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REVIEW

Heterogeneity in the lymphatic vascular system and its origin

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Abstract Lymphatic vessels have historically been viewed as passive conduits for fluid and immune cells, but this perspective is increasingly being revised as new functions of lymphatic vessels are revealed. Emerging evidence shows that lymphatic endothelium takes an active part in immune regulation both by antigen presentation and expression of immunomodulatory genes. In addition, lymphatic vessels play an important role in uptake of dietary fat and clearance of cholesterol from peripheral tissues, and they have been implicated in obesity and arteriosclerosis. Lymphatic vessels within different organs and in different physiological and pathological processes show a remarkable plasticity and heterogeneity, reflecting their functional specialization. In addition, lymphatic endothelial cells (LECs) of different organs were recently shown to have alternative developmental origins, which may contribute to the development of the diverse lymphatic vessel and endothelial functions seen in the adult. Here, we discuss recent developments in the understanding of heterogeneity within the lymphatic system considering the organ-specific functional and molecular specialization of LECs and their developmental origin.

Keywords Lymphatic vessel • Lymphatic vascular development • Lymphangiogenesis • Lymphvasculogenesis • Haemogenic endothelium

1. Introduction

The lymphatic vasculature has long been recognized for its vital function as a unidirectional drainage system for the clearance of interstitial fluid and transport of tissue-derived immune cells and antigens to the lymph nodes. However, it is becoming increasingly appreciated that lymphatic endothelial cells (LECs) not only provide a structural framework for transportation. Recent research has shown that LECs can present antigens and express immunoregulatory molecules that modulate immune cell activation and functions (reviewed in[1,2]). In addition, the lymphatic system plays an essential role in the uptake of dietary fat and has been implicated in clearance of cholesterol from peripheral tissues, and lymphatic dysfunction may thus contribute to the pathogenesis of obesity and arteriosclerosis (reviewed in[3–5]).

Lymphatic vessels within different organs and in different physiological and pathological processes show remarkable plasticity and heterogeneity, which likely reflects their tissue-specific specialization (*Figure 1*). In support of this, both blood endothelial cells (BECs) and LECs from different organs show unique expression profiles and transcriptional programmes.[6,7] In addition, BECs differentiated from embryonic stem cells adopt different molecular features depending on which tissue they were transplanted into,[6] suggesting that endothelium possesses an inherent phenotypic plasticity that allows organ-specific functional specialization. LECs in embryos compared with adult tissues, as well as LECs of different vascular beds, show differences concerning basic growth factor responses, but the molecular basis of this is not well understood. For example, postnatal deletion of vascular endothelial growth factor C (Vegfc),[8] which is the critical lymphatic growth factor during embryogenesis[9] was shown to result in regression of the specialized lacteal lymphatic vessels within the intestinal villi, without affecting the integrity or function of dermal lymphatic vessels.[8] Vascular bed-specific responses are further highlighted by the observations that Vegfc overexpression in the respiratory tract induced lymphatic vessel growth only during a critical period in perinatal development,[10] while in the skin Vegfc activated lymphangiogenesis also in adult stages.[11,12] To add another level of complexity, LECs in certain organs were recently shown to have multiple cellular origins,[13–15] which may contribute to the development of the diverse lymphatic vessel and LEC functions in the adult.

We are only beginning to understand the molecular differences that endow and functionally distinguish different types of lymphatic vessels in different organs and biological contexts. Although some of the signals

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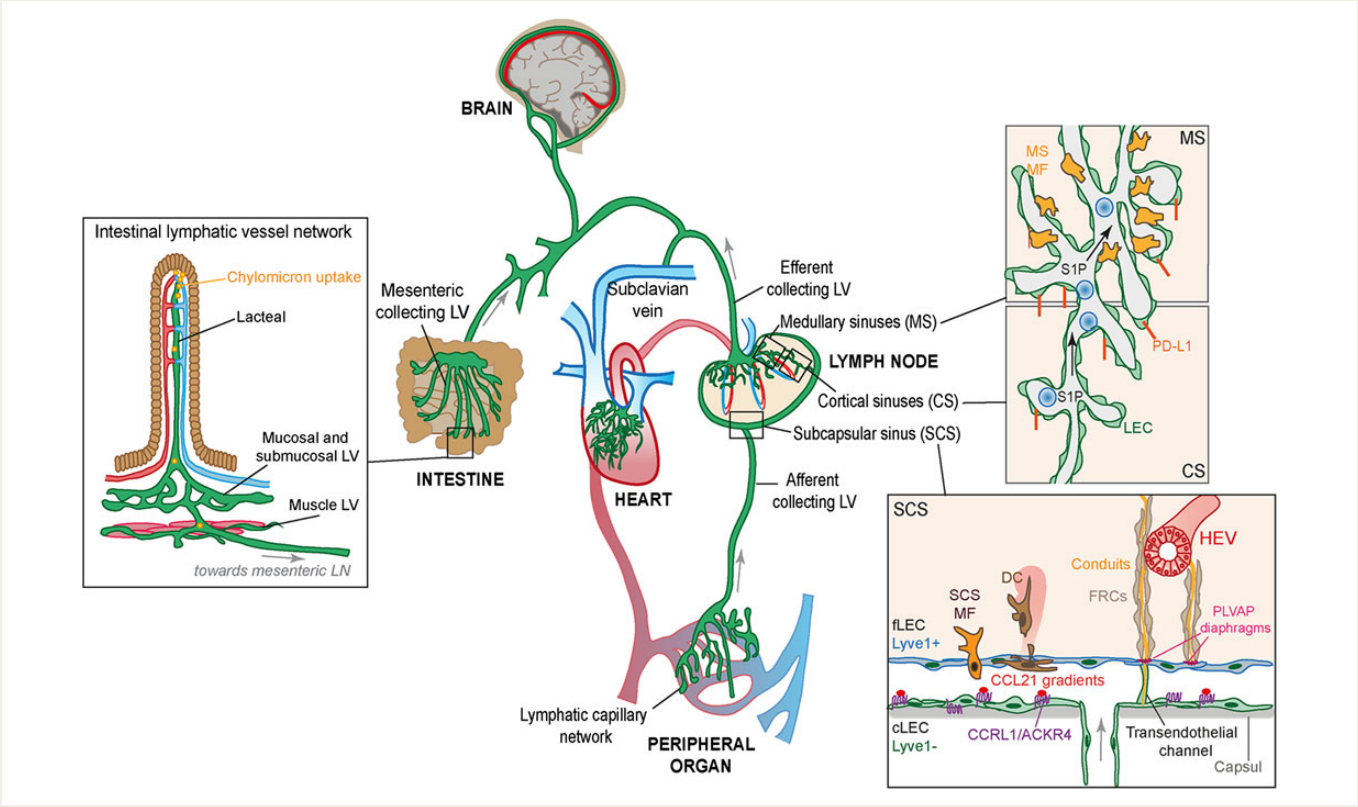

Figure 1 Organization of the lymphatic vasculature and the specialized lymphatic vessels within the intestine and the lymph node. The lymphatic capillary network (green) absorbs fluid that continuously leaks out from the blood capillary beds and returns it to the blood circulation (blue/red). On the left: The specialized capillary vessels in the intestinal villi called lacteals also take up dietary lipids as chylomicron particles. Both lacteals and capillary vessels in other peripheral organs provide a route for tissue-derived immune cells to the lymph nodes distributed along the collecting vessels; a function that is essential for induction of efficient immune responses and peripheral tolerance. On the right: Molecular and structural features of LECs in different parts of the lymph node (SCS, MS, and CS) are shown. LECs that build the ceiling of the SCSs (cLECs) can be molecularly distinguished from the LECs that form the floor (fLECs) by their low expression of the lymphatic marker Lyve1. cLECs instead express the atypical chemokine receptor CCRL1 (ACKR4) which through scavenging the chemokine CCL21 creates chemokine gradients for migration of tissue-derived DCs (brown) from the SCS into the lymph node parenchyma. Distinct populations of macrophages (MFs; yellow) are associated with SCS and MS but have a common role in scanning the lymph for pathogens and antigens. Small antigens, cytokines, and chemokines can enter the lymph node conduit system that descends from the floor of the SCS in the direction of high endothelial venules (HEVs). The size restriction of the conduit system is determined by PLVAP+ sieve-like diaphragms in transendothelial channels that connect the SCS to the conduits. CS and MS produce S1P essential for immune cell exit from the lymph node and express the immune check point molecule PD-L1 involved in LEC-induced antigen tolerance.

that regulate the developmental patterning and functional specialization of the lymphatic vasculature have been identified, little is known about how this patterning is maintained and how LEC plasticity is controlled. Here, we review recent insights into the heterogeneity within the lymphatic system focusing on the organ-specific functional and molecular specialization and the developmental origins of LECs.

2. Functional and molecular heterogeneity of lymphatic vessels

2.1 Vessel-type-specific specialization of lymphatic endothelium for fluid absorption and drainage

In most tissues, the return of extravasated tissue fluid back to the blood starts with absorption by specialized blind-ended capillary lymphatic vessels (also known as initial lymphatic vessels) (*Figure 2*). They converge into progressively larger collecting lymphatic vessels that transport the fluid, which inside the lymphatic vessels is called lymph, into the largest lymphatic vessels; the thoracic and right lymphatic ducts that empty into the subclavian veins. Despite their unique morphological features, lymphatic capillaries and collecting vessels share the expression of many of the genes that distinguish lymphatic from blood endothelium.[16–18] These include the master transcriptional regulator of lymphatic differentiation and identity, prospero homeobox 1 (Prox1), the transmembrane *O*-glycoprotein podoplanin (Pdpn, also known as gp38 and T1alpha), the tyrosine kinase receptor vascular endothelial growth factor 3 (Vegfr3 also known as FLT4), and neuropilin 2 (Nrp2), a receptor for Class III semaphorins, which similar to Vegfr3 binds Vegfc (*Figure 2*). These genes are not only markers of LECs but also critical regulators of the development of the lymphatic vasculature (reviewed in[17,18]). All lymphatic vessels also share the expression of the endothelial-specific junctional proteins VE-cadherin, Claudin5, and PECAM-1, but the organization of the cell–cell junctions is distinct. Lymphatic capillaries show discontinuous button-like junctions, while

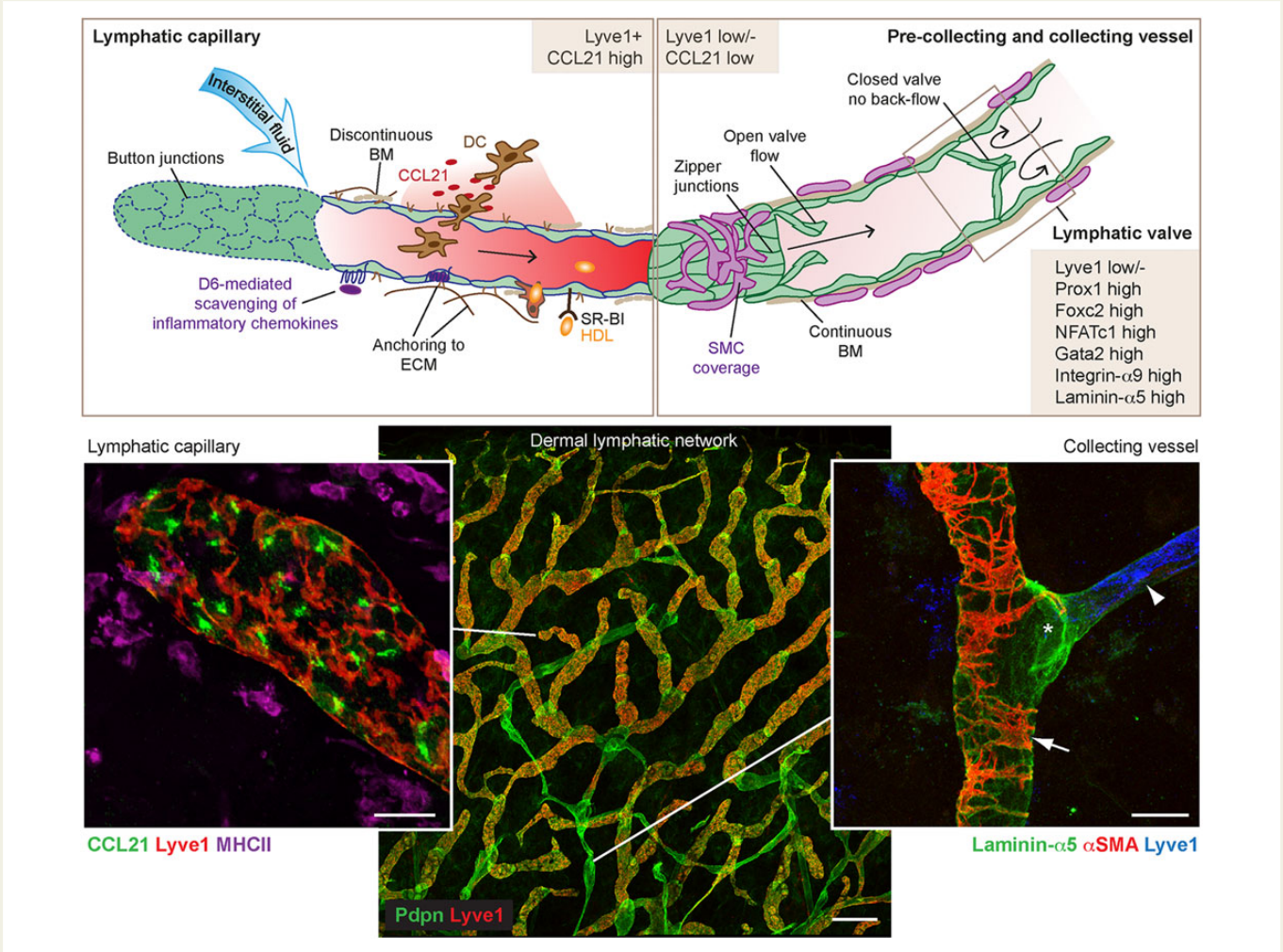

Figure 2 Structural and molecular features of capillary and collecting lymphatic vessels. The blind-ended lymphatic capillaries, designed to remove fluid and soluble molecules from the interstitial space, are characterized by button-like intercellular junctions, discontinuous basement membrane (BM), and anchoring filaments. High capillary expression of the CCR7-ligand CCL21 allows recruitment of DCs. Collecting lymphatic vessels that transport the lymph instead have zipper-like intercellular junctions, continuous basement membrane, smooth muscle cell coverage, and intraluminal valves. The main distinguishing molecular features of the endothelium of capillary vessels, collecting vessels, and valves are depicted in brown boxed areas. Additional functions of the capillary and collecting lymphatic networks include inflammatory chemokine scavenging through expression of the atypical chemokine receptor D6 (ACKR2) and RCT passively or through expression of the cholesterol carrier SR-BI or indirectly through macrophages. Bottom panels show whole-mount immunofluorescence images of mouse ear skin stained with the indicated antibodies. Arrowhead points to a Lyve1 positive capillary and arrowhead to a Lyve1 negative collecting vessel. Valve at a vessel branch point is indicated by an asterisk. Scale bars: 25 μm (capillary and collecting vessels) and 200 μm (lymphatic network).

collecting vessels have zipper-like junctions[19] (*Figure 2*). Lymphatic capillaries are surrounded by a thin discontinuous basement membrane. They readily sense changes in interstitial pressure by their anchoring to the extracellular matrix,[20] which can modulate the opening of 'flap valves' in between the button junctions to allow fluid entry.[19] It is also through these flap valves that immune cells enter the lymphatic vessels.[19,21]

Lymphatic capillaries can be distinguished from collecting vessels by their high expression of the hyaluronan receptor Lyve1[22] and the chemokine CCL21[23,24] (*Figure 2*). The latter allows recruitment of activated CCR7 positive dendritic cells (DCs) both in homeostasis and in inflammation through the formation of haptotactic CCL21 chemokine gradients, established by the immobilization of CCL21 on extracellular heparin sulfate, that guides migration of DCs towards the lymphatic vessels.[24] These gradients may act together with soluble gradients produced by LECs or by the DCs themselves as they migrate.[25] DC entry is followed by intraluminal crawling inside the lymphatic capillaries, a process which was recently described to depend on immobilized intraluminal CCL21 gradients formed in a flow-dependent manner.[26] In contrast, transfer of DCs within the collecting vessels to draining lymph nodes is thought to be passive. In addition to DCs, other immune cells including recirculating memory T-cells[27] and neutrophils[28] have been reported to enter lymphatic vessels in a CCR7-dependent manner. In contrast to CCL21, the role of Lyve1 in capillary vessels is still not clear. Although Lyve1 is expressed already in the earliest venous-derived LEC progenitors in E9.5-E10.5 embryos,[29] deletion of Lyve1 in mice does not result in major defects in lymphatic vessel morphogenesis or mobilization of DCs to lymph nodes when evaluated after FITC dibutyl phthalate skin painting.[30]

Whereas capillary vessels are designed for uptake of fluid and immune cell recruitment, collecting vessels are specialized for transport. They are surrounded by a continuous basement membrane and perivascular smooth muscle cells, which together with the zipper-like junctions make these vessels relatively impermeable for fluid diffusion[19] (*Figure 2*). Besides morphology and distribution of junctional proteins, collecting vessels can be distinguished by reduced expression of Lyve1[22] and CCL21[23] compared with capillary vessels, and it is generally not thought that collecting vessels allow transmigration of immune cells. However, recent data show that collecting vessels display an increased permeability during inflammation allowing exchange of lymph components with adipose tissue macrophages and DCs, which were shown to enter the lymph node.[31] The large collecting vessels and the connecting lymph nodes are embedded in subcutaneous or visceral fat, but the implication of this is largely unknown. In addition to an exchange of lymph components between the adipose tissue immune cells and the collecting vessels, the association may reflect the need for energy and other factors during adaptive immune responses in the lymph nodes[32,33] and could be a reservoir of common progenitors of lymph node mesenchymal stromal cells and adipocytes, active in development and possibly regeneration.[34] Another special feature of collecting vessels is the luminal valves that are essential for preventing retrograde lymph flow. Endothelial cells forming the valve leaflets show a unique spindle-like morphology and expression profile that is shared with venous valve endothelial cells.[35,36] They are characterized by high expression of the transcription factors Foxc2 and GATA2, and Integrin-α9 that are not only expressed by but also critically required for the formation of valves.[35,37,38] Valve endothelial cells also express high levels of Prox1, Connexin-37, and they show high calcineurin/NFATc1 signalling, while Lyve1 and Nrp2 levels are low or absent[35,39] (*Figure 2*).

There is not a clear border between capillary and collecting vessels, and sometimes the term pre-collecting vessel is used to describe the part of the lymphatic network in-between the capillary vessels and collecting vessels. Pre-collecting vessels express Lyve1 and contain valves but have no or very few smooth muscle cells. In human tissues, these vessels were described to display lower expression of PDPN together with distinct expression of chemokines and chemokine receptors including CCL27 and the atypical chemokine receptor Duffy antigen chemokine receptor DARC (also known as ACKR1).[23]

Studies on mouse models have uncovered molecular mechanisms that regulate the remodelling of the embryonic lymphatic vasculature into a fully functional network of capillaries and collecting vessels and acquisition of vessel-type-specific features. In addition, flow-induced mechanosignalling has been implicated in lymphatic vessel remodelling and maintenance of specific features including luminal valves.[38–41] For detailed information of the molecular regulation of lymphatic vessel morphogenesis and remodelling, we would like to refer the reader to excellent recent reviews.[16–18]

2.2 Specialized functions of lymphatic vessels in fat absorption and metabolism

The intestinal lymphatic system consists of two independent vessel networks: the lacteals that drain the intestinal villi interconnected with a submucosal lymphatic network, and the lymphatics that drain the muscular layer within the intestine[42] (*Figure 1*). Both of these connect in the mesentery with larger collecting vessels, which transport the incoming fluid to the mesenteric lymph node. The lacteals play a unique and vital role in the uptake of triglyceride-loaded particles known as chylomicrons and fat-soluble vitamins from the villi, for delivery to the blood via the cisterna chyli and thoracic duct. The mechanism of uptake of the chylomicrons, which can be very large up to 1 μm in diameter, is not entirely clear, and may involve both intercellular transport through LEC junctions and intracellular transport across LECs in vesicles (reviewed in[3,4]). Active contraction of the lacteals, which is mediated by smooth muscle cell contractions and controlled by the autonomic nervous system, allows drainage of absorbed lipids into the collecting vessel network.[43]

In many aspects, lacteals display similar features as skin dermal capillary vessels, including high expression of Lyve1 and CCL21,[7,44] while mesenteric collecting vessels are characterized by low expression of Lyve1 as well as the presence of valves and layers of extracellular basement membrane and smooth muscle cells.[39] Just as in the dermis, the lymphatic vessels within the villi and submucosa provide a path for tissue-derived DCs to reach the draining mesenteric lymph node, which constitutes a fundamental mechanism for induction of oral tolerance towards ingested antigens and the microbiota (reviewed in[45]). However, lacteals do not have the same uniform button-like junctional organization as, e.g. skin dermal capillary vessel but a mix of both continuous zipper junctions and discontinuous button-like junctions.[44] The lacteals in adult intestine in contrast to adult dermal lymphatic vessels display a low but detectable proliferation also under homeostasis.[44] Reflecting the more active proliferative state of the lacteals, deletion of the Notch ligand DLL4[44] or postnatal deletion of Vegfc[8] resulted in lacteal regression and reduced uptake of lipids without affecting the integrity of quiescent dermal lymphatic vessels. Cultured human intestinal and dermal LECs display a distinct set of differentially expressed genes, despite sharing the expression of known LEC markers.[7] However, further studies profiling specifically the transcriptome of lacteals are needed to understand the molecular mechanisms underlying their functional specialization.

Although the lacteals have a unique role in the uptake of dietary fat, lymphatic vessels outside the gastrointestinal tract also affect fat metabolism and defects in lymph transport may contribute to cholesterol-driven diseases like atherosclerosis (reviewed in[3–5]). Atherosclerosis is a chronic inflammatory disease of larger arteries and a major cause of myocardial infarction and stroke. It involves formation of plaques in the subendothelial intimal space with recruitment of monocytes that accumulate as lipid-loaded foam cells. Reverse cholesterol transport (RCT) refers to the removal of cholesterol from the tissue and its delivery back to the liver. Lymphatic vessels have been shown to contribute to RCT, either by acting as conduits for macrophages[46] or by cell free uptake of cholesterol passively or actively through expression of the HDL receptor SR-BI.[47] Hence, lymphatic vessels could be expected to have a protective effect against atherosclerosis by affecting both RCT and the local inflammatory environment. This notion is supported by data from *in vivo* animal experiments. Mice with lymphatic hypoplasia due to systemic defects in Vegfr3 signalling either due to a kinase inactivating mutation in *Vegfr3* (*Chy* mice) or expression of soluble Vegfr3-Ig show higher levels of lipoprotein and faster progression of atherosclerotic lesions in genetic models of atherosclerosis ($LDLR^{-/-}/ApoB^{100/100}$).[48] However, it should be noted that lymphatic vessels associated with arteries are rarely found in the intima where the arteriosclerotic plaques develop but instead localize to the adventitial layer. Further studies are needed to fully understand the impact of lymphatic vessels on the local atherogenic process.

Accumulation of adipose tissue has been reported as a clinical manifestation in patients suffering from chronic lymphoedema,[4,5,49] which

suggests direct or indirect roles of lymphatic vessels in local fat metabolism. In support of this notion, haploinsufficient *Prox1* mice and the *Chy* mice carrying a kinase inactivating mutation in *Vegfr3* that both develop oedema were also reported to display accumulation of fat.[50,51] However, conflicting data for the *Chy* model exist,[5] and other mouse models with lymphatic dysfunction such as *K14-Vegfr3-Ig* mice do not display increased body weight compared with controls.[5,51] It is possible that differences in genetic background of the mice and thereby differences in the immunological phenotype[52] contribute to different outcomes on fat metabolism. Inflammatory cytokines such as tumour necrosis factor alpha are known to affect fat metabolism,[53] and lymphatic vessels may influence adipose tissue biology indirectly due to their role in clearance of cytokines and immune cells from the tissue environment.

2.3 Fluid homeostasis in the brain and the eye

No lymphatic vessels are found inside the brain in contact with neuronal cells, where an alternative system for fluid drainage, the so-called glymphatic system, exists. The glymphatic system is named after its dependence on glial cells and a similar draining function as the lymphatic system, but there are no structural similarities between the two (reviewed in[54]). However, in 2015, two groups independently demonstrated that the mouse brain meninges contain a lymphatic network.[55,56] Their data confirmed and extended a forgotten anatomical observation by the Italian physician Mascagni in 1787 and observations of dural lymphatic vessels in rat published in 1987.[57] The new studies by Louveau *et al.*[55] and Aspelund *et al.*[56] showed that meningeal lymphatic vessels absorb cerebrospinal fluid allowing antigen and immune cell trafficking to cervical lymph nodes but only have a limited role in fluid drainage from the brain.[55,56] Phenotypically the meningeal lymphatic vessels were shown to have features of lymphatic capillaries, including expression of Lyve1 and CCL21, with lymphatic valves observed only in vessels near the base of the skull.[55,56] The re-discovery of meningeal lymphatic vessels has put new focus on the immune regulation of the brain and could have implications for the understanding of immune-mediated diseases of the brain such as encephalomyelitis.[58] It remains to be determined if the brain lymphatic vessels are molecularly different from other lymphatic vessels in the body.

The cornea of the eye is completely avascular, which has been linked to expression of a secreted splice variant of Vegfr2 and truncated soluble Vegfr3 inhibiting the effects of Vegfl/Vegfc.[59,60] However, lymphatic vessels are present in the ciliary body, and the anterior segment of the eye and abnormal lymphangiogenesis plays a role in pathological eye conditions (reviewed in[61]). In addition, it was recently shown that the so-called Schlemm's canal (SC) that has an important function in draining the aqueous humour in-between the lens and the cornea, has lymphatic-like properties.[62–66] The SC expresses Prox1 and forms by postnatal migration of venous-derived progenitors analogous to the formation of venous-derived lymph sacs during embryogenesis.[62–64] ECs lining the SC also express other typical lymphatic markers including Integrin-α9, Vegfr3, and CCL21 and respond to Vegfc with increased sprouting and proliferation. However, they have very low or no expression of Lyve1 and Pdpn,[62,63] and the SC is thus not considered a true lymphatic vessel. Interestingly, the expression of Prox1 was found to correlate with SC integrity and function, suggesting that it could be used as a biosensor for pathological changes.[62] In addition, due to the stimulatory effect on SC growth, Vegfc treatment was suggested as a potential novel strategy for treatment of the degenerative disease glaucoma, which is coupled to increased intraocular pressure due to inadequate drainage of the aqueous humor.[63] Angiopoietin/Tie2 may also represent a target for glaucoma treatment due to a reported selective dependence of the lymphatic vessels in the eye and the SC on this signalling pathway.[65] Genetic deletion of the two Tie2 ligands angiopoietin 1 and 2 during late embryogenesis at E16.5 caused a specific loss of eye lymphatic vessels and the SC resulting in development of glaucoma within 3 months after birth.[65] It is possible that the late embryonic requirement of angiopoitin/Tie2 may reflect, at least in part, the postnatal formation of the SC, but it could also indicate diversification of the signalling pathways regulating different vascular beds.

2.4 Molecular and functional heterogeneity of the lymph node lymphatic vessel network

The lymphatic vessels are closely integrated with the lymph nodes, which are distributed, often in chains, along the collecting lymphatic vessels. The lymphatic connection to the lymph node is crucial for the establishment of efficient contacts between tissue and blood-borne immune cells, and the lymph nodes are essential not only for induction of effective immune responses but also their resolution and for maintenance of tolerance (reviewed in[45,67,68]). The lymph nodes consist of a complex network of lymphatic sinuses, surrounding a highly organized parenchyma of immune cells, specialized blood vessels, and stromal cells which together provide an optimal environment for induction and regulation of immune responses[67,68] (*Figure 1*). The lymphatic sinuses of the lymph node can be seen as an extension of the peripheral lymphatic network but differ from both capillary and collecting vessels with unique structural and molecular features.

2.4.1 Lymph node subcapsular sinus

The incoming lymph is drained by afferent lymphatic vessels into the large subcapsular sinus (SCS) located underneath the collagenous capsule that surrounds the lymph node. The lymphatic cell layer forming the floor of the SCS (fLECs) allows cellular trafficking into the lymph node parenchyma but is at the same time highly selective with regard to the molecules transported, excluding those >70 kDa or with a radius >4 nm.[69] Smaller antigens and soluble molecules like chemokines and cytokines can gain access through the highly specialized conduit system, which consists of a network of tubular collagen fibres ensheathed by fibroblastic reticular cells (FRCs).[69,70] The reticular fibres descend from the fLEC layer in the direction of the high endothelial venules (HEVs), where blood derived immune cells enter the lymph node parenchyma. The conduit system allows crosstalk between lymph and blood[71] but still shields the immune cell compartment of the lymph node from direct contact to the lymph. Access of antigens to the lymphoid compartment of the lymph node instead has to go through DCs or other antigen presenting cells[70] (reviewed in[45,68,72]).

The features of the sinus floor that allow exclusion of fluid transport but still permit cellular transmigration have been elusive for a long time. Recently, expression of the glycoprotein plasmalemma vesicle-associated protein (PLVAP) (also known as PV-1 and MECA-32) by lymph node LECs was shown to be essential for the barrier function of the sinus, affecting both the size-selective entry of lymph-borne antigens to the conduits and the cellular transmigration.[73] PLVAP was shown to form sieve-like diaphragms in transendothelial channels in LECs that bridge the sinus to the conduits.[73] PLVAP was not restricted to fLECs but displayed a widespread expression in lymph node LECs, while no expression was reported in peripheral skin LECs.[73] PLVAP has earlier been thought to be confined to BECs and is the only protein that has been associated

with formation of diaphragms in endothelial cells.[74] The fLECs are also lined by a basement membrane-like extracellular matrix and are in close contact with sinus-lining-specialized stromal cells called marginal reticular cells (MRCs)[75] and metallophilic CD169+ macrophages (MMF) that scan the lymph for antigens.[76,77]

The same chemokine axis CCR7–CCL21 that guides entry of DCs into peripheral lymphatic vessels[24,26] have intriguingly also been shown to be essential for their exit from the SCS into the lymph node parenchyma.[78] Whereas transendothelial channels restricted by PLVAP diaphragms in fLECs may provide a path for the lymph-borne immune cells into the lymph node, the CCR7-driven directional transit was shown to be regulated by the LECs that form the ceiling of the SCS (cLECs), dependent on their expression of the atypical chemokine receptor CCRL1 (ACKR4).[79] CCRL1 is a scavenging receptor for the CCR7-ligands CCL19 and CCL21, efficiently targeting them for internalization and degradation. Expression of CCRL1 was shown to allow formation of CCL21 chemokine gradients reaching from the sinus into the lymph node parenchyma. Conversely, the absence of CCRL1 resulted in a dramatic accumulation of CCL21 at the floor of the SCS, creating a reversed gradient and preventing efficient exit of DCs.[79] Expression of CCRL1 is mainly confined to the SCS cLECs, and no expression is found in the primary dermal lymphatic plexus. Another marker that has been specifically assigned to LECs in the SCS is the chemokine CCL1,[80,81] which was shown to allow CCR8 chemokine receptor-driven entry of tumour cells into the lymph node.[80] The absence of CCL1 still allowed tumour entry into the afferent lymphatics but blocked transmigration of tumour cells across the sinus. The immunological functions of sinus-derived CCL1 is not entirely clear, but CCR8 has been shown to be involved in monocyte-derived DC migration from the skin to lymph nodes.[81]

2.4.2 Lymph node cortical and medullary sinuses

Most of the lymph that enters the SCS will never enter the conduits but will be channelled out through the cortical and medullary sinuses (CSs and MSs, respectively) and leave the lymph node through the efferent lymphatic vessels. The MSs form a complex irregularly shaped network and are transversed by fine reticular strands. Similar to fLECs, MS LECs intimately interact with macrophages. The medullary macrophages are phenotypically distinct from the macrophages found in the SCS but similarly contribute to clearance of pathogens and antigens from the lymph (reviewed in[72]). The areas of CSs that connect to the MSs contain fewer macrophages. The factors that determine the distinct spatial relationship between different macrophage populations and different part of the lymph node LEC network are not known.

Both CSs and MSs have been shown to be involved in lymphocyte egress from the lymph node by their production of sphingosine-1-phosphate (S1P). S1P binds the sphingosine-1-phospate receptor type 1 (S1PR1) expressed by T-cells, which is necessary for T-cell egress both from the thymus into the blood and from lymph node into the lymph.[82–85] The important source of S1P in the blood is haematopoietic cells,[83] whereas the important source of S1P in lymph was shown to be Lyve1 expressing cells,[82] which will include fLECs and large parts of the CS and MS network. Deficiency in the production of S1P in Lyve1 expressing cells also affects patterning of the peripheral lymphatic vessels,[82] and S1P may thus have a dual role in lymphatic vessel development and LEC-mediated immune regulation. Another molecule involved both in developmental patterning of lymphatic vessels and in the regulation of S1P egress is Integrin-α9.[86] Besides its role in the development of lymphatic valves,[35] Integrin-α9 was also shown to regulate secretion of S1P from the LECs.[86]

2.5 Lymph node LECs in antigen presentation and regulation of immune cell activation

Besides expressing proteins that can affect immune cell access to and exit from the lymph node parenchyma, multiple data support that lymph node LECs express both MHC class I and class II molecules and contribute to antigen presentation and regulation of immune cell activation.[87–93] Tewalt *et al.*[88] showed that lymph node LECs lack expression of co-stimulatory molecules but express the immune check point molecule programmed cell death 1 (PD-1) ligand (PD-L1), with the highest expression in CSs and MSs. In line with this finding, several studies have linked lymph node LECs to peripheral and tumour-induced tolerance,[87–89,91,93] but lymph node LECs may also act as a reservoir for antigens facilitating induction of protective immune responses in vaccination and viral infection, an effect that was shown to be dependent on the proliferative response of LECs in infection.[92] Lymph node LECs have been suggested to present antigens directly, but it is also possible that they induce tolerance indirectly by transferring antigens to DCs[93] or that they receive antigen complexes from DCs.[91] Most studies do not differentiate *in vivo* effects of antigen presentation by LECs from other lymph node stromal cells, and many questions remain.

The lymphatic network of the peripheral skin-draining lymph node is much simpler in a newborn mouse compared with an adult[94] and will grow and remodel extensively during the first postnatal weeks. Neonatal lymph nodes were shown to express much lower levels of PD-L1 compared with adult lymph nodes,[95] and it is likely that crosstalk between recruited immune cells and ECs in conjunction with other stromal cells shapes postnatal lymph node lymphatic patterning. It is also an interesting possibility that peripheral lymphatic vessels can adopt a lymph node-like specialization in disease. This may occur during the formation of tertiary lymphoid organs, also known as ectopic lymphoid structures, which are an accumulation of immune cells and stromal cells with a lymph node-like organization in chronically inflamed tissues and tumours.[96]

2.6 Peripheral lymphatic vessel heterogeneity and plasticity in immune regulation

Reduced peripheral lymphatic drainage will in addition to oedema be expected to lead to increased accumulation of inflammatory mediators, including cytokines, which are normally transported away with the lymph and affect immune cell trafficking to the lymph nodes. This can disrupt the immune regulatory pathways that are dependent on the communication between the tissue and the lymph nodes.[1,45,67] Supporting this notion, inhibition of the Vegfc/Vegfr3 growth factor axis has been shown to result in increased and prolonged inflammatory responses,[97,98] defects in peripheral immune tolerance,[45,99] and can change the resting immunological environment within the tissue.[50] It should be noted that the experimental strategies have been based on targeting of the Vegfc/Vegfr3 growth factor axis globally, and it cannot be excluded that part of the effects may be due to effects on other Vegfr3 expressing cells including macrophages[100] and BECs.[101] Interestingly, the effect on Vegfr3/Vegfc inhibition may also be context dependent, and treatment with soluble Vegfr3 receptor in inflammation-driven carcinogenesis was shown to reduce the inflammatory response and inhibit cancer development.[102]

Both lymph node and peripheral lymphatic vessels express cytokine and toll-like receptors (TLRs)[1,2] in a pattern that differs between LECs in different organs[103,104] and respond to inflammatory stimuli not only through changes in proliferation and fluid permeability (reviewed in[2]). For example, inflamed dermal lymphatic endothelium was reported to suppress DC maturation through up-regulation of Mac-1/ICAM-1.[105] Peripheral LECs can also influence leukocyte migration by modulating chemokine availability through expression of the atypical chemokine receptor D6 (ACKR2),[106] a scavenging receptor for inflammatory chemokines. Human LECs in the skin, gut, and lymph nodes express D6,[107] but any potential differences in the expression of D6 between different types of vessels or organs are unknown. D6 deficient mice display exuberated and prolonged responses in phorbol ester-induced skin inflammation,[108] which was linked to accumulation of leukocytes around peripheral lymphatic vessels and in draining lymph nodes leading to lymphatic congestion and impaired fluid and cell drainage.[106] Dermal LECs display different gene expression profiles in response to treatment of the skin with a contact sensitizer oxazolone vs. complete Freund's adjuvant.[109] The plasticity of LECs in disease is also supported by reported changes in LEC gene expression profiles in experimental models of tumour-associated lymphatic vessels.[110] It will be of high relevance to understand how LECs adapt to different conditions during infection, inflammation, and cancer.

3. Heterogeneous origin of LECs in different organs

3.1 Discovery of LECs of different origins

Florence Sabin, an American anatomist, postulated already in 1902 that mammalian lymphatic vessels originate from the venous ECs.[111] Based on ink injection experiments in embryonic pigs, she proposed that two primitive jugular lymph sacs originate from ECs budding from large veins, which is followed by sprouting of vessels from the lymph sacs (the centrifugal model).[111] An alternative model by Huntington and McClure in 1910 proposed that LECs form from mesenchymal precursors (the centripetal model).[112] Based on the analysis of serial tissue sections, Huntington and McClure concluded that lymphatic vessels initially develop along embryonic veins and centripetal extensions are formed to connect them first with the lymph sacs and then with the venous system.[112] The debate on the two competing theories continued until more than 100 years later, in 2007, Srinivasan *et al.* provided convincing evidence in support of Sabin's theory of the venous origin of lymphatic vessels by genetic tracing of *Prox1* lineage cells.[113] The venous source of LECs was shown to be evolutionary conserved in teleosts based on studies in zebrafish.[114,115] Reports from avian embryos[116,117] and amphibians[118] suggested an additional mesodermal source of LECs. An alternative origin of LECs was also suggested in the mouse.[119] However, these studies were based on marker expression[118,119] or chimeric transplantation experiments,[116,117] and not lineage tracing, and functional evidence was lacking. In 2015, our group[14,15] and Klotz *et al.*[13] surprisingly found compelling evidence for at least two alternative non-venous sources of LECs in the mouse embryo, which contributed, alongside the venous-derived LECs, to the development of the lymphatic vasculature in an organ-specific manner (*Figure 3*). The new data could be seen as settling the old controversy concerning the origin of lymphatic vessels, but it also opens up many new questions.

3.2 Venous origin of lymphatic vessels

During the past decade, the stepwise process of LEC commitment, which is driven by Prox1 expression in a subset of venous EC, and

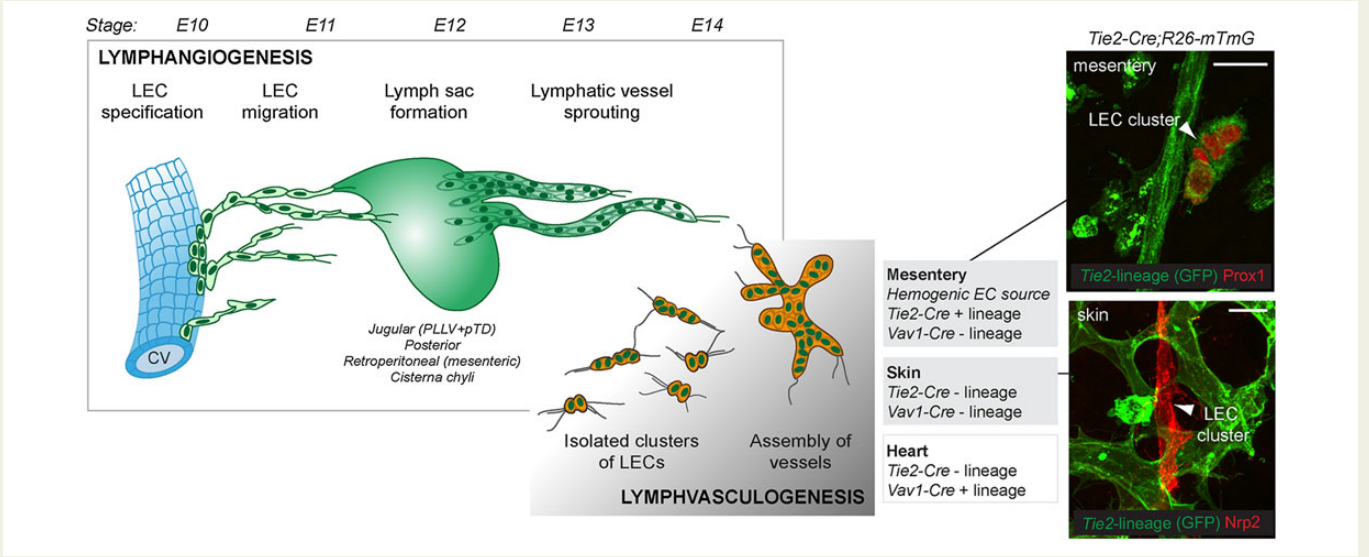

Figure 3 Key developmental steps in the formation of the embryonic lymphatic vasculature. The first LECs arise through transdifferentiation from venous endothelial cells. Venous-derived LECs exit the veins and form primitive lymphatic structures, so-called lymph sacs, from which vessels sprout further to peripheral organs (lymphangiogenesis). Alongside, in certain organs, non-venous-derived LEC progenitors give rise to clusters of LECs that assemble into vessels (lymphvasculogenesis). Stages (E, embryonic days) of mouse development are shown on the top and key differences in the cellular lineages of non-venous-derived LECs in the mesentery, skin, and heart are depicted. Mesenteric non-venous-derived LECs form from a HemEC source and are traced (GFP+) by the pan-endothelial *Tie2-Cre* (top panel on the right). In contrast, both the non-venous dermal LECs of lumbar skin (bottom panel on the right) and part of the cardiac lymphatic vasculature form from *Tie2* negative lineage (GFP-), and their precise cellular origin within the embryo still remains to be determined. Scale bars: 25 μm.

the formation of the first venous-derived lymphatic vessels have been carefully characterized in the mouse[113,120,121] and zebrafish.[114,115] Although some of the upstream regulators of Prox1 have been identified, it is still unclear how Prox1 expression and thus selection of LECs is restricted into specific veins to allow the formation of six primitive lymph sacs [i.e. paired jugular and posterior lymph sacs, retroperitoneal (mesenteric) lymph sac and cisterna chyli] in precise locations in the mammalian embryos. A recent study in zebrafish demonstrated that the commitment of LECs within the posterior cardinal vein (PCV) is linked with asymmetric division of Prox1 positive BECs, with daughter cells adopting different fates; one becomes LEC and up-regulates Prox1, while the other remains in the vein and down-regulates Prox1 expression.[122] Intriguingly, another recent study reported that the zebrafish PCV contains specialized mesodermal-derived angioblasts that can give rise to LECs but also to other endothelial cell populations.[123] These angioblasts were shown to reside on the ventral wall of the PCV and transit dorsally within the vein before giving rise to lymphatic sprouts.[123] Together, these data demonstrate a previously unknown heterogeneity within the developing cardinal vein and suggest that LEC fate can be specified before budding from the PCV. It remains to be shown whether mammalian veins also harbour such angioblasts with capacity to differentiate into LECs.

3.3 *Tie2* lineage negative, non-venous origin of the dermal lymphatic vasculature

Studies on the early development of the murine lymphatic vasculature have been focused on the analysis of the 'jugular lymph sac', i.e. the peripheral longitudinal lymphatic vessel (PLLV) and primordial thoracic duct (pTD)[120] formation in the cervical and thoracic regions of the embryo. Lineage tracing and high-resolution visualization of the developing vasculature in this region,[113,120] together with functional studies assessing the consequence of *Prox1* deletion using the endothelial restricted *Tie2-Cre* line,[113] suggested that all LECs descend from a *Tie2* lineage endothelial source, consistent with their venous origin. However, we reported that although the cervical and thoracic dermal LECs were efficiently traced with the *Tie2-Cre* in E13 embryos, isolated clusters of LECs forming in the lumbar region of the skin were negative[14] (*Figure 3*). Fluorescence-activated cell sorting analysis revealed that the *Tie2* lineage negative LECs accounted for more than 30% of the entire dermal lymphatic vasculature in E13 embryos. Consistent with the major contribution of non-venous-derived LECs to the vessels of the lumbar skin, LEC clusters in this region could not be initially targeted and depleted by *Tie2-Cre*-induced deletion of *Prox1*. This was despite the efficient *Tie2-Cre*-induced recombination in the blood vasculature and in early venous-derived LEC progenitors, which lead to the conclusion that an alternative *Tie2* lineage negative LEC progenitor source gives rise to the isolated clusters of LECs in the lumbar region of the skin.[14]

The isolated *Tie2* lineage negative non-venous-derived LEC clusters rapidly coalesced to continuous vessel networks in a process we termed lymphvasculogenesis,[14] analogous to the process of vasculogenesis occurring during early establishment of the blood vasculature system (reviewed in[124]). The source of the dermal non-venous LEC progenitors still remains to be discovered. Although lineage tracing data suggests a non-endothelial source of dermal LECs, it cannot be excluded that the *Tie2* negative lineage reflects vessel formation from a so far non-characterized immature vascular bed. Several studies have suggested a haematopoietic origin of lymphatic progenitors,[125–128] mainly based on the expression of shared markers, including, e.g. Pdpn, Lyve1, and Vegfr3, which are expressed by both LECs and haematopoietic cells including monocytes and macrophages. However, our studies excluded transdifferentiation of LECs from haematopoietic progenitors since *Vav1-Cre*, a pan-haematopoietic Cre line, did not show any tracing of dermal LECs.[14] This is consistent with previous studies that also excluded *Vav1-Cre* lineage tracing of LECs.[129]

3.4 Non-venous haemogenic endothelial source of the mesenteric lymphatic vasculature

Similar to the lumbar skin, mesenteric lymphatic vessels were reported to form from isolated clusters of LECs that assemble into vessels, in contrast to the sprouting of venous-derived LECs from the mesenteric lymph sac.[15] Intriguingly, in contrast to the dermal LEC progenitors, the mesenteric progenitors were efficiently traced by the *Tie2-Cre* transgene (Lukas Stanczuk and TM, unpublished data; *Figure 3*), which supports an endothelial origin of these LECs. However, rather than venous endothelium, the origin of the mesenteric LECs was traced to haemogenic endothelial cells (HemECs).[15] This conclusion was based on two major observations: (1) Positive lineage tracing using the endothelial-specific *Pdgfb-CreER*^{T2} line, which, when induced at embryonic day (E)8-E9, labels efficiently all major haemogenic vessels including the dorsal aorta, vitelline artery, and the yolk sac (YS) vasculature, but not the venous-derived LEC progenitors, and (2) positive lineage tracing of E10–E11-induced *cKit-CreER*^{T2}. cKit is a marker associated with HemECs and HemEC-derived haematopoietic progenitors from all known haemogenic sites of the embryo and the YS.[130] Using these strategies, it was not, however, possible to distinguish between the different haemogenic sites. Like in the skin, the mesenteric LECs could not be traced by *Vav1-Cre,* which suggests a HemEC-derived lineage separate from known haematopoietic lineages.

Whether distinct mechanisms differentially regulate lymphatic vessel formation from venous and non-venous LEC progenitors is largely unknown. Mahadevan *et al.*[131] reported that in the chick and mouse, gut lymphatic vessel formation relies on prior growth of arteries that coincides with the asymmetric gut tube looping and is restricted to the left side of the developing dorsal mesentery. Asymmetrically expressed Pitx2 drives expression of the Cxcr4 ligand Cxcl12 on the left side, thereby directing asymmetric arteriogenesis, and regulates mesenteric lymphatic vessel formation in an organ-specific manner. In particular, *Pitx2* deficiency was shown to specifically inhibit the formation of the mesenteric lymphatic vessels, but not the venous-derived mesenteric lymph sac, which was concluded to indicate the existence of an alternative non-venous-derived LEC progenitor population.[131] Organ-specific effects of genetic targeting of the Vegfr3/PI3K kinase pathway further indicated differences in the molecular regulation of mesenteric compared with dermal lymphatic vessel formation. Deletion of p110α catalytic subunit of the PI3K specifically in the LECs, or combined haploinsufficiency for genes encoding p110α and Vegfr3 resulted in organ-specific defects in the development of mesenteric and intestinal lymphatic vessels.[15] In addition, global deletion of the p110α regulatory subunits *p85α*, *p55α*, and *p50α* resulted in organ-specific lymphangiectasia of intestinal lymphatic vessels with less pronounced defects in other lymphatic vascular beds.[132]

Although the dermal and mesenteric non-venous LEC progenitors have distinct embryonic sources, they display similarities in marker expression. Non-venous-derived LECs can be differentiated by their initial low expression of Lyve1 both in the mesentery and skin.[14,15] Lyve1

is, however, quickly up-regulated as the clusters assemble, and the non-venous-derived LECs share expression of several markers of venous-derived LECs, including Vegfr2, Vegfr3, Prox1, Nrp2, and Pdpn. Also Tie2, which is initially not expressed in the dermal LEC clusters, is induced as the vessels assemble and mature.[14]

3.5 Non-venous origin of cardiac LEC progenitors

Klotz *et al.* reported that part of the lymphatic vasculature in the heart, similar to the dermal LEC clusters, could not be traced by the *Tie2-Cre* transgene.[13] This suggests that local blood vessels, which are presumably efficiently targeted by the *Tie2* transgene, are unlikely to provide a source of cardiac LECs, but they are instead derived from a non-endothelial cell origin or a *Tie2* negative vascular bed. Based on positive lineage tracing with the haematopoietic *Vav1-Cre* and E7 induced *Csf1r1*^{MeriCreMer}, YS HemECs were suggested as the source of cardiac LECs.[13] It was previously shown that early induction of *Csf1r1*^{MeriCreMer} labels erythro-myeloid progenitors (EMPs), which are the major haematopoietic progenitors produced in the YS.[133,134] However, YS-derived EMPs can also be labelled in *Tie2* ^{MeriCreMer} by induction at E7.5,[134] and it would thus be expected that YS-derived haematopoietic progenitors and their progeny, in contrast to the non-venous-derived cardiac LECs, are similarly targeted by the constitutive *Tie2-Cre* transgene. Supporting this notion, constitutive *Tie2-Cre* labels the majority of CD11b+ myeloid cells present in the E10 embryo which at this stage mainly originate from the YS.[135] Further evidence for cardiac LEC origin was provided by lineage tracing by *Pdgfrb-Cre*, which was postulated to support a HemECs YS-derived lineage.[13] This was based on an earlier report of Pdgfrβ immunoreactivity in the YS haemogenic endothelium,[136] which is in contrast to the absence of Pdgfrβ expression in other BECs.[137] Our recent data, however, indicate that the *Pdgfrb-Cre* transgene does not significantly label YS haemogenic endothelium or YS-derived haematopoietic progenitors[135] and the conclusion on the *Pdgfrb-Cre*-induced tracing of LECs as a proof for a YS-specific lineage of non-venous-derived cardiac LEC progenitors may need re-evaluation. Based on the available data, cardiac non-venous LECs must thus be concluded to derive from a specific haematopoietic *Vav1* positive cell lineage with a link to an early YS *Csf1r1* lineage originating from a unique, previously unknown *Tie2* negative cell population. However, as discussed above, *Tie2* negative lineage tracing may alternatively indicate a non-endothelial rather than endothelial origin of cardiac LECs. Klotz *et al.* excluded several non-endothelial sources, including the WT1$^+$ pro-epicardial organ, Mesp1$^+$ or Nkx2.5$^+$ mesoderm and Wnt1$^+$ neural crest,[13] and the precise identity of the cardiac LEC progenitor thus remains to be determined.

4. Concluding remarks

Reducing or increasing lymphatic vessel density and the lymphangiogenic response have been put forward as possible therapeutic tools to modulate inflammatory responses and cancer metastasis. However, in light of our new understanding of lymphatic diversification in the regulation of vessel- and vascular bed-specific functions, new strategies to tune lymphatic responses in disease by regulating the molecular patterning of vessels may prove to be as important as regulation of vessel density alone. However, we still lack an adequate understanding of the organ- and vessel-type-specific molecular differences of endothelium, how they contribute to specific functions of lymphatic vessels, and how the establishment and maintenance of these features are regulated.

The discovery of (an) alternative non-venous origin(s) of LECs that contribute to the lymphatic vasculature of the skin, mesentery, and heart has changed our understanding of the mechanisms of embryonic lymphatic vessel formation. Intriguingly, the source of the non-venous LEC progenitors appears to be different in different organs, potentially contributing to the development of organ-specific features within the lymphatic network. We currently lack tools that allow isolation of non-venous-derived LEC progenitors or their specific tracing in embryonic and postnatal tissues. These tools will be important for assessing the contribution of different types of LEC progenitors to specific vascular beds and for studying their role in lymphatic vessel homeostasis, regeneration, and organ-specific molecular adaptation.

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