

Cardiac lymphatic dysfunction and repair in cardiovascular disease

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Abstract

The field of cardiac lymphatic research has expanded considerably over the past decade. Clinical studies have uncovered lymphatic remodelling in a wide range of cardiovascular diseases, and experimental research has demonstrated that these structural alterations often lead to dysfunction of lymphatic transport. Given the vital physiological role of lymphatics, insufficient lymphatic drainage can affect several aspects of cardiac pathophysiology, including myocardial fluid balance, the immune microenvironment, collagen turnover and lipid handling. In this Review, current knowledge on cardiac lymphatics is summarized, including the structural and molecular specializations underlying their diverse homeostatic functions, and how these features can be altered in cardiovascular diseases. The latest research on the effects of inflammation on lymphatics is presented, together with the mechanisms by which lymphatics modulate immunity. The regulation of cardiac lymphangiogenesis is discussed, including accumulating evidence of immune cell–lymphatic crosstalk in the heart, the role of metabolic and biomechanical stimulation of lymphangiogenesis, and examples of experimental approaches to therapeutic lymphangiogenesis and their current limitations. Finally, areas for future research are highlighted, including the translation of lymphatic imaging and lymphangiogenic therapies to the clinic for patients with cardiovascular disease.

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Key points

- Cardiac lymphatic dysfunction is emerging as a hallmark of cardiovascular disease, contributing to insufficient clearance of fluid, waste products and immune cells from the heart.
- New molecular insights into cardiac lymphatic endothelial cells reveal promising therapeutic targets to restore lymphatic flow in cardiovascular diseases.
- Beyond draining immune cells, cardiac lymphatics might influence local immune responses in the heart through antigen presentation coupled to the production of immunomodulatory factors.
- Cardiac fibroblasts and immune cells orchestrate lymphatic remodelling in cardiovascular diseases, with both pro-lymphangiogenic and anti-lymphangiogenic immune cell subsets having been identified.
- Mechanosensory signalling in lymphatics, triggered by stretch or flow, could link cardiac stiffening to lymphatic dysfunction in cardiovascular diseases.
- Metabolic cues, including lipids and amino acids, fine-tune lymphatic endothelial cell gene expression profiles, including *VEGFR3* and other key genes.

Introduction

The cardiac lymphatic system is a network of vessels in the heart that maintains fluid balance, removes waste products, contributes to immune surveillance and responds to inflammation and injury. Cardiac lymphatic research has advanced considerably over the past decade, although the cardiac lymphatics remain heavily understudied compared with lymphatics in other organs^{1,2}. The considerable effect of chronic inflammation on the progression of most cardiovascular diseases (CVDs) is now well established, and the potential to resolve inflammation and prevent fibrosis through stimulation of cardiac lymphatics is beguiling. Promisingly, experimental studies have demonstrated the validity of this concept through gene, protein and cell therapy to boost cardiac lymphangiogenesis, notably after myocardial infarction (MI)¹. In addition, clinical investigations using cardiac biopsy or autopsy samples have shown that cardiac lymphatics are remodelled in a range of CVDs, and circulating levels of lymphangiogenic growth factors have

been proposed to have prognostic value in patients with coronary artery disease³. Moreover, single-cell RNA sequencing (RNA-seq) and spatial transcriptomic studies of cardiac cell subpopulations, including rare lymphatic endothelial cells, in both experimental and clinical settings, are leading to major advances in our understanding of cardiac lymphatics. One notable shift in research focus is linked to the discovery in various experimental models of CVD that, despite the presence of extensive lymphangiogenesis (including increased lymphatic vessel size and density) in the heart, the cardiac lymphatics seem to be dysfunctional^{4–8}.

In this Review, I first outline the structural and molecular bases for the functional properties of the lymphatic system in general, with a focus on the vessels that drain the heart. Second, I discuss the currently available evidence on the regulation of cardiac lymphangiogenesis, including the contribution of metabolic and biomechanical factors and immune cells. I also review the molecular mechanisms involved in lymphatic-mediated resolution of inflammation and potential therapeutic targets for the modulation of immunity and highlight the latest experimental data on the functional effects of therapeutic lymphangiogenesis. Finally, I address the clinical outlook in the field, including important open research questions.

Cardiac lymphatic structure and function

The lymphatic vasculature of the heart shares many similarities with that of other organs⁹ but also has unique structural and functional features. The cardiac lymphatic network is composed principally of lymphatic capillaries, which are limited to the ventricular subepicardial layers and the interventricular septum in small mammals, such as rodents^{1,10} (Fig. 1a), but penetrate all layers of the heart in large mammals, including humans¹¹. These blind-ended, highly permeable lymphatic capillaries (Fig. 1b) drain towards the epicardium, where they join to form impermeable lymphatic precollector vessels (Fig. 1c), which have unidirectional bicuspid valves (similar to those found in precollector vessels in other organs) to prevent back-flow (Fig. 1d–f). The precollector vessels subsequently drain into larger, extra-cardiac lymphatic collectors, which connect to the thoracic duct via chains of periaortic and mediastinal lymph nodes. Each segment of the lymphatic vasculature has a distinct role in the regulation of lymphatic drainage, and their unique features in the heart are discussed in the sections below, together with the potential mechanisms underlying lymphatic dysfunction in CVD.

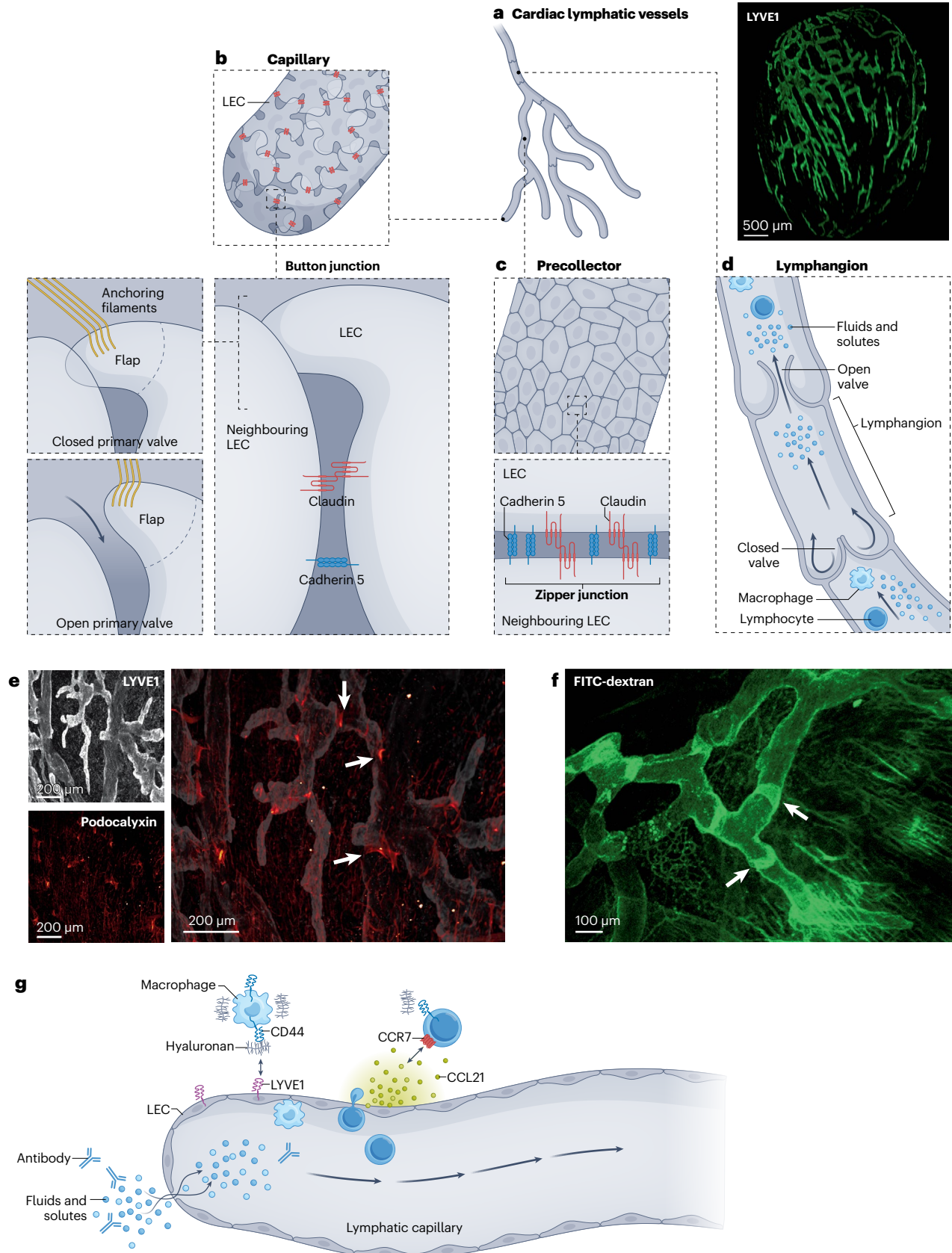
Lymphatic capillaries

Lymphatic capillaries in all organs are composed of a monolayer of oak-leaf-shaped lymphatic endothelial cells (LECs) that, unlike endothelial cells in blood vessel capillaries, are not ensheathed in a

Fig. 1 | Specialized structural and functional organization of cardiac lymphatics.

a, Lymphatic vessels cover the superficial cardiac layers in mice, as shown by immunostaining for lymphatic vessel endothelial hyaluronin acid receptor 1 (LYVE1; green). **b**, Lymphatics originate as blind-ended capillaries, formed from a monolayer of oak-leaf-shaped lymphatic endothelial cells (LECs), connected by button-like junctions with intermittent overlapping cell membrane flaps ('primary valves') that can be pulled back through the tension of anchoring filaments attached to the extracellular matrix to increase intercellular space. **c**, The downstream precollector vessels have continuous cell–cell zipper junctions, which prevent lymph leakage during transport towards the lymph nodes. Both button (panel **b**) and zipper (panel **c**) junctions contain cadherins (such as cadherin 5) and claudins. **d**, The functional element in lymphatic vessels is referred to as a lymphangion – a vessel segment between two consecutive

lymphatic valves – which maintains unidirectional flow in the system. **e**, Mouse cardiac lymphatic capillaries with valves. Arrows point to podocalyxin^{high} structures (red) found in LYVE1^{high} (grey) lymphatic capillaries. **f**, Lymphatic capillary valves (arrows) can also be visualized by intramyocardial injection of lymphatic tracers, as shown in this image of a healthy rat heart injected with fluorescein isothiocyanate (FITC)-dextran (green). **g**, Fluids and solutes enter lymphatic capillaries through passive absorption. By contrast, immune cells are actively recruited to lymphatic vessels by expression of chemotactic factors in LECs, including C-C motif chemokine 21 (CCL21) to attract C-C chemokine receptor type 7 (CCR7)-expressing immune cells, and by expression of LYVE1 and other adhesion molecules in LECs that allow interactions with CD44⁺ immune cells through a hyaluronan glyocalyx. Panels **a**, **e** and **f** were obtained from whole-mount imaging using lymphatic tracers or staining for podocalyxin and/or LYVE1.



continuous basement membrane. These capillary LECs partially overlap with neighbouring cells, creating ‘flaps’ that act as primary valves enhancing fluid entry under elevated interstitial pressure. The high permeability of lymphatic capillaries, which enables efficient uptake of fluid and solutes, depends on the presence of specialized, discontinuously sealed cell–cell junctions, interspaced between the flaps to form ‘button-like’ junctions that loosely connect neighbouring LECs (Fig. 1b). By contrast, precollector and collecting lymphatic vessels in all organs display continuously sealed junctions referred to as ‘zippers’ (Fig. 1c). Both button and zipper junctions contain the same types of molecules found in blood vascular endothelial cell–cell junctions, including cadherins (such as cadherin 5) in adhesion junctions, and claudins (such as claudin 5) and tight junction protein 1 in tight junctions. Therefore, the primary difference between button and zipper junctions is the membrane spacing of these components.

Capillary LECs are tethered to collagens and elastic microfibrils in the extracellular matrix (ECM) by specialized anchoring filaments that bind to adaptor proteins, including elastin microfibril interface-located proteins (EMILINs), multimerin 1 (MMRN1) and MMRN2, localized in the ECM. This anchoring of capillary LECs prevents vessel collapse during stretching of the ECM induced by tissue movements. Moreover, in response to increased interstitial pressure, these anchoring filaments pull on the flaps (unimpeded by button junctions) to transiently increase the intercellular space between adjacent capillary LECs, allowing increased uptake of fluid, solutes, nutrients and other macromolecules (Fig. 1b). Once the capillary bulb is filled, the increase in intraluminal pressure returns flaps to the relaxed position, trapping the lymph within the capillary until the lymph is aspirated into the precollector vessels. This entry of fluids and solutes occurs through passive drainage (Fig. 1g), driven by favourable pressure gradients in lymphatic capillaries (negative pressure propagated from downstream collecting vessels).

Table 1 | Structural and molecular profiles of cardiac LEC subpopulations

Characteristic	Capillary LEC	Precollector LEC	Valvular LEC
Button junctions	+	–	ND
Zipper junctions	–	+	ND
<i>Lyve1</i>	+++	+/-	+/-
<i>Ccl21</i>	+++	+	++
<i>Vegfr3 (Flt4)</i>	+++	+	++
<i>Nrp2</i>	+++	+	+
<i>Pdgfb</i>	–	++	–
<i>Egr1</i>	–	++	+
<i>Foxp2</i>	+	++	+
<i>Cldn5</i>	++	++	+++
<i>Klf2</i>	+	++	+++
<i>Prox1</i>	+	+	+++
<i>Foxc2</i>	–	–	++
<i>Gata2</i>	–	–	++
<i>Cldn11</i>	–	–	++

Characteristics of cardiac lymphatic subpopulations shared between healthy adult female C57BL/6J and BALB/c mice¹⁷. –, no expression; +/-, weak expression; +, moderate expression; ++, strong expression; +++, very strong expression; LEC, lymphatic endothelial cell; ND, not determined.

By contrast, immune cell drainage, which also mostly occurs at the level of lymphatic capillaries, is actively guided in all organs by chemokine signals emitted by LECs. Immune cells transmigrate at LEC junctional areas rich in chemokines and immune cell adhesion molecules (such as the intercellular adhesion molecules (ICAMs), P-selectin and vascular cell adhesion molecule 1 (VCAM1)) or the lymphatic vessel endothelial hyaluronin acid receptor 1 (LYVE1)¹² (Fig. 1g). Experimental studies in LYVE1-deficient mice, which have unaltered cardiac lymphatic density and structure, have revealed that the absence of this hyaladherin is associated with a marked reduction in cardiac immune cell clearance by the lymphatic system¹³. In addition, a mouse study showed that dendritic cells can enter lymphatic collectors in the skin during acute inflammation, a process that is dependent on the upregulation of VCAM1 (ref. 14).

Immunohistochemistry and single-cell RNA-seq analyses of human embryonic hearts^{15,16} and mouse adult cardiac LECs^{9,17} have revealed that capillary LECs in the heart (such as those in other organs¹⁸) have elevated expression of *AQP1*, *CCL21*, *LYVE1*, *MMRN1*, *NRP2* and *VEGFR3* (also known as *FLT4*) and reduced expression of *EGR1* (which encodes the shear-stress-induced transcription factor early growth response protein 1) compared with LECs in precollector vessels (Table 1). The level of *EGR1* might reflect differences in fluid shear stress in the various vascular branches of the lymphatic network, with mostly slow laminar flow in capillaries and high oscillatory flow in the precollector and collector vessels. These differences contribute to molecular, structural and functional vessel specialization.

Experimental studies in other organs have revealed some essential regulators of the ultrastructural characteristics of lymphatic capillaries, including angiopoietin 2 (ref. 19), which binds the angiopoietin 1 receptor (also known as TEK), and vascular endothelial growth factor C (VEGFC), which binds to vascular endothelial growth factor receptor 3 (VEGFR3). Mouse studies indicate that VEGFR3-mediated upregulation of NOTCH1 signalling in capillary LECs is crucial for the prenatal and postnatal development of button junctions in the lymphatic capillaries of several organs²⁰. Emerging data show that angiopoietin 2 might act indirectly on this process by upregulating *Vegfr3* expression in LECs²¹. Similarly, in mouse intestinal lymphatic capillaries (lacteals), VEGFC production by gut macrophages was essential for maintenance of lymphatic button junctions²², whereas elevated VEGFA–VEGFR2 signalling induced zippering of lacteal LEC junctions, rendering these lymphatic capillaries less permeable^{23,24}. Remarkably, zippering of lacteals, which reduced lipid absorption in the gut, was sufficient to prevent diet-induced obesity in mice²³. The hormone adrenomedullin is another lymphangiogenic factor that stimulates zippering of lymphatics through upregulation of cadherin 5 expression²⁵. Of note, cadherin 5 has been shown to reduce VEGFR3 endocytosis, which is required for optimal receptor signalling²⁶. This finding highlights the important role of VEGFC and VEGFD signalling in the maintenance of junctional specialization in lymphatic capillaries, underlying the ability of these vessels to drain fluid, solutes and macromolecules. Whether the molecules discussed here also affect lymphatic capillary button junction formation in the heart is currently unknown.

Precollector and collector vessels

Lymphatic precollector and collector vessels are distinguished from lymphatic capillaries by the presence of zipper-type LEC junctions (Fig. 1c), rendering them less permeable than capillaries, which protects against leakage of lymph during transport. In most organs, these vessels (especially collectors) are characterized by a mural layer of lymphatic muscle cells ensheathed in a continuous basement membrane and

by regularly spaced bicuspid lymphatic 'secondary' valves, ensuring unidirectional lymph flow (Fig. 1d).

At the molecular level, single-cell RNA-seq analyses have revealed that mouse cardiac LECs in precollectors, similar to those in other organs¹⁸, have lower expression of *Aqp1*, *Ccl21*, *Lyve1* and *Vegfr3* and elevated expression of *Egr1* and *Pdgfb* compared with capillary LECs¹⁷. Cardiac LECs of lymphatic valves are characterized by elevated expression of genes encoding valve-regulating transcription factors, such as *Gata2*, *Prox1* and *Sox18*, as well as other genes, including *Cldn11* and *Itga9* (ref. 17) (Table 1), findings that are similar to those for lymphatic valves in other organs^{18,27}. Intriguingly, *Foxc2*, which encodes another major lymphatic valve transcription factor, is expressed not only in cardiac valvular LECs but also in cardiac mitral valves and endocardial cells^{28,29}.

Functionally, lymphatic precollector and collector vessels are organized into serial lymphangions, which are lymphatic vessel segments situated between two valves (Fig. 1d). Each lymphangion contracts in turn, which allows sequential filling of lymphangions to propagate lymph downstream in the direction of the lymph nodes. When a lymphangion is emptied, the drop in intraluminal pressure allows the upstream valve to open for refilling. This cyclic process of contraction and relaxation is reminiscent of the cardiac cycle, and, therefore, the terms 'systolic' and 'diastolic' volumes and 'ejection fraction' are used to characterize lymphatic pumping efficiency³⁰.

In most tissues, the propagation of lymph in collecting vessels is mainly generated by the coordinated rhythmic actions of lymphatic muscle cells, with the contribution of surrounding tissue motion and pressure changes that further promote lymph drainage. The amplitude and frequency of lymphatic muscle cell spontaneous phasic contractions are regulated by mechanosensory pathways, including LEC-mediated signalling of lymph flow rates in a nitric oxide (NO)-dependent manner, to ensure adequate and coordinated lymphatic drainage³⁰. However, cardiac lymphatic precollectors differ from those of other organs in that they do not have lymphatic muscle cells^{4,27,31}. Thus, lymphatic propulsion in the heart fully depends on the surrounding cardiomyocytes for sequential compression of the lymphatic vessels. Consequently, fluid enters lymphatic capillaries during cardiac diastole, and, in systole, the lymph is propagated in subepicardial precollector lymphatics from the apex towards the base of the heart.

In rats and humans, collector vessels in the periphery contract 1–10 times per minute^{30,32}, whereas contractile frequency in the heart is much higher (60–100 bpm in humans and 250–600 bpm in rats). Given that the distance from the apex to the base of the heart is too long, even in rodents, to allow lymph drainage in a single cardiac cycle, the presence of closely interspaced valves might be vital to prevent the back-flow of lymph. In adult mouse hearts, cardiac lymphatics have shorter inter-valve distances than dermal precollector vessels, but similar distance as dermal lymphatic capillaries^{17,33,34}. Indeed, similar to those in human skin^{33,34}, cardiac lymphatic capillaries in mice (identified as LYVE1^{high} structures) have lymphatic valves, with only the outermost tips of capillaries lacking valves¹⁷. Similarly, in mouse hearts, the presence of PROX1^{high} valvular LECs has been demonstrated in both LYVE1^{low} precollectors and LYVE1^{high} blunt-ended lymphatic capillaries¹⁰. Whether these findings also apply to lymphatics in the human heart remains to be investigated.

Lymphatic alterations in CVD

Early experimental studies (1960s–1990s) on the alteration of lymphatic transport in CVD used large animal models (dogs, pigs and rabbits)³⁵,

whereas more recent experimental research has focused on models of MI in mice, rats and fish¹. Lymphatic remodelling has also been demonstrated in mouse models of atherosclerosis, venous pressure-induced acute myocardial oedema, pressure overload-induced cardiac hypertrophy and following heart transplantation^{1–3,36}. Notably, cardiac lymphatic occlusion in mice led to rapid cardiac dysfunction³⁷, a finding similar to observations in larger mammals³⁵. In models of atherosclerosis, including *Apoe*^{-/-} or *Ldlr*^{-/-} mice, lymphatic enlargement and dysfunction occurred in arterial vessel walls and also in the dermis, where poor lymphatic uptake contributed to cholesterol accumulation in the skin and reduced dendritic cell transit to lymph nodes³⁸. Interestingly, in a mouse model of viral myocarditis, transient stimulation of cardiac lymphangiogenesis was linked to increased VEGFC production in cardiac macrophages³⁹.

Clinical indications that cardiac lymphatics are altered in CVD came from histological analyses using an antibody to podoplanin (D2-40). Elevated lymphatic vessel density and altered lumen sizes were seen in biopsy and autopsy samples from patients with acute or chronic ischaemia, chronic heart failure or valvular endocarditis^{1,40,41}. Expansion of cardiac lymphatics has also been described in patients with hypertensive obstructive cardiomyopathy⁴². By contrast, no significant increase in myocardial lymphatic density (except in perivascular niches) was found in septal myectomy samples from patients with primary or secondary hypertrophic or dilated cardiomyopathy⁷. Instead, a reduction in lymphatic vessel lumen sizes was evident in patients with end-stage heart failure. Moreover, in a study of lymphatic markers, lower cardiac gene expression of *LYVE1*, *VEGFC* and *VEGFR3* was reported in patients with end-stage heart failure compared with healthy donor hearts⁵. Intriguingly, in a large-scale prospective cohort study of patients with coronary artery disease, patients with the highest circulating levels of VEGFC had better outcomes⁴³, whereas those with elevated levels of VEGFD had poorer outcomes⁴⁴. Similarly, in patients with acute heart failure, low serum levels of VEGFC were associated with increased severity of tissue congestion⁴⁵. Whether these findings reflect the function of lymphatics in the heart, or in other organs (for example, in congested lungs), currently remains to be determined. In the following sections, I will discuss the current evidence pertaining to structural alterations of lymphatic capillaries induced by inflammation and CVD, as well as functional alterations and molecular changes in cardiac lymphatics demonstrated in experimental models of CVD.

Structural alterations in lymphatic capillaries

The ultrastructural organization of cardiac lymphatic capillaries can be altered by myocardial inflammation in CVDs, contributing to insufficient lymphatic uptake. Indeed, both experimental and clinical studies of lymphatics in organs other than the heart have demonstrated that lymphatic uptake and transport are often reduced in response to inflammation, contributing to the cardinal sign of tissue swelling. This process has been linked to the actions of immune mediators, such as IL-1 β , which has been demonstrated to revert button junctions into zipper junctions (zippering) in tracheal lymphatic capillaries⁴⁶. In addition, virus-induced upregulation of the expression of *Vegfa* in the skin of mice has been shown to result in zippering of lymphatic capillary junctions⁴⁷, similar to the effects of this angiogenic growth factor in lacteals²³. The pathophysiological effect of this ultrastructural change in dermal lymphatics was a reduction in passive lymphatic uptake and the spread of virions. By contrast, these structural changes did not impede active dendritic cell transport to lymph nodes for initiation of adaptive immune responses⁴⁷.

Investigations into the ultrastructural properties of cardiac lymphatics in CVD, including electron microscopy studies in dogs and rabbits, have revealed substantial changes in lymphatic morphology during acute or chronic myocardial oedema⁴⁸. Structural changes in cardiac lymphatics were investigated in a mouse model of metabolic syndrome (genetic obesity linked to mild angiotensin II-induced hypertension), characterized by myocardial chronic low-grade inflammation, hypertrophy and fibrosis⁴⁹. Striking disorganization of anchoring filaments was observed, with widening of intercellular LEC junctions and thickening of the basement membrane in capillary lymphatics of hearts from deceased animals compared with healthy controls⁴⁹. Such loss of anchoring filaments, coupled with stiffening of the ECM, in hypertensive heart disease could lead to inefficient capillary LEC flap opening, thus impeding lymphatic fluid uptake, aggravating pressure overload-induced chronic myocardial oedema and triggering cardiac fibrosis and dysfunction⁵⁰. Promisingly, in a mouse model of MI, loss of lymphatic capillary button junctions in non-infarcted areas of the heart could be rescued by adeno-associated virus 9 (AAV9)-mediated *Vegfc* gene delivery⁵¹. In parallel, selective expansion of lymphatic capillaries has been found in several models of cardiac hypertrophy induced by pressure overload through either chronic angiotensin II infusion⁵² or transverse aortic constriction (TAC)^{6,7}. Initial reports indicated that, although this lymphatic remodelling was associated with reduced lymphatic transport after TAC⁶, there was no clear loss of button junctions in these expanded lymphatic capillaries⁷. In future studies, investigators should seek to determine whether adrenomedullin or pro-inflammatory mediators, such as VEGFA, regulate capillary LEC permeability in CVD and thus potentially contribute to lymphatic insufficiency, affecting fluid and macromolecule uptake.

Functional alterations in precollector vessels

Several risk factors associated with CVD, including metabolic syndrome (obesity, hyperglycaemia, hypercholesterolaemia and hypertension) and ageing, are associated with generalized lymphatic dysfunction, as revealed in experimental models. For example, mice deficient in apolipoprotein E with hypercholesterolaemia or mice with obesity and hyperlipidaemia have leaky lymphatic collector vessels^{38,53}. The effects of ageing on the lymphatic system include rarefaction of lymphatic capillaries, leakage from collectors and loss of lymphatic muscle cell contractile activity^{54–56}. Ageing in mice leads to dilatation of both capillaries and precollector lymphatics in the heart, coupled with reduced levels of cadherin 5 in collector vessels⁸. Promisingly, exercise training (voluntary wheel running) in aged mice was sufficient to restore cardiac lymphatic structure and function⁸. This lymphatic rejuvenation was associated with reductions in myocardial inflammation, fibrosis, lipid deposits and, promisingly, diastolic dysfunction.

Inflammation can also directly alter precollector transport function. Research in organs other than the heart has demonstrated that activated CD11b⁺ myeloid immune cells (monocytes and mast cells), which are recruited during inflammation, produce high levels of NO because they express inducible NO synthase (iNOS)⁵⁷. This process overrides endogenous endothelial NO synthase (eNOS)-dependent mechanisms in LECs regulating the relaxation of lymphatic muscle cells in collectors, thus reducing lymphatic transport⁵⁷. However, this mechanism is not applicable in the heart because cardiac lymphatics lack lymphatic muscle cells. By contrast, many pro-inflammatory cytokines have deleterious effects on cardiomyocyte contractility and relaxation (such as the negative inotropic effects of IL-1 β)⁵⁸. Therefore, inflammation-induced impairment of cardiac diastolic or systolic

function could be expected to induce reductions in lymphatic precollector and collector transport capacity in the heart. Of note, in a rat model of ischaemia–reperfusion injury, lymphangiography revealed that lymphatic insufficiency persisted for several months after the MI⁴. However, therapeutic lymphangiogenesis accelerated the resolution of this condition, thereby reducing cardiac dysfunction⁴.

Taken together, these findings suggest that dysfunction in cardiac lymphatic transport, which has been demonstrated in diverse experimental models of CVD, could be caused by impaired function of lymphatic capillaries, precollector vessels or both, combined with the direct lymphatic effects of cardiac dysfunction. To better discern lymphatic-specific disease mechanisms, with the aim of identifying tractable therapeutic targets, transcriptomic analyses could be used to investigate whether the molecular profiles of cardiac LEC are altered in various CVDs.

Molecular changes in cardiac lymphatics

Few of the available single-cell RNA-seq studies of mouse or human hearts have captured sufficient numbers of rare cardiac LECs to allow comparative analyses^{9,15,16,59}. One notable example is a single-nucleus RNA-seq analysis, in which non-ischaemic left ventricular samples from patients with end-stage ischaemic cardiomyopathy were compared with samples from non-failing hearts (organ donation)⁶⁰. A higher yield of LECs in failing than non-failing hearts was reported, a finding supported by increased densities of *CCL21*-expressing LECs in fibrotic areas, as observed using *in situ* hybridization. An analysis comparing cardiac LEC expression profiles between groups (415 LECs from non-failing hearts and 1,012 LECs from failing hearts) revealed a reduction in *MMRNI* expression and an increase in *STAB2* and *UNC5B* expression in heart failure LECs⁶⁰. Although potential changes in the balance between cardiac LEC subpopulations were not addressed in this study, on the basis of the well-established higher relative abundance of capillary LECs compared with precollectors in the heart, the reduction in *MMRNI* expression could reduce capillary ECM anchoring, thus altering the permeability of lymphatic capillaries; promote lymphatic hyperplasia; or both⁶¹. Conversely, the observed upregulation of *STAB2* expression, which encodes stabilin 2 (a hyaladherin similar to LYVE1), could promote lymphatic-mediated clearance of both hyaluronan and heparin⁶². The role of *UNC5B*, a receptor for the netrin family of neural guidance molecules, has not yet been investigated in cardiac lymphatics, but *in vitro* data indicate that this receptor is upregulated by NOTCH1 signalling in endothelial cells, where it helps to stabilize cell junctions in conditions of elevated shear stress⁶³.

Bulk RNA-seq analyses of cardiac LECs in a mouse model of angiotensin II-induced chronic pressure overload (associated with only minor cardiac lymphatic remodelling but apparent lymphatic transport dysfunction linked to cardiac inflammation) revealed upregulation of Toll-like receptor pathways in LECs, associated with changes in lymphatic chemokine profiles (reduced *Ccl21* and increased *Cxcl1* and *Cxcl12* expression)⁵. However, in this model, no obvious changes in LEC junctional molecules or lymphatic marker genes could explain the observed reduction in lymphatic drainage. Thus, cardiac dysfunction per se might have driven poor lymphatic transport in this model.

Cardiac lymphatic expression profiles have been investigated in female BALB/c mice with cardiac pressure overload induced by TAC¹⁷. Extensive lymphatic capillary expansion, coupled with insufficient lymphatic transport, indicative of lymphatic dysfunction, had previously been demonstrated in this model⁷. Single-cell RNA-seq was used to characterize transcriptomic changes in cardiac LEC subpopulations

8 weeks after TAC¹⁷. The key findings included upregulation of *Lyve1* and *Ccl21* expression and loss of *Cldn5* expression in both capillary LECs and precollector LECs. These alterations were validated at the protein level¹⁷, which suggests that LEC junctional properties linked to claudin 5 are likely to be altered, even though immune cell attraction and adhesion involving C-C motif chemokine 21 (CCL21) and LYVE1, respectively, might be increased in remodelled cardiac lymphatics to help to resolve inflammation after TAC. Whether such molecular changes cause a shift between button and zipper junctions, contributing to lymphatic dysfunction in pressure overload-induced heart failure, remains to be determined. Another major discovery in the transcriptomic analysis of cardiac LEC subpopulations was a relative reduction in the number of valvular LECs after TAC¹⁷. Whole-mount imaging revealed selective rarefaction of lymphatic valves in the expanded lymphatic capillary network after TAC in BALB/c mice, but not in C57BL/6J mice¹⁷. Future studies should investigate the factors regulating cardiac lymphatic valvulogenesis, as well as the role of inflammatory mediators in this process. Importantly, whether lymphatic valves are lost in CVD-related remodelling of cardiac lymphatics in patients is currently unknown.

As already mentioned, stimulation of lymphangiogenesis in CVD is not limited to the ventricles but can also occur in the aortic and mitral cardiac valves. Lymphatic expansion and dysfunction in mitral valves have been reported in a mouse model of myxomatous valve degeneration²⁸. Similarly, a single-cell RNA-seq study revealed expansion of lymphatics in mitral valves, driven by local VEGFC production by CD206⁺ macrophages, during autoimmune valvulitis in mice, as well as in patients with rheumatic valvular heart disease²⁹. The lymphatic remodelling in mice included expansion of not only *Lyve1*⁺*Ccl21*⁺ capillary LECs but also *Foxc2*-expressing valvular LEC-like cells. However, preferential expansion of capillary LECs during chronic inflammation in mitral valves was also reported²⁹. Notably, these inflamed capillary LECs in cardiac valves lost expression of *Cldn5* (ref. 29) (similar to subepicardial capillary LECs in the TAC model¹⁷).

Lymphatic immune modulation

Lymphatics can modulate immunity in several ways, for example, by evacuating immune cells from tissues to lymph nodes and by interacting with immune cells locally in the tissue, thus influencing immune responses. A new subtype of LECs, termed immune-interacting LECs (iLECs), has been identified using single-cell RNA-seq analyses in a mouse model of dermal lymphatic malformations associated with chronic inflammation¹⁸. This population of LECs was characterized by selective expression of an acute phase response protein, pentraxin-related protein PTX3, encoded by *Ptx3*. In addition, the iLECs displayed elevated levels of other immune-related lymphatic markers, such as the hyaladherin and scavenger receptor stabilin 1 (encoded by *Stab1*), the mannose receptor C type 1 (also known as CD206; encoded by *Mrc1*) and atypical chemokine receptor 2 (also known as D6; encoded by *Ackr2*), which scavenges C-C motif chemokines, including CCL2. Transcriptomic studies of developing human hearts have demonstrated *PTX3* expression in expanding cardiac LECs¹⁵. However, whether iLEC subpopulations are induced in cardiac lymphatics during inflammation in patients with CVD is currently unknown. Additionally, spatial transcriptomics of adult human hearts has revealed a distinct subepicardial 'immune niche' enriched for LECs and immune cells⁶⁴. A CellPhoneDB analysis to identify ligand–receptor pairs between different cardiac cell populations indicated that LEC-derived CCL2 and CCL28 might specifically stimulate recruitment to this niche of IgA⁺ plasma B cells expressing C-C chemokine receptor type 2 (CCR2) and CCR10 (ref. 64).

The T-box transcription factor TBX1 has a key role in cardiac lymphatics after MI in animal models, mediated by the interaction with VEGFR3 to upregulate immune-interacting molecules, such as CCL21 and ICAM1 (ref. 65). In parallel, functional experimental studies have indicated that LYVE1 also has an important role in the mechanisms involved in cardiac lymphatic immune uptake in CVD^{13,66}. Indeed, LYVE1 binds to CD44⁺ immune cells, including macrophages, through a hyaluronan coat (cellular glycolyx), which allows bridging of LYVE1 and CD44, promoting immune cell adhesion and transmigration into lymphatic vessels (Fig. 1g). Furthermore, the atypical chemokine receptor 3 (ACKR3), which scavenges C-X-C motif chemokines, including stromal cell-derived factor 1 (CXCL12), is important in mediating inflammation resolution in cardiac lymphatics after MI in mice¹⁰. In addition to influencing immune responses, deletion of *Ackr3* led to a reduction in myocardial oedema after MI¹⁰, potentially associated with increased permeability of capillary LEC junctions.

Most of the available evidence on lymphatic regulation of adaptive immune responses (outside the lymph nodes) comes from studies of tumour models in mice. Indeed, upregulation of antigen-presenting major histocompatibility complex class I or II molecules, together with immune-suppressive molecules, including transforming growth factor- β 1 and the immune checkpoint programmed cell death 1 ligand 1, in tumour-draining lymphatics has been reported in several investigations^{50,51}. Consequently, lymphatic antigen presentation can induce anergy in cytotoxic CD8⁺ T cells and promote expansion of regulatory T cells^{57,68}, which is deleterious in a tumour setting given that it allows tumour immune escape. In vitro, T helper 1-type cytokines, notably interferon- γ (IFN γ), drive these molecular changes in LECs⁶⁷. Whether myocardial inflammation in CVD activates similar immunosuppressive mechanisms in cardiac lymphatics remains to be investigated.

Regulation of cardiac lymphangiogenesis

Diverse cardiac cell types, including cardiomyocytes, coronary endothelial cells, vascular smooth muscle cells and cardiac fibroblasts, have been proposed to contribute to the regulation of lymphangiogenesis during fetal development and to the pathophysiology of CVD through the production of lymphangiogenic growth factors^{1,16,69,70}. Moreover, several immune cell populations, notably macrophages^{6,22,71–73} and B cells^{74,75}, are rich sources of lymphangiogenic growth factors and have been shown to contribute substantially to the regulation of lymphangiogenesis in various tissues, including the heart (Fig. 2a and Box 1). Furthermore, although VEGFC and VEGFD, acting through VEGFR3 in LECs, are well established as the main lymphangiogenic growth factors, many other molecules can directly or indirectly (for example, through upregulation of VEGFR3) stimulate lymphangiogenesis. Notable examples include hormones, such as adrenomedullin⁷⁶ and oestrogen⁷⁷, both present at elevated levels in women and female animals. Of note, some experimental studies are emerging on sex-specific differences in cardiac lymphatics^{77,78}. LECs also express receptors for hormones of the renin–angiotensin–aldosterone system (RAAS), such as angiotensin II, which has been reported to stimulate LEC proliferation in vitro via the type 1 angiotensin II receptor⁷⁹. The interaction between the RAAS and the lymphatic system has been reviewed in detail elsewhere⁸⁰. Lymphangiogenesis as well as VEGFR3 activation can also be directly stimulated by biomechanical factors, such as elevated luminal shear stress or vessel stretching (Box 2), and by arterial or neuron-derived factors, including CXCL12, which binds to C-X-C chemokine receptor type 4 on LECs to stimulate VEGFR3 expression^{81,82}. In addition,

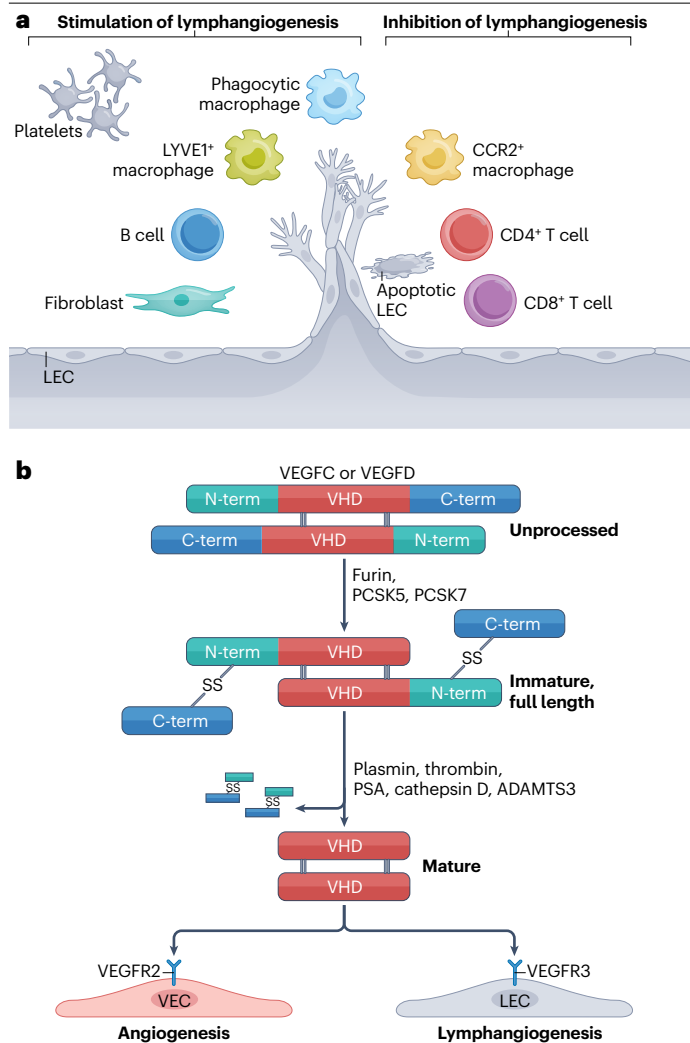


Fig. 2 | Cellular and molecular regulation of lymphangiogenesis. **a**, Cardiac fibroblasts, B cells, activated platelets, cardiac resident macrophages expressing lymphatic vessel endothelial hyaluronin acid receptor 1 (LYVE1) and alternatively polarized, monocyte-derived phagocytic macrophages can all stimulate lymphangiogenesis by releasing vascular endothelial growth factor C (VEGFC). By contrast, cardiac-infiltrating classically polarized, monocyte-derived CCR2⁺ macrophages and T cells secrete pro-inflammatory mediators that reduce lymphangiogenesis and harm lymphatic endothelial cells (LECs). **b**, Several proteases regulate the bioactivity of VEGFC and VEGFD by cleavage of a C-terminal sequence from the unprocessed intracellular forms, followed by removal of N-terminal sequences from the secreted, immature full-length protein forms. Finally, the mature form of the dimeric growth factors is generated, containing only the VEGF homology domains (VHD) that bind with high affinity to vascular endothelial growth factor receptor 2 (VEGFR2) or VEGFR3 to selectively activate angiogenesis and lymphangiogenesis, respectively. ADAMTS3, a disintegrin and metalloproteinase with thrombospondin motifs 3; PCSK, proprotein convertase subtilisin/kexin; PSA, prostate-specific antigen (also known as kallikrein-related peptidase 3); VEC, vascular endothelial cell.

Intriguingly, pretreatment with rosuvastatin before MI induction in mice increased cardiac VEGFR3 protein expression after MI compared with no pretreatment⁸⁴.

Endogenous inhibition of lymphangiogenesis is an understudied area in lymphatic biology. Many known angiostatic factors, including the pro-inflammatory cytokines IFN γ and tumour necrosis factor, as well as C-X-C motif chemokine 10, suppress both angiogenesis and lymphangiogenesis^{85–87}. Intriguingly, another angiostatic factor, thrombospondin 1, has been identified as a key inhibitor of lymphangiogenesis in atherosclerotic aortas in mice⁸⁸. Remarkably, in these mice, prevention of thrombospondin 1 binding to LECs, by conditional deletion of *Cd47*, which encodes a cell surface receptor for thrombospondin 1, was sufficient to increase periaortic lymphatic density and reduce atherosclerosis⁸⁸. Another matricellular protein, tenascin C, has also been proposed as an endogenous inhibitor of cardiac lymphangiogenesis and might act, in part, by promoting macrophage pro-inflammatory polarization⁸⁹.

Experimental *in vitro* and *in vivo* data have demonstrated that stimulation of lymphangiogenesis in various organs (for example, induced by wound healing or tissue remodelling) mediated by endogenous VEGFC and VEGFD is strictly regulated by proteases⁹⁰. Both VEGFC and VEGFD require post-translational processing by intracellular and extracellular proteases to reach their mature forms and to efficiently bind to and activate VEGFR2 or VEGFR3. These essential growth factor-processing enzymes include members of the proprotein convertase subtilisin/kexin (PCSK) family, such as the serine proteases furin (PCSK3), PCSK5 and PCSK7, which cleave the C-terminal propeptide domains of VEGFC and VEGFD⁹⁰. Once secreted from the cell, the immature full-length VEGFC or VEGFD proproteins are processed by extracellular or cell membrane-bound PCSKs and other proteases, including prostate-specific antigen (PSA; also known as KLK3) and cathepsin D⁹¹, which cleave the N-terminal domain of the proteins to generate mature VEGF homology domain dimers (Fig. 2b). In addition, the platelet-derived serine proteases plasmin and thrombin can also contribute to the maturation of secreted pro-VEGFC and pro-VEGFD^{90,92}. Of note, the cleavage sites for these proteases differ slightly, and they each generate distinct isoforms of mature VEGFC and VEGFD. Interestingly, VEGFD maturation by cathepsin D leads to an isoform that is selective for VEGFR2, thus displaying higher angiogenic than lymphangiogenic activity⁹¹. Conversely, fully matured VEGFC loses its affinity for VEGFR2 and gains selectivity for VEGFR3 (ref. 91).

Unlike VEGFD, the maturation of VEGFC seems to be mainly regulated by the extracellular enzyme ADAMTS3 (a disintegrin and metalloproteinase with thrombospondin motifs 3), which cleaves VEGFC under the guidance of the secreted protein CCBE1 (collagen and calcium binding EGF domain-containing protein 1)⁹³. Indeed, CCBE1, which can also guide PSA-mediated activation of both VEGFC and VEGFD, is essential for developmental lymphangiogenesis, and variants in *CCBE1* cause lymphatic malformations in humans⁹⁴. Intriguingly, although many studies have demonstrated that maintenance of mature lymphatic vessels in most organs does not require constant growth factor signalling (unlike in blood vessels), one investigation showed that inducible deletion of *Ccbe1* leads to age-related regression of meningeal lymphatics in mice⁹⁵. Notably, CCBE1, which provides spatial guidance cues for lymphangiogenesis, is produced by many different cell types, including fibroblasts⁹⁶, smooth muscle cells and macrophages.

As discussed, although many factors directly promote lymphangiogenesis, several act indirectly by reinforcing VEGFR3 signalling in LECs, highlighting the crucial role of VEGFC and VEGFD in lymphatics.

emerging data indicate that metabolic factors, including monounsaturated free fatty acids and ketone bodies, promote LEC survival and proliferation and stimulate lymphangiogenesis *in vivo*⁸³ (Box 3).

VEGFC has many clear advantages as a therapeutic target, such as selectivity for LECs, induction of button junctions in capillary LECs to maintain their hyperpermeability, and stimulation of pumping in collector vessels⁹⁷. However, whether delivery or overexpression of VEGFC is the optimal therapy to stimulate lymphangiogenesis in settings of chronic cardiovascular inflammation or in ageing remains to be determined. Moreover, whether additional targets (such as biomechanical, enzymatic, immune, lymphangiostatic or metabolic factors) could provide synergistic effects to improve lymphatic function and, therefore, restore lymphatic drainage in CVD remains unknown.

Effects of therapeutic lymphangiogenesis

Most studies of therapeutic lymphangiogenesis in CVD or other diseases have focused on the delivery of VEGFC or VEGFD⁹⁸. Modalities include repeated systemic injection or biopolymeric intramyocardial delivery of recombinant VEGFC⁵, antibody-mediated delivery of fibronectin-targeted recombinant VEGFC⁹⁹, or cardiac implantation of either VEGFC-loaded gelatine-based hydrogels or VEGFC-expressing adipose tissue-derived mesenchymal stem cells^{37,100}. As a robust alternative for controlled delivery, gene therapy with viral vectors (adenovirus or AAV) is often used¹. Adenovirus-mediated VEGFD delivery is being investigated in the phase I KAT301 clinical trial¹⁰¹ in patients with coronary artery disease and refractory angina.

Cardiac infarct remodelling

Indication that therapeutic delivery of VEGFC might improve cardiac function after MI came from a study¹⁰² in mice published in 2015. The investigators used repeated intraperitoneal injections of recombinant VEGFC protein to stimulate cardiac lymphangiogenesis acutely after MI and observed reductions in infarct size, associated with a striking reduction in cardiac dysfunction after MI¹⁰². Conversely, a study in mice with severely reduced lymphangiogenic capacity, caused either by expression of a soluble form of VEGFR3 (sVEGFR3; which acts as a VEGFC–VEGFD trap) or by an inactivating mutation in one copy of the *Vegfr3* gene (Chy mice), revealed that infarct scar maturation (healing)

after MI was reduced, frequently resulting in cardiac rupture, compared with mice with wild-type VEGFR3 (ref. 103).

In addition to the classical role of lymphatics in the drainage of excess interstitial fluids and the resolution of inflammation, a new role has been proposed, with LECs identified as a source of paracrine factors influencing the viability of cardiomyocytes after MI in mice¹⁰⁴. This ‘lymphangiocrine’ effect in the heart was linked to reelin, a secreted ECM protein. Reelin has an important role in neuronal patterning during brain development, as well as many essential functions in the periphery in adults¹⁰⁵. Intriguingly, in mice, LEC-selective deletion of the gene encoding reelin (*Reln*) during fetal development led to cardiac hypoplasia, whereas early postnatal deletion of *Reln* reduced cardiac regeneration after MI¹⁰⁴. In vitro, reelin released from LECs stimulated the viability of cardiomyocytes¹⁰⁴, indicating that cardiac lymphatics could be directly involved in limiting infarct expansion and remodelling after MI via secreted factors.

Other studies have revealed opposing findings related to lymphatics in the infarcted myocardium. For example, AAV9-mediated delivery of *sVegfr3* after MI in adult female mice prevented infarct lymphangiogenesis but did not lead to an increase in infarct size nor cause cardiac rupture⁵¹. In addition, neither intramyocardial VEGFC delivery after MI in rats nor AAV9-mediated delivery of *Vegfc* after MI in mice led to increased lymphatic density in the infarct scar nor to reduction of infarct sizes^{4,51}. Similarly, loss of function studies in adult mice showed that conditional lymphatic deletion of *Vegfr3* or composite loss of *Vegfc* and *Vegfd*, although sufficient to almost fully abrogate cardiac infarct lymphangiogenesis, did not lead to increases in the infarct size or the number of immune cells in the scar and did not worsen cardiac dysfunction after MI¹⁰⁶. Taken together, these studies indicate that endogenous VEGFC and VEGFD are key regulators of infarct lymphangiogenesis after MI and that, whereas infarct lymphangiogenesis does not reduce infarct size, VEGFC and VEGFD might affect the scar maturation kinetics.

Cardiac lymphatics depend intimately on the surrounding cardiomyocytes for their function. Therefore, the abnormally dilated lymphatic capillaries that are found inside the cardiac infarct zone,

Box 1 | Immune cell regulation of lymphangiogenesis

During fetal development in mice, resident cardiac macrophages guide lymphangiogenesis through the production of hyaluronic acid⁷², which promotes lymphatic endothelial cell (LEC) proliferation via lymphatic vessel endothelial hyaluronic acid receptor 1 (LYVE1)¹²¹. Similarly, in adult mice, myeloid immune cells, notably neutrophils and macrophages infiltrating the heart, are key sources of vascular endothelial growth factor C (VEGFC)⁷³. In addition, cardiac macrophages, including resident LYVE1⁺CCR2⁻ populations, are essential for cardiac lymphatic expansion in mice after myocardial infarction⁷². Release of VEGFC has also been linked to the process of efferocytosis⁷³, a characteristic shared by resident macrophages or alternatively polarized monocyte-derived macrophages. Other experimental studies have shown that various macrophage subpopulations can exert opposing effects on lymphangiogenesis. For example, inhibition of lymphangiogenesis has been linked to cardiac-infiltrating, monocyte-derived, pro-inflammatory CCR2⁺ macrophages, whereas stimulation of lymphangiogenesis has been associated with cardiac resident, LYVE1⁺ macrophages during

pressure overload-induced heart failure in mice⁶. In addition, stimulation of mouse macrophages with the pro-inflammatory cytokine IL-1 β increased not only VEGFC production but also the expression of *Adamts3*, *Ccbe1*, *furin* and *Pcsk5* (ref. 122). This finding indicates a coordinated action, whereby IL-1 β upregulates vascular endothelial growth factor receptor 3 levels in LECs¹²³ and promotes the expression and maturation of VEGFC in macrophages. Moreover, antibody-mediated blockade of IL-1 β signalling reduced cardiac pressure overload-induced cardiac lymphangiogenesis in mice¹²². Platelets are also a rich source of VEGF family members, including VEGFC, and platelet aggregation during tissue repair has been shown to lead to stimulation of both angiogenesis and lymphangiogenesis in various organs⁹². Conversely, several T cell populations exert inhibitory effects on lymphatics, inducing lymphatic dysfunction, suppression of lymphangiogenesis or LEC apoptosis¹²⁴. Accordingly, prevention of T cell mobilization or neutralization of the T helper 1 cytokine interferon- γ were shown to improve cardiac lymphangiogenesis after myocardial infarction in mice⁵¹.

Box 2 | Mechanosensory regulation of lymphatics

Lymphatic endothelial cells (LECs) in all organs are more sensitive to fluid shear stress than vascular endothelial cells (VECs) in blood vessels. This property, which reflects the lower range of shear stress found in lymphatic vessels, is attributable in part to increased expression of vascular endothelial growth factor receptor 3 (VEGFR3) in LECs⁶¹. Moreover, the generation and maintenance of lymphatic valves have both been shown to depend on mechanosensory pathways activated by oscillatory shear stress¹²⁵. This finding has been linked to suppression of the lymphatic valve-inhibiting transcription factor forkhead box protein O1, mediated by RAC- α serine/threonine-protein kinase (also known as AKT), in LECs¹²⁵. As in VECs, the molecular machinery that couples fluid flow to LEC responses includes platelet endothelial cell adhesion molecule, cadherin 5, vascular endothelial growth factor receptor 2 (VEGFR2) and VEGFR3, in interaction with integrin β 1, leading to the activation of transcriptional coactivator YAP1 and WW domain-containing transcription regulator protein 1 (also known as TAZ)⁶¹. In addition, shear stress is sensed by the ion channel complex of PIEZO1 (piezo-type mechanosensitive ion channel component 1) and ORAI1 (calcium release-activated calcium channel protein 1), which activate calcium-sensitive transcriptional regulators, such as Krüppel-like factor 2 (ref. 126).

Intriguingly, stretch-induced activation of integrin β 1 has been found to activate VEGFR3 in a ligand-independent manner¹²⁷. In homeostatic settings, the direct interaction between integrin β 1 and VEGFR3 is blocked by the mechanosensitive scaffold protein ILK¹²⁷. However, with stretching of the surrounding extracellular matrix, such as that induced by myocardial oedema, ILK is displaced intracellularly, enabling integrin β 1 to connect with VEGFR3, leading to receptor activation by autophosphorylation. Consequently, increased interstitial pressure could directly drive lymphatic expansion, even in the absence of external ligands, potentially contributing to the observation that some lymphatics are formed postnatally in the heart in mice lacking VEGFC and VEGFD⁷⁸. The importance of coordination between biochemical (growth factors, hormones and chemokines) and biomechanical (shear stress and stretch-induced) signals in the regulation of cardiac lymphangiogenesis has been demonstrated by the finding that LEC-specific deletion of *Ilk* potentiated lymphatic expansion in the heart in mice after myocardial infarction¹²⁷. Future research should address how changes in cardiac collagen content, and other drivers of left ventricular stiffness, contribute indirectly to cardiac lymphatic (dys) function and remodelling in heart failure.

which lack contractile elements, are probably severely dysfunctional and contribute little to drainage of the scar tissue. This hypothesis is supported by findings of severe oedema and inflammation in infarcted regions in patients and animal models⁴, as seen on T2 magnetic resonance imaging in the clinic¹⁰⁷. Given the emerging non-lymphatic role of VEGFC and VEGFD on immune cells expressing VEGFR2 and/or VEGFR3 (refs. 51,108,109), the effects on myocardial scar maturation observed with sVEGFR3 therapy in mice might reflect a direct effect on cardiac immune cell recruitment after MI. Notably, delivery of *sVegfr3* was associated with reduced CD4⁺ T cell infiltration into the myocardium in a mouse model of cardiac allograft transplantation¹¹⁰ and in a mouse model of MI⁵¹. Moreover, stimulation of lipopolysaccharide-primed macrophages with VEGFC suppressed pro-inflammatory cytokine expression *in vitro*⁷³. These emerging, potentially direct immunomodulatory effects of lymphangiogenic growth factors increase the complexity of the causal link between therapeutic lymphangiogenesis and the resolution of inflammation, highlighting a topic in need of further research.

Viable myocardium

Cardiac lymphangiogenesis also occurs in non-infarcted areas after MI, albeit at a much slower rate and lower amplitude than within the infarct scar, owing to the weaker increase in VEGFC, VEGFA and activated immune cells in non-*ischaemic* regions⁴. Of note, the border zone and remote areas of the left ventricle also display substantial molecular, structural and functional changes in the weeks and months after MI, including low-grade chronic myocardial oedema and inflammation promoting interstitial fibrosis and compensatory cardiomyocyte hypertrophy^{4,51}. Therefore, lymphangiogenesis outside the infarct scar has been proposed to have long-term functional consequences for cardiac remodelling and the development of heart failure. Indeed, therapeutic acceleration of endogenous cardiac lymphangiogenesis in non-infarcted areas (through biopolymeric intramyocardial slow release of VEGFC in rats⁴, or AAV9-mediated *Vegfc* gene therapy in mice⁵¹)

has been shown to reduce myocardial oedema, inflammation, interstitial fibrosis and cardiac dysfunction. Similar findings were reported with the use of daily systemic injections of recombinant human VEGFC in a mouse model of cardiac pressure overload¹¹¹.

Systemic effects

Lymphatics in the periphery participate in the control of blood pressure. A 2009 study in rats and mice demonstrated that salt-induced hypertension led to increased dermal VEGFC production and local expansion of lymphatics⁷¹. Moreover, inhibition of lymphangiogenesis in these animal models aggravated hypertension¹¹². Subsequent studies revealed that lymphangiogenesis also occurs in the kidney in experimental models of hypertension¹¹³. Moreover, selective stimulation of renal lymphangiogenesis, either by nanoparticle-mediated delivery of VEGFC or transgenic overexpression of VEGFD, reduced blood pressure in mouse models of hypertension, including chronic angiotensin II infusion^{114,115}. These findings are exciting for their potential to inform new treatments for antihypertensive drug-resistant hypertension. However, investigating the benefits of therapeutic cardiac lymphangiogenesis in models driven by chronic arterial hypertension has important limitations. Unless the delivery of the therapeutic agent or transgene can be restricted to the heart, it could promote systemic lymphatic expansion, contributing to the reduction in hypertension. Consequently, any observed cardiac benefit of therapeutic lymphangiogenesis in a kidney-dependent model of hypertension is likely to be due to decreased cardiac afterload, angiotensin II activation or both. Indeed, in a study of therapeutic lymphangiogenesis during angiotensin II-induced pathological cardiac hypertrophy in mice, the cardiac benefit of the therapy was associated with reduced chronic arterial hypertension⁵.

Clinical outlook

Although clinical trials of VEGFC therapy in patients with coronary artery disease were performed more than 20 years ago¹ and a clinical

Box 3 | Metabolic regulation of lymphangiogenesis

Stimulation of lymphangiogenesis is characterized by increased β -oxidation of fatty acid substrates in lymphatic endothelial cells (LECs)¹²⁸. In addition to generating ATP needed for cell division, fatty acid metabolism yields increased cellular levels of acetyl-CoA, used by LECs for epigenetic histone acetylation mediated by the transcription factor prospero homeobox protein 1 (PROX1) to drive the expression of lymphangiogenic target genes, including *Vegfr3* (also known as *Flt4*)¹²⁸. PROX1, which is essential for LEC identity, also upregulates the expression of genes encoding fatty acid transporters, such as *Cd36* and *Cpt1a*, ensuring that LECs have an ample mitochondrial supply of fatty acids to produce acetyl-CoA and maintain LEC fate. LECs store excess triglycerides in the form of intracellular lipid droplets, which are released on demand as fatty acids through the process of lipophagy. Indeed, prevention of lipophagy in LECs severely reduced lymphangiogenesis in vitro and in vivo in experimental mouse models¹²⁹. In addition, studies in experimental tumour settings have revealed synergistic stimulation of lymphangiogenesis by vascular endothelial growth factor C and the monounsaturated free fatty acid oleic acid¹³⁰. At present, the only evidence that lipid metabolism regulates cardiac lymphatics in vivo

comes from a study in mice showing that increased circulating levels of ketone bodies (in mice fed a high-fat, low-carbohydrate ketogenic diet) led to improved cardiac lymphangiogenesis after myocardial infarction⁸³. A key role for altered amino acid metabolism during cardiac lymphangiogenesis has been reported in a two-hit mouse model of heart failure with preserved ejection fraction¹³¹. Defective branched-chain amino acid catabolism was observed in LECs, which led to reduced levels of vascular endothelial growth factor receptor 3 and rarefaction and dysfunction of cardiac lymphatics¹³¹. Despite these various lines of evidence coupling cardiac lymphatic remodelling to energy metabolism, whether alterations in myocardial metabolism in cardiovascular disease affects lymphatic function or lymphangiogenesis remains to be determined. In addition, given the role of lymphatics in the drainage of metabolic waste products, including lactate¹³² and, potentially, excess lipids secreted by cardiomyocytes¹³³, in addition to lymphatic-mediated cholesterol efflux from atherosclerotic plaques¹³⁴, the question remains whether insufficient lymphatic drainage in cardiovascular disease contributes to deregulated metabolic states, such as cardiac steatosis.

trial of VEGFD therapy in patients with coronary artery disease and refractory angina is ongoing¹⁰¹, whether these treatments restore cardiac lymphatic function in patients remains unknown. Indeed, one of the many current limitations for rapid clinical translation of this approach is that little is known about cardiac lymphatic function in human health or disease. The invasive nature of lymphangiography and lymphoscintigraphy means that the only available data on lymphatic transport in the heart of large mammals come from experimental studies in rabbits and dogs³⁵ and magnetic resonance imaging-based research in pigs¹¹⁶. However, these techniques for lymphatic imaging are routinely used in the clinic to investigate lymphatics in other organs, for example, to trace lymph node metastasis in patients with cancer and to evaluate

limb lymphatic function in patients with suspected lymphoedema¹¹⁷. Given that pulmonary or peripheral congestion is a key clinical finding in patients with heart failure, the question of whether systemic lymphatic dysfunction contributes to congestive signs and symptoms is intriguing. A first line of evidence in support of this hypothesis comes from an elegant clinical study in patients with heart failure with preserved ejection fraction, in whom dermal lymphangiography revealed reduced lymphatic reserve capacity compared with aged-matched and sex-matched healthy individuals¹¹⁸. This finding, which is concordant with experimental studies demonstrating peripheral lymphatic dysfunction in mouse models of metabolic syndrome⁵³, warrants follow-up studies to investigate the extent of lymphatic dysfunction in patients

Box 4 | Open research questions

Experimental research

- Does improved lymphatic transport of immune cells and cardiac antigens alter adaptive immunity (autoimmune mechanisms in heart failure or prevention of cardiac transplant rejection)?
- What is the role of the lymphatic system in lipoprotein handling in the heart? Does lymphatic-mediated improvement of lipoprotein handling have the potential to limit cardiac steatosis in cardiometabolic syndrome?
- Is there a special immunometabolic niche in perivascular lymphatics that regulates coronary atherosclerosis?
- What are the effects of menopause on the remodelling capacity and functions of cardiac lymphatics?
- What therapies are needed to restore cardiac lymphatic structure and function in cardiovascular diseases?
- Does the combination of therapeutic angiogenesis and lymphangiogenesis lead to a functional synergistic effect in ischaemic heart disease?

Clinical research

- Is cardiac lymphatic drainage altered in patients with cardiovascular diseases? Are lymphatic valves altered in cardiac lymphatics in these patients?
- Do sex-related differences in cardiac lymphatic remodelling and function exist in cardiovascular diseases?
- What current pharmacological treatments for heart failure or metabolic syndrome have the potential to alter peripheral or cardiac lymphatic drainage capacity?
- What clinical (imaging or circulating) biomarkers can be used to specifically assess cardiac lymphatic function or predict poor cardiac lymphangiogenesis in patients?
- Are patients with lymphatic disease at an increased risk of cardiovascular disease onset or progression?

with CVD. An improved understanding of the systemic role of lymphatics in patients with heart failure could lead to the development of new targeted therapies for congestion. Whether patients with congenital variants in lymphatic-related genes that lead to lymphatic dysfunction have an increased risk of CVD remains unknown. Notably, genome-wide association studies have revealed links between single-nucleotide polymorphisms in *VEGFC* and idiopathic dilated cardiomyopathy¹¹⁹ and left ventricular wall thickening¹²⁰.

Conclusions

Our knowledge of the development, organization, molecular fingerprint and diverse functions of the cardiac lymphatic system has expanded considerably over the past 5 years. Multiple experimental studies have highlighted the dynamic effect of immune cell-derived mediators on lymphatic function and remodelling in the heart in various diseases. In addition, evidence is emerging on the molecular mechanisms underlying lymphatic regulation of inflammation, including the adaptive immune response, in CVD. Although the field has clearly advanced, many important challenges and important open research questions remain (Box 4). With the availability of advanced tools for lymphatic research (molecular markers, inducible transgenic mouse models, targeted therapies, functional imaging approaches and in vitro models), together with emerging data on the molecular profiles of cardiac lymphatic cell subpopulations (RNA-seq, single-cell RNA-seq and spatial transcriptomic data from LECs in humans and rodents), the field is poised for further breakthroughs in our understanding of the regulation and role of cardiac lymphatics in health and disease. Ultimately, the aim will be to identify new therapeutic targets to maintain or restore lymphatic drainage in the heart, including the coronary arteries and valves, to limit the development and progression of CVDs.

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