

The evolving cardiac lymphatic vasculature in development, repair and regeneration

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Abstract | The lymphatic vasculature has an essential role in maintaining normal fluid balance in tissues and modulating the inflammatory response to injury or pathogens. Disruption of normal lymphatic vessel development or function can have severe consequences. In the heart, reduced lymphatic function can lead to myocardial oedema and persistent inflammation. Macrophages, which are phagocytic cells of the innate immune system, contribute to cardiac development and to fibrotic repair and regeneration of cardiac tissue after myocardial infarction. In this Review, we discuss the cardiac lymphatic vasculature with a focus on developments over the past five years [Au: OK] arising from the study of mammalian and zebrafish model organisms. In addition, we examine the interplay between the cardiac lymphatics and macrophages during fibrotic repair and regeneration after myocardial infarction. Finally, we discuss the therapeutic potential of targeting the cardiac lymphatic network to regulate immune cell content and alleviate inflammation in patients with ischaemic heart disease.

[Au: Au: Please ignore the [H1], [H2] I have inserted in front of the headings, and the [G] added after the glossary terms. These notes are included to aid in the correct formatting later in the production process.]

[Au: I have highlighted red and marked with [G] some additional terms that readers might be unfamiliar with. If you agree that it would be useful to define the new terms, please add definitions to the revised manuscript (terms listed at the end of the manuscript). Please feel free to define other terms that you think readers might be unfamiliar with.]

[H1] Introduction

The circulatory system of vertebrates is composed of two complementary vasculatures, the blood and lymphatic vascular systems¹. The blood vasculature is a closed system responsible for transporting gases, fluids, [Au: OK] nutrients, metabolites and cells to the tissues². This extravasation of fluid and macromolecules [Au: OK] results in a continuous accumulation [Au: OK] of extracellular fluids and increased interstitial pressure². Tissue fluid balance is maintained by the lymphatic vasculature, an open circulatory system that transports fluids and cells from organs back to the blood circulation³. In addition to regulating interstitial fluid homeostasis, lymphatic vessels have essential roles in the immune response through the uptake and transport of pathogens, antigens and immune cells from tissues to regional lymph nodes before the return of the extravasated fluid and solutes to the blood circulation [Au: OK] .

In the heart, an extensive lymphatic network contributes to normal cardiac function in steady-state conditions and to myocardial healing after injury⁴. An increasing number of

studies have determined the lineage heterogeneity of cardiac lymphatics during development and their essential role in fibrotic repair after myocardial infarction (MI) in non-regenerative animal models, such as adult mice^{5–9}, and regenerative models such as zebrafish [Au: OK]^{10–13}. These studies hold great promise for ongoing efforts to develop therapies for cardiovascular diseases, highlighting the lymphatic vessels as a potential therapeutic [Au: OK] target to reduce myocardial oedema and modulate the immune response after MI [Au: OK]. In this Review, we summarize the current knowledge on the development, structure and function of the cardiac lymphatic vasculature, with an emphasis on breakthroughs over the past five years [Au: OK] in the study of cardiac lymphatic heterogeneity in mice and zebrafish [Au: OK]. We also discuss emerging findings on the immunomodulatory role of cardiac lymphatics and their functional interaction with immune cells during the fibrotic repair process after injury in the adult heart, as well as during cardiovascular tissue restoration and regeneration in neonatal mice and in adult zebrafish. Finally, we describe ongoing preclinical studies investigating the lymphatic vessels as a potential therapeutic target in acute MI.

[H1] Cardiac lymphatic structure and function [Au: OK]

Cardiac lymphatics run alongside the blood vessel network and have many of the functional features of the systemic lymphatic vasculature (FIG. 1), specifically the maintenance of homeostasis of interstitial fluid pressure^{14,15} and the modulation of the immune response¹⁶. Disruption of these processes can lead to severe health problems; for example, a 3.5% increase in myocardial fluids can lead to a 40% reduction in cardiac output^{17,18}. Lymphatic vessels are lined by a monolayer of oak-leaf-shaped lymphatic endothelial cells (LECs), and

are composed of three compartments: the initial lymphatics, the pre-collector lymphatics and the collector lymphatics^{1,19}. Interestingly, the localization of the lymphatic capillaries and the routes of collector vessels in the heart are not fully conserved between species²⁰.

[H2] Interspecies differences [Au: OK]

[H3] Initial lymphatics

Initial lymphatics or capillaries are thin, blind-ended and highly permeable vessels that are ideal for draining cells, fluid and macromolecules. In most mammals, such as humans, dogs and pigs, initial lymphatics located in the area from the subepicardium to the subendocardium drain extracellular fluids, cells and macromolecules that make up the lymph^{21,22}. However, in rabbit and mouse hearts, lymphatics are absent from the endocardium^{23,24}. The different mechanisms for immune cell uptake by the initial lymphatics have been previously described in detail¹⁶. Briefly, a primary valve system at the level of the LECs allows the entrance of cells, fluids and macromolecules to the initial lymphatics while preventing their escape back to the interstitial space^{25–28}. This primary valve system is created by flaps of adjacent LECs that interconnect and loosely overlap with one another^{25,29}. These LECs have specialised cell–cell junctions, called buttons, which are discontinuous, thereby allowing fluid entry to the lymphatic vessels while also securing the structural integrity of the endothelium²⁹. Furthermore, the abluminal side of the LECs is overlaid with extracellular-matrix-anchoring filaments that prevent the initial lymphatics from collapsing under increased interstitial pressure³⁰. As pressure increases, the lymph is formed inside the initial lymphatics and is transported to the pre-collector and collector lymphatic vessels.

[H3] Pre-collector and collector lymphatics [Au: OK]

Once inside the lymphatic vessel, the lymph travels through the subepicardial pre-collectors and collectors to the mediastinal lymph nodes (MLNs) and then back to the systemic lymphatic [Au: OK] circulation³¹. In the systemic lymphatic vasculature, the LECs have continuous seams of zipper-like cell–cell junctions that make the vessels impermeable to fluids and cells²⁹. In the systemic lymphatics, each collecting vessel is arranged in a functional pumping unit called a lymphangion, which is defined as the section between two consecutive secondary intraluminal valves that is overlaid with contracting lymphatic smooth muscle cells [Au: OK]^{14,15}. Of note, cardiac lymphatics do not have a lymphatic smooth muscle cell layer, and lymph flow is solely dependent on passive propulsion powered by the periodic motion of cardiac contraction^{5,6,32,33}. As the lymph flows towards the MLNs, at each lymphangion the upstream valve closes preventing retrograde flow, while the downstream valve opens resulting in positive flow^{14,15}.

In the human, dog and pig heart, collectors composing the left and right lymphatic trunks run along the major coronary arteries^{34,35}. The left trunk collects lymph from the anterior and posterior interventricular branches and from the left marginal branch. The left trunk then passes behind the left atrial appendage [Au: OK] and ascends onto the posterior surface of the pulmonary trunk and up to the pretracheal lymph nodes near the aortic arch. From the pretracheal lymph nodes [Au: OK], a single vessel travels behind the aorta to the cardiac lymph node, which lays between the superior vena cava and the right brachiocephalic artery, then a number of lymphatic vessels [Au: OK] lead to the right lymphatic duct, which terminates in the right venous angle. The right trunk collects lymph

from the right area of the heart and proceeds on the anterior surface of the aorta. The right trunk then enters the left anterior mediastinal chain and left paratracheal lymph nodes, from where it passes to the thoracic trunk and terminates in the left venous angle.

In contrast to the cardiac collector lymphatics of humans, pigs and dogs, which accompany coronary arteries, in the mouse and rat hearts these vessels accompany cardiac veins²⁰. Specifically, in the mouse heart the left collector drains the paraconal interventricular sulcus (left and right ventricles) around the left conal vein towards the left side of the pulmonary trunk and upwards to the MLNs³³. The second collector drains the lymph from the left ventricle running along the left cardiac vein followed by the coronary sinus and then upwards towards the MLNs³³. The lymph then reaches the draining lymph node via afferent collector lymphatics³⁶. The unique functions and adaptations of lymphatic vessels in lymph nodes have been thoroughly reviewed previously³⁷. In the MLNs, cells of the innate and adaptive immune system reside in specific locations, poised to be activated^{37,38}. Following activation of adaptive immunity, lymphocytes enter the lymph and exit the lymph node via efferent lymphatic vessels^{39,40} until the lymph flow reaches either the right duct or the thoracic duct³⁹. From the right duct or thoracic duct [Au: OK], the lymph eventually returns to the venous circulation at the level of the jugular and subclavian veins, where specialized lymphovenous valves ensure the unidirectional drainage of the lymph to the blood^{41–44}.

[H1] Development of cardiac lymphatics

Over the past two decades [Au: OK], our knowledge on the embryonic development of the lymphatic vasculature has increased substantially [Au: OK]. During embryogenesis, the

development of the lymphatic vessels takes place after the major blood vessels (the dorsal aorta and the cardinal vein) [Au: OK] have been formed. Two distinct mechanisms have been proposed for the development of the lymphatic network: **lymphvasculogenesis** [G]^{12,45,46} and **lymphangiogenesis** [G]^{47–50}. The origin of the lymphatic vasculature has been the subject of some controversy for many decades. An initial report from Florence Sabin, dating back to the 1900s, suggested that the lymphatic endothelium buds directly from the venous endothelium⁵¹. By contrast, Huntington and McClure proposed that LECs originate from the mesoderm and subsequently form connections with the venous endothelium⁵².

Experimental work published in 2019 [Au: OK] has shed some light on this historic debate. Stone and Stainier provided evidence that the fate of LECs in mice [Au: OK] is hard-wired early on during embryogenesis at the level of the mesoderm⁵³. Specifically, cell lineage tracing using *Pax3*–Cre and *Myf5*–Cre [Au: OK] transgenic mice showed that, in addition to lateral plate mesoderm, the paraxial mesoderm also contributes endothelial cells during embryonic blood vessel development in mice⁵³. Around embryonic day (E) 9.5, these paraxial mesoderm-derived endothelial cells selectively differentiate from the dorsolateral part of the anterior cardinal vein to form the first precursor LECs [Au: OK], characterized by the expression of the transcription factor prospero homeobox protein 1 (PROX1). These LECs subsequently give rise to the majority of the lymphatic endothelium, including systemic and organ-based (for example, the heart) lymphatics, with only limited contribution to the blood endothelium⁵³. *Prox1* is necessary and sufficient for LEC specification^{54,55}, and requires the transcription factors SOX18 and COUP transcription factor 2 (COUP-TF2) to be expressed^{56,57}. Both SOX18 and COUP-TF2 bind and activate expression of *Prox1* directly, whereas COUP-TF2 also promotes lymphatic differentiation indirectly by repressing the arterial fate driven by the Notch signalling pathway^{56,58} [Au: clarified that *Prox1* gene

expression requires SOX18 and COUP-TF2 that bind the transcription site of *Prox1* and activate its transcription.]. Once PROX1 has been expressed, a positive feedback loop between PROX1 and vascular endothelial growth factor receptor 3 (VEGFR3) maintains the precursor LEC identity⁵⁹. Concomitantly, precursor LECs start expressing lymphatic vessel endothelial hyaluronic acid receptor 1 (LYVE1), a protein that is essential for the lymphatic modulation of the immune response during inflammation^{60,61}, but is redundant during embryogenesis^{62,63}. At approximately E10.5, clusters of precursor LEC start aggregating and budding off along the length of the cardinal vein to form the lymph sacs, which are the lymphatic vessel precursors. For the budding process to take place, the VEGFR3–VEGFC signalling pathway is essential, although VEGFD also contributes to a lesser extent⁴⁸. At around E12.5, adjacent LECs budding from the cardinal vein are connected by continuous, impermeable zipper junctions, providing strong structural integrity to the forming lymphatic vessels [Au: OK]^{64,65}. Starting at E16.5 and continuing during postnatal development, the junctions of the initial lymphatics transform into permeable buttons^{29,64,65}. The role of lymphatic junctions in health and disease has been reviewed previously⁶⁶. Two different mechanisms maintain lymphovenous haemostasis, that is, prevent blood from flowing into the lymph sacs^{67,68}. First, PROX1 activates the expression and production of podoplanin, which subsequently interacts with the platelet receptor C-type lectin domain family 2 (CLEC2)⁴², inducing platelet aggregation and thereby lymphovenous haemostasis⁴². Second, blood backflow can be prevented by the lymphovenous valves formed by specialized PROX1⁺ LECs residing in the cardinal vein, connecting the lymphatic vessels with the jugular and subclavian veins^{41,44}. After the lymph sacs have been formed and separated from the cardinal vein, the lymph sacs start expanding into the developing embryonic tissues and organs by lymphangiogenesis, where the lymphatic vasculature continues to mature (Table

1). Detailed reviews of the formation of the systemic lymphatic network have been published previously^{69,70}.

[H2] Mouse cardiac lymphatics [Au: OK]

The cardiac lymphatic system has received substantial attention over the past 5 years. In particular, the development of the lymphatics in the forming heart, the characterization of the embryonic origin and the roles in adult heart disease have been appraised in a review published in 2019 [Au: OK]⁴ (FIG. 2). In mice, the first cardiac LECs emerge at E12.5 from the extracardiac region near the outflow tract on the ventral side of the heart and from the sinus venosus on the dorsal side of the heart⁵. Whole-embryo staining at E10.5 and E12.5 identified a LEC population emerging from the cardinal vein and migrating towards the sinus venosus and outflow tract, suggesting that cardinal vein-derived endothelial cells might be the venous source of coronary lymphatics⁵. By E14.5, cardiac lymphatics extend along the base-to-apex axis on both sides of the heart, with the ventral side displaying a slight delay compared with the dorsal side⁵. Interestingly, the epicardium and the outflow tract have been identified as sources of VEGFC for coronary vasculature development⁷¹, making these structures potential signalling hubs to control VEGFR3-dependent lymphatic sprouting. Studies investigating the spatiotemporal development by whole-mount and tissue-section staining, as well as by Indian ink injection into the myocardium, suggest that during embryogenesis, lymphatic vessels appear only in the subepicardial layer^{5,32,33}. From birth to approximately postnatal day (P)15, lymphatic vessels continue to grow and branch laterally to adequately cover both dorsal and ventral surfaces of the mouse heart, and also grow deeper into the underlying myocardium, without reaching the endocardium^{32,33}. Although these studies are informative, they lack some of the imaging depth and detail that more modern approaches provide. The combination of tissue clearing, which renders large tissue

samples transparent, with 3D imaging has proven to be a powerful tool for in-depth, multiview visualization of the whole vascular structure in the developing kidneys⁷² and injured heart⁷³ of mice. Therefore, adopting similar methods in the future could provide more insight into the development of the cardiac lymphatic vasculature, for instance, to reveal how venous and non-venous derived LECs integrate into a single vascular network (see below).

[H3] Non-venous origin of mouse cardiac lymphatics [Au: OK]

The majority of cardiac LECs originate from the **paraxial mesoderm [G]**-derived *Tie2*⁺ endothelium of the **cardinal vein [G]**⁵³, although recent studies have identified the contribution of non-venous sources^{5,8,9}. Fate mapping using genetic drivers to trace *Mesp1*⁺ mesoderm, *Nkx2-5*⁺ cardiac mesoderm, *Wt1*⁺ epicardium and *Wnt1*⁺ (neural crest cells) excluded all of these as potential LEC sources. Further lineage-tracing experiments using Cre recombinase (Cre)-driver lines under the control of the *Vav1*, *Pdgfrb* and *Csf1r* promoters identified the **haemogenic endothelium [G]** of the **yolk sac [G]** as a potential contributor of LECs^{5,74}. Strikingly, two complementary studies have reported that the ventral and dorsal lymphatic endothelium have distinct origins and develop through different mechanisms^{8,9}. The first study used genetic lineage tracing to show the contribution of the *Isl1*⁺ lineage to cardiac LECs around the outflow tract and ventral side, suggesting a contribution from the **second heart field [G]** (SHF)⁹. In the second study, a series of experiments proved that non-venous SHF-derived precursors contribute LECs exclusively to the ventral vascular network⁸. Initially, clonal analysis indicated that the origin of the cardiac lymphatic vasculature is different in the two sides of the heart⁸. Subsequently, genetic lineage-tracing experiments showed that about half of the ventral lymphatic endothelium originates from *Isl1*⁺ and *Mef2c*⁺ precursors⁸. Moreover, the cardiac neural crest was excluded as a source, and the

contribution of the *Is/1⁺* lineage to the LECs of the cardinal vein was minimal⁸. Finally, both constitutive deletion of *Tbx1*, which results in the lack of the SHF, and conditional deletion of *Prox1* in the SHF in mice led to complete agenesis of the ventral lymphatics, with no effect on the dorsal lymphatics, supporting the notion that the SHF is an essential cellular source for the ventral lymphatic endothelium⁸.

Although lineage-tracing experiments have proven valuable for determining the origin of LECs, issues around the specificity of the Cre drivers and incomplete or even ectopic recombination of Cre reporters have complicated data interpretation, leading to conflicting reports. Therefore, cross-validating new information with a combination of genetic knockout experiments, multiple lineage reporters and clonal analyses is crucial [Au: OK]⁷⁵. The relevance of each of the aforementioned origins of cardiac lymphatics with respect to the role of lymphatics in cardiac diseases is currently unclear. Further investigation of the molecular cues that drive specification of LECs from different sources will provide insight into the selective targeting of LEC subpopulations to invoke therapeutic lymphangiogenesis.

[H2] Zebrafish cardiac lymphatics

In zebrafish, cardiac lymphatics are found only in the subepicardial layer and drain into large collecting vessels in the outflow tract, which connect to the facial lymphatic vasculature¹². Similarly to mouse cardiac lymphatics, zebrafish cardiac lymphatic vessels derive from both venous and non-venous (angioblast) sources^{10,76}. Specifically, the cardiac lymphatic endothelium is first established on the outflow tract, or bulbus arteriosus, from facial lymphatic vessels that originate from sprouts of the cardinal vein and primary head sinus (lymphangiogenesis), as well as from a population of lymphangioblasts

(lymphvasculogenesis)^{12,76,77}. This process takes place relatively late in development, but before the initiation of the coronary vasculature, at about 3–4 weeks post fertilization (wpf)^{12,13}. However, the expansion of cardiac lymphatic vessels over the ventricle takes place after the formation of the coronary vasculature in juvenile zebrafish, at approximately 12–16 wpf^{12,13}. Over subsequent months, the bulbus arteriosus lymphatic vessels sprout towards the ventricle in close proximity to the major coronary vessels and continue to grow along the base-to-apex axis, analogous to mouse cardiac lymphatics^{12,13}. Interestingly, hearts with an underdeveloped coronary plexus caused by CXC-chemokine receptor type 4 (*Cxcr4a*) deficiency have severe ventricular lymphatic abnormalities, whereas the bulbus arteriosus lymphatics were normal^{12,13}. This finding suggests that the presence of a mature coronary tree is required for the lymphatics to migrate down the ventricle, with potential coronary artery-derived signals (such as CXC-chemokine ligand 12 (*Cxcl12a*) promoting this developmental process^{12,13}. Apart from sprouting lymphatics, clusters of isolated LECs of unknown origin have been reported to connect and contribute to the cardiac lymphatic vasculature¹². Comparable to the systemic lymphatic vessels, cardiac lymphatics require *Vegfr3*–*Vegfc* signalling to develop, with genetic models such as *vegfr3*^{−/−}, *vegfc*^{+/-} or hypomorphic *vegfc*^{hy/hy} zebrafish models displaying a severe lymphangiogenic phenotype^{11–13}.

Overall, the cardiac lymphatic system seems to be more heterogeneous than previously thought, with contribution of both venous and non-venous sources, such as the yolk sac haemogenic endothelium and SHF progenitors, and with contributions differing across dorsal versus ventral aspects of the forming heart. Understanding this diversity in more

detail and assessing its implications in the adult heart and in response to pathological insult could reveal new therapeutic avenues for the treatment of cardiovascular diseases.

[H1] Cardiac lymphatic responses to injury

During adult homeostasis, the cardiac lymphatics have functions akin to systemic lymphatics in modulating tissue fluid and immune surveillance (as discussed above). Following injury, such as MI, the cardiac lymphatics respond according to whether the default wound healing is via fibrotic repair, as in adult mammals including humans, or via a regenerative response, as occurs during neonatal stages in mammals **[Au: OK]** and in adult zebrafish.

[H2] Cardiac fibrotic repair

[H3] Oedema after myocardial infarction

MI is caused by coronary artery occlusion caused, for example, by the formation of atherosclerotic plaques in the arterial wall⁷⁸, which results in reduced blood flow to the heart and can lead to prolonged ischaemia and to cardiomyocyte death **[Au: OK]**. The endothelium in the ischaemic region is also affected, leading to increased vascular permeability and a substantial loss of lymphatic vessels, causing poor myocardial fluid draining and persistent oedema^{7,17,79}. Adult humans **[Au: OK. We changed it to 'adult humans' as there are reported cases of regeneration in human neonates.]** and other adult mammals lack the ability to regenerate the heart⁸⁰. Therefore, after myocardial ischaemia, the heart remodels in an attempt to compensate for the loss of cardiovascular tissue and healing occurs by replacing the dead myocardium with scar tissue⁸¹ (see below). During the remodelling phase, the cardiac lymphatics undergo lymphangiogenesis by growing and expanding in the infarcted area^{7,60,82–84}. However, despite the endogenous lymphatic

response, the myocardial oedema and inflammation persist^{7,60,82–84}. An important contributing factor for the insufficient drainage by the cardiac lymphatics after MI could be the reduced cardiac contractility caused by the death of cardiomyocytes, which acts as the major extrinsic force for lymph propulsion from the heart to the MLNs^{85,86}. The increase in interstitial pressure, combined with the loss of myocardium, eventually leads to fibrosis, impaired heart function and ultimately heart failure^{17,18,87}.

[H3] The immune response to heart injury

In adult mice, shortly after the induction of MI through surgical ligation of the left anterior descending coronary artery⁸⁸, circulating pro-inflammatory stimuli, such as damage-associated molecular patterns and cytokines, activate and recruit innate immune cells to the injury site⁸⁹. Neutrophils and monocytes are the first to infiltrate the infarcted myocardium to clear debris and dead cells by **phagocytosis [G]** and **efferocytosis [G]**, respectively⁹⁰. In mice, neutrophil numbers peak 3 days after MI, followed by a biphasic response of monocytes and monocyte-derived macrophages up to 5 days post-injury (dpi), with gradual reduction to baseline levels thereafter⁹¹. During these phases, the embryonic-derived tissue-resident macrophages die and are replaced by monocytes and monocyte-derived macrophages⁹². The first phase (1–4 dpi) of the immune response after injury the adult heart is an inflammatory phase, with an increase in the number of pro-inflammatory monocyte-derived CCR2⁺ macrophages **[Au: OK]**^{84,93}. These cells secrete inflammatory and proteolytic factors, and have increased phagocytosis and efferocytosis^{84,93}. By contrast, the second phase (≥ 5 dpi) is an anti-inflammatory phase, with pro-reparative, tissue-resident CCR2[−] macrophages contributing to angiogenesis and scar formation^{84,93,94}. Interestingly, macrophages were previously thought to contribute only indirectly to scar formation by

supporting the activation of cardiac fibroblasts into myofibroblasts⁸⁴. However, a study published in 2020 demonstrated that macrophages can directly contribute to scar formation in the adult heart after MI by expressing and depositing collagen⁹⁵. Comparable to the mouse model, hearts from adult patients with heart failure are also populated by tissue-resident CCR2⁻ macrophages and monocyte-derived CCR2⁺ macrophages⁹⁶. After MI in mice [Au: OK], the epicardium and the pro-inflammatory macrophages secrete VEGFC, which drives lymphangiogenesis and the extensive remodelling of the cardiac lymphatic network^{5,7,60}. This endogenous response of the cardiac lymphatics attempts to maintain an optimal immune cell load, which is necessary for effective tissue repair^{7,60,82–84}. However, the response of the cardiac lymphatics is insufficient to clear the immune cells, which results in chronic inflammation and increased scar formation^{6,60}. Apart from neutrophils, monocytes and macrophages, other leukocytes, such as T cells, infiltrate the heart during the first week after MI in adult mice⁹¹. Although the response of the adaptive immune system to MI has not been well studied, the current view is that regulatory T cells have a beneficial role in cardiac healing^{97,98}. By contrast, CD4⁺ effector T cells produce pro-inflammatory cytokines and CD8⁺ T cells have direct cytotoxic effects^{99,100}. The immune response to MI in adult mice has been previously described in detail⁸⁹.

[H3] Augmentation of injury-induced cardiac lymphangiogenesis

The endogenous lymphangiogenic response is insufficient to clear the myocardial oedema and the infiltrated immune cells after MI. This inefficient endogenous response has prompted attempts to increase lymphangiogenesis and lymphatic function in the injured heart. Augmentation of the lymphangiogenic response with administration of recombinant VEGFC-C156S, which specifically interacts with VEGFR3, has been shown to improve cardiac

function after MI in animal models [Au: OK] , as assessed by echocardiography and cine-MRI^{6,7,60} (FIG. 3). Different routes of VEGFC-C156S administration, such as protein therapy^{7,60} or gene therapy with either adenovirus or adeno-associated virus (AAV) vectors⁶, have yielded varying outcomes that could potentially be attributed to the short half-life of the VEGFC-C156S protein⁴⁷. Injection of microparticles loaded with VEGFC-C156S in the left ventricle after MI induction [Au: OK] led to an increased clearance of myocardial oedema in rats⁷. Intraperitoneal injection of AAV–VEGFC-C156S 7 days before MI induced an increased clearance of T cells in female mice and male rats⁶. Lastly, intraperitoneal injection of recombinant VEGFC-C156S after MI in mice increased macrophage clearance via a LYVE1-dependent mechanism compared with vehicle-treated mice [Au: OK] ⁶⁰. LYVE1 is highly expressed at the surface of initial lymphatics and interacts specifically with the ubiquitous glycosaminoglycan polymer hyaluronan that coats the surface of leukocytes¹⁰¹. Engagement of LYVE1 with hyaluronan promotes the docking and transmigration of human [Au: OK] macrophages to lymphatic vessels in vitro¹⁰². In addition, dendritic cells dock and transmigrate to LECs in a LYVE1–hyaluronan-dependent manner in a mouse [Au: OK] model of dermal injury¹⁰³. Disruption of this interaction led to impaired migration of dendritic cells to draining lymph nodes and reduced the CD8⁺ T cell response to antigens¹⁰³. Whereas administration of VEGFC–C156S has been proven to increase lymphangiogenesis and improve cardiac function after MI in animal models, trapping of VEGFC and VEGFD with the use of the soluble decoy VEGFR3 (sVEGFR3) has provided contradicting results. Initially, a study reported extensive [Au: OK, changed to extensive] cardiac lymphatic defects, intramyocardial haemorrhage and higher mortality in sVEGFR3-transgenic mice compared with control littermate mice after MI¹⁰⁴. However, intraperitoneal injection of AAV—sVEGFR3 7 days before MI in female mice and male rats⁶ did not affect lymphangiogenesis,

but led to reduced infarct region thinning and T-cell infiltration to the heart 7 dpi, as well as to improved cardiac function 21 dpi⁶. Of note, surviving cardiomyocytes at the border zone and infarcted area express high levels of *Vegfr3* and undergo hypertrophy in the first days after MI^{105,106}. In the same regions, *Vegfc* and *Vegfd* are upregulated within 3 dpi, and in vitro studies show that VEGFC contributes to cardiomyocyte hypertrophy and survival^{105,106}. These studies collectively point to non-lymphangiogenic roles for VEGFC in the infarcted heart, which have to be taken into consideration when interpreting experimental outcomes following interference of the VEGFR3–VEGFC pathway. Furthermore, sex-dependent differences have been reported in the cardiac lymphatic vasculature under normal and MI conditions¹⁰⁷, highlighting another variable that needs to be accounted for when assessing the lymphatic responses after MI.

[H2] Cardiac regeneration

[H3] Cardiac regeneration in neonatal mice

In contrast to adult mammals, which are incapable of functional recovery after heart injury, the neonatal mammalian heart has an evolutionarily conserved regenerative capacity^{80,108–111}. The widely accepted notion is that in mice, the heart fully regenerates after left anterior descending coronary artery ligation at postnatal day 1 (P1), whereas the same injury at P7 leads to fibrotic scarring^{112–114}. Of note, anecdotal evidence from clinical case reports described cardiac regeneration in neonate **[Au: OK]** patients with MI caused by congenital heart disease^{115,116}. In neonatal mice, the immune response triggered by MI is markedly **[Au: OK]** different from that in adult animals, and these differences have been thoroughly reviewed previously^{117,118}. Briefly, the macrophages found in normal heart at early postnatal

stages are primarily tissue-resident CCR2⁻ macrophages that originate from embryonic sources and are maintained through local proliferation^{93,119,120} [Au: OK]. By contrast, circulating CCR2⁺ monocytes and monocyte-derived CCR2⁺ macrophages have little contribution to the cardiac monocyte–macrophage population at these stages [Au: OK, added the word ‘cardiac’]^{93,120}. In response to cardiac injury in the neonatal heart, the number of tissue-resident CCR2⁻ macrophages expands without additional infiltration of CCR2⁺ monocytes⁹³. Interestingly, general depletion of macrophages with the use of clodronate liposome treatment after MI at P1 inhibited cardiac regeneration and favoured fibrotic scar formation with significantly depressed cardiac function¹²¹. This lack of regeneration [Au: OK] was attributed to impaired angiogenesis¹²¹, which is consistent with the growing evidence supporting direct and indirect macrophage contributions to angiogenesis¹²². Although clodronate liposomes can specifically target macrophages¹²³, they target phagocytic cells in general, such as dendritic cells [Au: OK], and as such these results need to be interpreted with caution. Moreover, different macrophage depletion strategies can produce contrasting effects¹²⁴. Therefore, it is important to examine the effect of depleting specific immune cell subpopulations on cardiac regeneration in neonatal mice with the use of genetic models. The essential function of macrophages in heart regeneration in neonatal mice¹²¹ together with the immunomodulatory role of lymphatic vessels in the adult mouse heart⁶⁰ highlight an interesting area of further study as to how the cardiac lymphatics respond in a regenerative setting in mammals and to what extent the cardiac lymphatics interact with macrophages in neonatal hearts.

[H3] Cardiac regeneration in fish

Whereas cardiac lymphatics have not been examined in the neonatal mouse heart regeneration model, they have [Au: OK] received attention in adult zebrafish models of cardiac injury during the past two years^{11–13}. Zebrafish can fully regenerate their heart without scar formation after apical resection¹²⁵, or via a temporary scar following cryoinjury¹²⁶. During the first week after apical resection, *vegfc* expression increases transiently in the adult zebrafish, with no signs of cardiac lymphangiogenesis^{11,13}. By contrast, elevated *vegfc* expression after cryoinjury lasted for weeks and was accompanied by enlargement and migration of lymphatic vessels into the wound site^{11–13}. This observation suggests that different types of cardiac injury can initiate diverse healing responses. Similar to the mammalian immune response, macrophages and neutrophils were reported to migrate to the injured site during the first week after cryoinjury in zebrafish¹³. The immune response and debris were cleared by lymphatics from the wound area¹³. Moreover, disruption of the *Vegfr3–Vegfc* pathway blocked the lymphatic response to cryoinjury, which resulted in inefficient immune cell clearance and increased scar formation^{11–13}. Surprisingly, the cardiac regenerative capacity was not completely lost in the absence of lymphatics, because a subset of zebrafish was able to fully recover after cryoinjury^{11,13}. Nevertheless, data from RNA sequencing and immunostaining suggest that a lack of lymphatic response shifts the cardiac microenvironment from pro-regenerative to pro-inflammatory after cryoinjury in zebrafish, thereby affecting cardiac healing^{11,13}. By contrast to zebrafish, in *Oryzias latipes* (medaka), another teleost fish, the response to cardiac injury is excessive fibrosis and an unresolved scar¹²⁷. Comparative analyses between the two species suggests that reduced and delayed immune response affects the regenerative ability of medaka after cryoinjury compared with zebrafish¹²⁸. *Astyanax*

mexicanus is a single fish species comprising surface-dwelling and cave-dwelling populations, which have altered physical and metabolic phenotypes while evolving independently in surface rivers versus caves in northern Mexico¹²⁹. After surgical removal of the ventricular apex, surface-dwelling fish are able to fully regenerate their heart, whereas cavefish form a permanent fibrotic scar¹²⁹. To date, the cardiac lymphatic vessels in medaka and *Astyanax mexicanus* have not been investigated in terms of either their development or response to injury. Comparing the immune and lymphatic response to cardiac injury between neonatal versus adult mammals, zebrafish versus medaka, and surface-dwelling versus cave-dwelling *Astyanax mexicanus* holds great promise for understanding the mechanisms that lead to cardiac regeneration versus fibrotic repair after cardiac injury.

These studies raise important questions regarding the interaction of cardiac lymphatics with immune cells and their contribution to heart repair after MI. For instance, is improved healing caused by the general clearance of immune cells or by selective clearance of subpopulations? How does alteration of lymphangiogenesis affect the clearance of adaptive immune cells, such as dendritic cells, and subsequently antigen presentation and recruitment of T cells to the infarcted region? In the future, an important objective will be to investigate in more detail the time-dependent interaction between lymphatic vessels and different immune cell types after MI. Finally, do the cardiac lymphatics in neonatal mammals respond and function differently to the cardiac lymphatics in adult mammals in order to retain pro-regenerative macrophages after MI [Au: OK, simplified sentence] ? In summary, in adult rodents, endogenous lymphangiogenesis in response to cardiac injury seems to be insufficient to clear interstitial fluids and immune cell build-up, leading to chronic inflammation, myocardial oedema, fibrosis and impaired healing. However, this

endogenous response can be augmented by increasing lymphangiogenesis, for instance, by VEGFC-C156S administration, as discussed above. Therefore, a better understanding of the molecular mechanisms by which lymphatic vessels respond to clear the oedema and infiltrated innate and adaptive immune cells [Au: OK] after MI could provide the basis for developing therapies for patients with heart disease.

[H1] Clinical opportunities

MI is currently a major cause of mortality worldwide and currently no treatments are available to revert the cardiac damage [Au: OK]. Current treatments focus on early re-establishment of the blood flow to prevent further tissue damage and therapy with drugs such as angiotensin-converting enzyme (ACE) inhibitors and β -blockers to support the surviving myocardium¹³⁰. Restoration of blood flow is initially accomplished by percutaneous coronary intervention or administration of thrombolytic drugs, which in severe cases involves invasive surgeries, such as coronary artery bypass graft surgery or even heart transplantation¹³⁰ [Au: OK, included percutaneous coronary intervention as a reperfusion strategy]. Therefore, the development of new treatments to repair or regenerate [Au: OK] the damaged myocardium continues to receive great interest. Initial studies focused on cell-based therapies involving the injection of cardiac or non-cardiac cells [Au: OK] into the infarct area with the aim of replacing the lost cardiomyocytes and improve heart function after MI^{131,132}. However, the evidence indicates that cell-based therapies alone are ineffective and require complementary approaches to make the cardiac microenvironment conducive to regeneration^{133–135}. A study published in 2020 showed that intracardiac injection of different types of adult stem and progenitor cells, dead cells or a

chemical inducer of the innate immune response all improved heart function, which was attributed to an acute and beneficial immune response¹³⁶. Therefore, early inflammation combined with a balanced innate and adaptive immune response seems to be crucial for optimal repair and potential regeneration of the infarcted heart, whereas broad immunosuppression has adverse effects^{137,138}. As a result, a time-dependent, drug-mediated manipulation of the lymphatic response could help modulate the inflammatory content in myocardium and promote both myocardial survival and restoration. Proof-of-principle is provided by the aforementioned studies targeting recombinant VEGFC-C156S to invoke increased lymphangiogenesis and improved outcome after MI⁵⁻⁷. However, VEGFC or its isoforms are not an optimal drug of choice given their very short half-life in serum⁴⁷.

Currently, pre-clinical studies are investigating lymphangiogenesis as a potential drug target for immunomodulation after MI^{107,139-141}. Most studies are focusing on the VEGFR3 signalling pathway, because the induction of this pathway has been shown to promote lymphangiogenesis and lead to better outcomes after MI in experimental animal models^{5,7,60,104}. A phase I/IIa clinical trial assessing the efficacy and safety of intramyocardial adenovirus vector-mediated VEGFD-ΔNΔC gene therapy in patients with refractory angina showed promising results, with significant improvement in myocardial blood flow compared with placebo¹³⁹. However, this positive finding is compromised by the need for repeat invasive gene therapy administration and the cost per patient. Therefore, exploring additional pathways that promote a cardiac lymphatic response is important. For instance, the epicardial-specific peptide adrenomedullin (encoded by *Adm*) has been identified to be cardioprotective through a beneficial effect on cardiac lymphatic permeability and lymphangiogenesis^{107,140} **[Au: OK, inserted references and clarification]**. In a pilot clinical

study, intravenous injection of adrenomedullin in patients with acute MI resulted in significantly improved cardiac structure and function, as evaluated by MRI, compared with baseline¹⁴⁰. Additionally, overexpression of *Adm* in mice results in reduced oedema, dilated cardiac lymphatic vessels and improved cardiac function after MI¹⁰⁷. In this study, adrenomedullin was found to regulate the gap-junction protein connexin 43 in cardiac LECs, promoting their coupling and potentially increasing the permeability of lymphatics¹⁰⁷. This study highlights that pre-clinical research focusing on inducing cardiac lymphatic growth by lymphangiogenesis, as well as improving their functional maturation, has therapeutic potential.

Targeting lymphangiogenesis could also have clinical applications beyond cardiovascular disease¹⁴². While this Review was under preparation, the global pandemic of coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged. The vast majority of patients with COVID-19 [Au: OK] have heart and lung complications, and post-mortem analysis suggests that an excessive inflammatory response (known as a “cytokine storm”) and associated damage to organ microcirculation is a major contributor to disease severity and mortality¹⁴³. In addition, patients with pre-existing cardiovascular disease develop a more severe COVID-19 response owing to direct viral effects on a compromised myocardium (myocarditis) and/or indirect effects via cardiac hyperinflammation and impaired coronary microvasculature [Au: OK]^{144–147}. Notably, children usually present with more mild COVID-19 symptoms than adult patients¹⁴⁸. [Au: OK] This difference has been hypothesised to be due to a more immature immune response at younger ages¹⁴⁸. Considering this circumstantial evidence, one might speculate that augmentation of lymphatic growth and function could assist in clearing the oedema and

immune cell accumulation in the hyperinflamed tissue environment in patients with COVID-19, thereby improving the disease outcome.

[H1] Conclusions

The development and function of the lymphatic vasculature, often described as the secondary circulatory system, has received increasing [\[Au: OK\]](#) attention in the last decade [\[Au: OK\]](#). Studies using state-of-the-art imaging technologies and genetic models have focused on the lymphatic endothelium in more detail, revealing tissue-specific heterogeneity in origin, function and response to injury. In contrast to the pre-existing dogma, several non-venous sources have been shown to contribute to cardiac lymphatics, indicating that further research is required to characterize fully the ontogeny of cardiac lymphatics in different settings. In the context of adult cardiac injury [\[Au: OK\]](#), the lymphatics of the heart [\[Au: OK\]](#) respond by growth and sprouting in an attempt to clear the oedema and immune cells from the damaged tissue. In animal models, augmentation of the cardiac lymphatic response after cardiac injury has proven to be beneficial for wound healing, whereas general immunosuppression leads to severe adverse effects [\[Au: OK\]](#). This finding supports the notion that controlled immunomodulation by lymphangiogenesis could be of great clinical value for treating patient with ischaemic heart disease and preventing or even reversing heart failure. Nevertheless, open questions remain regarding the response and function of cardiac [\[Au: OK\]](#) lymphatics in the disease setting, which requires close collaboration between basic and clinical researchers to deliver effective therapies. Overall, the focus needs to be on implementing a combinatorial approach to tackle the complexity of both restoring lost cardiovascular tissue and conditioning the local injury environment.

Targeting the regulation of cardiac lymphangiogenesis to improve fluid balance and modulate the immune response and downstream fibrosis might emerge as viable strategy to contribute to combined therapy for cardiac repair and regeneration [\[Au: OK\]](#).

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Authors contributions

K.K. researched data for article and wrote the manuscript. J.M.V. and P.R.R. edited the manuscript before submission. All authors provided substantial contribution to the discussion of content.

Competing interests

P.R.R. is co-founder and equity holder in OxStem Cardio, an Oxford University spin-out that seeks to exploit therapeutic strategies stimulating endogenous repair in cardiovascular regenerative medicine. The other authors declare no competing interests. [\[Au: OK\]](#)

Key points

- The cardiac lymphatic vasculature appears during embryonic development and continues to mature structurally and functionally until late postnatal stages.

- Both venous and non-venous sources contribute to the lymphatic endothelium of the heart, and the identity of the venous-derived lymphatic endothelial cells is defined very early in development during specification of the embryonic mesoderm.
- In adult mice, the cardiac lymphatics respond to cardiac injury by sprouting within the damaged area in an attempt to clear the immune cells and excess tissue fluid (oedema).
- Drug-induced augmentation of the lymphatic response to injury improves heart repair and function in adult mice, suggesting that lymphatics could be a possible drug target for myocardial infarction and for other diseases.
- The neonatal mouse heart has regenerative capacity that requires the presence of pro-reparative macrophages, which suggests an alternate function for cardiac lymphatics during neonatal heart regeneration.
- Adult zebrafish can regenerate their hearts after cryoinjury and the lymphatics respond to the site of injury to clear infiltrating immune cells, a process that is essential for complete regeneration.

Table 1 | Developmental origin of organ-specific lymphatic vessels in mice [Au: OK]

Organ	Development	Origin (lineage marker) [Au: OK, changed gene with lineage, as these markers are no longer expressed in LECs]	Refs
Heart	E12.5	Venous endothelium (<i>Tie2</i>) Haemogenic endothelium (<i>Csf1r</i> , <i>Vav1</i> and <i>Pdgfrb</i>) Second heart field (<i>Isl1</i>)	5,8,9
Meningeal	Perinatal	Unknown	149–152
Mesenteric	E12.5	Haemogenic endothelium (<i>cKit</i>) Arterial development initiates the assembly of non-venous-derived lymphatic endothelial cells in the left dorsal mesentery	28,46,153–155
Kidney	E14.5	Unknown	72,156,157
Bones	Disease	Pre-existing lymphatic endothelial cells	158
Dermal	E12.5	Lymphatic endothelial cell clusters from local dermal blood capillary plexus Venous endothelium (<i>Tie2</i>) Possible non-venous source	45,50,159–161
Corneal	Transient during disease	Limbal pre-existing lymphatics Potential contribution from macrophage populations	162–169

E, embryonic day.

Fig. 1 | **Structure and function of cardiac lymphatic vessels.** **a** | Schematic illustration of the mediastinal lymph nodes, where the cardiac lymphatics drain the lymph. **b** | Dorsal and ventral aspect of the adult mouse cardiac lymphatic network. **c** | The path of the lymph begins at the initial lymphatics, where lymphatic endothelial cells (LECs) connected by permeable button junctions drain immune cells, fluids, macromolecules and pathogens from the interstitial space, making up the lymph. The lymph is transported through the pre-collector and collector that are connected by impermeable zipper junctions to the mediastinal lymph nodes.

Fig. 2 | **Origin and development of the mouse cardiac lymphatic vasculature.** **a** | The cardiac lymphatic vasculature is visible at the dorsal and ventral side of the forming mouse heart from embryonic day (E) 14.5. **b–d** | Different cell lineages contribute to the generation of cardiac lymphatic endothelial cells. A subpopulation of paraxial mesoderm-derived venous endothelial cells bud from the cardinal vein to give rise to lymphatic endothelium (panel b). Other non-venous populations contribute to the cardiac lymphatics, such as the yolk sac-derived haemogenic endothelium (panel c) and an *Is/1⁺* second heart field population that is found specifically on the ventral cardiac lymphatics (panel d).

Fig. 3 | **Endogenous and VEGFC-C156S-augmented cardiac lymphatic response to myocardial infarction** [Au: OK]. Schematic illustration of the endogenous response of the lymphatic vessels to myocardial infarction and the augmented response induced by administration of vascular endothelial growth factor C (VEGFC)-C156S. **a** | After induction of myocardial infarction through surgical ligation of the left anterior descending coronary artery, accumulation of fluids leads to oedema and infiltration of immune cells, such as

macrophages, dendritic cells and T cells, into the myocardium. **b** | In response to myocardial infarction, cardiac lymphatic vessels undergo lymphangiogenesis in an attempt to clear the excessive tissue fluid and the inflammatory cells. However, this response is insufficient and the heart is repaired through fibrotic scar formation. **c** | Augmentation of the lymphangiogenic response through administration of VEGFC-C156S [Au: OK] leads to decreased oedema and increased immune cell clearance, and subsequently to improved cardiac healing and function.

Glossary terms

Lymphvasculogenesis

De novo formation of new lymphatic vessels from progenitor cell clustering.

Lymphangiogenesis

Formation of new lymphatic vessels arise from pre-existing ones, typically by sprouting.

Paraxial mesoderm

Sub-population of mesoderm containing progenitor cells that give rise to somites, which form muscle, connective tissue and the dermis.

Cardinal vein

The basis for the intraembryonic venous circulation consisting of anterior and posterior cardinal veins, which drain blood from the head and body into a pair of common cardinal veins and subsequently empty into the sinus venosus of the primitive heart.

Haemogenic endothelium

A special subset of vascular endothelium that acquires haematopoietic potential and can differentiate to haematopoietic stem and progenitor cells.

Yolk sac

The first membranous sac that is attached to and envelopes the developing embryo, provides early nutrition and the first site of blood cell production.

Second heart field

Cardiac progenitor cells in splanchnic mesoderm that contribute myocardium and smooth muscle to the formed heart tube at either the arterial or venous pole.

Phagocytosis

The process by which a cell uses its plasma membrane to engulf a large particle or another cell.

Efferocytosis

The process by which dying cells are removed by phagocytic cells, before their membrane integrity is breached.