lymphatic development was proposed by Huntington and McClure in 1910, who suggested that lymph sacs arise in the mesenchyme, independently of veins, via distinct progenitor cells². More recent evidence has supported Sabin's model, such that trans-differentiation of venous into lymphatic endothelial cells (LECs) is now widely accepted, with the veins regarded as the sole origin of the entire lymphatic vasculature in mammals^{3–7}. To date, studies which support a venous origin have focused exclusively on the development of the systemic lymphatic vasculature. Organ-based lymphatics have received little attention and in the heart, while the presence of cardiac lymphatic vessels has been described⁸, virtually nothing is known about their role during development or in the healthy or failing adult heart. We therefore sought to characterize the formation of the cardiac lymphatic vessels through developmental stages, to identify their embryonic origin and effect during organogenesis and to assess their response to pathological insult in the adult setting.

Development of the cardiac lymphatic vasculature

Whole-mount staining of murine hearts for early LEC markers VEGFR-3 (ref. 9) and *Prox1* (ref. 10), revealed the emergence of lymphatic vessels at embryonic day 12.5 (E12.5), sprouting from extra-cardiac regions proximal to the outflow tract, on the ventral side (Fig. 1a, increased magnification in Fig. 1b). At E14.5, lymphatic vessels were observed on the ventricular surface sprouting from the region of the sinus venosus, on the dorsal side (Fig. 1c, increased magnification in Fig. 1d and Extended Data Fig. 1a, increased magnification in Extended Data Fig. 1b). At E16.5 the major dorsal vessels spread inferiorly from the inflow region (Fig. 1e, increased magnification in Fig. 1f), while ventrally smaller vessels arose between the atria (Extended Data Fig. 1c, d). By E18.5, the vessels continued to expand and projected towards the apex of the heart on both dorsal and ventral

surfaces (Fig. 1g, h and Extended Data Fig. 1e, f). From birth (post-natal day 0 (P0)), the vessels developed a more extensive branched network and expanded further over the ventral side of the neonatal heart (Fig. 1i, j). By P10, the cardiac lymphatics provided superficial coverage of the majority of the epicardial surface of the heart (Extended Data Fig. 1g, h) and appeared fully developed by P15 (Extended Data Fig. 1i, j). The lymphatic identity of the VEGFR-3- and *Prox1*-labelled cardiac vessels (Fig. 1a–j and Extended Data Fig. 1a–n) was further validated by co-immunostaining for the lymphatic vessel endothelial hyaluronan receptor 1 (Lyve-1), which also labels tissue macrophages¹¹. Coronary LECs within the expanding plexus on both dorsal and ventral sides of the developing heart co-expressed VEGFR-3, *Prox1* and Lyve-1 (Extended Data Fig. 1o–v). Cardiac lymphatic vessels aligned with the endomucin (Emcn)-positive coronary veins during late gestation (E15.5–18.5) (Fig. 1k–m) and established extensive inter-vessel connections analogous to blood vessel anastomosis (Fig. 1n–p). At birth (P0) lateral Lyve-1⁺ sprouts beneath smooth-muscle-actin-positive coronary veins (Fig. 1q–s) were indicative of a close anatomical relationship between the coronary veins and developing lymphatic vasculature (Fig. 1t).

A venous and non-venous contribution of LECs

Prox1⁺ LECs did not appear to emerge or bud-off from Emcn-expressing coronary vessels between E12.5–14.5 (Extended Data Fig. 2a–i). Instead, extra-cardiac LECs migrated into the sinus venosus on the dorsal side, and outflow tract on the ventral side of the heart by E12.5 (Extended Data Fig. 2a, b, also Fig. 1a, b) and expanded to form a network proximal to Emcn⁺ veins from E13.5 and E14.5 (Extended Data Fig. 2d–i) through to E17.5 (Extended Data Fig. 2j–o). Whole embryo staining at E10.5 and E12.5 (Extended Data Fig. 3a–f) revealed a *Prox1*/VEGFR-3-expressing LEC population emerging from the Emcn⁺ common cardinal vein and migrating towards the neighbouring sinus venosus and outflow tract (Extended Data

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*These authors contributed equally to this work.

Appendix III

Authorship on a paper published in *Blood* in 2014. I performed analysis of CD68 embryos during a 1st year rotation project

e-Blood

PHAGOCYTES, GRANULOCYTES, AND MYELOPOIESIS

Human CD68 promoter GFP transgenic mice allow analysis of monocyte to macrophage differentiation in vivo

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Key Points

- CD68-GFP reporter mice show GFP transgene expression in both monocytes and tissue resident macrophage populations.
- Adoptively transferred CD68-GFP monocytes maintain GFP expression after recruitment in an ongoing inflammatory response.

The recruitment of monocytes and their differentiation into macrophages at sites of inflammation are key events in determining the outcome of the inflammatory response and initiating the return to tissue homeostasis. To study monocyte trafficking and macrophage differentiation in vivo, we have generated a novel transgenic reporter mouse expressing a green fluorescent protein (GFP) under the control of the human CD68 promoter. CD68-GFP mice express high levels of GFP in both monocyte and embryo-derived tissue resident macrophages in adult animals. The human CD68 promoter drives GFP expression in all CD115⁺ monocytes of adult blood, spleen, and bone marrow; we took advantage of this to directly compare the trafficking of bone marrow-derived CD68-GFP monocytes to that of CX₃CR1^{GFP} monocytes in vivo using a sterile zymosan peritonitis model. Unlike CX₃CR1^{GFP} monocytes, which downregulate GFP expression on differentiation into macrophages in this model, CD68-GFP monocytes retain high-level GFP expression for 72 hours after differentiation into macrophages, allowing continued cell tracking during resolution of inflammation. In summary, this novel CD68-GFP

transgenic reporter mouse line represents a powerful resource for analyzing monocyte mobilization and monocyte trafficking as well as studying the fate of recruited monocytes in models of acute and chronic inflammation. (*Blood*. 2014;124(15):e33-e44)

Introduction

Immune cells of the mononuclear phagocyte system (MPS) play a key role in host immune responses. Tissue-resident macrophages play a sentinel role in initiating acute inflammatory responses and, together with monocyte-derived macrophages that differentiate from recruited monocytes, they regulate local inflammatory responses. Macrophages play an important role in initiating wound repair and restoring tissue homeostasis after infection or tissue injury.^{1,2} In some cases, tissue homeostasis is not restored, and macrophages can promote continuing tissue damage and chronic inflammation, such as in rheumatoid arthritis and atherosclerosis.³ Monocytes are circulating CD115⁺ myeloid cells that develop from the common monocyte precursor in the bone marrow downstream of the macrophage dendritic cell (DC) precursor in the bone marrow before being released into the bloodstream.⁴⁻⁷ Factors that enhance monocyte release from bone marrow into blood include chemokines derived from sites of inflammation,⁸⁻¹¹ and recent studies have identified hyperglycemia and hyperlipidemia as important factors that regulate monocyte release by acting on bone marrow progenitor cells.¹²⁻¹⁶ Blood

monocytes fall into 2 populations—Ly6C^{hi} and Ly6C^{lo} subsets that have been predicted to have different roles in vivo because of differential expression of chemokine receptors and different behaviors in intravital microscopy studies in which Ly6C^{lo} monocytes have a vascular patrolling behavior.¹⁷⁻¹⁹

Human CD68 and its murine homolog, macrophage (CD68), are both heavily glycosylated type I transmembrane proteins that belong to the lysosomal/endosomal-associated membrane proteins.²⁰ The exact function of CD68 has yet to be confirmed, but a role in antigen processing and as a scavenger receptor have been previously proposed.^{20,21} In terms of expression, CD68 has been reported to be restricted to cells of myeloid lineage, specifically monocyte/macrophages.²² CD68 tissue staining is commonly used as a marker for infiltrating monocyte-derived macrophages in atherosclerotic lesions and other sites of chronic inflammation.²³

The majority of studies that involve monitoring leukocyte trafficking and inflammatory cell recruitment use transgenic animals in which specific cell types have been labeled by genetic “knock-in”

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A.J.I. and E.M. contributed equally to this study.

This article contains a data supplement.

There is an Inside *Blood* Commentary on this article in this issue.

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Appendix IV

Manuscript in preparation:

Developmental programming of the cardiac lymphatics towards immunomodulation and improved heart repair

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The lymphatic vasculature is a blind-ended network covering most tissues and organs of the body and is an essential component of vertebrate development and homeostasis. During pathological conditions lymphatic vessels expand via the process of lymphangiogenesis in order to aid the clearance of interstitial fluid and reduce inflammation. Whilst the response of the lymphatics to injury and inflammation has been well documented at peripheral sites, there is minimal insight with respect to their role during pathophysiology of organ systems such as the heart. We investigated the response of the cardiac lymphatic vessels to myocardial infarction (MI) and observed a significant lymphangiogenic response, which was underpinned by reactivation of the developmental lymphatic programme. VEGF-C treatment significantly augmented lymphangiogenesis post-MI resulting in improved cardiac function. To determine whether this effect might be mediated by an immunomodulatory role, we flow-sorted immune cells from VEGF-C treated hearts and observed increased clearance of macrophages to mediastinal lymph nodes by day 7 post-MI. The molecular phenotype of cleared versus retained macrophages was equivalent, suggesting that a reduction in macrophage load in the heart alone was sufficient to correlate with improved outcome. Finally, we examined macrophage trafficking in two genetic mouse models with compromised lymphatic vessels: LYVE-1 knock-out mice which have impaired immune cell uptake and PROX1 haploinsufficient mice which have a restricted lymphangiogenic response to injury. In both cases we observed significantly reduced macrophage clearance, and are currently testing whether this correlates with reduced cardiac output, elevated fibrosis and increased pathological remodelling. These data suggest that invoking developmental lymphangiogenesis to modulate the innate immune response may represent a therapeutic target to promote optimal cardiac repair following injury.

Appendix V

First author of a review article published in *Clinical Anatomy* in 2016. I wrote the literature review and included edits as suggested by Professor Paul Riley.

Clinical Anatomy 29:305–315 (2016)

REVIEW

Anatomy and Development of the Cardiac Lymphatic Vasculature: Its Role in Injury and Disease

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Lymphatic vessels are present throughout the entire body in all mammals and function to regulate tissue fluid balance, lipid transport and survey the immune system. Despite the presence of an extensive lymphatic plexus within the heart, until recently the importance of the cardiac lymphatic vasculature and its origins were unknown. Several studies have described the basic anatomy of the developing cardiac lymphatic vasculature and more recently the detailed development of the murine cardiac lymphatics has been documented, with important insight into their cellular sources during embryogenesis. In this review we initially describe the development of systemic lymphatic vasculature, to provide the background for a comparative description of the spatiotemporal development of the cardiac lymphatic vessels, including detail of both canonical, typically venous, and noncanonical (hemogenic endothelium) cellular sources. Subsequently, we address the response of the cardiac lymphatic network to myocardial infarction (heart attack) and the therapeutic potential of targeting cardiac lymphangiogenesis. *Clin. Anat.* 29:305–315, 2016. © 2015 Wiley Periodicals, Inc.

Key words: cardiac lymphatics; lymphatic development; cellular origins; lymphangiogenesis

INTRODUCTION

Over the last 15 years our understanding of the structure and function of the systemic lymphatic vasculature has grown exponentially. Identification of key lymphatic genes has allowed developmental mechanisms to be investigated, and various disease models and clinical studies have revealed the importance of lymphatic vessels during inflammation and as a route for cancer metastasis. These advances have raised important questions as to how organ based lymphatics develop and whether they could be therapeutically targeted in disease.

Investigation of the cardiac lymphatic vasculature dates back to the 17th Century, as first described in the dog heart (Rudbeck, 1653). Despite the knowledge of the presence of a lymphatic plexus within the heart, little was known until recently regarding the anatomy of these vessels, their development or their role in cardiovascular disease. The anatomy of the cardiac lymphatics has been the subject of relatively few studies; historically, basic descriptive anatomical

studies in several species, including humans, have revealed the location of lymphatics within the heart and their route of drainage. More recently, molecular insight into the key lymphatic genes alongside functional genetic studies on model organisms, have led to insight into the development of the cardiac lymphatic vasculature. Lineage tracing studies have revealed that unlike systemic lymphatic vessels, the cardiac lymphatics are heterogeneous in origin. Recent work has also highlighted the significance of the lymphatic vasculature in cardiovascular disease and has indicated that treatments which stimulate lymphangiogenesis may be beneficial.

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