Lymphatic System in Cardiovascular Medicine

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Abstract: The mammalian circulatory system comprises both the cardiovascular system and the lymphatic system. In contrast to the blood vascular circulation, the lymphatic system forms a unidirectional transit pathway from the extracellular space to the venous system. It actively regulates tissue fluid homeostasis, absorption of gastrointestinal lipids, and trafficking of antigen-presenting cells and lymphocytes to lymphoid organs and on to the systemic circulation. The cardinal manifestation of lymphatic malfunction is lymphedema. Recent research has implicated the lymphatic system in the pathogenesis of cardiovascular diseases including obesity and metabolic disease, dyslipidemia, inflammation, atherosclerosis, hypertension, and myocardial infarction. Here, we review the most recent advances in the field of lymphatic vascular biology, with a focus on cardiovascular disease. (Circ Res. 2016;118:515-530. DOI: 10.1161/CIRCRESAHA.115.306544.)

Key Words: cardiovascular diseases ■ endothelial cells ■ lymphangiogenesis ■ vascular endothelial growth factor C ■ vascular endothelial growth factor receptor-3

The recognition of the existence of lymphatic vessels has evolved slowly over the course of history, most importantly because of the difficulty of visualizing these transparent vessels. The first records of lymphatic vessels date back to ancient Greece, when Hippocrates (c. 460 to c. 370 BC) and Artistotle (c. 384 to c. 322 BC) documented vessels that may have been lymphatic vessels. Unequivocal reference to lymphatic vessels came from Alexandria, when dissenters including Erasistratus (c. 304 to c. 250 BC) described milky arteries in the mesentery of dogs that had consumed lipid-rich meals. In the 17th century, after a 2000-year gap, Gaspar Aselli can be credited for being the first to document lymphatic vessels. Aselli’s initial observations, it was established that these vessels made up a distinct vascular network that was separate from but connected to the blood vascular system. The gross anatomy of the lymphatic vessels was finally settled from the beginning of the 19th century.1,2

Because of the challenge of their visualization, lymphatic vessels have been historically ignored in research. Early anatomic studies relied primarily on intravascular injection of contrast agents.3 However, the visualization of lymphatic endothelial cells (LECs) was revolutionized during the late 1990s through the identification of vascular endothelial growth factor receptor (VEGFR)-3,3 prospero homeobox 1 (PROX1) transcription factor,4 integral membrane glycoprotein podoplanin (PDPN),5 and lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1)6 as lymphatic-specific markers. Research in the field has bloomed during the 21st century through molecular genetic studies of developing embryos that have revealed >50 genes involved in the specification, expansion and maturation of lymphatic vessels, and in lymphovenous separation.7 Although classical studies have considered the lymphatic vasculature as a passive transit system from the extracellular space to the blood circulation, the lymphatic system has been identified to actively regulate numerous physiological and pathological processes. Moreover, lymphatic vessels have been identified in organs where they were previously not thought to exist, including the eye, where they are involved in intraocular pressure regulation,8–11 and in the central nervous system, where they drain cerebral interstitial fluid, cerebrospinal fluid, macromolecules, and immune cells.12,13 In addition, pioneering research has revealed lymph node (LN) LECs as antigen-presenting cells involved in the induction of peripheral tolerance.14 These seminal findings have opened unexpected avenues for research on the lymphatic vasculature.

In this review, we will update the state-of-the-art of the lymphatic system in development and disease pathogenesis with a special focus on cardiovascular diseases. For lymphangiogenesis in cancer, detailed mechanisms of developmental lymphangiogenesis, and the physiology of lymph propulsion, we would like to refer the reader to several excellent reviews.7,15,16

Lymphatic Physiology

The lymphatic organ system is unique to vertebrates and is composed of draining lymphatic vessels, LNs, and associated lymphoid organs. Unlike the blood vessels in the circulatory system, lymphatic vessels are blind-ended unidirectional absorptive vessels that transport interstitial fluid, immune cells, and macromolecules to the LNs, and from these back to the blood circulation (Figure 1). The lymphatic vessels are found in almost every vascularized tissue except neural tissue and bone marrow. On the basis of their morphology, function,
Tissue Fluid Homeostasis

Under physiological conditions, some of the intravascular blood plasma is constantly filtered through the semipermeable blood EC (BEC) layer into the extracellular space. The majority of the extravasated interstitial fluid and macromolecules are absorbed back by the lymphatic vessels, whereas only transient reabsorption may occur in the venules. Some tissues are exceptions, for example, the kidney and the intestinal mucosa, where venous fluid absorption is sustained by local epithelial secretions. Overall, it has been estimated that the total plasma volume of the human body (≈3 L) extravasates from the blood circulation every 9 hours, and the great majority of this fluid is transported back to systemic circulation through the lymphatic system. The lymphatic system is thus a major contributor to tissue fluid homeostasis (Figure 1). Mice with severe lymphatic defects often exhibit massive embryonic edema and lethality; and if they survive until the neonatal period, they have severe problems caused by pulmonary edema and lethality; and if they survive until the neonatal period, they have severe problems caused by pulmonary edema and lethality.21 Contrary to the popular belief, lymphatic vessels do not function simply as a passive transit system but must actively overcome net pressure gradients that oppose flow. To do so, the collecting vessels contain intraluminal valves to prevent backflow and are covered by smooth muscle cells, which periodically contract to drive lymph forward. In addition, extrinsic compression by the surrounding tissue during muscle activity significantly contributes to lymph propulsion. The lymphatic vessel segment flanked by 2 valves is called a lymphangion, a contractile unit that propels lymph into the next lymphangion through the interposed valve in a unidirectional manner (Figure 1C). These intricate features of lymphatic physiology have been recently reviewed elsewhere.

Immune Cell and Soluble Antigen Trafficking

Lymphatic vessels are crucial conduits not only for the trafficking of leukocytes from peripheral tissues to their draining LNs but also for the drainage of soluble antigens. Although tissue resident dendritic cells (DCs) take up antigens and migrate to LNs for antigen presentation, soluble antigens transit to LNs faster than DCs, which is thought to prime the LN for the arrival of the antigen-presenting cells. Interestingly, the entry of soluble antigens from the LN lymphatic sinuses to the reticular LN conduits occurs in a size-dependent manner. Although large antigens are taken up by subcapsular macrophages and paracortical DCs, small antigens (<70 kDa) can directly enter the T- and B-cell zones. Recently, LN LECs were found to contain plasmalemma vesicle–associated protein–positive transendothelial pores that regulate the size-selective entry of lymph-borne antigens to the reticular conduits. Plasmalemma vesicle–associated protein–deficient mice lacked the pore-associated diaphragms, which markedly facilitated the entry of antigens and lymphocytes through the floor of the subcapsular sinus.

The entry of leukocytes and antigen-presenting cells into lymphatic vessels and their emigration from the LN subcapsular sinus into the parenchyma is actively regulated by LECs through the expression of several chemokines and adhesion molecules. Perhaps the best studied of these are the lymphoid homing chemokines, CCL21 and CCL19, that potently guide and recruit activated DCs and certain other leukocytes that express the cognate receptor CCR7. Mice lacking CCR7 ligands have impaired DC and T-cell homing to LNs and cannot mount adaptive immune responses. In the LN, LECs generate functional CCL21 gradients through the expression of the CCL21/CCL19 scavenger receptor CCR1L to allow emigration of DCs into LN parenchyma. LN LECs also express CCL21, which may promote the entry of CCR7+ DCs into the LNs, and several adhesion molecules, such as intercellular adhesion molecule-1, which may synergize with CCL21 to promote lymphocyte binding and transmigration.
The lymphatic system plays a critical role in the body’s immune system. It is an important component in the surveillance and presentation of antigens to the immune system, aiding in the maintenance of self-tolerance and the induction of peripheral tolerance. Lymphatic endothelial cells (LECs) are active contributors to the induction of peripheral tolerance by presenting peripheral tissue antigens on MHC I together with low levels of costimulatory molecules and high levels of coinhibitory PD-L1, resulting in inactivation of the CD8+ T cells that recognize these peripheral antigens. The deletion was selectively mediated by PD-L1 expressed on LECs and blockade of this receptor prevented the deletion of tyrosinase-specific CD8+ T cells, resulting in autoimmune vitiligo.

Peripheral Immune Tolerance

T cells sometimes escape thymic mechanisms of central immune tolerance. Previous work has attributed peripheral immune tolerance to the cross-presentation of tissue-derived antigens by quiescent tissue-resident DCs to self-reactive T cells, leading to anergy or deletion. However, several recent studies have identified that LN stromal cells and LN LECs are active contributors to the induction of peripheral tolerance by expressing peripheral tissue antigens, major histocompatibility complex (MHC) class I and MHC class II molecules, and a variety of immunoregulatory factors including high levels of the coinhibitory receptor programmed death-ligand 1 (PD-L1) and low levels of the costimulatory factors. LECs present peripheral tissue antigen on MHC I together with rather low levels of costimulatory molecules and high levels of coinhibitory PD-L1, resulting in inactivation of the CD8+ T cells that recognize these peripheral antigens. The deletion was selectively mediated by PD-L1 expressed on LECs and blockade of this receptor prevented the deletion of tyrosinase-specific CD8+ T cells, resulting in autoimmune vitiligo.

Absorption of Dietary Lipids

The absorption of dietary nutrients is critically dependent on intestinal villi, which consist of finger-like enterocyte-lined extensions of the gut wall filled with connective tissue containing a cage-like blood capillary network and a 1 or 2 central lymphatic vessels called the lacteals. Most nutrients are absorbed by blood vessels, but the passive diffusion of particles of high molecular weight or colloidal nature is limited across BECs. Therefore, the lacteals are essential for the uptake of dietary fats and fat-soluble vitamins. Advances in...
intravital imaging of lacteals have revealed that they contract through the activity of the surrounding smooth muscle cells regulated by the autonomic nervous system.\textsuperscript{18}

Although the mechanisms of lipid processing by the enterocytes have been explored in detail, the mechanism on how these lipid particles are delivered into the lacteals is still unclear. Early transmission electron microscopic studies have revealed that both passive paracellular and active transcellular transport mechanisms could contribute to lymph production in lacteals.\textsuperscript{40} The transcellular transport of lipids has also been shown in a tissue engineered model of intestinal enterocyte and lacteal interaction.\textsuperscript{25} However, cultured LECs do not form button-like junctions, and thus do not mimic the junctional profile of lacteals in vivo.\textsuperscript{39}

In mice, intestinal lacteals develop during the early postnatal period. Mice with genetic defects causing lymphatic dysfunction, such as the Chy mice that have a heterozygous missense mutation in Vegfr3 that inactivates its kinase activity,\textsuperscript{41} Vegfc heterozygous,\textsuperscript{41,42} and Prox1 heterozygous mice\textsuperscript{43} as well as mice deleted of the ANG2 ligand of the endothelial TIE2 receptor tyrosine kinase,\textsuperscript{44} accumulate milky peritoneal fluid known as chylous ascites. In adults, ascites most often results from primary lymphedema, malignancies affecting the abdominal lymphatic system, such as ovarian carcinoma or lymphoma, or surgical trauma to lymphatic vessels.\textsuperscript{45}

Importantly, owing to their special uptake and transport properties, the lacteals provide an appealing drug-delivery route. Drugs taken up by lacteals bypass the hepatic first-pass metabolism. Therefore, engineering drugs that are selectively taken up by the lacteals constitutes an elegant strategy to increase the oral bioavailability of rapidly liver-metabolized drugs.\textsuperscript{46} Below, we discuss aspects of lymphatic development, and later return to lacteals in the context of obesity and cardiovascular disease.

**Development of the Lymphatic Vascular System**

**Origins and Mechanisms of Development**

A widely accepted dogma in the field has been that the lymphatic vessels first arise from embryonic veins and thereafter expand by sprouting and proliferation, as initially postulated in 1902 by the American anatomist Florence Sabin\textsuperscript{47,48} and shown by using, eg, Tie2\textsuperscript{+} cell lineage tracing in mice.\textsuperscript{49} However, in 1910, a seemingly contradictory theory by Huntington and McClure proposed that lymphatic vessels first arise from embryonic veins and thereafter coalesce to form the lymphovenous valves that in lacteals.\textsuperscript{40} The transcellular transport of lipids has also been shown in a tissue engineered model of intestinal enterocyte and lacteal interaction.\textsuperscript{25} However, cultured LECs do not form button-like junctions, and thus do not mimic the junctional profile of lacteals in vivo.\textsuperscript{39}

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In the avian lymphatic vasculature has a dual origin, with contribution from both venous- and mesenchymal-derived cells.\textsuperscript{54–56} Recent cell lineage tracing studies have now unequivocally contributed to the lymphatic vasculature also in mice.\textsuperscript{54–56}

Development of the lymphatic vasculature is regulated by the autonomic nervous system.\textsuperscript{38} Intravital imaging of lacteals have revealed that they contract through the activity of the surrounding smooth muscle cells regulated by the autonomic nervous system.\textsuperscript{18}

LEC Specification

In the vasculature, PROX1 has been considered a master-regulator of the lymphatic phenotype although it is also expressed in venous valves\textsuperscript{46} and on the concave side of cardiac valves.\textsuperscript{65} In Prox1 null embryos, LECs in the cardinal vein wall bud off in an unpolarized manner, but fail to fully differentiate and proliferate.\textsuperscript{4} In contrast, overexpression of PROX1 in BECs upregulates the expression of LEC-specific markers.\textsuperscript{61,67}

The signals resulting in polarized PROX1 expression in the cardinal vein remain somewhat unclear. SRY-box (SOX)-18 transcription factor, which shows uniform expression in the cardinal vein and is required for LEC specification upstream of Prox1, but only in the C57BL/6 background.\textsuperscript{60} In other genetic strains, the related SOX7 and SOX17 transcription factors were able to substitute for the lack of SOX18.\textsuperscript{68} In addition, the COUP transcription factor II (COUP-TFII) is also required for the induction of Prox1.\textsuperscript{69} Furthermore, retinoic acid may play a role as it has been shown to promote lymphangiogenesis.\textsuperscript{70} The expression of the retinoid acid degrading enzyme CYP26B1 is polarized in the areas where the initial LECs bud off. Enhanced retinoic acid signaling in Cyp26b1 null-mice resulted in an aberrant increase of LEC progenitors in the cardinal vein and in hyperplastic lymph sacs and lymphatic vessels.\textsuperscript{71} Similarly, Notch signaling, which orchestrates cell-fate decisions, seems to play a role in
Figure 2. Development of the lymphatic vascular tree. A. The common cardinal vein (CCV) at E9.0. B. Specification of lymphatic endothelial cells (LECs) at E9.5, identified by PROX1 expression in the CCV, in the intersomitic veins and in the superficial plexus. C. Budding of initial LECs (iLECs) from the CCV at E10 to E10.25. D. Formation of the lymph sacs, comprising the primordial thoracic duct (pTD) and primordial longitudinal lymphatic vessel (PLLV) between E10.5 and E11.5. E. Two mechanisms of expansion of the lymphatic vascular tree: lymphangiogenesis and lymphvasculogenesis. F. Lymphatic vessel maturation and remodeling from E14.5 onward: recruitment of smooth muscle cell (SMC) coverage and valve formation. Panels A–D adapted from Hägerling et al59 with permission. Panel E derived from Stanczuk et al55 BEC indicates blood endothelial cell.

**Expansion of the Lymphatic Vascular Tree**

The budding of the LECs from the lymph sacs is absolutely dependent on VEGFC signaling as demonstrated by the lack of lymphatic vessels in Vegfc-deficient mice, whereas the related Vegfd is dispensable for lymphatic development. VEGFC and VEGFD are both ligands for VEGFR3, but upon proteolytic processing, they can also bind and activate VEGFR2. During development, VEGFR3 is also expressed in the blood vasculature and Vegfr3 gene-targeted mice die at E10.5 due to defective development of the cardiovascular system. Later in the development VEGFR3 expression is downregulated except in lymphatic vessels and fenestrated BECs, while being dynamically upregulated in angiogenic tip cells. Interestingly, compound deletion of both Vegfc and Vegfd does not recapitulate the early embryonic lethality observed in Vegfr3-null mice, suggesting that VEGFR3 may have other yet to be identified mechanisms of activation, for example, via β integrin signaling.

The interaction of collagen and calcium binding EGF domains 1 (CCBE1) protein with VEGFC/VEGFR3 signaling has recently received much attention. Ccbe1 was initially identified in zebrafish forward-genetic screens as being indispensable for lymphangiogenesis. Subsequent work associated Ccbe1 mutations with Hennekam syndrome, a form of hereditary lymphedema. Ccbe1 is expressed near developing lymphatic vessels and highly in the developing heart in embryos, and it acts in an LEC nonautonomous manner to enhance the activity of VEGFC. In mice that lack Ccbe1, the budding of initial LECs from the cardinal vein is halted, and the lymph sacs fail to form.

Mechanistically, CCBE1 was shown to bind select pericellular matrix proteins and to enhance VEGFR3 signaling by promoting the cleavage of VEGFC, but not VEGFD, into its active, fully mature form by the ADAM metallopeptidase with thrombospondin type 1 motif 3 (ADAMTS3) metallopeptidase and possibly
Recently, Adamts3 knockout embryos were shown to be massively edematous and embryonically lethal after E15 and to lack any peripheral lymphatic vasculature.87 Surprisingly, there was no evidence of a connective tissue phenotype although procollagen has been shown to be a major substrate for ADAMTS3.87 Additional studies should elucidate the detailed cellular mechanisms of ADAMTS3 function, especially its effect on the directionality of LEC sprouting.

In addition to VEGFR3, VEGFC also binds neuropilin-2 (NRP2), an axon guidance receptor expressed in veins and lymphatic vessels. NRP2 has a specific role in lymphatic vessel development because Nrp2 mutant mice have lymphatic capillary hypoplasia without blood vascular defects.88 Interestingly, NRP2 can bind not only VEGFC and VEGFD but also VEGF, simultaneously with VEGFR2 or VEGFR3 in vitro.41,89,90 Nrp2 and Vegfr3 compound heterozygotes, but not Nrp2 and Vegfr3 compound heterozygotes, display defective lymphatic vascular development,91 indicating that in vivo, NRP2 genetically interacts with VEGFR3, but not with VEGFR2. Thus, NRP2 functions as a coreceptor for VEGFR3 and may cooperate to increase the affinity of LECs toward VEGFC/D to enable maximal sensing of growth factor gradients.

Maturation of the Lymphatic Vascular Tree

After the establishment of the primitive lymphatic plexus, the lymphatic vessels undergo maturation to form a hierarchical tree composed of lymphatic capillaries, precollectors, and collecting vessels (Figure 2F). Collecting lymphatic vessels form valves, recruit smooth muscle cells, and deposit basement membrane.7 Furthermore, from E17.5 to postnatal day (P) 28, the initial lymphatic vessels transition from zipper-like junctions to button-like junctions,92 a process in which ANG2 signaling has been implicated.93 Several signaling pathways are involved in the maturation and maintenance of the collecting lymphatic vessels. Perhaps the best characterized is FOXC2/calcineurin/NFATC1 signaling, which is indispensable not only for both the maturation of collecting lymphatic vessels and for the formation of valves but also for the maintenance of lymphatic valves and vessel integrity during postnatal life.94–97 Mechanistically, FOXC2 cooperates with calcineurin/NFTAC1 transcription factor during the maturation of collecting lymphatic vessels. Several other molecules involved in the maturation of lymphatic vessels have been identified, including CX26, CX37, and CX43,98 Reelin,99 elastin microfibril interfacier 1 (EMILIN1),100,101 semaphorin
3A (SEMA3A)-NRP1, ephrin-B2/EPH receptor B4 (EFNB2/EPHB4) signaling, activin A receptor type I (ALK1), transforming growth factor beta receptor II (TGFBR2), GATA binding protein 2 (GATA2); these and the role of lymphatic flow–induced mechanotransduction have recently been reviewed elsewhere.

Since the discovery of the first LEC-specific marker proteins several years ago, there has been a dramatic increase in our understanding of the molecular mechanisms involved in lymphatic vascular development. Importantly, this knowledge has furthered our understanding of the involvement of lymphatic vasculature in human diseases, as highlighted below.

**Lymphatic System in the Pathogenesis of Cardiovascular Diseases**

**Lymphedema**

Impaired lymphatic drainage results in an abnormal accumulation of interstitial fluid defined as lymphedema. On the etiological basis, lymphedemas are classified as inherited (primary) or acquired (secondary) lymphedemas. Primary lymphedemas result from defects in genes involved in lymphatic vessel development, involving most often the VEGFC/VEGFR3 signaling axis. Secondary lymphedemas arise from damage or physical obstruction of lymphatic vessels or LNs.

**Secondary Lymphedemas**

Typical causes include chronic inflammation with fibrosis, malignant tumors, physical disruption, radiation damage, and certain infectious agents. Severe edema of the upper limb may complicate the effective treatment of breast cancer. The surgical removal and irradiation of the breast and certain infectious agents. Severe edema of the upper limb may complicate the effective treatment of breast cancer. The surgical removal and irradiation of the breast and associated axillary LNs results in lymphedema in 6% to 30% of patients. GJC2 (CX47) mutations are associated with a predisposition toward the development of postmastectomy lymphedema.

Perhaps the most dramatic example of secondary lymphedema is seen in lymphatic filariasis, a neglected tropical disease that affects ≈40 million people in the endemic areas of Africa, South America, and South-East Asia. Lymphatic filariasis is caused by mosquito-transmitted parasitic nematodes, such as Wuchereria bancrofti (in 90% of the cases), which specifically target and dwell in lymphatic vessels and LNs for years, resulting in extensive fibrosis. This can result in stigmatizing edema of the external genitalia and lower limbs that is so massive as to earn the appellation elephantiasis. William C. Campbell and Satoshi Ōmura were awarded one half of the 2015 Nobel Prize in Physiology or Medicine for the discovery of a class of anthelmintics that have radically lowered the incidence of lymphatic filariasis (and onchocerciasis) via annual mass administrations.

Another tropical lymphedema is podoconiosis (endemic nonfilarial elephantiasis), a noninfectious geochemical disease of the lower limb lymphatic vessels resulting from chronic barefoot exposure to red-clay soil derived from volcanic rock. Our limited knowledge of its pathogenesis suggests that mineral particles in red-clay soils are absorbed through the skin of the foot and engulfed by macrophages in the lymphatic system of the lower limbs, inducing an inflammatory response in the lymphatic vessels resulting in fibrosis and vessel obstruction. The heritability of podoconiosis accounts for 63% of the cases, and the association with variants in the HLA class II locus suggests that the condition is an abnormal T-cell–mediated inflammatory reaction to the mineral particles.

**Primary Lymphedemas**

Primary lymphedemas have been previously subclassified on the basis of their onset into congenital, peripubertal, and late-onset lymphedema. Some lymphedemas occur as a part of a syndrome. To date, at least 19 different genes have been associated with different isolated or syndromic lymphedemas. The diagnostic workup of primary lymphedemas involves a complex algorithm.

**VEGFC-VEGFR3**

VEGFR3 was the first lymphedema gene to be identified, and its mutations account for about one half of primary human lymphedemas. Heterozygous mutations in the tyrosine kinase domain of the receptor inhibit VEGFR3 signaling, often in a dominant negative manner, and cause anatomic and functional defects in the lymphatic system, resulting in primary congenital lymphedema also known as Nonne–Milroy lymphedema (OMIM 153100). These patients typically have bilateral lower limb lymphedema, which is usually apparent at birth. VEGFC mutations have also been identified in a Milroy-like disease, which is indistinguishable from Nonne–Milroy lymphedema.

**CCBE1**

Heterozygous or compound heterozygous CCBE1 mutations were recently associated with Hennekam lymphangiectasia-lymphedema syndrome (OMIM 235510), which is characterized by lymphedema, lymphangiectasia with systemic/visceral involvement, and mental retardation. CCBE1 impacts the development of lymphatic vessels by regulating VEGFC/VEGFR3 signaling via a complex mechanism (Figure 3), but defects in other organ systems suggest that CCBE1 also exerts functions outside of the lymphatic system.

**PTPN14**

The protein tyrosine phosphatase PTPN14 has been linked to lymphedema-choanal atresia syndrome (OMIM 608911). PTPN14 seems to interact with VEGFR3 on VEGFC stimulation, but in contrast to the previously mentioned genetic defects in which hypoplastic lymphatic vasculature is evident, mice with a Ptpn14 gene trap show hyperplasia of the lymphatic vessels with lymphedema. Therefore, hyperactive VEGFR3 signaling resulting from the absence of a tyrosine phosphatase could also result in lymphedema development.

**FOXC2**

The transcription factor FOXC2 is downstream of VEGFR3 signaling and has been associated with late-onset lymphedema (hereditary lymphedema II; OMIM 153200), which is often associated with distichiasis and ptosis (OMIM 153400), and yellow nails (OMIM 153300). Mouse studies indicate that an abnormal interaction between lymphatic vessels and mural cells and lack of valves may underlie the pathogenesis of this disease.
SOX18
As introduced earlier, the Sox18 transcription factor is involved in the induction of PROX1 expression in LECs. Mutations in SOX18 are associated with hypotrichosis-lymphedema-telangiectasia syndrome (OMIM 607823). Ragged mice, which have Sox18 mutations, also display sparse hair, lymphedema, and cutaneous telangiectasias.\(^{50,68,122}\)

Other Lymphedema Genes
Several other primary lymphedemas have been linked to GATA2, which was recently shown to regulate PROX1 expression.\(^{109,123}\) The connexins GJC2 (CX47)\(^{124,125}\) and GJA1 (CX43),\(^{126}\) the mechanically activated ion channel PIEZO1\(^{127}\) and other genes including HGF,\(^{128}\) KIF11,\(^{129}\) PTPN11,\(^{130}\) HRAS,\(^{138,139}\) and KRAS,\(^{130}\) SOS1,\(^{130}\) RAF1,\(^{130}\) IKBKG,\(^{131–134}\) RASA1,\(^{135–137}\) and HRAS,\(^{138,139}\) and a locus in the human chromosome 15q.\(^{140}\)

Treatment
Lymphedema remains a relatively common debilitating lifelong disease with limited treatment options, including controlled compression stockings/bandages and physiotherapy. Thus, novel curative treatment options are needed. Preclinical studies indicate that reconstitution of damaged lymphatic vessels with the aid of lymphangiogenic growth factors provides a promising treatment strategy. In a mouse model of Nonne–Milroy disease, VEGFC gene therapy generated functional lymphatic vessels.\(^{31}\) In a mouse model of postmastectomy lymphedema, local and transient VEGF overexpression induced functional lymphatic network restoration in the damaged areas.\(^{141}\) VEGFC initially induces aberrant and leaky vessels, which is followed by a prolonged period of remodeling, differentiation, and maturation, resulting in a functional network of collecting lymphatic vessels containing valves and mural cells. Network restoration was further enhanced by coconimtant autologous LN transplantation.\(^{145}\) The therapeutic value of LN transfer with perinodal VEGFC treatment has been validated in large animals\(^{142}\) and is currently advancing to clinical trials for the treatment of postmastectomy lymphedema.

Obesity
Excess body weight is the fifth most important risk factor contributing to the overall disease burden worldwide and especially to cardiovascular diseases.\(^{143}\) Obesity is caused by an imbalance between calorie intake and energy expenditure. The first evidence of lymphatic vessel malfunction as a cause of obesity came from the paradoxical observation that Prox1\(^{−/−}\) mice develop adult-onset obesity without changes in energy intake or expenditure.\(^{65}\) Fat accumulation around the lymphatic vessels in the Prox1\(^{−/−}\) mice suggested that the leakage of lymph, which effectively promotes adipogenesis in vitro, is the key mechanism by which lymphatic vessels promote adipocyte hypertrophy. Adipose tissue accumulation observed in patients with lymphedema and in the skin of the Chy mice fits this idea.\(^{41,144}\) However, the interpretation of the obesity phenotype in the Prox1\(^{−/−}\) mice is difficult because PROX1 is also expressed in the liver and skeletal muscle, which are key regulators of energy metabolism. Furthermore, fat accumulation does not occur in K14-VEGFR3-Ig mice, and increased body weight is not observed in the Chy mice, Vegfc\(^{−/−}\) or K14-VEGFR3-Ig mice, which all have lymphatic vessel dysfunction (Table). VEGFR3 expression in inflammatory cells and blood vessels as well as the role of VEGFC in lipid absorption also introduce confounding variables.\(^{146–148}\)

We recently found a surprising novel mechanism that regulates dietary lipid uptake and obesity development in the VEGFC-deficient mice.\(^{149}\) Deletion of VEGFC in adult mice, which have a normally developed lymphatic system, does not result in adverse effects on animal health even 6 months after gene deletion. Surprisingly, VEGFC deficiency had no effect on lymphatic vasculature in the skin, trachea, or LNs, but it caused a slow regression of intestinal lymphatic vessels. The atrophy of the intestinal lymphatic vessels reduced lipid uptake, increased lipid excretion into feces, counteracted obesity, and improved glucose metabolism in mice fed a high-fat diet.\(^{145}\) However, no accumulation of lipid in the villus interstitium was observed, suggesting a feedback mechanism to restrict lipid absorption by the enterocytes. A continuous low-level VEGFC-mediated sprouting of lacteal vessels could be responsible for the maintenance of lacteal vessel structure.\(^{39}\) Furthermore, VEGFC could regulate smooth muscle cell contractions in the villus\(^{150}\); such contractions were recently shown to play an important role in lipid absorption.\(^{148}\) These findings could open possibilities for the development of new drugs to treat dyslipidemia and obesity. An overview of the lymphatic vessels roles in lipid absorption and transport is presented in Figure 4.

Inflammation
Inflammation is a part of a complex biological response associated with protection of tissues against harmful stimuli such as pathogens, damaged cells, and irritants.\(^{151}\) Depending on the inductive signals, cellular sensors, secreted mediators, and target tissue, the associated inflammatory response comes in

### Table. Body Weight Measurement in Mouse Models With Lymphatic Defects

<table>
<thead>
<tr>
<th>Mouse Model</th>
<th>Gen. Bkgd.</th>
<th>Age, wk</th>
<th>Diet† (length)</th>
<th>WT Littermates</th>
<th>Gene Targeted</th>
<th>PValue‡</th>
<th>Value‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chy(^{1})</td>
<td>NMRI</td>
<td>20–22</td>
<td>HFD (14 wk)</td>
<td>45.7±1.1 (n=10)</td>
<td>43.5±1.2 (n=5)</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>Vegfr3(^{−/−})</td>
<td>BL/6J</td>
<td>18–20</td>
<td>HFD (12 wk)</td>
<td>33.2±1.5 (n=5)</td>
<td>34.7±1.2 (n=7)</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>K14-VEGFR3-Ig(^{−/−})</td>
<td>FVB</td>
<td>19</td>
<td>HFD (12 wk)</td>
<td>36.4±1.6 (n=5)</td>
<td>34.3±1.2 (n=4)</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>Vegfc(^{−/−})</td>
<td>ICR×BL/6J</td>
<td>52–54</td>
<td>SD</td>
<td>51.5±0.6 (n=2)</td>
<td>51.0±1.2 (n=4)</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>Vegfc(^{−/−})</td>
<td>ICR×BL/6J</td>
<td>25–27</td>
<td>SD</td>
<td>45.3±0.9 (n=8)</td>
<td>41.5±1.2 (n=5)</td>
<td>0.02</td>
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</tbody>
</table>

Only male mice were analyzed. The data is presented as average±SEM. HFD indicates high-fat diet; and WT, wild-type.

*Age when body weight was measured.
†HFD (Research diets, Cat no. D-12451, 45% calories from fat).
‡P value, unpaired 2-tailed test. We thank Harri Nurmi for collecting these data.
has been inhibited or induced experimentally. Furthermore, as evidenced in studies in which lymphangiogenesis in mice profoundly alters the course of inflammation and tissue repair. IAL is not merely a bystander, but may also regulate the contraction of lacteal-associated smooth muscle fibers, which have an important role in lipid absorption. VEGFR3 signaling with monoclonal antibodies extended the inflammatory responses. On the contrary, the induction of lymphangiogenesis has beneficial effects in many models. The improved lymphatic drainage increases during inflammation, and particularly during its resolution; it is of pivotal importance to remove the soaring numbers of inflammatory cells, noxious antigens, excess cytokines and cellular debris, and to resolve the edema resulting from increased blood vascular permeability. Accumulating evidence suggests that inflammation-associated lymphangiogenesis (IAL) is not merely a bystander, but profoundly alters the course of inflammation and tissue repair as evidenced in studies in which lymphangiogenesis in mice has been inhibited or induced experimentally. Furthermore, newly formed lymphatic vessels that do not regress may leave behind a permanent inflammatory memory.

**Role of IAL in Inflammation**

The effects of the inhibition of lymphangiogenesis in inflammation have been studied in many mouse models. Blocking VEGFR3 signaling with monoclonal antibodies extended the duration of inflammation, aggravated the inflammation or edema in several experimental models, such as in ultraviolet B irradiation–induced skin inflammation, oxazolone–induced contact hypersensitivity, Mycoplasma pulmonis–induced airway inflammation, chronic inflammatory arthritis and inflammatory bowel disease. Overall, these results indicate that IAL is necessary to mount appropriate inflammatory responses. On the contrary, the induction of lymphangiogenesis has beneficial effects in many models. The improved lymphatic clearance in K14-VEGFC and K14-VEGFD mice significantly limited the severity of acute inflammation and edema in oxazolone–induced contact hypersensitivity and ultraviolet B irradiation–induced skin inflammation. Moreover, transgenic expression of VEGFC in a model of chronic cutaneous inflammation completely inhibited the development of chronic skin inflammation. However, a concern in the translatability of these approaches relates to inflammatory conditions where pathogens could potentially hijack the lymphatic system to gain systemic access.

**Regression of Lymphatic Hyperplasia**

In the resolution of inflammation, the newly generated blood vessels undergo pruning and regress back to the basal state. Unlike blood vessels, the lymphatic vessels do not always completely regress after the removal of an inflammatory stimulus, for instance, in mice after Mycoplasma pulmonis infection, despite administration of steroids. However, in an experimental model of skin inflammation, the formed LN lymphatic vessels almost completely regressed in coordination with changes in LN volume. In the normally avascular cornea that may contain antilymphangiogenic factors, lymphatic vessels are much faster at regressing than blood vessels during the resolution of suture-induced corneal neovascularization. Therefore, the regression of lymphatic vessels seems to be tissue and insult dependent. A question of particular importance for future studies is what types of inflammatory cascades are capable of inducing lymphatic remodeling of permanent nature and what is the functional outcome.

**Transplant Rejection**

A potentially devastating aspect of IAL relates to organ transplantation and particularly to transplant rejection. Transplant rejection is associated with an extensive lymphangiogenic response, in the context of cardiac allografts, blocking VEGFR3 signaling increases graft survival, possibly by modulating immune cell trafficking. Similarly, the cornea has been extensively used to analyze the role of lymphangiogenesis in transplant immunology. A recent report demonstrated the crucial role of lymphatic vessels in mediating corneal allograft rejection and showed that antilymphangiogenic therapy increases graft survival.

**Origins of LECs During IAL**

Postnatal lymphangiogenesis has, by definition, been thought to arise from pre-existing LECs. However, as introduced in the context of embryonic development, recent studies have indicated that non-LEC (and non-BEC) progenitors may also contribute to lymphvascularogenesis, raising the possibility that similar mechanisms may also be reactivated in adults. Macrophage transdifferentiation into LECs has been suggested to occur in...
some models of IAL, but these studies been inconclusive because of the lack of lineage-tracing approaches. However, myeloid lineages did not seem to contribute to lymphangiogenesis in the skin or mesentery during embryogenesis. In a study by Kerjaschki et al in male-to-female sex-mismatched rejected kidney transplants, some 13% of the newly formed LECs contained a Y chromosome and were thus derived from an undetermined source of host cells, indicating a dual (or higher tier) origin for the neovessels. Future research should address which cell types specifically contribute to the newly formed LECs in adult lymphvasculogenesis.

Atherosclerosis and Myocardial Infarction

Atherosclerosis involves a chronic inflammatory disease of the arterial wall, and complications related to this condition represent the most common cause of morbidity and mortality in Western societies. The disease develops silently over decades, evolving from fatty streaks characterized mainly by macrophages loaded with cholesterol esters to advanced plaques with several secondary changes. Continuous recruitment of monocytes into plaques drives the progression of this chronic inflammatory condition, and atherosclerotic inflammation is sustained at least in part by the deposition of cholesterol crystals and undesirable immunity against cholesterol-associated apolipoproteins. Although the link between cholesterol and inflammation that drives disease progression is not completely understood, it is established that removal of cholesterol from the arterial wall comprises a step toward regression of atherosclerosis.

The multistep process of cholesterol mobilization from extravascular tissues to biliary and nonbiliary excretion is termed reverse cholesterol transport (RCT). Cholesterol removal from macrophage stores involves hydrolysis, mobilization, and efflux of cholesterol esters to lipoprotein acceptors such as apoAI, which results in the formation of HDL. HDL leaves the interstitial tissue and is transported through bloodstream into the liver for disposal as biliary cholesterol and bile salts or to the intestinal wall for transintestinal cholesterol efflux. Although the initial and final steps of RCT have been well characterized, it was only recently shown that HDL primarily uses lymphatic vessels in the efflux from the intestitium to the bloodstream. Induction of lymphangiogenesis by the administration of VEGFC into the footpad improved lymphatic function, decreased footpad cholesterol content, and improved RCT in ApoE−/− mice. In contrast, surgical disruption of collecting lymphatic vessels in the popliteal area reduced RCT from the footpad by as much as 80%. In another study, surgical ablation of lymphatic vessels in the tail also blocked RCT. In Chy mice, which selectively lack dermal lymphatic vessels, RCT from the rear footpad was impaired by ≤77%.

The relevance of lymphatic RCT in the atherosclerotic aortic

Figure 5. Lymphatic vessel role in cholesterol metabolism, atherosclerosis, and myocardial infarction. A, Whole mount staining of adult heart showing epicardial lymphatic vessels stained for VEGFR3 and LYVE1. B, Schematic overview of the heart with myocardial infarction caused by the occlusion of the atherosclerotic coronary artery. Proliferation of lymphatic vessels occurs in the affected area. C, Cross section of an atherosclerotic coronary artery and an adventitial lymphatic vessel. D, Hypothetical model for the role of lymphatic vessels in high-density lipoprotein (HDL)–mediated cholesterol removal from atherosclerotic plaques. Plasma-derived HDL enters the atherosclerotic plaque, interacts with ABCA1 or ABCG1 translocases at the plasma membrane of a cholesterol-loaded macrophage (foam cell, enlarged), binds cholesterol, and may exit via lymphatic vessels located in the vicinity of the coronary artery. Possible roles of VEGFC in atherosclerosis and myocardial infarction are highlighted with the bullet points.
wall was highlighted in an experiment in which atherosclerotic aortas of donor ApoE−/− mice were loaded with radiolabeled cholesterol and transplanted into recipient ApoE−/− mice that were treated with VEGFR3-blocking antibodies to block the regrowth of adventitial lymphatic vessels. This prevented the cholesterol efflux from the aortic plaques.180

Although the lymphatic uptake of macromolecules is primarily considered paracellular and passive, enabled by the unique button-like inter-EC junctions and flaps that can open under tension from anchoring filaments,17,23 active transcellular routes could possibly also contribute to lymph production.25 Interestingly, in vitro LECs expressed functional HDL transporters, including scavenger receptor class B member 1 (SR-BI) and ATP binding cassette subfamily (ABC)-A1, but not ABCG1.26 Internalization and transcytosis of HDL by LECs were mediated by SR-BI, and this was suggested to contribute to lymph production. In vivo, inhibition of SR-BI with blocking antibodies inhibited lymphatic uptake of HDL by as much as 75%, indicating that active transcellular SR-BI–mediated uptake of HDL into lymphatic vessels is a critical step for RCT.26 This is a surprising finding, and it would be important to determine why HDL prefers lymphatic vessels instead of the postcapillary venous system to exit the interstitial space.

Overall, these data indicate that RCT is critically dependent on lymphatic vessels, and that the venous system is not enough to sustain RCT. Furthermore, inducing lymphangiogenesis could constitute a strategy to enhance RCT. This could be especially important in the case of hypercholesterolemia and obesity that were shown to directly impair lymph vessel function.182–185 However, in the context of the arterial wall, RCT becomes more difficult as lymphatic vessels are normally localized in the adventitia and do not occur in the intima even in advanced atherosclerotic plaques.166 The relevance of lymphatic vessels in cholesterol metabolism and development of atherosclerosis was further demonstrated in K14–VEGFR3-Ig/low-density lipoprotein receptor−/−/ApoB100/100 mice.187 The established lymphatic defects in these 2 models were associated with increased levels of atherogenic lipoproteins, reduced periaortic lymphatic vessels, and more numerous atherosclerotic plaques. It remains to be assessed whether inducing intimal or adventitial lymphangiogenesis could enhance RCT and reverse atherosclerosis.

The most important complication of atherosclerosis is acute coronary syndrome, often culminating to myocardial infarction (MI). MI is followed by a robust inflammatory reaction characterized by the coordinated mobilization of different leukocyte subsets, which aid in scavenging dead cardiomyocytes and released macromolecules while promoting granulation tissue formation and remodeling.188 It was recently shown that after MI, cardiac lymphatic vessels undergo a profound lymphangiogenic response and that ectopic VEGFC stimulation augments the lymphangiogenic response resulting in a transient improvement in post-MI cardiac function.56 Therefore, inducing lymphangiogenesis could provide a pathway for inflammatory cell efflux to tip the balance in favor of wound healing within the injured adult heart.56 Recent studies demonstrated that VEGFC is an important regulator of coronary vasculature development, and it would be of great interest to determine whether this function relates to beneficial effects of VEGFC in a mouse model of MI.189,190 The roles of lymphatic vessels in MI and atherosclerosis are summarized in Figure 5. It is clear that much further analysis is needed to dissect the role of the lymphatic vessels in the pathogenesis of cardiovascular disease including dietary fat absorption and metabolism, adipose tissue inflammation, obesity, RCT, regulation of tissue inflammation, and innate and adaptive immunity. This work is now possible because of the many tools that have been created during the past decade, and it should lead to additional strategies to reduce cardiovascular morbidity and mortality.

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