Cardiac lymphatics in health and disease

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2019-01

Brakenhielm , E & Alitalo , K 2019 , ' Cardiac lymphatics in health and disease ' , Nature reviews. Cardiology , vol. 16 , no. 1 , pp. 56-68 . https://doi.org/10.1038/s41569-018-0087-8

http://hdl.handle.net/10138/313218
https://doi.org/10.1038/s41569-018-0087-8

acceptedVersion

19/06/2018
Nat Rev Cardiol.

Cardiac lymphatics in health & disease

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Keywords: Heart failure, edema, inflammation, lymphangiogenesis, myocardial infarction, transplantation, atherosclerosis

Main text words	5121 words + 1507 words in figure legends/boxes
References	167
Figures	4
Boxes	3

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The authors declared that no conflict of interest exists

Abstract

The lymphatic vasculature, which accompanies the blood vasculature in most organs, is indispensable in for maintenance of tissue fluid homeostasis, immune cell trafficking and nutritional lipid uptake and transport, as well as reverse cholesterol transport. In this review, we discuss the physiological role of lymphatics in the heart, in maintenance of cardiac health, and describe alterations of lymphatic structure and function that occur in cardiovascular pathology, including atherosclerosis and myocardial infarction. We further discuss briefly the role that immune cells may play in the regulation of lymphatic growth (lymphangiogenesis) and function. Finally, we provide recent examples of how the cardiac lymphatics may be targeted therapeutically to restore lymphatic drainage in the heart in order to limit myocardial edema and chronic inflammation.

Key points

- Cardiac lymphatics display a dynamic range of fluid uptake and transport, linked to cardiac contractility and heart rate
- Cardiac lymphatics undergo significant remodeling in several cardiovascular diseases, which can alter the lymphatic drainage capacity in the heart
- Insufficient lymphangiogenesis may contribute to the buildup of atherosclerotic lesions in large arteries due to accumulation of both lipids and activated immune cells
- Immune cells contribute to the process of lymphatic remodeling by stimulating or inhibiting lymphangiogenesis
- Therapeutic stimulation of cardiac lymphangiogenesis after myocardial infarction leads to accelerated resolution of myocardial edema and inflammation, promoting cardiac recovery

Introduction

The heart is endowed with an extensive lymphatic network[1,2] that plays an essential role in myocardial fluid and immune cell homeostasis, both of which are crucial for maintenance of cardiac health. For example, an imbalance between myocardial blood microvascular permeability and cardiac lymphatic drainage results rapidly in edema with profound detrimental short and long term effects on cardiac function[3,4]. Research on cardiac lymphatics has received very little attention in comparison to research of lymphatics in other organs[5–7]. Indeed, although it was shown over 40 years ago that cardiac *lymphatic function* may be altered in cardiovascular diseases[8], only very recently were the first studies published on the occurrence and impact of cardiac *lymphatic remodelling* in cardiovascular diseases, including myocardial infarction (MI) and chronic heart failure[9–16]. In this review, we summarize the current understanding of the cardiac impact of insufficient lymphatic function and remodelling (*lymphangiogenesis*) in the heart following cardiac injury, with emphasis on the role of myocardial edema in cardiac dysfunction. Further, we discuss the promise that therapeutic lymphangiogenesis holds for treatment of cardiovascular diseases, notably for post-MI heart failure, but also for atherosclerosis.

Lymphatic vasculature: structure & function & remodeling

Lymphatic vessels are found in almost all vascularized tissues and organs. The lymphatic vasculature is organized into capillaries (also referred to as initial lymphatics), which absorb interstitial fluid and solutes from the extracellular space, followed by precollectors and collecting vessels that transport lymph *via* the lymph nodes towards the thoracic ducts that empty into the superior vena cava at the junctions between the left and right subclavian and internal jugular veins (**Fig. 1**). Collecting lymphatic vessels run next to arteries and veins,

forming a unidirectional transport system indispensable for tissue and body homeostasis. The lymphatic vasculature consists of a monolayer of specialized lymphatic endothelial cells (LECs), derived mostly from blood vascular endothelial cells (BECs) during embryogenesis[17]. Hence, the lymphatic endothelium shares many structural and molecular characteristics of blood vascular endothelium, including zipper-like adherens junctions for endothelial barrier function, presence of mechanoreceptors sensing vascular shear-stress, and expression of many of the growth factor, cytokine, and hormone receptors found in blood vessels.

Lymphatic capillaries, which represent the functional "uptake" unit of the system, are highly branched, blunt, open-ended structures composed of oak leaf-shaped LECs having no or only discontinuous basement membrane but equipped with extracellular-matrix anchoring filaments, which act similarly to the *chordae tendineae* of cardiac valves to prevent lymphatic vessel collapse under settings of increased interstitial pressure. Further, the lymphatic capillaries display specialized intercellular junctions, called button junctions, that function in a flap-like manner to allow free passage of fluid, solutes, macromolecules, and immune cells between juxtaposed LECs. The capillaries coalesce into straighter precollectors and collector vessels, equipped with tight junctions and a solid, continuous basement membrane, as well as an adventitial layer in the larger truncs. Both precollectors and collectors are coated by a muscular layer in the form of specialized, autonomously contracting lymphatic smooth muscle cells (LMCs). Thus, the collecting vessels have a mural organization reminiscent of venules. Further, similar to small veins, precollector and collector vessels are equipped with bicuspid valves that prevent lymph backflow. The functional "transport" unit of lymphatics is called a *lymphangion*, defined as a lymphatic precollector or collector vessel segment situated in between two sequential lymphatic valves (**Fig. 1**). This unit behaves like a small heart (with systolic and diastolic-like contractile cycles) that acts to push the lymph forward, towards the thoracic duct. The contractile function of each lymphangion is ensured essentially by forces *intrinsic* to the vessel wall, generated in a flow-dependent manner by phasic and tonic contractions of the LMCs. The activity of each lymphangion is further modulated by *extrinsic* forces derived from surrounding tissue movements and pressure gradients, including skeletal muscle contractions, and thoracic pressure changes induced by respiratory cycles, bowel movements, heartbeats, and arterial pulsations[18]. The generated intralymphatic pressure spans from negative values (driving fluid absorption) in capillaries to 35-40 mmHg in major lymphatic truncs[18]. Thus, it is only due to the chain-like organization of the sequential lymphangions, which join forces, that lymph can be transported from the legs to the level of the heart, where the thoracic ducts meet central veins via lymphovenous valves in large animals. The function of lymphatic vessels in the meninges surrounding the central nervous system, and pressure relationships in the cephalic parts of the body are poorly known, but under intense investigation[19–21].

The main function of the lymphatic system is to ensure the return of extravasated fluid and solutes from the tissue interstitium to the blood circulation in order to maintain homeostasis of hydrostatic and oncotic interstitial pressures and to replenish the blood plasma volume. Indeed, it has been estimated in humans that every 9 hours, the entire plasma volume leaves the blood circulation by capillary ultrafiltration, and this fluid and associated electrolytes are returned to the blood vascular system essentially by reuptake through the lymphatics[22]. The lymphatic fluid, or lymph, is rich in tissue-derived proteins[23], including enzymes[24] (e.g. lactate dehydrogenase, malate dehydrogenase, and glutamate oxaloacetate transaminase) and metabolites (e.g. lactate), but also lipids (triglycerides, phospholipids and cholesterol)[25]. Whereas lacteal lymphatics ensure the uptake and transport of dietary lipids packed into

chylomicrons from the small intestine, lymphatics in other tissues are essential for HDL-mediated reverse cholesterol transport[26]. Furthermore, as the lymph is propelled from tissues back to the blood circulation *via* lymph nodes, its composition changes dynamically. Notably, a substantial (up to 50%) reuptake of afferent lymph fluid and solutes to the blood circulation may occur already in lymph nodes through their specialized high-endothelial venules[27,28].

Lymphatic vessels further play an important immunomodulatory role and participate in immune surveillance. Indeed, lymphatics actively regulate reuptake of tissue-infiltrating immune cells patrolling the various organs, and both antigen-presenting cells (dendritic cells and macrophages) and lymphocytes selectively home to lymphatics for further transport to draining lymph nodes (dLN) for fine-tuning of immune responses. In addition, lymphatic-mediated drainage of antigens and cytokines produced in tissues during inflammation significantly impact the amplitude and duration of both innate and adaptive immunity[16,29].

Lymphatic tissue drainage may be reduced or interrupted in conditions such as parasitic infection (e.g. filariasis), trauma, surgery, therapeutic radiation, transplantation, medication or venous insufficiency, or due to congenital structural alterations of lymphatics[6]. Insufficient lymphatic drainage results in protein-rich tissue edema referred to as *lymphedema*. Tissue edema occurs when the normal exchange between the blood circulatory system and the lymphatic network is disrupted, either due to increased blood capillary ultrafiltration exceeding lymphatic absorption, or due to inadequate uptake of tissue fluid by lymphatic capillaries and/or poor lymph drainage in precollectors and collecting segments. Increased blood microvascular permeability, induced by inflammation, ischemia, or arterial hypertension, leads often to edema, indicating that in such conditions, lymphatic outflow may be limiting.

Lymphatic vascular remodeling by the process of *lymphangiogenesis* is in many ways similar to the better known process of angiogenesis. The cellular and molecular mechanisms regulating lymphangiogenesis have been recently reviewed in detail elsewhere[17,30,31]. Briefly, the key drivers of LEC migration, proliferation, and differentiation are the vascular endothelial growth factor (VEGF) family members VEGF-C and VEGF-D that bind and active the VEGF receptor (VEGFR)-3, selectively expressed by LECs (**Box 1**). The proteolytically processed forms of these factors can also activate VEGFR2, expressed by BECs, LECs and a few other cell types. Among lymphatic transcriptional regulators, Prox1 has emerged as the master transcription factor responsible for inducing and maintaining LEC identity. Interestingly, Prox1 plays a parallel role in cardiomyocytes, where it regulates cardiac hypertrophic responses[32], as well as the switch between expression of fast-twitch and slow-twitch muscle genes in heart[33] and skeletal muscle[34]. Indeed, cardiomyocyte-specific Prox1-deficiency in mice leads to overexpression of fast-twitch muscle genes and development of early-onset dilated cardiomyopathy (DCM)[33]. In adults, lymphatic vessels are quiescent, similar to blood vessels, except during tissue remodeling, for example in the intestine[35,36]. In contrast, lymphangiogenesis is reactivated in many pathological conditions such as lymphedema, chronic inflammation, transplant rejection, and tumor growth[6]. Therapeutic modulation of lymphangiogenesis in humans is currently pursued in several clinical trials: whereas inhibition of tumor lymphangiogenesis shows promise in the prevention of metastatic disease, stimulation of lymphangiogenesis may reduce edema and limit chronic inflammation[37].

Lymphatic development & organization in the heart

The cardiac lymphatics were initially described by the Swede Olaus Rüdbeck in 1653, and further studied by the French anatomist Constant Sappey[38] in 1874, followed by the works of British globetrotter Dr. Lewis R Shore in the beginning of the past century[1,8]. Studies in monkeys, rodents and birds have since revealed that cardiac lymphatics are established soon after the development of the blood vasculature during embryogenesis[12,39–42]. In comparison, in fish, the cardiac lymphatics develop in the juvenile-adult stages, and sprout towards the ventricle following coronary vessel formation (personal communication *Pr Karina Yaniv*). In mice, the first cardiac lymphatic sprouts appear at the base of the heart just after the appearance of the first coronary vessels on embryonic day 12 (E12), but prior to the onset of coronary blood circulation at E14. Cardiac lymphangiogenesis during development depends critically on the ingrowth of cardinal vein BEC-derived Prox-1$^+$ VEGFR3$^+$ lymphatic precursor cells that first migrate onto the dorsal epicardial surface, progressively extending from the base towards the apex of the heart, along the coronaries, to eventually cover a large part of the surface of the heart by E14.5. In comparison, the mesenteric lymphatic vasculature develops around the same time, just prior to establishment of an active lymphatic drainage in the embryo by E15.5, when the first lymphatic valves appear[43]. These early cardiac Prox1$^+$ LECs further differentiate *in situ*, gain expression of mature lymphatic markers, including LYVE-1 and podoplanin, and organize into a net-like structure covering both the atria and the ventricles of the heart. In addition to these venous-derived lymphatic precursors, the development of cardiac lymphatics seems to involve the process of *lymphvasculogenesis* with recruitment and incorporation of LYVE-1$^+$ (and initially Prox-1$^-$) progenitor cells, or *lymphangioblasts,* whose origin remains to be further determined[12,44]. These potentially non-venous-derived LECs may constitute up to 20% of cardiac lymphatic vessels, indicating significant heterogeneity of the lymphatics of the heart, similar to what has been described for dermal and mesenteric lymphatic beds[45]. The cardiac lymphatic vessel remodeling and maturation processes continue postnatally, and appear to be fully completed by 2-3 weeks after birth in mice[12,41]. The key role of VEGF-C and VEGF-D in guiding cardiac lymphangiogenesis was revealed in a study of mice expressing a soluble VEGFR3 (sVEGFR3) construct that acts as a VEGF-C/VEGF-D trap[46]: these transgenic mice display lymphatic hypoplasia or aplasia in many organs, including the heart. As a consequence, the mice expressing the VEGF-C/VEGF-D trap develop severe pre- and postnatal edema, including pericardial fluid accumulation. However, the lymphatic vasculature eventually forms postnatally in these mice, indicating that other lymphangiogenic growth factors may, at least partially, compensate for the lack of VEGF-C and VEGF-D[17].

The mature cardiac lymphatic network in humans and dogs spans all layers of the heart[1,8,47](**Fig. 2a, b**). It covers both atria and ventricles, and extends at least to the mitral valve in humans[8,48,49]. The cardiac lymphatics of the ventricles are organized into a subendocardial plexus, notably rich in the papillary muscles, coupled to a sparser mid-myocardial lymphatic capillary network that drains centrifugally into straighter and valved lymphatic precollectors present in the subepicardium[11]. These superficial precollectors run next to the anterolateral coronary arteries and the posterior coronary sinus, from the apex towards the base of the heart, where extra-cardiac larger collector vessels empty into cardiac-draining mediastinal lymph nodes located under the aortic arch and around the trachea[1,12,13,39,50]. In other mammals, the subepicardial lymphatic network, composed of both capillaries and precollectors, is also rich on the ventricular and atrial external surfaces and in the subepicardial layers of the interventricular septum, but its intramyocardial ramifications

are more limited, notably in mouse hearts (**Fig. 2c-f**). The evolution of lymphatic penetrance into the deeper myocardial layers may relate to ventricular wall thickness (left ventricular systolic wall thickness: ~1 mm in mice, ~3 mm in rats, and ~1 cm in humans) *versus* the free diffusion of fluid and solutes in the myocardium.

A distinguishing feature of cardiac lymphatics is the almost complete absence of LMCs in the subepicardial valved precollectors, as noted in human, rat and mouse hearts[13,39,49]. Thus, lymph propulsion from the heart is driven essentially by extrinsic factors, *i.e.* cardiac muscle contraction and twist forces. In accordance, the rate and force of cardiac contractions and the duration of diastole have significant impact on cardiac lymph flow: when cardiac contractility decreases or heart rate increases, cardiac lymphatic transport is reduced[51–54]. Importantly, central venous pressure is another major determinant of cardiac lymphatic drainage, as it limits lymph return centrally[8].

Regulation and impact of cardiac lymphatic transport

The study of cardiac lymphatic drainage *in vivo* classically involves terminally-invasive approaches based on direct intramyocardial injection of lymphatic-selective dyes and tracers followed by macroscopic evaluation, as described in many species including in human cadavers[8], anaesthetized pigs, dogs, and rabbits[55]. Alternatively, macroconfocal fluorescence imaging can be used (**Fig. 3a**), as we recently demonstrated in rodents[13]. The ongoing development of new multimodal lymphatic imaging agents holds great promise for clinical translation of cardiac lymphangiography[56–58]. Notably, a minimally-invasive approach to monitor cardiac lymphatic transport has been described in pigs based on dual MRI/NIRF probes[59] (**Fig. 3b**), and by lymphoscintigraphy in dogs[60]. Investigations of lymphatic transport function *ex vivo,* which has been used to assess lymphatic precollector and collector vessels in many different tissues via modified wire- and pressure-myographs[61], has not yet been applied to cardiac lymphatics. However, given the apparent lack of LMC coating in cardiac precollectors, and thus absence of intrinsic lymphatic contractility, such *ex vivo* approaches may not be pertinent for studies of the drainage function in cardiac lymphatics.

Starting in the 1960s, studies of the regulation of lymph drainage in many different organs, including the heart in humans, dogs, pigs, and rabbits[8,50,55,62,63], revealed the essential pathophysiological role of lymphatics in upholding tissue homeostasis. These ground-breaking studies, based on direct cannulation of large extra-cardiac collectors, demonstrated that the cardiac lymphatic system displays a dynamic range of lymph flow in physiology, varying between 1-7 mL per hour in dogs[8,64]. In anaesthetized pigs, the baseline cardiac lymph flow rate was estimated to 1-3 mL per hour[55]. Importantly, cardiac lymph drainage may increase up to 6-fold in healthy hearts during acute, experimentally-induced increases in blood vessel ultrafiltration[65]. For example, in a model of acute myocardial ischemia in dogs that leads to cardiac blood microvascular hyperpermeability, almost a doubling of cardiac lymph flow rate was noted[66]. However, this was not sufficient to prevent build-up of myocardial edema, indicating that lymphatic drainage capacity may be insufficient under pathological conditions[67].

Insufficient cardiac lymphatic drainage may not only lead to myocardial edema, but it may also aggravate inflammation. Indeed, reduced lymphatic clearance of tissue-infiltrating immune cells and cytokines has been shown in experimental studies to increase and prolong the inflammatory reaction in response to acute inflammatory stimuli in organs such as the skin, intestine, and trachea[16,29,68] (**Box 2**). Conversely, other studies have revealed that immune cells contribute to regulation of lymphatic function and remodeling in skin, intestine

and lymph nodes [16,29,69] (**Box 3**). Thus, the functions of the immune system and the lymphatic system appear closely and dynamically linked, and the heart should be no exception. Given the well-recognized importance of cardiac inflammation in cardiovascular diseases, we direct the reader to recent excellent reviews on this topic[70–73]. In the next section, we will instead focus on the cardiac impact of edema, which remains a less appreciated, albeit common complication of cardiovascular diseases.

Functional impact of myocardial edema

As discussed above, cardiac lymphatics play an essential role in counteracting myocardial edema[64]. While the presence of peripheral edema is a well-known clinical telltale sign of heart failure, it has been less commonly recognized that myocardial edema may also occur in these patients. Clinical detection of myocardial edema is based on cardiac magnetic resonance (CMR) imaging using late gadolinium enhancement (LGE) T1 mapping, T2-weighted imaging, or more recently, native T2 mapping[74–76]. Myocardial edema has thus been evidenced in the clinic in many different cardiovascular conditions[64,77], including acute MI, acute decompensated heart failure[78], acute and chronic arterial hypertension[79], non-ischemic dilated cardiomyopathy (DCM)[80], and acute myocarditis[76,81], but also following cardioplegia induced by cardiac surgery. Clinically-detectable myocardial edema, extending beyond the infarct, may persist for 6-12 months post-MI in humans[82,83]. In experimental MI (induced by either permanent coronary ligation or ischemia-reperfusion injury in rats), tissue edema may additionally be directly quantitated using microgravimetry (wet-dry weight ratios). Significant myocardial edema was found to persist in the non-infarcted left ventricular wall and interventricular septum for up to 6 weeks after MI and longer still in the infarct scar (**Fig. 3c**)[13]. In rats, microgravimetric evaluation of cardiac water content is well correlated with MRI native T2 mapping signals, indicating MRI signal specificity for water (**Fig. 3d**)[13].

Importantly, myocardial edema is not only a consequence of cardiac injury, but it also *causes* microvascular and cardiomyocyte dysfunction and damage. Indeed, extensive studies in dogs have revealed that the heart is exquisitely sensitive to variations in interstitial fluid pressure, which directly impacts cardiac compliance[54,84]. Thus, even small increases in cardiac water content reduce cardiac compliance, meaning stiffening of the heart, and may significantly reduce cardiac function. Notably, it has been demonstrated in dogs that an acute increase in cardiac water content, from physiological 75% to pathological 77.6%, may reduce cardiac output by 30-40%[51,85]. Similarly, myocardial edema induced by selective experimental obstruction of cardiac lymphatics rapidly leads to cardiac dysfunction and coronary vasculopathy in dogs[86,87]. Further, *chronic* myocardial edema contributes to reactive interstitial fibrosis[3,9,64,88]. In rabbits, experimental obstruction of cardiac lymphatics stimulates pro-collagen I and III synthesis in the heart, resulting in increased collagen deposition[18]. Interestingly, studies in dogs have suggested that such interstitial fibrosis, accompanied by a switch in the production of collagen isoforms, may represent an initially adaptive mechanism set to limit the impact of interstitial pressure increase on cardiac compliance during prolonged or recurrent myocardial edema[51,84]. It is possible that the cardiac fibrosis and dysfunction observed in many cardiovascular diseases is in part due to myocardial edema that remains unresolved because of insufficient cardiac lymphatic drainage.

As previously mentioned, stimulation of lymphangiogenesis has been proposed as a treatment to resolve peripheral edema of different etiologies, including secondary lymphedema[30]. Although the recent development of molecular markers for lymphatic vessels has fueled investigations into lymphatic anatomy, function, and growth in many organs,

considerably advancing our understanding of this parallel vascular transport system[30], only few studies to date have assessed the impact of endogenous or therapeutically-induced cardiac lymphangiogenesis on the heart.

Cardiac lymphangiogenesis & lymphatic drainage in pathology

Remodeling of cardiac lymphatics has been shown to occur in patients with ischemic heart disease, both in the acute and in the chronic phase, as well as at the terminal stage of chronic heart failure[10,11,54,89]. Further, cardiac lymphangiogenesis has been reported in patients suffering from severe cardiac inflammation, for example in the mitral valve and ventricles of patients with infective endocarditis[11,90], but also in the aortic valve in patients with degenerative calcified stenosis[11,91]. Notably, in humans, only the mitral, but not the aortic valve, is endowed with lymphatics under healthy conditions[8,39]. Finally, lymphatic remodeling has been evidenced in human hearts after cardiac transplantation[92,93]. In all these conditions, the observed alterations include focal lymphatic hyperplasia.

In experimental studies, cardiac lymphangiogenesis has so far been investigated only in rodent models of MI[10,12,13,94–96] or cardiac transplantation[97,98], and in dogs following cardiac lymphatic ligation[48]. Studies of mouse and rat hearts post-MI have demonstrated that cardiac lymphangiogenesis occurs in the infarct zone as well as in the non-infarcted regions of the heart[10,12,13,94–96]. We and others have shown that the endogenous lymphangiogenic response, driven mainly by increased cardiac expression of VEGF-C and VEGF-D during the first months post-MI, is characterized by lymphatic capillary expansion, especially in the infarct scar. However, MI also rapidly leads to cardiac precollector slimming and rarefaction, both potentially linked to acute cardiac inflammation[99]. As a consequence, cardiac lymphatic transport remains severely limited for several months post-MI, as revealed by cardiac lymphangiography in rats[13]. Thus, whereas the *acute* myocardial edema post-MI is largely due to blood microvascular hyperpermeability induced by acute ischemia (which overwhelms adaptive cardiac responses of acutely increased lymphatic drainage, as mentioned above[66]), in the *chronic* phase post-MI, the lymphatic remodeling of precollectors, in both infarcted and non-infarcted areas of the left ventricle, leads to a deterioration of cardiac lymph transport capacity, and hence, the establishment of chronic myocardial edema (**Fig. 3c**).

In the setting of experimental cardiac transplantation, allograft lymphangiogenesis and increased cardiac VEGF-C and VEGFR3 expression have been noted in rats[97] and in patients. Lymphatic activation in the allograft was found to be aggravated by ischemia-reperfusion injury to the graft prior to transplantation[98]. In addition to stimulating lymphangiogenesis, VEGF-C led to increased immune cell homing to the heart, aggravating cardiac inflammation. Conversely, treatment with a sVEGFR3 trap reduced lymphatic CCL21 chemokine expression[97] and limited cardiac inflammation, notably T cell infiltration, in the graft[98]. As a result, treatment with sVEGFR3 reduced transplant arteriosclerosis and increased graft survival. These findings highlight the dynamic role of lymphatics in bridging innate and adaptive immune responses (**Box 3**). However, given the potential aggravating effect of lymphangiogenic inhibition on myocardial edema also in cardiac transplants, it would seem safer to control host-versus graft immune rejection by direct immunomodulatory therapies. For example, targeting regulatory T cell expansion or application of lymphatic-formulated immune suppressants could be envisaged[100].

Finally, in view of the prevalence of cardiac inflammation and myocardial edema in many other cardiovascular diseases, including DCM, chronic hypertensive or diabetic cardiomyopathy, acute Kawasaki or Takotsubo cardiomyopathy, and metabolic syndrome-

induced heart failure with preserved ejection fraction (HFpEF), further investigations are sorely needed to determine the potential impact of cardiac lymphatics in the disease-specific etiology. Interestingly, elevated VEGF-D expression and cardiac lymphatic hyperplasia has been reported in patients with acute Kawasaki disease[101]. Further, given that lymphatics are located close to the conduction system bundles in the heart, it is possible that lymphatic dysfunction, induced by ageing, metabolic syndrome, elevated venous pressure, or surgical intervention around the base of the heart, may contribute to development of atrial fibrillation[102].

Vascular lymphangiogenesis in atherosclerosis

In the heart, lymphatic vessels are also found in the adventitial layer of the coronary arteries at sites where *vasa vasorum* are found[103], and in the adventitia of most major arteries[104,105] (**Fig. 4a, b**). Interestingly, experimental studies have revealed an essential function of lymphatic vessels in mediating *reverse cholesterol transport* (RCT) from tissues, even from the aortic wall in pigs, to the liver[94–96] . This indirectly suggests a role for periarterial lymphatics in the process of atherosclerosis[109] (**Fig. 4c**). Although adventitial lymphatic vessel density was not increased in human atherosclerotic coronary arteries[11,110], in progressive atherosclerotic coronary lesions, lymphatic-like vessels were detected in the medial and intimal layers in the vicinity of calcium deposits[11]. The phenotype of LEC-like cells in such vessels should be further confirmed by use of other LEC markers, such as Prox1. In atherosclerotic lesions, macrophage foam cells have been suggested as a significant source of lymphangiogenic VEGF-C[110]. Similar findings have been reported for other atherosclerotic arteries in humans, including iliac arteries[111,112], and the abdominal aorta, where adventitial lymphatic densities were increased and correlated with intimal thickness, notably in advanced or ruptured atherosclerotic lesions[113], where also VEGF-D expression may be increased[114].

Experimental studies in atherosclerosis mouse models have yielded comparable findings, including increased lymphatic density in carotid atheroma plaques in mice[113]. However, one recent study instead found decreased adventitial lymphatic vessel density in atheromatous plaques in the abdominal aorta of aged ApoE-deficient mice, despite locally elevated VEGF-C expression[115]. This was linked to increased aortic expression of soluble VEGFR2 that acts as a trap for VEGF and processed forms of VEGF-C and VEGF-D, and hence may reduce both angiogenic and lymphangiogenic responses. Interestingly, therapeutic inhibition of plaque lymphangiogenesis, by overexpression of a VEGF-C/VEGF-D trap, led to accelerated atherosclerosis in the descending aorta in both ApoE-deficient and in atherogenic $LDLR^{-/-}/ApoB^{100/100}$ mice[108,116]. Similarly, it was recently reported that surgical dissection of aortic plaque-draining lymph nodes led to aggravated plaque formation in ApoE-deficient mice, including increased intraplaque and adventitial T cell densities[113]. Further, a potential role for additional lymphangiogenic factors in plaque lymphangiogenesis was shown by local silencing of the CXCL12 (SDF-1α) chemokine in the carotid artery of ApoE-deficient mice, which resulted in reduced adventitial lymphangiogenesis and increased T cell density[113]. Taken together, these studies suggest a beneficial role of peri-advential lymphatics in limiting both cholesterol accumulation and chronic plaque inflammation during atherosclerosis. Future studies should reveal whether therapeutic lymphangiogenesis targeting the arterial wall confers protection against atherosclerosis in humans.

Therapeutic stimulation of cardiac lymphangiogenesis

Research over the last 20 years has shown that the success of therapeutic *angiogenic* approaches is largely determined by the mode of growth factor delivery[117]. Indeed, control over growth factor concentrations in tissues, as well as the duration of their expression and distribution are important for safe therapeutic induction of stable and functional blood vessels. Arguably, the same may hold true for therapeutic *lymphangiogenesis*. In order to improve the spatiotemporal control over therapeutic growth factors, a plethora of biopolymeric delivery systems have been developed for localized tissue delivery of recombinant growth factors[118,119]. In parallel, adeno-associated (AAV) viruses have emerged as vectors of choice for durable and organ-targeted gene delivery[120]. Interestingly, co-expression of two other secreted proteins (the extracellular matrix-anchoring protein CCBE1 and the VEGF-C-activating enzyme ADAMTS3)[121] is required for full activation and maturation of VEGF-C. Thus the physiological tissue gradients of these two "activators" that guide functional lymphatic growth may be usurped for therapy when the native full-length and cleavable VEGF-C protein or gene constructs, rather than the preprocessed mature form, are employed for therapy[122,123].

Most experimental studies of therapeutic lymphangiogenesis have so far focused on the delivery of VEGF-C gene or protein, which stimulates both lymphangiogenesis and angiogenesis in its proteolytically-processed mature form[123,124]. Promisingly, VEGF-C gene therapy, delivered by adenoviral or AAV vectors, was shown to reduce edema and inflammation in several different experimental models[37,125]. However, VEGF-C may also increase blood and lymphatic vessel permeability, depending on its proteolytic processing and binding to VEGFR2 or VEGFR3[126,127]. To enable selective stimulation of lymphangiogenesis, without concurrent effects on angiogenesis or on lymphatic vascular permeability, VEGFR3-selective VEGF-C designer mutants have been developed[124,128]. Similarly, viral vectors have been used that produce "pre-activated" recombinant forms of VEGF-C or VEGF-D (VEGF-$C^{\Delta N \Delta C}$ and VEGF-$D^{\Delta N \Delta C}$, respectively), which display improved *in vivo* efficacy[129,130].

The first studies investigating the cardiac effects of therapeutic lymphangiogenesis were performed in experimental MI models in rodents[131]: *Klotz et al* used repeated intraperitoneal injections in mice of naked recombinant human VEGFR3-selective VEGF-C_{C156S} mutant[12], whereas *Henri et al.* used intramyocardial spatiotemporally-controlled biopolymeric delivery in rats of recombinant rat VEGFR3-selective VEGF-C_{C152S} mutant[13]. Modulation of cardiac lymphangiogenesis post-MI by apelin[96] or by cell therapy with bone marrow-derived endothelial progenitor cells (EPC)[94] have also been reported. Promisingly, we recently demonstrated that targeted VEGF-C_{C152S} protein therapy, in an ischemia-reperfusion MI model in rats, led to dose-dependent acceleration of subepicardial lymphatic capillary expansion, and reduced deleterious precollector remodeling in the non-infarcted LV[13]. In contrast, the angiogenic and arteriogenic responses in the heart were unaltered. As a result of selectively-improved cardiac lymphangiogenesis, the resorption of chronic myocardial edema, in both non-infarcted left ventricular free wall and interventricular septum, was accelerated by 3 weeks post-MI, and cardiac macrophage infiltration levels were reduced. Furthermore, both interstitial cardiac fibrosis and cardiomyocyte hypertrophy, which developed by 8 weeks post-MI in control rats, were prevented, leading to an improvement of cardiac function, as evaluated by hemodynamic analyses including of left ventricular end-diastolic pressure, and systolic and diastolic left ventricular pressure-volume relations[13]. Our unpublished data from a mouse MI model, using AAV-9-mediated myocardial VEGF-C gene

delivery, similarly indicate the beneficial cardiac effects of targeted lymphangiogenic therapy. Taken together, these findings suggest that therapeutic stimulation of cardiac lymphangiogenesis with VEGF-C represents a novel approach to prevent deleterious cardiac remodeling post-MI and to limit heart failure development in patients.

Of note, percutaneous intramyocardial plasmid gene therapy with VEGF-C has been investigated in in coronary artery disease (CAD) patients[132]. The rationale at the time was however therapeutic stimulation of cardiac angiogenesis/arteriogenesis with full-length native VEGF-C to reduce angina[133]. Unfortunately, these early trials were halted due to catheter-based issues, and there is no data available on its potential effects on cardiac lymphatics, myocardial edema, or cardiac fibrosis in these patients.

Future perspectives & Conclusion

During the last decade, many growth factors have been found to stimulate lymphangiogenesis, including VEGF-A, Angiopoietins, Platelet Derived Growth Factors (PDGFs), Insulin-like Growth Factors (IGFs), Fibroblast Growth Factor (FGF)-2, and Hepatocyte Growth Factor[17]. Several of these may work via an indirect mechanism, for example by stimulating VEGF-C production, or recruitment of VEGF-C producing leukocytes. It is possible that growth factor *combinations* could be beneficial for the therapeutic creation of functional lymphatics, similarly as in functional blood vessel growth[134,135]. Developmental studies have shown that multiple growth factors are necessary for the differentiation and patterning of LECs into a functional, hierarchical lymphatic system[30]. Interestingly, the combination of VEGF-C with FGF-2 provides additive effects on stimulation of both angiogenesis and lymphangiogenesis[136]. Such dual stimulation of both vascular systems may be more beneficial in cardiovascular diseases characterized by insufficient perfusion, chronic edema and inflammation.

Currently, there is an ongoing phase I/IIa clinical trial (KAT301/NCT01002430) to evaluate dual angiogenic/lymphangiogenic adenoviral gene therapy with a recombinant VEGF-D mutant (VEGF-$D_{\Delta N\Delta C}$) in CAD patients with refractory angina[120,137]. The safety of the NOGA-guided intramyocardial gene delivery has been confirmed, although an increase was noted in anti-adenoviral titers in the treated patients. Promisingly, the myocardial perfusion reserve was significantly improved by the intramyocardial-targeted VEGF-D gene therapy at 3 and 12 months, especially in patients with the highest baseline lipoprotein a plasma levels. Larger, randomized trials are thus warranted to further confirm therapeutic efficacy. It is however, as yet unknown whether the VEGF-D gene therapy influenced cardiac lymphatics, edema, inflammation or fibrosis in these patients.

In conclusion, both clinical and experimental research over the last 20 years has uncovered the fundamental role that lymphatics play in the pathogenesis of many different diseases, revealing the potential that modulation of lymphangiogenesis holds for therapeutic intervention. Indeed, experimental studies have yielded ample proof-of-principle that whereas *inhibition of lymphangiogenesis* limits tumor metastasis, and potentially graft rejection, *stimulation of lymphangiogenesis* accelerates resolution of inflammation and edema. Most current therapeutic studies have been focused on the VEGF-C/VEGF-D/VEGFR3 pathway. However, a deeper understanding of the cellular and molecular pathways involved in disease- and tissue-specific alterations of lymphatics should provide additional therapeutic tools to restore lymphatic health. Such therapeutic targeting of the lymphatic vessels in the heart and its coronary arteries and valves holds great promise to limit the development and progression of cardiovascular diseases.

Acknowledgment

We thank Dr. Anna Ratajska (Warsaw Medical University, Poland) for critical reading of the manuscript, and we acknowledge the work of colleagues and collaborators in providing the research background for this review. Special thanks goes to David Godefroy (Inserm UMR1239-DC2N Laboratory, Rouen, France) and Damien Schapmann (PRIMACEN, Rouen, France) for expert assistance with cardiac light sheet and confocal microscopy, respectively. This work was supported by the Academy of Finland (Centre of Excellence Program 2014–2019 [271845 and 307366]), the European Research Council (ERC) under the European Union's Horizon 2020 Research and Innovation Programme (grant agreement no. 743155), the Novo Nordisk Foundation, the Sigrid Juselius Foundation, the Helsinki Institute for Life Sciences (HiLife) and the Finnish Cancer Society (all to K.A.), the ERA-CVD (LYMIT-DIS project, a transnational R&D programme jointly funded by national funding organisations within the framework of the ERA-NET ERA-CVD, E.B), FHU REMOD-VHF (Inserm U1096 laboratory), and generalized institutional funds (E.B) from French Inserm and the Normandy Region together with the European Union: "*Europe gets involved in Normandie*" with European Regional Development Fund (ERDF): CPER/FEDER 2015 (DO-IT) and CPER/FEDER 2016 (PACT-CBS).

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Figures & Boxes

Fig. 1. Structure of lymphatic vessel drainage pathway. Schematic illustration of the organization of lymphatic capillaries (a) and a collector vessel (b) including outline of a lymphangion and schematic view of its contractile function that drives lymph propagation towards the draining lymph node (dLN), illustration of major cardiac lymphatic trunks and dLNs around the base of the heart (c), and the lymphatic return to the venous system through the thoracic duct/subclavian vein junctions (d).

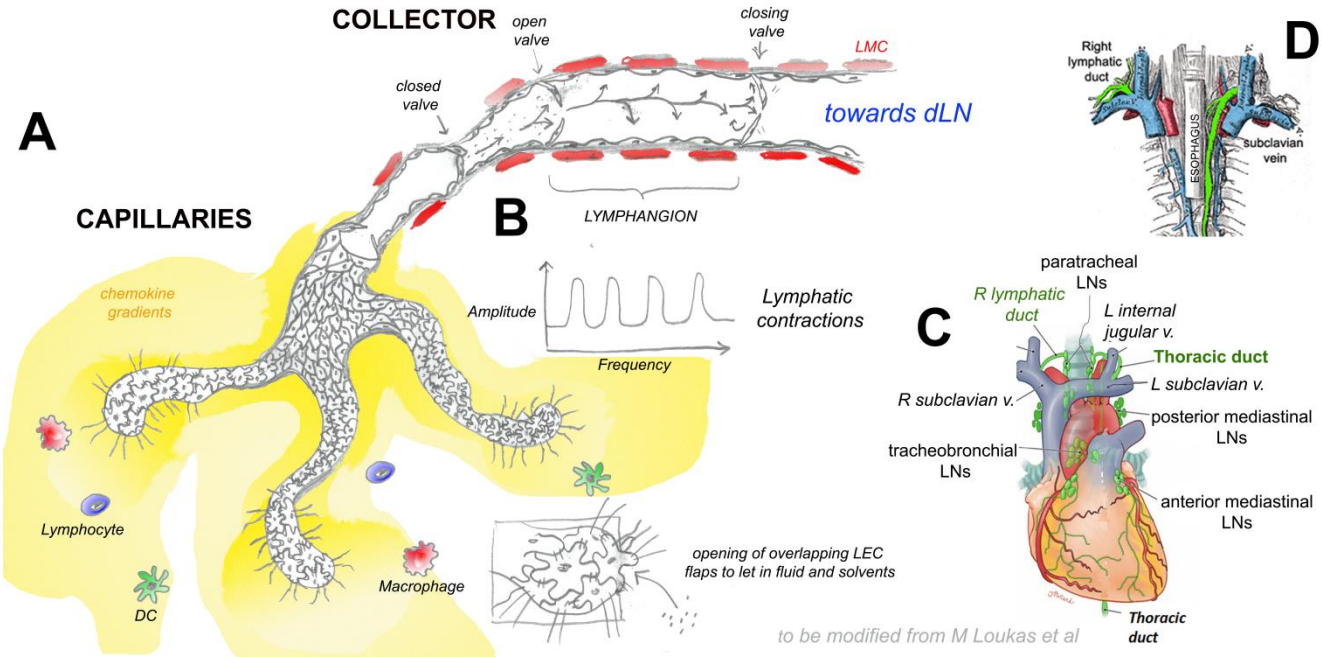


Fig. 2. Lymphatic vasculature in the heart. Illustration of superficial lymphatics (a) in a human heart[38] (lymphatic vessels in *white*), and of the intramyocardial plexus (b) in a dog heart[2] (lymphatic vessels in *green*), and examples of the superficial lymphatic network in healthy rat (c, lymphatic vessels in *brown*), and mouse (d-f, lymphatic vessels in *green*) hearts visualized by immunohistochemical whole-mount staining of the lymphatic marker LYVE1 (c-f) revealed by light transmission microscopy (c), light sheet ultramicroscopy (d, e), and confocal microscopy (f). Lymphatics (LYVE1$^+$) are shown in green and arteries (alpha-smooth muscle actin$^+$) in red (f). Scale bar: 1 mm in *d*; 200 µm in *e*, and 50 µm in *f*.

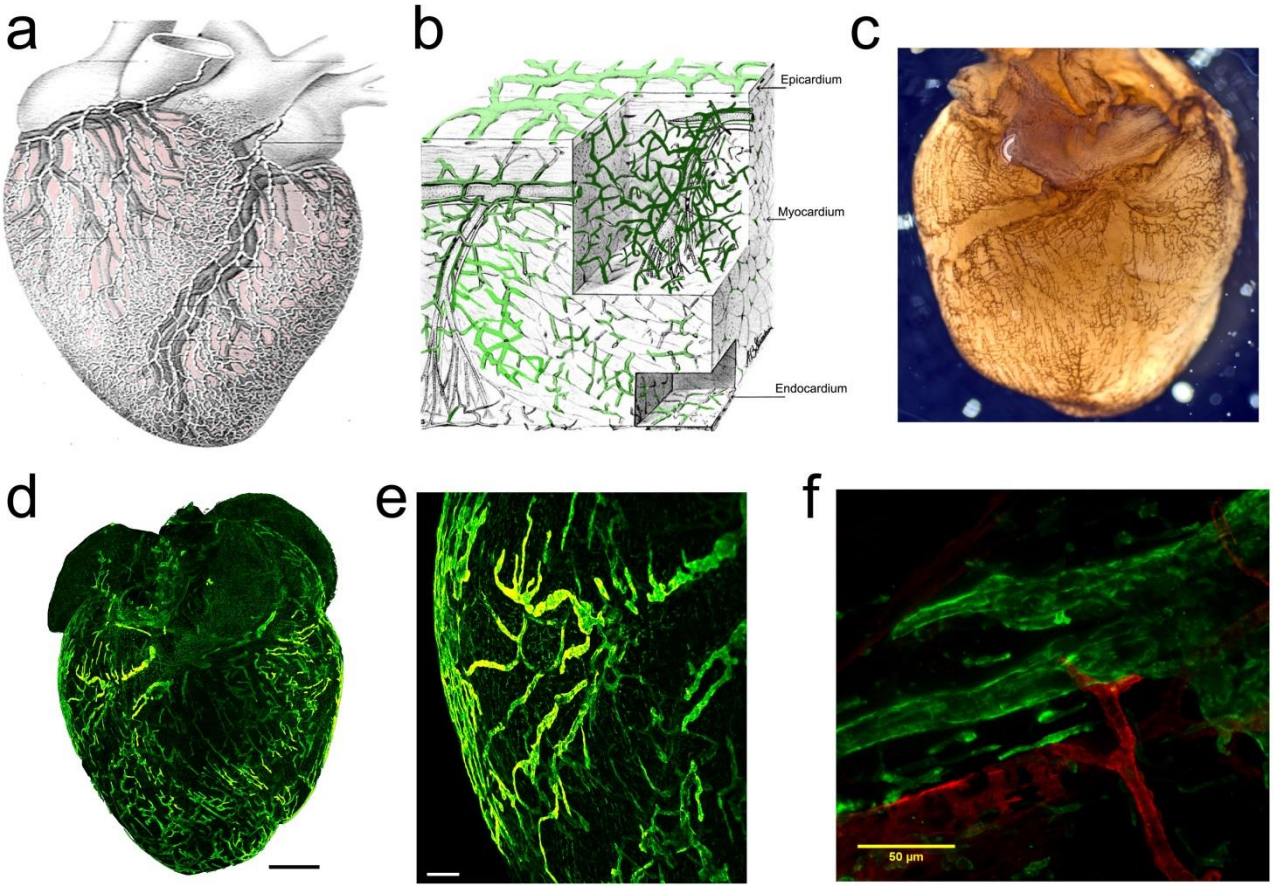


Fig. 3. Lymphatic function vs. edema in the heart. Examples of macroconfocal lymphangiography by intramyocardial injection of quantum dots as a lymphatic tracer[13], and FITC-Dextrane as blood vascular tracer, in a healthy mouse heart (***a***). Scale bar 1 mm. Arrows point to actively draining precollector vessels. Illustration of cardiac lymphatic transport towards the dLNs (*yellow*) in an infarcted (*blue zone*) pig heart (**b**) visualized by MRI following intramyocardial injection of macromolecule-based gadolinium complexes coupled to a near-infrared probe (PG-Gd-NIRF813) contrast agent[59]. Microgravimetric evaluation (wet weight/dry weight ratios) of cardiac edema in the infarct versus in non-infarcted left ventricle in a rat model of ischemia-reperfusion injury (***c***, adapted from[13]). Comparison with healthy sham controls using One-Way ANOVA showed significance at $p<0.01$, **; $p<0.001$ ***. Linear correlation of native MRI T2 mapping signal vs. microgravimetric measurement of cardiac water content in rat hearts at 1 or 16 weeks post-MI (***d***, adapted from[13]).

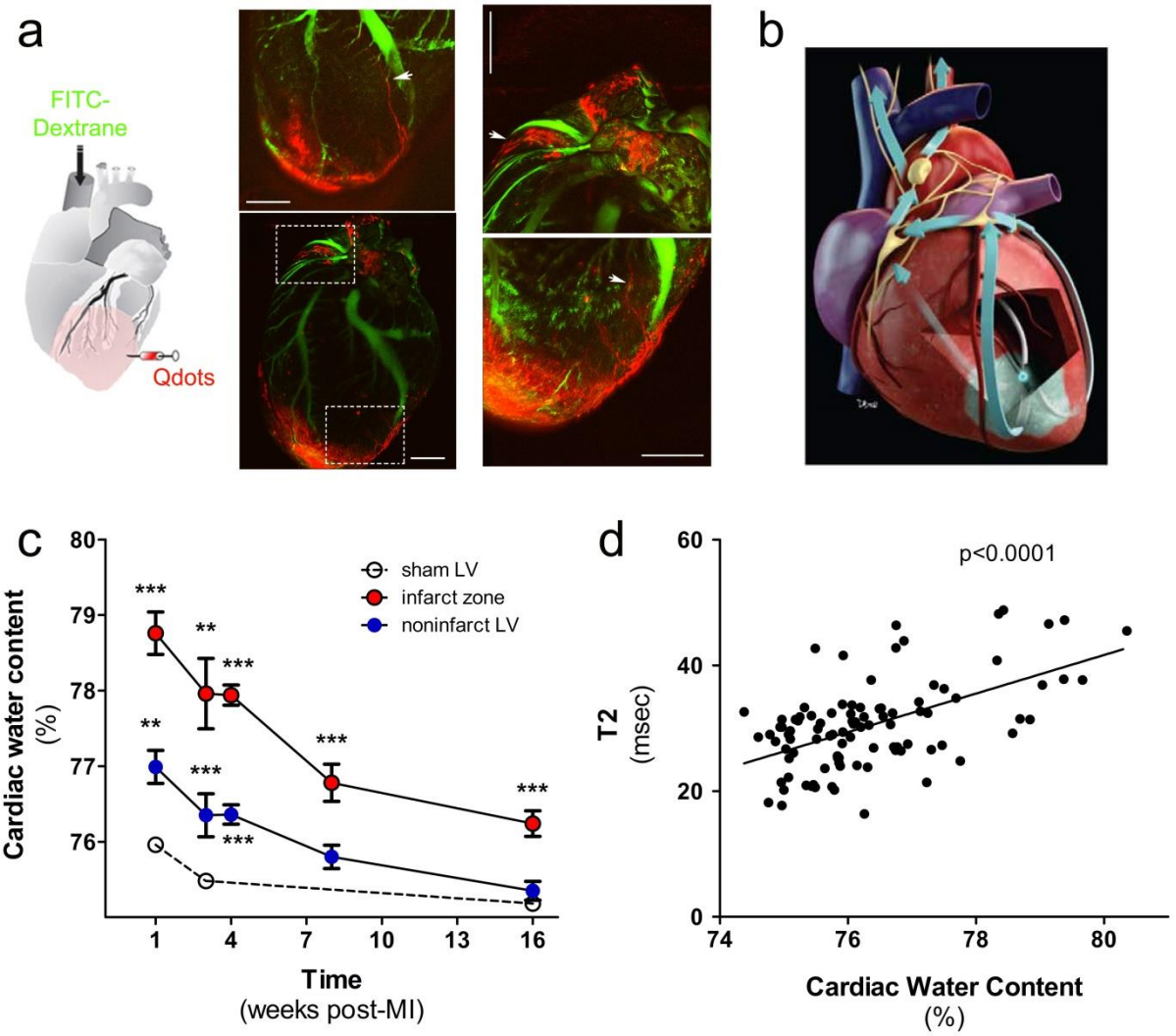
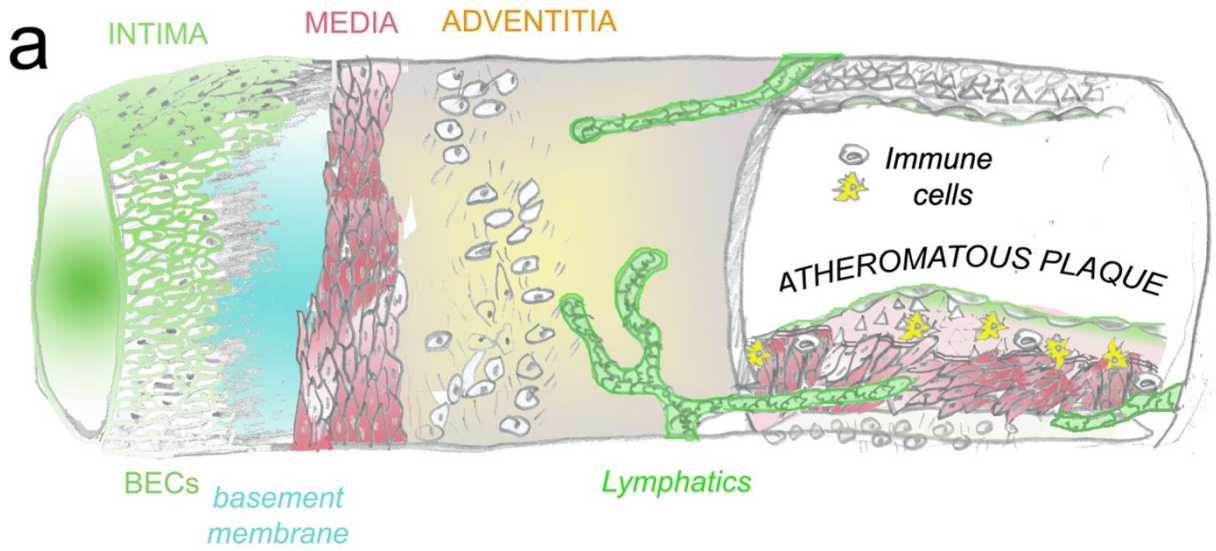
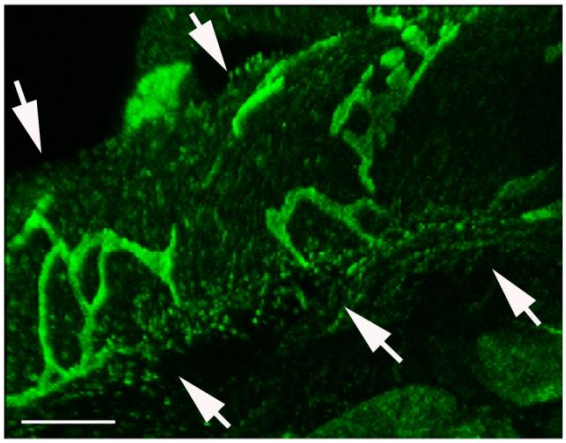
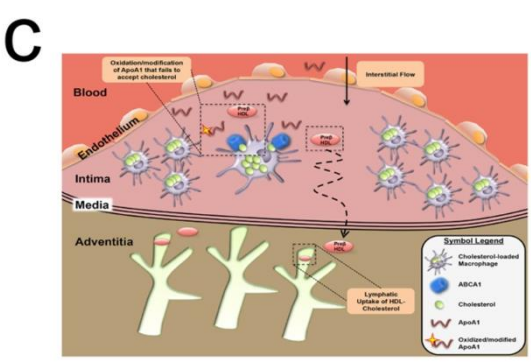


Fig. 4. Perivascular lymphatics in atherosclerosis. Schematic illustration of the organization of the lymphatic vasa vasorum in the adventitial layer of a large artery with extension of lymphatic capillaries towards the expanded inflamed media of an atherosclerotic plaque (**a**), example of peri-aortic lymphatics in the ascending aortic arch in mouse revealed by whole mount staining for the lymphatic marker LYVE1 (**b,** lymphatics in *green*, white arrows indicate aortic wall limits), Scale bar= 500 µm. Adventitial lymphatics participate in reverse cholesterol transport from the artery wall by uptake of HDL particles (**c**, adapted from[26]).







representation of a zoomed view of p**anel a**

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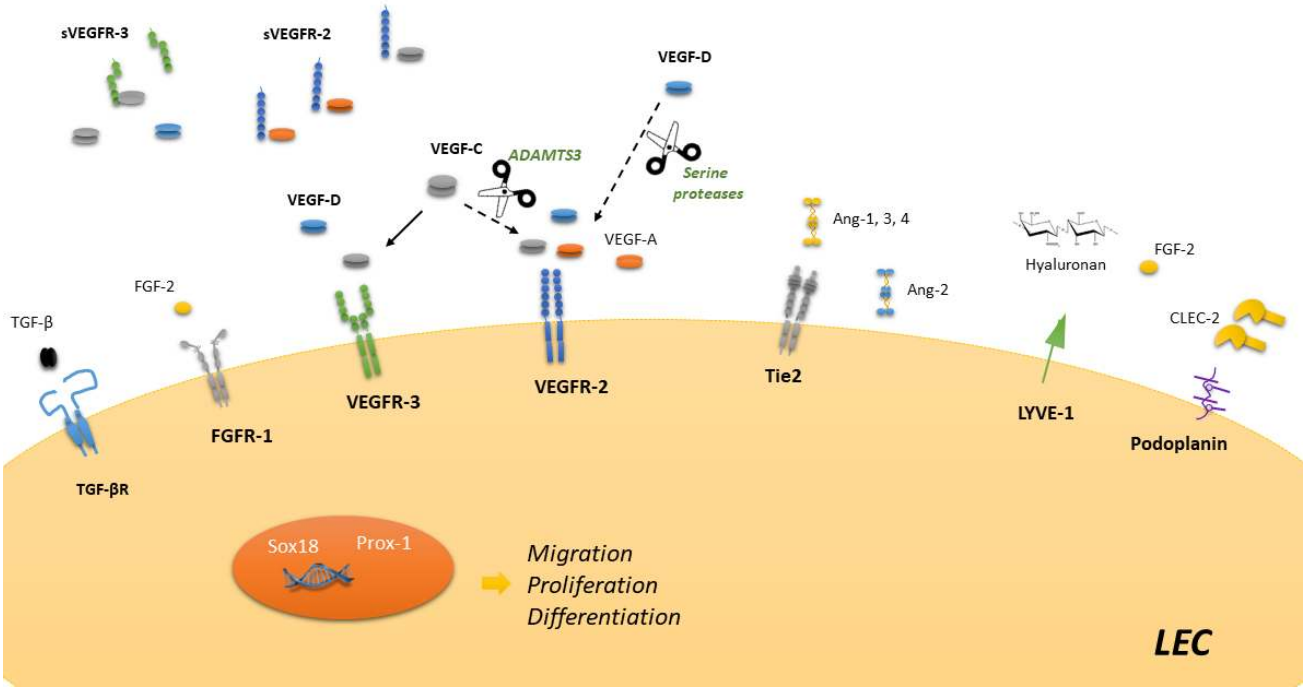
BOX 1. Molecular regulation of lymphangiogenesis.
Lymphatic endothelial cells (LEC) selectively express vascular endothelial growth factor receptor (**VEGFR**)**-3**, which is activated by VEGF-C and VEGF-D, leading to stimulation of cell migration, proliferation and lymphatic development, controlled by the **SOX18** and **Prox1** transcription factors. The receptor-specificity and affinity of VEGF-C and VEGF-D are regulated by extracellular proteases, ADAMTS3 and as of yet partially characterized serine proteases, respectively[121], which cleave the growth factor pro-proteins to generate fully mature forms that also can activate VEGFR2, expressed by both blood vascular endothelial cells (BEC) and LECs. The bioactivity of VEGF-C and VEGF-D is can be inhibited by soluble VEGFR2 (sVEGFR2) and sVEGFR3 that modulate extracellular growth factor gradients. Other LEC markers include **Podoplanin**, which binds platelet-derived CLEC-2[138], and LYmphatic VEssel hyaluronan receptor (**LYVE-1**), which binds hyaluronan. LECs also express many other common transmembrane growth factor receptors including fibroblast growth factor receptor (FGFR1), angiopoietin receptors Tie1/Tie2, and transforming growth factor β receptor (TGFβR).



BOX 2. Lymphatic regulation of immune responses.
The uptake and drainage by lymphatics of immune cells patrolling tissues is controlled by several processes[68]: *1)* Capillary LECs secrete chemotactic molecules that selectively attract different immune cell populations expressing their cognate receptors[139] (e.g. CCL21 and CX3CL1 to attract CCR7- and CX3CR1-expressing cells, notably DCs[140,141] and T cells[69,142]; CXCL12, CCL2, and CXCL10 to attract CXCR4-, CCR2- or CXCR3-expressing myeloid cells[143]; but also lipid mediators such as sphingosine 1-phosphate (S1P) that attracts S1P-receptor-expressing lymphocytes, notably memory T cells[144]); *2)* Capillary LECs express adhesion molecules essential for immune cell crawling during the initial steps of intralymphatic diapedesis[145]; *3)* depending on the structure and function of the lymphatic precollector and collector vessels, intralymphatic pressure gradients are generated to further propel the immune cells towards draining lymph nodes (dLN)[146]. In addition to **lymphatic modulation of immune cell reuptake and transport**, the passive absorption and drainage of tissue-derived antigens and pro-inflammatory cytokines and tissue-infiltrating pathogens in lymph[147], influences the duration of the local inflammatory process as well as the type and

amplitude of the immune response initiated in the dLN[148,149]. Indeed, efficient lymphatic transport of antigen presenting cells, such as DCs, to the dLN, complemented by **direct antigen presentation by LECs** in the lymph node lymphatics[150], is required to generate adaptive immune responses that ensure adequate host defense. Activated LECs may also produce and release anti-inflammatory mediators, including prostacyclins[151], that modulate locally the maturation and activation of infiltrating immune cells including DCs and $CD8^+$ T cells. In strong support for the key roles of lymphatics in immune regulation, a multitude of studies have revealed that inhibition of lymphangiogenesis delays, and stimulation of lymphangiogenesis accelerates, inflammatory resolution[16,29,68,151]. Furthermore, as a consequence of this potent immune-modulatory activity, lymphatic-targeted vaccines have shown great potential for future development[152,153]. Conversely, graft lymphangiogenesis may adversely contribute to transplant rejection[92,97,154]. Interestingly, inefficient immune responses due to downregulation of costimulatory molecules and upregulation of the T-cell inhibitory molecule PD-L1 in LECs in tumor-draining lymphatics may actively contribute to tumor immune evasion[155]. This suggests that host immune responses can be modulated therapeutically without compromising the other homeostatic functions of lymphatics in the graft during organ transplantation.

BOX 3. Immune cell regulation of lymphatic function and remodeling.

Experimental studies have revealed that immune cell-derived pro-inflammatory mediators released during an acute immune reaction, including TNFα, IL1β, and IFNγ but also nitric oxide ($NO^{\cdot}$) produced at high levels by inducible nitric oxidase synthase (iNOS)-expressing cells, leads to **lymphatic transport dysfunction**[156]. Indeed, *in vivo* imaging studies have shown that inflammatory cytokines induce button to zipper transformation of LEC junctions and acute inflammation is often associated with reduced precollector/collector pumping with a reduction of the frequency and/or amplitude in lymphangion contractions. For example, in a mouse model of acute contact dermatitis, skin-infiltrating monocyte-mediated regional overproduction of $NO^{\cdot}$ overwhelms local lymphatic physiological gradients created by LECs. This leads to inhibition of LMC contractility and thus reduced lymphatic drainage[157]. Together with *increased ultrafiltration,* due to blood capillary hyperpermeability accompanying tissue injury, infection or ischemia, the stagnation of tissue fluid due to *inefficient lymphatic drainage* causes the build-up of regional edema, one of the cardinal signs of inflammation. Interestingly, this increase in interstitial fluid and osmotic pressures may act to amplify local immune responses through activation of osmotically-sensitive immune cells[158]. The physiological explanation for this phenomenon of lymphatic transport shutdown during acute inflammation is the sequestration of danger signals (PAMPs and DAMPs) at the site of injury in order to limit systemic disease in the case of infection. Further, this mechanism will also act to limit the spread of auto-antigens following tissue injury, that otherwise could cause a break of self-tolerance with induction of autoimmune responses initiated in dLNs. Interestingly, a determinant role for cardiac DC subsets was recently shown in a mouse MI model in inducing autoreactive T cell expansion in cardiac dLNs[159]. This would suggest that initial reduction of cardiac lymphatic drainage acutely post-MI may be beneficial to limit T cell-mediated targeting of myocardial autoantigens, although it comes at the expense of increasing local inflammation and edema in the heart. Beyond impacting lymphatic function, immune cells also actively participate in **regulation of lymphangiogenesis**: while B cells[160] and myeloid cells[161] may be a rich source of VEGF-C, they also secrete other factors that stimulate lymphangiogenesis. For example, tumor-associated macrophages may produce pro-angiogenic and pro-lymphangiogenic factors, including VEGFs, IGFs and PDGFs, driving both tumor angiogenesis and lymphangiogenesis[162]. On the other hand, T lymphocytes, notably $CD4^+$ helper T cells, suppress lymphangiogenesis in lymph nodes and in other tissues[163,69]. Further, although many pro-inflammatory mediators reduce lymphatic function, they may paradoxically also stimulate lymphangiogenesis indirectly. For example, S1P signaling in non-classical $CD206^+$ tumor-associated macrophages was recently shown to induce IL1β-mediated upregulation of VEGF-C in LECs, leading to autocrine stimulation of

VEGFR3 signaling[164]. Similarly, in a model of airway inflammation in mice, IL1β overexpression potently drives VEGF-C/-D-dependent lymphatic expansion[165]. Another example is TNFα that induces VEGF-C production[166], for example in macrophages, leading to stimulation of lymphangiogenesis[167]. In conclusion, it is now evident that the immune system and the lymphatic network are dynamically linked at multiple levels, with a complex interplay that is highly context dependent in that **inflammation may either drive or suppress lymphatic function and remodeling**, and conversely that **lymphatic drainage and lymphangiogenesis may accelerate inflammatory resolution, but also promote immunity**. It remains to be determined how cardiac lymphatics impact acute or chronic inflammation in the heart, and conversely what the roles are for immune cells and inflammatory mediators in cardiac lymphangiogenesis during development and in cardiovascular diseases.