An overview of lymphatic vessels and their emerging role in cardiovascular disease

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ABSTRACT

Over the past decade, molecular details of lymphatic vessels (lymphatics) have been rapidly acquired due to the identification of lymphatic endothelial-specific markers. Separate from the cardiovascular system, the lymphatic system is also an elaborate network of vessels that are important in normal physiology. Lymphatic vessels have the unique task to regulate fluid homeostasis, assist in immune surveillance, and transport dietary lipids. However, dysfunctional lymphatic vessels can cause pathology, while normal lymphatics can exacerbate pathology. This review summarizes the development and growth of lymphatic vessels in addition to highlighting their critical roles in physiology and pathology. Also, we discuss recent work that suggests a connection between lymphatic dysfunction and cardiovascular disease.

Key words: Cardiovascular disease, inflammation, lymphedema, lymphatic vessels, vegf receptor-3

INTRODUCTION

Lymphatic vessels were described dating back to the 17th century.[1] As the second component of the human vasculature, they are less well characterized relative to blood vessels. At the turn of the 21st century, the identification of lymphatic endothelial markers such as Prox-1,[2] podoplanin,[3] and lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1)[4] has advanced study of lymphatic endothelial cells (LECs) during the past decade. For some time, it has been known that lymphatic vessels complement blood vessels by absorbing fluid, proteins, and cells (collectively known as lymph) from the interstitial space. Therefore, lymphatic vessels are found in most, but not all, vascularized tissues. However, it is appreciated that the lymphatic vasculature serves critical and nonredundant roles apart from the blood vascular system. It assists in immune surveillance, transport of nutrients from the intestine, and regulation of tissue pressure. Furthermore, the blood and lymphatic vasculatures are fundamentally different in their mode of operation. The blood system is a closed circuit system of high pressure that transports its content throughout the body, with the heart providing the force necessary for circulation. On the other hand, the lymphatic system is a unidirectional, low-pressure system that is relatively "passive" in its mode of action. Hence, lymph is propelled forward by respiration, skeletal muscle contraction, and intrinsic contraction of smooth muscle cells that surround the larger collecting lymphatic vessels. Unlike blood capillaries, lymphatic capillaries are more firmly attached to the extracellular matrix by anchoring filaments. Despite their functional and morphological differences, they do have some characteristics in common. Similar to blood vessels, larger lymphatic vessels contain a basement membrane in addition to valves that aid in unidirectional flow. In addition, certain signaling molecules common to both vasculatures are necessary during development and tissue remodeling, as discussed later.

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Lymphatic vessel development

For the most part, the molecular mechanisms that dictate

the development and growth of lymphatic vessels contrast to those of blood vessels. In the early 20th century, two models were put forward regarding how the lymphatic system develops. In 1902, Florence Sabin proposed that the blood vasculature gave rise to the lymphatic vasculature. Through meticulous ink-injection experiments, she concluded that the lymphatics originated in the cardinal vein of fetal pigs, form lymph sacs that migrate toward the periphery, and form lymph vessels that spread throughout the body.[5] A few years later, Huntington and McClure proposed that lymphangioblasts form the original lymph sacs and later establish venous connections.[6] While there is evidence in non-mammalian species that suggest embryonic veins and mesenchymal lymphangioblasts contribute to lymphatic vessels, much molecular support for Sabin's model has been generated thus far, in addition to lineage tracing experiments that further support the existing data that lymphatics are derived from the venous system.[7]

In mouse development, a subset of venous endothelial cells in the anterior cardinal vein express Sox-18, a member of the Sox family of transcription factors, which have been shown to have a pivotal role in cardiovascular and blood vascular development.[8,9] Sox-18 is expressed in the cardinal vein at E9.0 in a subpopulation of endothelial cells. Sox-18 can then bind to the promoter of Prox-1, also a homeobox transcription factor, to initiate the lymphatic specification program [Figure 1a]. In addition, Coup-TFII, an orphan nuclear receptor, assists in turning on and maintaining the expression of Prox-1 [Figure 1a].[10] Prox-1 expression in this subset of cells in the anterior cardinal vein around embryonic (E) 9.75 of mouse development is necessary for these blood endothelial cells to subsequently commit to the LEC lineage.[2] After commitment to the lymphatic cell lineage, vascular endothelial growth factor receptor-3 (VEGFR-3) is critical for sprouting and migration in response to its ligand, vascular endothelial growth factor-C [VEGF-C, Figure 1b].[11] Sprouting is necessary for the formation of the primary lymph sacs [Figure 1c]. Peripheral lymphatic vessels are thought to subsequently form by centrifugal sprouting from the primary lymph sacs, followed by maturation of large collecting lymphatic vessels [Figure 1e].

A cadre of genes have been found to be important for each stage of lymphatic development, which include lymphatic commitment, migration and proliferation, separation, and remodeling/maturation. These molecular mediators are thoroughly summarized elsewhere.[12] Furthermore, recent work has suggested that micro-RNAs are involved in the regulation of lymphatic vascular lineage-specific differentiation from blood ECs *in vitro* and lymphatic vascular development *in vivo*.[13,14] Also, attention has refocused on the role of the hematopoietic system and its role in allowing lymphatic vessels to separate from blood vessels. Mice lacking certain signaling mediators have been shown to develop blood-lymphatic mixing during embryonic development.[15-18] This is the product of misconnections between lymphatics and blood vessels. Podoplanin, a transmembrane glycoprotein and surface marker for lymphatic endothelium, has recently gained interest. Podoplanin allows aggregation of platelets through interaction with the C-type lectin-like receptor 2 (CLEC-2) on platelets.[19,20] Recently, it was found that podoplanin-deficient mice phenocopy the blood-lymphatic mixing found in knockout mice other genes that are critical for separation of lymphatic vessels from blood vessels.[21-23] Therefore, platelets are also important for lymphatic separation from the blood endothelium [Figures 1c and 1d]. This has raised interesting questions concerning the nature of the interactions between platelets and the endothelium during lymphatic separation. Further work is needed to characterize mechanisms of lymphatic development and determine if similar mechanisms are recapitulated in pathological lymphangiogenesis.

THE PHYSIOLOGICAL ROLE OF LYMPHATICS

Lymphatics and fluid/fat uptake

Our cardiovascular system forces blood through the microcirculation. The dynamics of blood pressure and osmotic pressure is responsible for leakage of a relatively small amount of fluid from the blood into the interstitial space. However, collectively, in humans, tissue fluid and lymph make up a volume of approximately 12 liters.[24] In normal physiology, the blind-ended lymphatic vascular system drains this extravasated interstitial fluid from peripheral tissue and returns it to the blood. Through specialized junctions, the lymphatic capillaries are responsible for uptake of this fluid along with immune cells, antigens, lipids, macromolecules, and particulate matter, collectively referred to as lymph,[24] once inside lymphatic vessels. Having little or no basement membrane, lymphatic capillaries are composed of a single layer of thin-walled LECs[25] and are attached to the extracelluar matrix by anchoring filaments.[26,27] From the capillaries, lymph travels toward larger collecting lymphatic vessels, which are significantly different from lymphatic capillaries in that they have a basement membrane, contain intraluminal valves to ensure unidirectional flow, and are surrounded by smooth muscle cells, which serve as an intrinsic pump for lymphatic flow.[28] Indeed, removal of this fluid is critical in order to conserve tissue homeostasis, as will be discussed later.

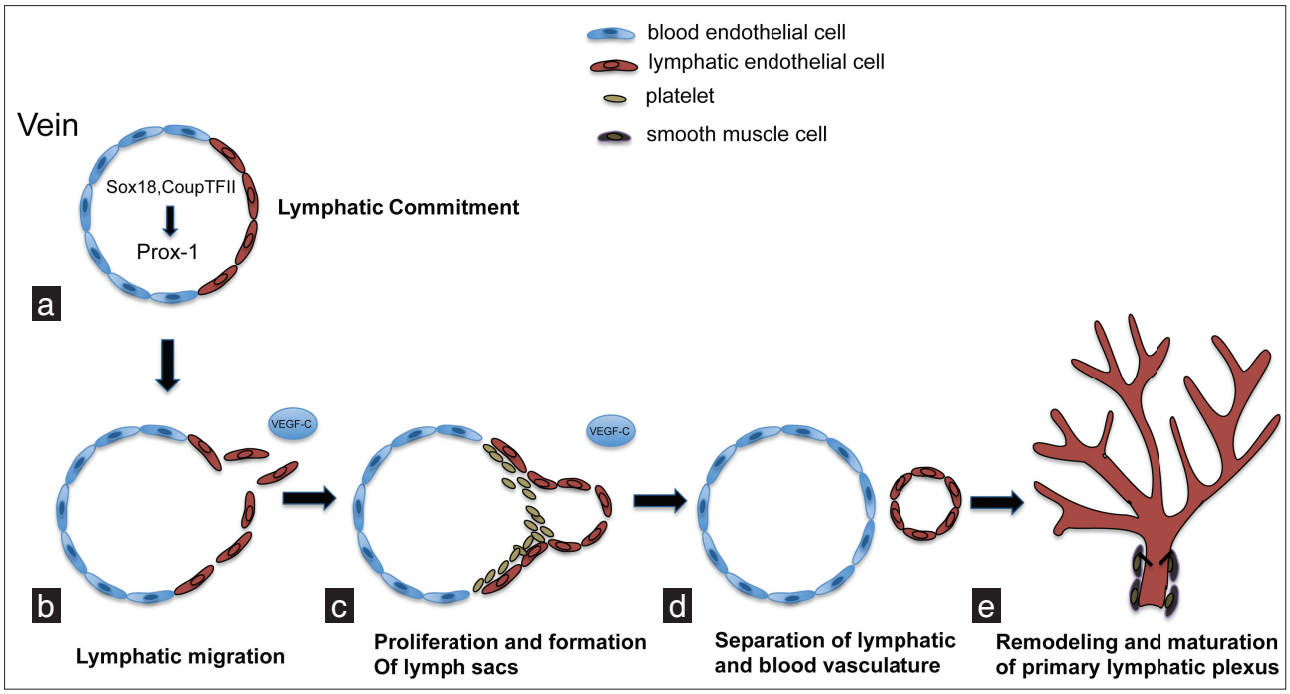

Figure 1: Development of the murine lymphatic vasculature. (a) Lymphatic competent "precursors" begin to express Sox-18 and CoupTFII, which subsequently induce expression of Prox-1, which is responsible for induction of and maintenance of the LEC phenotype. (b) A mesenchymal source of VEGF-C initiates sprouting of LECs and formation of primitive lymph sacs, through VEGFR-3. With the assistance of platelets and a continued source of VEGF-C, lymphatic cells separate from veins and proliferate to form a primitive lymphatic network (c, d), which is later remodeled into the mature lymphatic vascular system that features organization into capillaries and larger collecting vessels (e)

The discovery of lymphatic vessels dates back hundreds of years. They were reported to be found in the mesentery of well fed dogs in 17th century by Aselli.[1] Consumption of foods rich in lipids have been shown to increase lymph flow.[29] This observation underscores the role of the lymphatics in lipid transport. In the Western diet, the majority of dietary lipids are long-chain triglycerides that are digested and absorbed in distinct steps. Luminal hydrolysis in the intestine produces fatty acids and monoglycerides, which form mixed micelles with bile salts and enter mucosal enterocytes, where endoplasmic reticulum enzymes convert them back into triglycerides. Through mostly unknown mechanisms, these dietary lipids, known as chylomicrons, enter the lymphatic system in intestinal villi through lacteals, which are specialized lymphatic vessels. From the lacteals, these large lipoproteins then travel through the submucosal lymphatics and larger mesenteric lymphatics. Next, chylomicrons enter the blood to deliver triglycerides to adipose and muscle before going to the liver to deliver cholesterol. Due to the important role in fat absorption, lymphatic dysfunction often leads to accumulation of fat in mice and humans.

Lymphatics and immunity

Lymphatic vessels are abundant in many organs of the body, such as the skin. Perhaps, this is an evolutionary advantage to protect the host from foreign microbes, as lymphatic vessels are a critical component of the immune response. It is known that dendritic cells (DCs) in peripheral tissues upregulate the chemokine receptor, CCR7, after encountering pathogen-associated molecular patterns (PAMPS). This upregulation increases responsiveness to its ligand chemokine (C-C motif) ligand 21 (CCL21), which is expressed on lymphatic vessels.[30,31] DCs and other antigen-presenting cells (APCs) travel through afferent lymphatic vessels en route to lymph nodes where they present antigen to prime T cells and mount an adaptive immune response. Efferent lymphatic vessels are critical for allowing activated lymphocytes to exit from lymph nodes and return to the blood where they are transported to tissues throughout the body to serve their effector functions. In addition to priming T cells, lymph node-resident and incoming DCs are important for T-cell education and maintenance of peripheral tolerance. After entry into the lymph nodes, DCs via CCR7 migrate to the paracortex region of the lymph node, which produces CCL19 and CCL21.[32] Here, they interact with naive T cells that have also exited peripheral tissue via CCR7.[33] It is appreciated that the continuous sampling of antigen by DCs is necessary for the maintenance of peripheral tolerance.[34] However, DCs and other APCs do not have to gather all peripheral antigens. The flow of lymph, via lymphatic vessels, can assist in peripheral tolerance by bringing soluble antigen

to lymph node resident APCs. Furthermore, this afferent lymphatic flow is critical for maintaining lymph node architecture,[35] an additional positive regulator of peripheral tolerance. Recent work has shown that lymphatic vessels in the lymph node can directly maintain peripheral tolerance through expression of certain peripheral tissue antigens that cause CD8 T-cell deletion upon presentation by MHC class I.[36,37] Furthermore, the relationship between lymphatic vessels and lymphocytes in the lymph node appears to be even more dynamic and complex. T cells, through the production of interferon gamma (IFNγ), inhibit lymph node lymphangiogenesis.[38] On the other hand, B cells can positively regulate lymphangiogenesis,[39] through production of lymphangiogenic factors. These results indicate that the lymphatic vasculature is more than a passive conduit or a waste disposal for but is very active in immunological surveillance and lymph node homeostasis, thereby enhancing immunity.

LYMPHATIC SIGNALING

VEGF family

One of the most potent inducers of angiogenesis, vascular endothelial growth factor (or VEGF-A), was discovered more than two decades ago.[40] Since then, the family has grown and consists of additional members including placenta growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D, and VEGF-E. Collectively, the VEGFs and their receptors are critical regulators of angiogenesis and lymphangiogenesis. Of interest to the lymphatic system, VEGF-A, VEGF-C, and VEGF-D have been shown to stimulate lymphangiogenesis, through binding specific receptors. VEGF-A binds to VEGFR-1/fms-like tyrosine kinase 1 (FLT-1) and VEGFR-2/human kinase insert domain receptor (KDR), while VEGF-C and VEGF-D bind to VEGFR-3/FLT4 and upon proteolytic processing can bind to VEGFR-2.[41] VEGFR-1 and VEGFR-2 are abundantly expressed on blood endothelial cells and function mainly in angiogenesis, while VEGFR-2 and VEGFR-3 are highly expressed in LECs and primarily function in lymphangiogenesis.[42] VEGFRs contain an extracellular domain consisting of seven immunoglobulin (Ig)-like domains, a transmembrane domain, and an intracellular tyrosine kinase domain that undergoes dimerization and autophosphorylation at several tyrosine kinase residues after binding of ligand.[43,44] This recruitment of downstream signaling molecules leads to biological responses such as survival, proliferation, and migration [Figure 2].

VEGFR-3 is critical for lymphangiogenesis. The first ligand identified for VEGFR-3 was VEGF-C.[45] The spatiotemporal expression of VEGF-C with lymphatics suggested a role for VEGF-C in lymphatic development.[46] Indeed, a critical role

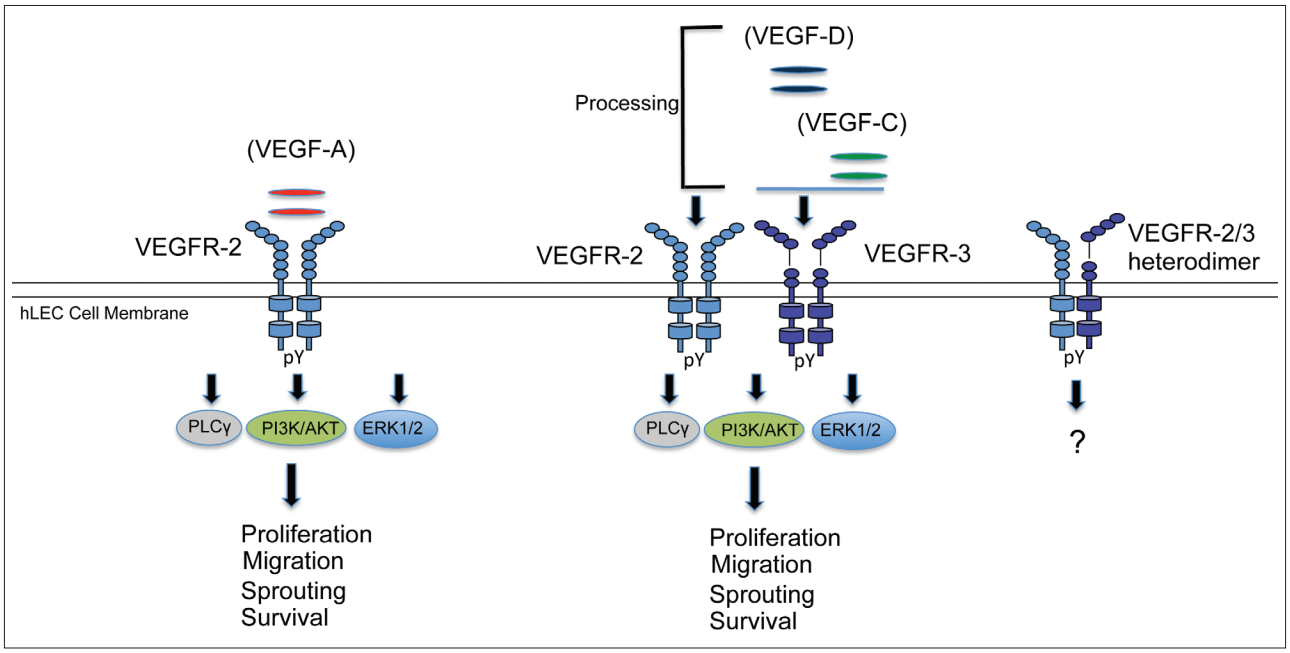

Figure 2: VEGF-VEGFR signaling in lymphangiogenesis. Of the VEGFR family proteins, VEGFR-2 and VEGFR-3 are strongly expressed in hLECs *in vivo* and exist as homodimers and heterodimers. VEGF-A activates VEGFR-2, whereas VEGF-C and VEGF-D activate both VEGFR-2 (after proteolytic cleavage) and VEGFR-3. In human lymphatic EC, VEGF-A preferentially induces phosphorylation of PLC-γ. In contrast, VEGF-C and D preferentially activate the Akt pathway. Both growth factors strongly activate the MAPK pathway. These signaling pathways have been shown to lead to effector functions of LECs, namely migration, sprouting, proliferation, enlargement (growth), survival and tube formation *in vivo* and *in vivo*. pY, tyrosine phosphorylation

for VEGF-C in lymphangiogenesis was seen in VEGF-C null mice, which die before birth due to an inability of committed lymphatic vessels to migrate and proliferate.[11] In addition to VEGF-C, VEGF-D has also been identified as a ligand for VEGFR-3.[47] Additional proof of the ability of VEGF-C and VEGF-D to stimulate lymphatic growth can be seen in individual overexpression of these ligands using transgenic mice. Under the control of a skin-specific promoter, VEGF-C and VEGF-D induce lymphatic hyperplasia.[48,49] Similarly, a mutant form of VEGF-C (VEGF-C156S, which only binds VEGFR-3) was sufficient for lymphatic growth. *In vitro*, VEGF-C/VEGFR-3 signaling is important for proliferation, migration, and survival of LECs through the activation of Akt and MAPK pathways.[50] It is worth noting that VEGFR-3 is expressed on certain tumor-associated blood vessels and blood vessels undergoing active angiogenic sprouting.[51] Furthermore, VEGFR-3-deficient mice die from failure of the primary vascular network to remodel, suggesting an important role for VEGFR-3 in blood vessels.

While VEGF-A is an undisputed inducer of angiogenesis, several lines of recent evidence support its role in lymphangiogenesis. Transgenic overexpression of VEGF-A promoted lymphangiogenesis.[52] Moreover, local injection of VEGF-A adenovirus into mouse ears induced significant lymphatic vessel growth, locally and systemically.[53,54] In addition, chronically inflamed tissue produced VEGF-A, which resulted in lymphangiogenesis.[55] It is unclear whether all these effects are accounted for through the VEGFR-2 receptor, or indirectly through recruitment of another cell type or upregulation of VEGF-C/D. However, it has been documented that systemic blockade of VEGFR-2 or VEGF-A prevented VEGF-A-induced lymphatic vessel formation *in vivo*.[55,56] *In vitro*, LECs signal through VEGFR-2, similarly to blood endothelial cells, but VEGF-C appears to be more potent for tube formation.[54] Our results also show that VEGF-A strongly activates the PLCγ pathway, while VEGF-C strongly activates the Akt pathway. Differential signaling "downstream" of VEGF receptors may lead to differences in functional outcomes. One molecule that we have identified downstream of VEGFR signaling is Bmx, a nonrecptor tyrosine kinase. Upon silencing Bmx, VEGFR -2/3 signaling was partially reduced, suggesting additional mediators of VEGF signaling. However, mechanistically, Bmx can interact with VEGFR-2 and VEGFR-3 to mediate downstream signaling of VEGF-A and VEGF-C.

It is known that VEGFR-2 and VEGFR-3 can form heterodimers, potentially in response to VEGF-A and VEGF-C.[57] It will be worthwhile to further dissect the differential roles of VEGFR-2/3 heterodimers on LECs *in vitro* and *in vivo*, as this interaction has shown to contribute to lymphangiogenic sprouting.[57] In addition, there may be unique signaling pathways activated in response to heterodimer formation, compared with either receptor alone. As mentioned earlier, VEGF/VEGFR-2 signaling can promote lymphangiogenesis in lymph nodes. However, VEGF/VEGFR-2 signals seem to mainly promote lymphatic vessel enlargement but not vessel sprouting in the skin. It will also be interesting to investigate if organ-specific differences account for the contrasting phenotypes. If so, what is the difference in the signaling repertoire between skin and lymph node lymphatics? These answers may be relevant for therapeutic organ-specific targeting of lymphatic vessels.

OTHER REGULATORS OF LYMPHANGIOGENESIS

While the VEGF family of receptors is well characterized, a plethora of other molecules are known to stimulate lymphangiogenesis. Other growth factors such as insulin-like growth factor (IGF) 1 and 2, fibroblast growth factor (FGF)-2, platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), and angiopoietin-1 have all been shown to be lymphangiogenic.[58-63] In addition, cytokines such as tumor necrosis factor (TNF-α) and lymphotoxin-α have been shown to drive lymphangiogenesis.[64,65] Unexpected inducers of lymphangiogenesis have been found such as Netrin-4, a secreted protein involved in axon guidance.[66] Adrenomedullin and endothelin-1, peptide vasodilator and vasoconstrictor, respectively, contribute to lymphangiogenesis.[67,68] Hormones such as luteinizing hormone, follicle-stimulating hormone, and growth hormone can also stimulate lymphangiogenesis.[69] Some aforementioned effects are direct, while others have been indirect, through upregulation of VEGF-C, for example. Conversely, transforming growth factor -beta (TGF-β), IFN γ, and thrombospondin-1 have been shown to inhibit lymphangiogenesis.[38,70-73] While the molecular mechanism of TGF-β-mediated inhibition is unknown, IFNγ downregulates Prox-1 and expression of LEC-specific genes, thus leading to decreased lymphangiogenesis *in vitro*. Thrombospondin-1 inhibits expression of VEGF-C/D via a CD36-dependent mechanism in monocytes. With knowledge of many ligands involved in lymphangiogenesis, further insight will be gained by exploring intracellular signaling pathways in response to these molecules.

LYMPHATICS IN PATHOLOGY

Lymphedema

When lymphatic vessels are dysfunctional, the importance

of the lymphatics in tissue drainage is manifested by lymphedema, in which the main symptom is persistent (chronic) swelling, usually of extremities. Lymphedema is classified as two forms: primary and secondary lymphedema. Primary lymphedema has a genetic etiology that leads to inadequate functioning of the lymphatic vasculature. It can present at birth or arise later in life. The oldest report of congenital lymphedema is known as Milroy's disease. Multiple reports have linked Milroy disease with a mutation in the tyrosine kinase domain of the VEGFR-3 gene.[74-76] Interestingly, the *Chy* mouse mutant, a model for congenital lymphedema that contains a heterozygous mutation to deactivate VEGFR-3, has abnormal cutaneous lymphatic vessels and symptoms of lymphedema.[77] A list of genes related to primary lymphedema is summarized elsewhere.[12]

Secondary lymphedema is the most common cause of edema. It is caused by obstruction or damage to normal lymphatic vessels. In industrialized countries, a common cause of edema is from surgery.[78] In tropical and subtropical countries, pathogenic filarial parasites are the major cause of lymphedema.[79] These mosquito-borne parasites reside in and cause damage to the lymphatic vessels, leading to an inhibition of lymphatic function.[79]

Currently, there is no cure for lymphedema. Therapeutic approaches include massage therapy, exercise, dietary restrictions, compression garments, skin care, manual drainage, and liposuction. VEGF-C/D-based therapeutics appear to be a promising alternative, as VEGF-C/D regenerated collecting lymphatic vessels improved the outcome of lymph node transplantation.[80]

Lymphatic vessels in chronic inflammation and cancer

In adulthood, lymphangiogenesis occurs primarily during tissue regeneration, tumor growth, and acute and chronic inflammation. Much attention has been focused on lymphangiogenesis in the context of inflammation. Inflammation is a well-known phenomenon that occurs in response to infection or injury. During inflammation, a variety of cell types are recruited to the inflamed sites. Among these, macrophages have been extensively implicated in the production of VEGF-C and VEGF-D, leading to lymphangiogenesis.[81] In addition to secretion of growth factors, macrophages have been reported to transdifferentiate and incorporate into lymphatic vessels in the context of inflammation in the cornea.[84] Although lymphangiogenesis is well documented during inflammation, the biological role of lymphangiogenesis during inflammation is not well understood. Presumably, one would expect lymphangiogenesis to be beneficial by allowing immune trafficking and clearance of pathogen infection and inflammation. This would resolve inflammation and enhance antigen presentation. Indeed, activation of lymphatic vessels by overexpression of VEGFR-3-specific ligands inhibits acute and chronic inflammation.[82,83] However, several reports suggest that lymphatic vessels generated during inflammation are not beneficial to the host. For example, a murine ovarian cancer model resulted in significant lymphangiogenesis. However, lymphatic vessels in this case were nonfunctional, as determined by functional assays.[85] Crohn's disease, an autoimmune inflammatory bowel disease, is often associated with lymphatic vessel dysfunction. Similarly, lymphatic contractile activity was compromised in a model of intestinal inflammation.[86] In addition, results suggest that even in acute inflammation, the function of the endothelial barriers in the initial lymphatics may be compromised.[87] Interestingly, during inflammation, cytokines are produced which have been shown to have negative effects on lymphatic vessels directly.[88] The presence of these cytokines may have a detrimental effect on lymphatic vessels during inflammation. Further work is needed to determine the temporal and spatial function of these cytokines in specific pathologies.

In addition to angiogenesis, tumors stimulate lymphangiogenesis in experimental murine models and in human cancers.[89] Lymphatic vessels have also been found to contribute to the metastasis of primary tumor cells to draining lymph nodes and distant organs. Tumor cells can disseminate through preexisting lymphatic vessels. In addition, it has been found that tumors can secrete prolymphangiogenic growth factors such as VEGF-A, VEGF-C, and VEGF-D which can directly induce lymphangiogenesis in order to advance their spread to the draining lymph nodes and distant organs.[90] In particular, VEGF-C has been found to upregulate CCL21 production in lymphatic endothelium, which in turn can promote lymphatic entry, by CCR7 -expressing tumor cells.[91] VEGF-C is also a chemoattractant for macrophages via VEGFR-3, which is expressed by a population of peripheral blood monocytes and activated tissue macrophages.[92] This recruitment may lead to further lymphangiogenesis. Therefore, the presence of VEGF-C (and VEGF-D) is associated with increased metastasis and poor prognosis in human patients.[89]

Lymphatic vessels and cardiovascular disease

The metabolic syndrome (MetS) is characterized by a cluster of metabolic risk factors and includes abdominal obesity, dyslipidemia, hypertension, insulin resistance, and

proinflammation and prothrombiotic conditions. Those with MetS are at increased risk for cardiovascular disease.[93] Obesity is considered a key contributing factor leading to the increased prevalence of the metabolic syndrome. Interestingly, several mouse models and cases of human pathology show the association between dysfunctional lymphatic vessels and obesity [Figure 3].

The 17th century observation of "milky veins" in fed dogs is now known to be chyle, a milky fluid rich in chylomicrons that consists of cholesterol, phospholipids, triglycerides and apolipoproteins. Chyle is transported by lacteals, which are specialized lymphatic capillaries that absorb dietary fats in the small intestinal villi. One can envision that a failure of lymphatics to absorb or transport lipids can start a cascade of metabolic disorders. Individuals that have genetic mutations associated with genes critical for lymphatic signaling often present with subcutaneous edema and chylothorax as summarized by Schulte-Merker *et al*.[12] Moreover, several mutant mice created to study lymphatic vessels have abnormal fat accumulation. The *Chy* mouse, mentioned earlier, is characterized by the accumulation of chylous ascites in the abdomen.[77] In addition, the K14-VEGFR-3-Ig transgenic mouse, which blocks VEGF-C-mediated signaling, develop a lymphedematous-like phenotype that includes increased deposition of subcutaneous fat.[94] An extensive list of murine genes that lead to lymphatic-associated fat accumulation is reviewed in Cueni *et al*.[95] Perhaps the most striking example is seen with the loss of one allele of Prox-1. Prox-1 heterozygosity resulted in defects of the lymphatic vasculature that lead to chylous ascites and adult onset obesity.[96] Interestingly, the integrity of the lymphatic vasculature was compromised in these mice, notably in the mesenteric lymphatic vessels, suggesting obesity may be a consequence of malfunctioning lymphatic vessels. It is also tempting to speculate that these mice may also have increased inflammation, leading to altered integrity of lymphatic vessels. Nonetheless, lymph was shown to promote differentiation of 3T3-L1 preadipocytes into adipocytes by unknown factors, indicating extravasated lymph, due to compromised lymphatic vessels, may directly influence

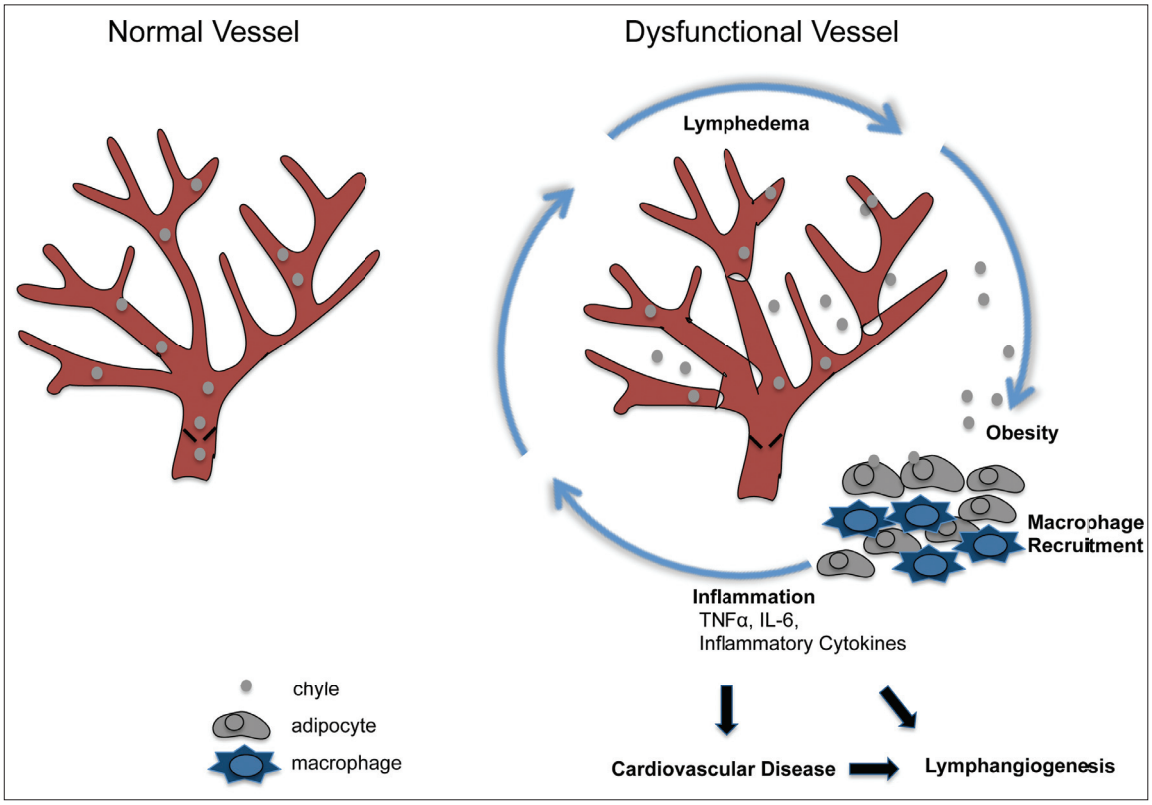

Figure 3: Proposed working model for dysfunctional lymphatic vessel contribution to cardiovascular disease (evidence from literature in text). Persons diagnosed with metabolic syndrome are at greater risk to develop cardiovascular disease. The metabolic syndrome is a condition diagnosed with the appearance of several risk factors in an individual, and obesity is considered a major risk factor. Dysfunctional lymphatic vessels are unable to properly drain lymph and chyle (shown here). This can cause edema and increased fluid volume leading to chylous ascites and chylothorax. Furthermore, chyle (including chylomicrons) can leak from compromised lymphatics and influence surrounding reservoirs of fat. Lymph can cause maturation of preadipocytes and growth of adipose tissue. Secretion of inflammatory cytokines from adipose tissue and macrophages is exacerbated in obese individuals. Recruitment of macrophages to adipose tissue is increased and excess inflammation can increase lymphangiogenesis and add further detriment to lymphatic vessels, amplifying the cycle, and may eventually contribute to cardiovascular disease

fat deposition. Indeed, these mice have subcutaneous and intra-abdominal fat accumulation, which has been implicated in metabolic disorders to a greater extent than total body fat.[97] It is known that adipose tissue can function as an endocrine organ, not to mention its function as the major storage site for triglycerides.[98] Adipokines, which are adipocyte-derived factors such as adiponectin and leptin, affect body energy homeostasis through autocrine and endocrine functions. Interestingly, Prox-1 heterozygous mice have increased leptin, an adipose-derived satiety hormone that acts on the hypothalamus to inhibit feeding. Moreover, visceral fat adipokine secretion is associated with systemic inflammation, insulin resistance, and diabetes in obese humans.[99-101] However, it is unclear if inflammation is a cause or consequence of obesity. Data have suggested that an increase in adiposity is accompanied by an increase in the inflammatory response.[99] Interestingly, there was an increased accumulation of macrophages in adipose tissue in the mesentery of the Prox-1 heterozygous mice. In addition to inflammatory cytokines such as TNFα and IL-6 secreted by adipose cells, macrophages have also been shown to be a source of proinflammatory factors in adipose tissue.[102] Collectively, these adipokines are able to recruit and activate more macrophages, which have been associated with insulin resistance.[103] This cyclical dynamic may be in part responsible for the low-grade inflammation linked to MetS.

In addition to inflammatory cytokines, adipose- associated macrophages can secrete angiogenic factors such as VEGF.[104] It is also possible that these adipose- macrophages can stimulate lymphangiogenesis, as macrophages also release growth factors including VEGF-C and VEGF-D, which elicit the formation of lymphatic vessels. Stimulation of lymphangiogenesis would likely lead to enhanced transport of lipids. However, several lines of evidence suggest the contrary. As previously mentioned, lymphatic function was shown to be impaired in several models of inflammation, suggesting that lymphatic function might be compromised in some inflammatory diseases, leading to further exacerbation of edema, inflammation, and obesity.[85,86]

Environmental factors including diet are thought to represent the etiology of the MetS.[105] Diets rich in long-chain triglycerides depend on the lymphatics for absorption after being packaged into chylomicrons. It is plausible that individuals with lymphatic abnormalities are more prone to developing symptoms of the MetS. Evidence has shown that circulating chylomicrons trigger an inflammatory response with the recruitment of neutrophils and activation of monocytes.[106] Furthermore, postprandial lipoproteins can upregulate expression of leukocyte adhesion molecules on the blood endothelium, thereby orchestrating adhesion and migration of inflammatory cells into various tissues.[107] It is possible that a higher level of circulating chylomicrons, due to impaired lymphatics, may exacerbate an otherwise normal physiological response leading to chronic inflammation that negatively affects lymphatic vessels, causing increased deposition of fat. Circulating lipids can then accumulate in both adipocytes and macrophages (foam cells), as found in atherosclerosis. Lipid storage in macrophages is an important step in the development of atherosclerosis, where plaque lesion progression is correlated with accumulation of foam cells.[108] Apolipoprotein E null mice are commonly used to investigate the biological mechanisms of plaque development in arteries. Interestingly, lymphatic vessel functions are also compromised as dyslipidemia advances in apolipoprotein null mice.[109] Elevated levels of chylomicrons may be a significant and independent risk factor for the development of cardiovascular disease by enhancing inflammation, leading to dysfunctional lymphatic vessels, which in turn exacerbates inflammation-associated pathology [Figure 3].

Hypertension is another major risk factor for cardiovascular disease. An excess of dietary salt has been commonly linked to hypertension. Recent work in rats on a high salt diet has shown that excess sodium accumulates in the interstitium of the skin, leading to a hypertonic state.[110] Mononuclear phagocyte system cells, importantly macrophages, sense hypertonicity and produce VEGF-C, which promotes lymphatic vessel growth, providing an additional buffer in response to high salt intake. In cultured macrophages, VEGF-C was regulated through tonicity enhanced binding protein (TonEBP), an osmotic stress responsive transcription factor. This phenomenon was due to VEGFR-3, as blocking VEGFR-3 resulted in increased blood pressure in mice on a high salt diet. In agreement, human subjects with refractory hypertension had higher concentrations of plasma VEGF-C compared with normotensive control subjects, providing further evidence of lymphatic vessels and macrophage contribution to interstitial fluid and blood pressure homeostasis.

In addition to indirect damage to the heart from resulting pathological sequelae, the lymphatic vessels of the heart proper have received little attention, although their existence has been known for some time.[111] The lymphatic system is also involved in fluid homeostasis of the cardiac interstitium, thereby preventing myocardial edema,[112] which can result in cardiac dysfunction. As heart function is significantly compromised with only a small increase in the interstitial fluid volume, it has been proposed that

impaired lymph drainage may lead to a variety of human myocardial diseases.[113] Large animal models, such as canines, are commonly used to investigate the outcome of impaired cardiac lymph flow on myocardial function. In an acute myocardial lymph flow impairment model, these animals developed cardiac edema and hemorrhage.[114] In a chronic cardiac lymph flow impairment model, these animals also developed edema, hemorrhages, deposition of fibrous and elastic tissue in addition to reduced cardiac function.[113] While the clinical data to link congenital lymphedema and cardiac dysfunction are sparse, cardiac transplantation serves as a high risk factor for damage of cardiac lymphatic vessels. It has been hypothesized that lymphatic disruption after cardiac transplantation may be a major cause for allograft failure and postoperative mortality.[115] Patients with at least one transplant rejection had a significantly lower density of VEGFR-3-positive vessels after transplantation.[116] Conversely, an investigation by Dashkevich *et al.* found a significantly higher density of Prox-1-positive lymphatic vessels in rejection grade A1 or A2 biopsy lung transplant recipients.[117] Similar findings were seen in kidney and cornea transplant recipients.[118,119] While increased lymphatic vessels can enhance antigen presentation and the subsequent adaptive immune response that may lead to organ rejection, decreased lymphatic vessels can lead to edema, which accompanies acute organ rejection in many cases. Further studies are warranted to prove the exact cause of organ rejection in specific cases. Such evidence will reveal if it is beneficial to stimulate lymphangiogenesis after cardiac transplantation. Also, to what extent is there a balance between too many or too few lymphatic vessels? Interestingly, several lines of evidence show that lymphatic vessels can grow or remodel in response to pathological changes of the heart. Infective endocarditis was shown to increase the number of lymphatic vessels,[120] but not blood vessels. In addition, lymphangiogenesis accompanies other major cardiac pathological changes, such as acute and chronic ischemia, progressive atherosclerosis, myocarditis, and hypertrophy.[120] Kawasaki disease (KD), characterized by systemic vasculitis, especially of the coronary arteries, results in tissue edema. Increased production of VEGF-D was associated with lymphangiogenesis in patients with acute KD. Consistent with findings in other tissues, coronary/cardiac inflammatory cell infiltration was accompanied by lymphangiogenesis.[121] It is possible that the lymphangiogenesis in certain pathologies may function as a compensatory mechanism to maintain physiologic conditions and reduce tissue edema during resolution of a particular insult, but lymphatic vessels may become compromised due to other inflammatory cytokines present.

CONCLUDING REMARKS

With the emergence of lymphatic-specific markers, further characterization of the underlying molecular mechanisms for lymphangiogenesis may provide a therapeutic avenue for selective inhibition of lymphatic vessels in diseases such as cancer. On the other hand, stimulation of lymphangiogenesis may be beneficial in diseases of lymphatic insufficiency. Additional study of lymphatic vessel regulation will yield further insight into recent implications of their contribution to transplant rejection, obesity, hypertension, and other metabolic and inflammatory disorders.

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