

Organ-specific lymphatic vasculature: From development to pathophysiology

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Recent discoveries of novel functions and diverse origins of lymphatic vessels have drastically changed our view of lymphatic vasculature. Traditionally regarded as passive conduits for fluid and immune cells, lymphatic vessels now emerge as active, tissue-specific players in major physiological and pathophysiological processes. Lymphatic vessels show remarkable plasticity and heterogeneity, reflecting their functional specialization to control the tissue microenvironment. Moreover, alternative developmental origins of lymphatic endothelial cells in some organs may contribute to the diversity of their functions in adult tissues. This review aims to summarize the most recent findings of organotypic differentiation of lymphatic endothelial cells in terms of their distinct (patho)physiological functions in skin, lymph nodes, small intestine, brain, and eye. We discuss recent advances in our understanding of the heterogeneity of lymphatic vessels with respect to the organ-specific functional and molecular specialization of lymphatic endothelium, such as the hybrid blood-lymphatic identity of Schlemm's canal, functions of intestinal lymphatics in dietary fat uptake, and discovery of meningeal lymphatic vasculature and perivascular brain lymphatic endothelial cells.

Introduction

Rich lymphatic vessel (LV) networks supply the skin dermis and mucosal membranes covering major organs, including the respiratory tract, nasopharyngeal cavity, intestine, mesentery, diaphragm, heart, and lung. LVs are lacking or very sparse in bone, bone marrow, adipose tissue, heart myocardium and skeletal muscles, and parenchymal tissues of brain, liver, kidney, and endocrine organs, such as the adrenal or thyroid gland. Presumably, these organs are devoid of LVs because of scarce interstitial fluid or the presence of an alternative drainage system, such as fenestrated blood vessels (BVs). Interstitial fluid is drained into specialized blind-ended lymphatic capillaries, which connect and converge into gradually larger collecting LVs and lymphatic ducts that empty into the subclavian vein. Lymphatic endothelial cells (LECs) of lymphatic capillaries are surrounded by a thin, discontinuous basement membrane, lack perivascular cells, and have discontinuous “button-like” cell junctions (Baluk et al., 2007). They readily sense changes in interstitial pressure via specialized anchoring filaments, which can modulate the opening of “flap valves” in-between the button junctions to allow fluid entry. It is also through these flap valves that immune cells enter lymphatic capillaries. Unidirectional lymph flow in collecting vessels is promoted by numerous intraluminal valves and coordinated contraction of LV smooth muscle cells (SMCs; Schulte-Merker et al., 2011; Sabine et al., 2016). LECs represent a distinct endothelial cell (EC) lineage, and

LVs are frequently distinguished from BVs based on their expression of the transcription factor prospero homeobox-1 (Prox1), transmembrane O-glycoprotein podoplanin (also known as gp38), vascular endothelial growth factor receptor 3 (VEGFR3; also known as Flt4), neuropilin-2, and lymphatic vessel endothelial hyaluronan receptor-1 (LYVE1; Tammela and Alitalo, 2010; Alitalo, 2011; Aspelund et al., 2016).

LVs have traditionally been regarded as passive conduits for fluid and some immune cells, but this perspective has been enormously updated with the discovery of novel structures, origins, and functions of LVs in several organs. Organ-specific lymphatic capillary LECs display remarkable heterogeneity and plasticity, and acquire specialized functional properties adapted to the local microenvironment. Understanding organotypic LEC differentiation and function can help in designing more effective therapeutic and regenerative strategies to cure a wider spectrum of common human diseases in which LVs have been shown to play major roles (Tammela and Alitalo, 2010; Alitalo, 2011; Aspelund et al., 2016). Advances in general physiology and pathology, development, lymphedema, and tumor lymphangiogenesis are already covered by excellent recent reviews (Tammela and Alitalo, 2010; Alitalo, 2011; Mortimer and Rockson, 2014; Aspelund et al., 2016; Dieterich and Detmar, 2016; Ulvmar and Mäkinen, 2016; Potente and Mäkinen, 2017). In this review, we aim to summarize the latest results and their significance in under-

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standing organ-specific lymphatic vasculatures, and highlight the key characteristics and uniqueness of their structure and functions in development, homeostasis, and diseases.

Developmental origins of organ-specific LVs

Since the discovery of VEGF-C and its receptor, VEGFR3, as the key lymphangiogenic growth factor pathway and the transcription factor Prox1 as the master of LEC specification, the development of lymphatic vasculature has been extensively studied and characterized in mouse and zebrafish models (Escobedo and Oliver, 2016; Venero Galanternik et al., 2016). The majority of LECs are produced by transdifferentiation from venous endothelium, when a subpopulation of venous ECs expressing Prox1 is specialized into LECs (Escobedo and Oliver, 2016; Venero Galanternik et al., 2016). Importantly, recent lineage-tracing studies highlighted an unexpected diversity of LEC origins in several organs. In skin, cervical and thoracic LVs are formed by lymphangiogenic sprouting of Tie2-lineage⁺ venous-derived LEC progenitors (Martinez-Corral et al., 2015). In contrast, most lumbar LVs are formed via the process of coalescence and vessel network formation (so-called lymphovasculogenesis) by isolated Tie2-lineage⁻ nonvenous LEC progenitors. Although the hematopoietic origin of LECs in such clusters has been conclusively ruled out (Martinez-Corral et al., 2015), the precise precursor cell type remains to be established. Similarly, during cardiac development, a proportion of LECs in the heart was shown to descend from Tie2-lineage⁻ nonvenous LEC progenitors (Klotz et al., 2015). Moreover, mesenteric LVs originate from two distinct LEC sources: Tie2⁺ venous ECs and isolated EC clusters arising from PDGFB⁺ and c-Kit⁺ hemogenic ECs (Stanczuk et al., 2015). During lymph node (LN) development, all stromal cells, including LECs, have been suggested to arise from nestin⁺ precursors (Koning et al., 2016). Nestin is a marker of mesenchymal stem cells, but it is also expressed by a variety of other cells types, including ECs; therefore, the precise origin of LN LECs remains to be fully investigated.

Prox1 and VEGF-C/VEGFR3/ADAM metalloproteinase with thrombospondin type 1 motif 3/collagen and calcium-binding EGF domains 1 are obligate molecular components that define LEC fate and LV expansion in all organs; however, there are important organ-specific differences in downstream pathways. For example, blockade of PI3K signaling, which acts downstream of activated VEGFR3, selectively affected the development of mesenteric, but not dermal, LVs (Stanczuk et al., 2015). Thus, the origins and heterogeneity of LECs in each organ are more diverse than previously expected, and further exciting findings on this topic are anticipated in the next decade.

Skin lymphatic vasculature

The skin is the body's first line of defense against a hostile environment, including pathogens that can enter upon skin injury. Although the epidermis is avascular, the skin dermis is

rich in BVs and LVs (Fig. 1 A). Dermal LVs are the main conduits for pathogens and immune cells from the periphery to draining LNs. They also play a major role in reverse cholesterol transport by transporting high-density lipoprotein-bound cholesterol from peripheral tissues to systemic blood circulation to regulate cholesterol metabolism (Fig. 1 B; Lim et al., 2013; Martel et al., 2013; Randolph and Miller, 2014). The migration rate of dendritic cells (DCs) and T lymphocytes via dermal afferent LVs is dramatically increased during the peak of inflammation (Fig. 1 C; Kim et al., 2014; Hunter et al., 2016). The majority of immune cells in the lymph of afferent LVs are T cells (80–90%) and DCs (10–15%; Hunter et al., 2016). CD4⁺ T effector memory cells are the main subset that migrates via dermal afferent LVs at steady state, as well as in inflammation, and Foxp3⁺CD4⁺ regulatory T cells (T reg cells) constitute up to 25% of the intralymphatic T cell population (Brinkman et al., 2016; Hunter et al., 2016). Tissue T reg cells are important regulators of dermal lymphatic function and repair LVs in inflammation and lymphedema (Gousopoulos et al., 2016); it is unclear whether T reg cells also contribute to maintaining normal LV integrity.

Cellular and molecular events during immune cell migration via dermal LVs have been extensively studied and reviewed (Randolph et al., 2017). C-C motif chemokine ligand 21 (CCL21) produced by LECs is required for DC and T cell trafficking in skin LVs, and likely in most LVs of other organs (Girard et al., 2012; Randolph et al., 2017). LECs secrete CCL21 abluminally and lumenally, and extracellular CCL21 gradients guide both recruitment and intraluminal directional crawling of DCs (Russo et al., 2016; Randolph et al., 2017). In addition, sphingosine-1 phosphate (S1P) signaling via S1P receptor 1 (S1PR1) regulates a T cell's decision to egress or remain in tissue (Baeyens et al., 2015). It is unknown whether S1P production by dermal LECs is as important for regulation of tissue lymphocyte egress as shown previously for LN LECs (Pham et al., 2010). Vascular cell adhesion molecule 1 (VCAM-1), expressed by inflamed LECs, promotes T cell and DC entry into LVs (Brinkman et al., 2016; Randolph et al., 2017; Teijeira et al., 2017). T reg cells produce especially high levels of lymphotoxin- α 2 β 1, which engages lymphotoxin receptor- β on LECs to promote cell transmigration (Brinkman et al., 2016). Subsequent intralymphatic T cell adhesion and crawling within capillaries are supported by intercellular adhesion molecule 1 (ICAM-1) on LECs and integrin lymphocyte function-associated antigen-1 on T cells (Teijeira et al., 2017). In addition to CCL21, C-X3-C motif chemokine ligand 1 and C-X-C motif chemokine ligand 12 are secreted from LECs to guide DC and other innate immune cells trafficking into inflamed LVs (Fig. 1 C; Kabashima et al., 2007; Johnson and Jackson, 2013). The spectrum of chemokines and adhesion receptors produced by dermal LECs differ depending on the nature of inflammatory stimuli; for example, ICAM-1, CXCL9, and CXCL10 are strongly induced in LECs in a contact hypersensitivity model, but not during innate immune response elicited by complete Freund's adjuvant

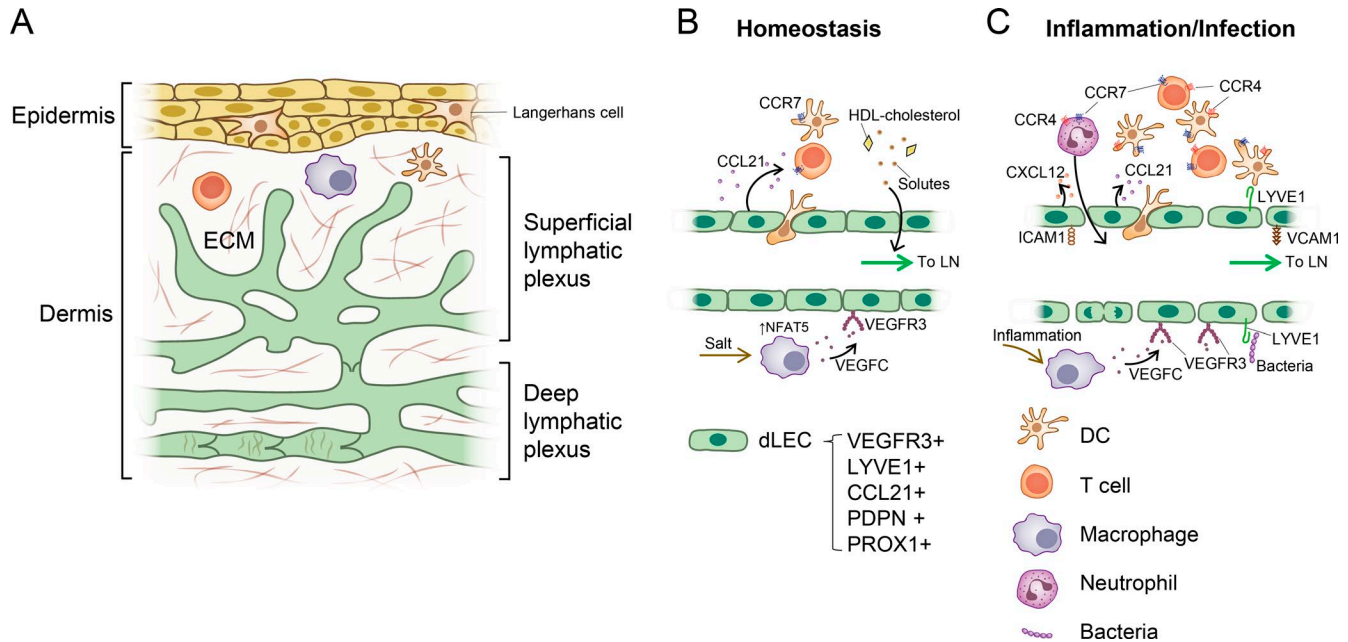


Figure 1. Organization and function of dermal lymphatic vasculature. (A) Skin LVs are organized in superficial and deep lymphatic plexuses. Superficial LVs are mostly capillaries, whereas deep lymphatic plexus contain collecting LVs draining to the regional LNs. ECM, extracellular matrix. (B) CCR7⁺ CD4⁺ T cells, Langerhans cells (specialized skin DCs residing in epidermis), and DCs are the main immune populations trafficking via dermal LVs in response to CCL21 gradient produced by capillary LECs. Dermal macrophages sense tissue osmotic pressure and maintain tissue fluid homeostasis and systemic blood pressure by activating transcription factor NFAT5 and producing VEGF-C, which induces expansion of dermal LVs and enhances tissue clearance. LVs also play a major role in reverse cholesterol transport through recirculation of high-density lipoprotein (HDL)-bound cholesterol from the interstitial space. dLEC, dermal LEC. (C) During bacterial infection, neutrophils, extravasated from BVs, also migrate through LVs to LNs to enhance adaptive immune response. Inflamed LECs produce adhesion receptors, such as VCAM-1 and ICAM-1, and chemokines (e.g., CXCL12) that enhance the attraction, adhesion, and intralymphatic crawling of immune cells. Lymphatic capillary hyaluronan receptor LYVE1 mediates interaction of LECs with hyaluronan-coated DCs and with the hyaluronan-containing capsule of some skin bacterial pathogens, such as GAS.

(Vigl et al., 2011). Such differential LEC responses are likely important for temporal coordination of tissue retention versus egress of specific immune cell populations during inflammation. Lately, a novel role of LYVE1 in DC transmigration has been uncovered (Johnson et al., 2017). In inflamed dermal interstitium, DCs are coated with hyaluronan and interact with LYVE1 on lymphatic capillary LECs, mediating docking between the two cells within discrete “transmigratory cups” that envelop transiting DCs, thereby facilitating DC transmigration into LVs (Fig. 1 C; Johnson et al., 2017). Bacterial hyaluronic acid capsule interaction with LEC LYVE1 is also involved in the spread of a common skin-resident pathogen group A streptococcus (GAS) from the infection site to the regional draining LN (Fig. 1 C; Lynskey et al., 2015). Lymphatic spread of GAS can be detrimental for the host, as it causes lymphangitis and lymphadenitis. However, blocking LN accumulation of GAS in *Lyve1*^{-/-} mice increased systemic dissemination of bacteria in blood circulation (Lynskey et al., 2015), indicating that high levels LYVE1 on LECs may be important for pathogen containment.

Neutrophil egress into dermal LVs is minimal both in the steady-state condition and in sterile inflammation. However, during bacterial infection, neutrophils are the first to

migrate from inflamed skin to draining LN, where they stimulate lymphocyte proliferation (Hampton et al., 2015). Neutrophil migration relies on lymphocyte function-associated antigen 1, an integrin/complement receptor CD11b and C-X-C or C-C chemokine receptors CXCR4 and/or CCR7 (Fig. 1 C; Gorlino et al., 2014; Hampton et al., 2015; Arokiasamy et al., 2017). Dermis also contains an abundant population of macrophages, which contributes to local control of lymphangiogenesis and lymphatic remodeling during development, inflammation, and wound healing (Harvey and Gordon, 2012; Kim et al., 2014). In adult skin, macrophages play a unique role in the regulation of salt-sensitive hypertension: in response to increased local osmotic stress, skin macrophages activate nuclear factor of activated T cell 5-dependent production and secretion of VEGF-C (Fig. 1 C). The resulting expansion of cutaneous lymphatic network enhances interstitial electrolyte clearance, reduces peripheral tissue fluid pressure, and restores homeostasis (Machnik et al., 2010; Wiig et al., 2013).

Lymphatic transport is important for normal skin homeostasis and is implicated in multiple skin inflammatory diseases, including atopic and contact dermatitis and psoriasis. The density and function of dermal LVs gradually re-

duces with age, which may contribute to the development of age-related skin disorders (Karaman et al., 2015). Impairment of dermal LV function leads to lymphedema, hyperpigmentation, keratosis, and papillomatosis (Carlson, 2014). Studies of animal models of chronic skin inflammation demonstrated that blocking LV expansion exacerbates the disease, whereas stimulation of lymphangiogenesis by VEGF-C administration reduces it (Huggenberger et al., 2010).

Meningeal lymphatic vasculature

The brain parenchyma lacks LVs and instead has an alternative but essential fluid drainage system, the so-called glymphatic system (Iliff et al., 2012). The glymphatic system is named for its dependence on glial cells for a draining function similar to the lymphatic system. The glymphatic system promotes waste clearance from the brain by expediting the flux of cerebrospinal fluid across the brain parenchyma, which is ~60% more efficient during sleep (Iliff et al., 2012). However, recent re-discovery of the mouse brain meninges lymphatic networks raised exciting questions and intriguing concepts (Aspelund et al., 2015; Louveau et al., 2015), and similar structures were also found in autopsy specimens of human meninges (Absinta et al., 2017). Importantly, high-resolution, clinical magnetic resonance imaging technique has enabled the visualization of human meningeal lymphatic networks (Absinta et al., 2017). In fact, the existence of lymphatic vasculature on the surface of the human brain had been proposed two centuries ago and displayed in anatomical wax model collections (the Josephinum Wax Models Museum, Vienna, Austria; Lukić et al., 2003), but it has been mostly ignored. Most meningeal LVs are located on the meningeal layer (also called “dura mater”) and are phenotypically similar to lymphatic capillaries in other organs (Fig. 2; Aspelund et al., 2015). However, valves are present only in LVs near the base of the skull alongside the cranial nerves. Activation of VEGFR3 by VEGF-C increases the diameter of meningeal LVs, and blocking VEGFR3 signaling completely abrogates meningeal LVs, indicating that these vessels, like peripheral LVs, are regulated by VEGFR3 (Aspelund et al., 2015). A recent study (Antila et al., 2017) demonstrated that VEGF-C–VEGFR3 signaling is crucial not only for development but also for maintenance of meningeal LV integrity during adulthood. Functionally, some cerebrospinal fluid and interstitial fluid of brain parenchyma flow through glymphatic system, drain into meningeal LVs, and reach the deep cervical LNs. Intriguingly, a significant decline in cerebrospinal fluid outflow via meningeal LVs was observed in aged mice (Ma et al., 2017), implying that meningeal lymphatic networks could be targeted for age-associated neurological conditions. Further research is required to map out the connections among this newly uncovered lymphatic circuit, the glymphatic system, and the rest of the lymphatic circuitry throughout the body (Louveau et al., 2017). It would also be intriguing to determine whether cerebrospinal fluid drainage into meningeal LVs also increases during sleep. Moreover, it is reasonable to speculate that impairment in this

lymphatic circuit could be related to brain edema, which is often detected during ischemia, concussions, inflammation, and brain tumors. Furthermore, it will be important to establish whether meningeal LVs are involved in the clearance of amyloid β and other misfolded proteins from the brain parenchyma, which are frequently and highly accumulated in patients with neurodegenerative diseases such as Alzheimer’s and Parkinson’s diseases.

The brain has also been regarded as an immune-privileged organ, in part because of the dearth of LVs. It was thought that infiltrated T cells in the brain exit through venous rather than lymphatic circulation, which is the case in all other organs (Ransohoff and Engelhardt, 2012). However, a large population of T cells was observed in the lumen of meningeal LVs, raising the intriguing possibility of an alternative gateway for T cells to egress from the brain to the deep cervical LNs (Louveau et al., 2015). Dissecting the lymphatic route for brain-resident T cells will advance our understanding of the communication between the central nervous system and the immune system and unveil new therapeutic targets for inflammatory neurological disorders, including encephalomyelitis and multiple sclerosis (Louveau et al., 2017). Another question requiring further investigation is whether meningeal LVs can act as a route for brain cancer metastasis. Thus, the re-discovery of meningeal LVs reveals new concepts regarding brain functions and diseases.

Brain mural LECs (muLECs)

In 2017, three research groups (Bower et al., 2017; van Lessen et al., 2017; Venero Galanternik et al., 2017) independently identified a novel population of isolated muLECs (also called mannose receptor-1⁺ perivascular cells) surrounding meningeal BVs using transgenic zebrafish models (Fig. 2). The muLECs express LEC markers such as LYVE1 and Prox1, and a perivascular macrophage marker, mannose receptor-1, but, unlike all other ECs, they do not form a lumenized vessel. muLECs are uniquely found on the inner side of the meninx, adjacent to the brain parenchyma. They originate from the lymphatic endothelium in the midbrain or the optic choroïdal vascular plexus by sprouting lymphangiogenesis during brain development, and differentiate into a dispersed, nonlumenized mural lineage. Similar to other LECs, muLEC development requires signaling through the VEGF-C–VEGF-D–collagen and calcium-binding EGF domains 1–VEGFR3 pathway. Mature muLECs are relatively large mural cells that produce vascular growth factors and accumulate low-density lipoproteins from the bloodstream. Although muLECs seem to be important for meningeal vascularization, they are dispensable for the maintenance of vessel integrity. These studies identified a novel lymphatic lineage that differentiates into mural cell-like cells that are necessary for establishing normal meningeal blood vasculature, and have raised the possibility that an equivalent cell type exists in the mammalian brain (Bower et al., 2017; van Lessen et al., 2017; Venero Galanternik et al., 2017). Although the three groups did not have

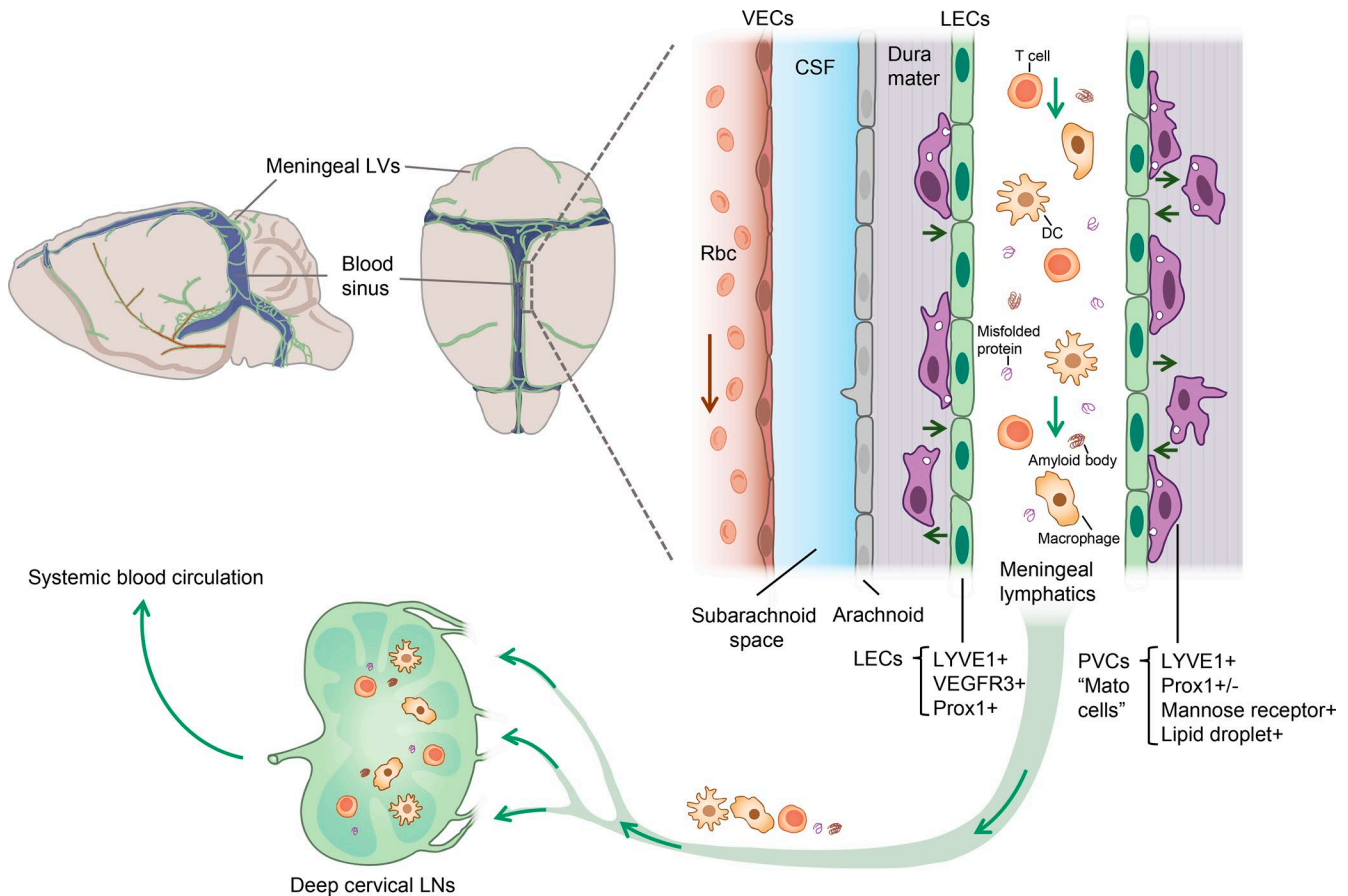


Figure 2. **Meningeal lymphatic vasculature and brain mural LECs.** Localization and distribution of LVs in dura mater of mouse brain. Cerebrospinal fluid (CSF) and interstitial fluid of brain parenchyma drain into meningeal LVs and reach the deep cervical LNs. Trafficking of brain immune cells, including T cells, DCs, and macrophages, and clearance of neurotoxic misfolded proteins and peptides, such as amyloid β , are among the potential important functions of meningeal LVs. A unique, LV-derived population of muLECs surrounding brain BVs was recently found in zebrafish. muLECs express Prox1, LYVE1, and mannose receptor 1 (MR1), and are highly endocytotic. Unlike all other EC, muLECs do not form cell-cell junctions or lumens. Whether muLECs are equivalent to perivascular Mato cells (LYVE1⁺, Prox1⁻, MR1⁺, and lipid droplet⁺), found in mammalian brain, remains to be established. Perivascular Mato cells are also detected in surrounding meningeal LVs.

the same consensus opinion, it is tempting to speculate that muLECs may be the equivalent of lipid-laden cells, perivascular macrophages, fluorescent granular perithelial cells, or 'Mato Cells' (Mato et al., 1981, 1996; Williams et al., 2001) that take up lipids and low-density lipoproteins from meningeal BVs in mammals. In fact, muLECs become elongated and accumulate a larger amount of lipid droplets after acute high-cholesterol diet. Moreover, they are able to take up and clear macromolecules through mannose receptors (van Lessen et al., 2017; Venero Galanternik et al., 2017). Further comparative investigation between fish and mammals is required to understand the roles and significance of muLECs.

Schlemm's canal (SC): Lymphatic intermediate and glaucoma

SC is an endothelium-lined channel that encircles the cornea and provides unique vascular route for aqueous humor out-

flow, which constantly refreshes the anterior chamber of eye (Fig. 3 A). Aqueous humor, continuously produced by the ciliary body, flows into the anterior chamber, and is drained into the aqueous and episcleral veins through trabecular meshwork and SC. SC has morphological, molecular, and functional similarities with LVs. In 2014, several independent research groups (Aspelund et al., 2014; Kizhatil et al., 2014; Park et al., 2014; Truong et al., 2014) found that SC represents a new intermediate vessel type, which expresses LEC markers Prox1, VEGFR3, and integrin $\alpha 9$, but not LYVE1 and podoplanin. The primitive SC is formed by blood ECs sprouting from the choroidal vein, and SC ECs are postnatally respecified to acquire lymphatic phenotypes through up-regulation of Prox1, which also appears to act as a molecular biosensor for aqueous humor outflow (Park et al., 2014). Importantly, Tie2 is expressed before Prox1 in SC ECs and is maintained at a high level to critically regulate SC integrity during adulthood

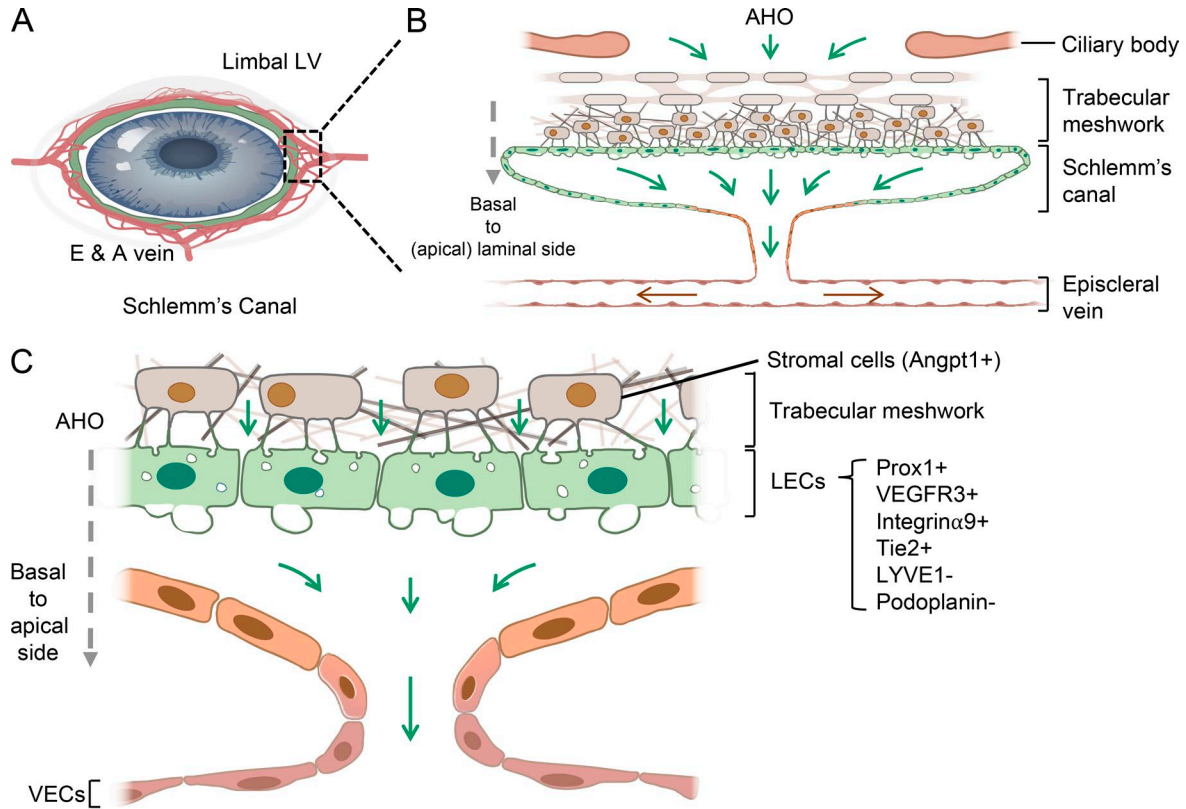


Figure 3. Schlemm's canal. (A) SC is an endothelium-lined channel that encircles the cornea and provides an exit route for aqueous humor. (B) Aqueous humor is produced from the ciliary body and drained into aqueous and episcleral veins through the trabecular meshwork and SC. (C) Aqueous humor is drained transcellularly and transported from the basal to luminal side through SC ECs, causing formation of giant vacuoles. SC ECs have an intermediate blood-lymphatic EC phenotype and express Prox1, VEGFR3, Tie2, and integrin α 9, but not LYVE1 or podoplanin. Angpt1⁺ stromal cells adjacent to the SC LECs may produce proteins of trabecular meshwork. When SC function is impaired, aqueous humor drainage is impeded and intraocular pressure is increased, ultimately leading to glaucoma. Angpt-Tie2 signaling maintains SC integrity, and loss of such signaling induces primary congenital and open-angle glaucoma. AHO, aqueous humor outflow; E & A vein, episcleral & aqueous vein; VEC, venous endothelial cell.

(Kim et al., 2017). Thus, SC ECs seem to originate from Tie2⁺ venous EC progenitors (Aspelund et al., 2014; Kizhatil et al., 2014; Park et al., 2014). Another important regulator of SC is VEGF-C/VEGFR-3 signaling, which is critical for the formation and differentiation of SC. Surprisingly, supplementation of VEGF-C can also induce SC growth in adults, leading to a reduction of intraocular pressure in the aged glaucoma model (Aspelund et al., 2014).

Aqueous humor is rapidly drained transcellularly through SC ECs causing formation of giant vacuoles (Fig. 3, B and C). When SC is impaired, aqueous humor drainage is impeded and intraocular pressure is increased, ultimately leading to glaucoma (Jonas et al., 2017). In this regard, glaucoma induced by defective SC could also be regarded as 'eye lymphedema'. However, the pathogenesis involving SC defects in glaucoma is still poorly understood. Intriguingly, primary congenital glaucoma phenotypes were detected in mouse models of double *Angpt1/Angpt2* deletions or *Tie2* deletion during postnatal periods, highlighting the impor-

tance of the angiopoietin (Angpt)-Tie2 system in SC development (Thomson et al., 2014). In fact, *Tie2* mutations have been identified in patients with primary congenital glaucoma (Souma et al., 2016). Nevertheless, although the incidence rate of primary congenital glaucoma is low, primary open-angle glaucoma is frequently observed in the elderly. A recent study (Kim et al., 2017) showed that double *Angpt1/Angpt2* deletions or *Tie2* deletion in adult mice severely impairs SC integrity and transcellular aqueous humor fluid transcytosis, leading to elevated intraocular pressure, retinal neuron damage, and impairment of retinal ganglion cell function, which are all hallmarks of primary open-angle glaucoma. Accordingly, Tie2 reactivation using a Tie2 agonistic antibody relieved the phenotype in double *Angpt1/Angpt2*-deleted mice and rejuvenated the SC in aged mice (Kim et al., 2017). These findings provide not only a novel molecular pathway in understanding pathogenesis of primary open-angle glaucoma but also a new therapeutic avenue for its treatment.

Sinusoidal LVs in LNs

LNs are highly dynamic secondary lymphoid organs where antigens, together with costimulatory signals, are delivered by afferent LVs (Fig. 4 A). LN LVs are extended lymphatic networks from peripheral afferent LVs, which continue to form the subcapsular sinus (SCS), stretch into the medullary sinus, and ultimately exit as efferent LVs. LVs traverse through densely packed aggregations of immune cells, predominantly B and T cells and such architecture facilitates intimate interaction between LN LVs and immune cells, directly influencing immune responses. Thus, LN LVs efficiently transport antigens and innate immune cells from various organs to naive lymphocytes in LNs, which is one of the crucial steps for the initiation and regulation of adaptive immune response as well as for the maintenance of immune tolerance (Junt et al., 2008; Randolph et al., 2017). During the acute phase of local tissue inflammation, robust lymphangiogenesis, stimulated by VEGF-A, C and D secreted from infiltrated, activated macrophages, occurs in the draining LN, and subcapsular LN LVs proliferate and penetrate deep into the cortex (Kataru et al., 2009). In this situation, activated B cells also contribute to LN lymphangiogenesis to promote dendritic cell (DC) mobilization from the inflamed tissue to LN (Angeli et al., 2006). As shown in helminth infection model, VEGF-A and VEGF-C production by B cells and mesenteric LN lymphangiogenesis relies on lymphotoxin-dependent feed-forward cross-talk of B-cells and surrounding follicular reticular cells (Dubey et al., 2017). Interferon- γ secreted from activated T cells can be a negative but balancing regulator that suppresses LN lymphangiogenesis during inflammation resolution (Kataru et al., 2011).

LN LECs produce several factors that regulate leukocyte trafficking, delivery of inflammatory signals, modulation of T cell activation, and development of secondary lymphoid organs. LECs regulate the exit of immune cells via efferent LVs by secreting S1P, generated by sphingosine kinases with the S1P lyase enzyme, creating an S1P gradient important for immune cell egress through efferent LVs (Fig. 4 A; Pham et al., 2010). In addition to its role in T cell trafficking, LN LEC-derived S1P promotes naive T cell survival by sustaining their mitochondrial function and oxidative phosphorylation (Mendoza et al., 2017). Integrin $\alpha 9 \beta 1$ on LECs is also necessary for LN lymphocyte egress, and the interaction between integrin $\alpha 9 \beta 1$ and its ligand tenascin-C has been shown to enhance the production of S1P by LECs (Ito et al., 2014). LN LECs are also an important source of interleukin-7, critical for lymphocyte expansion during LN remodeling (Onder et al., 2012). Interleukin-7 expression by LECs also provides a niche for memory T-helper cells in inducible bronchus-associated lymphoid tissues during allergic inflammation in lung (Shinoda et al., 2016). LN LECs produce immunosuppressive factors, such as TGF- β , indoleamine-2,3-dioxygenase, and nitric oxide to maintain peripheral tolerance to self-antigen in lymph (Fig. 4 B; Lukacs-Kornek et al., 2011; Malhotra et al., 2012). Of note, LN LECs directly express peripheral

tissue antigens and induce deletional tolerance in CD8⁺ T cell via PD-1–PD-L1 or LAG-3–MHC-II pathways (Rouhani et al., 2015). Moreover, a subset of LECs was shown to provide antigens to DCs, which then induced CD4⁺ T cell anergy (Rouhani et al., 2015). On the other hand, Dubrot et al. (2014) also reported that LECs can acquire preloaded peptide–MHC-II complexes from DCs to induce CD4⁺ T cell tolerance. Overall, LN LECs play multiple tolerogenic roles against self-antigens by directly presenting a variety of peripheral tissue antigens for CD8⁺ T cell deletional tolerance, whereas they also induce CD4⁺ T cell tolerance via antigen transfer to or from DCs (Fig. 4 B).

Tumors produce lymphangiogenic growth factors, tumor antigens, and premetastatic signals to tumor draining LNs even before LN metastasis (Karaman and Detmar, 2014; Ogawa et al., 2014; Dieterich and Detmar, 2016). COX-2/PGE₂–EP3-dependent induction of VEGF-C and VEGF-D in macrophages and DCs induces tumor-induced LN lymphangiogenesis (Ogawa et al., 2014). Interestingly, LECs of tumor draining LNs are capable of scavenging and cross-presenting tumor antigens, which induce dysfunction of CD8⁺ T cells (Lund et al., 2012). Tumor and tumor draining LN-derived VEGF-C promotes these processes and accelerates metastasis (Lund et al., 2012). In addition to follicular DCs, proliferating SCS LECs can capture or act as a reservoir for persistent viral antigens, contributing to the development of an effective memory CD8⁺ T cell pool with increased effector function and protective capacity (Fig. 4 B; Tamburini et al., 2014). This beneficial role of LN LECs can be exploited to improve efficacy of vaccines against viral infections.

SCS LECs and medullary sinusoidal LECs have distinct features including cellular organization, expression profiles, roles, and responses to inflammation (Fig. 4 A; Iftakhar-E-Khuda et al., 2016). For instance, macrophage scavenger receptor 1 is selectively expressed by SCS LECs and regulates the binding and transmigration of lymphocytes entering from peripheral tissues into LN parenchyma, whereas endomucin produced by medullary sinusoidal LECs presumably regulates lymphocyte trafficking via L-selectin (Iftakhar-E-Khuda et al., 2016). Medullary sinusoidal LECs also produced the highest levels of peripheral tissue antigens and PD-L1 and therefore play a key role in the deletion of alloreactive CD8⁺ T cells (Cohen et al., 2014). SCS LECs, but not peripheral LECs, express plasmalemma vesicle-associated protein (also known as MECA-32; Rantakari et al., 2015). The SCS floor allows the entrance of only small (<70 kD and <4 nm) solutes, which are then rapidly transported through the LN parenchyma to BVs by conduits, a specialized tubular meshwork formed by extracellular matrix from follicular reticular cells. Plasmalemma vesicle-associated protein plays a central role in such selective barrier function of subcapsular sinusoids by forming molecular “sieves” or diaphragms covering transendothelial channels in SCS LECs, which then prevents the passage of larger solutes to follicular reticular cell conduits (Fig. 4 A; Rantakari et al., 2015).

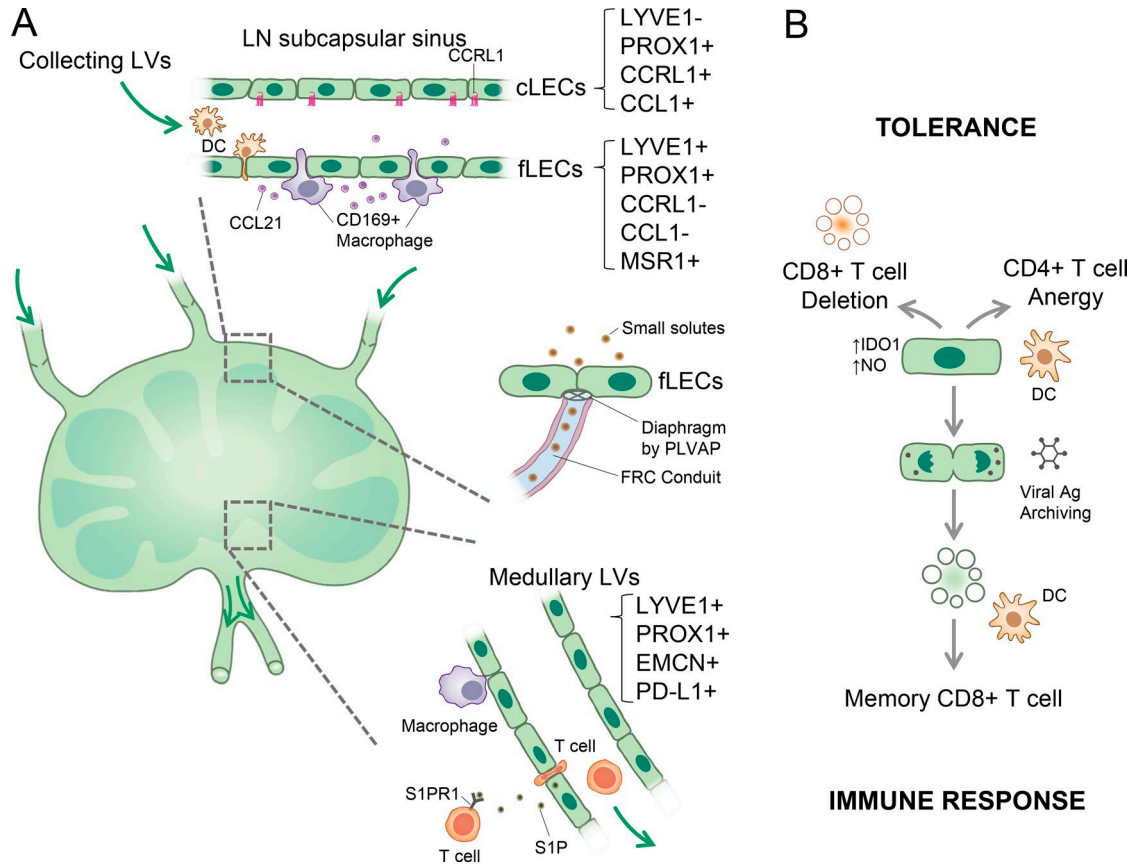


Figure 4. LN lymphatic vasculature. (A) Afferent LVs deliver lymph carrying antigens and immune cells to the LN SCS. From the SCS, lymph flows to the cortical and medullary sinuses and exits via efferent LVs. SCS “ceiling” LECs (cLECs) express the decoy CCL19/CCL21 receptor CCRL1, which creates a gradient of CCL21 enhancing transmigration of DCs into T zones. CD169⁺ SCS macrophages inserted in the “floor” LEC (fLEC) layer take up antigens and pathogens. The majority of lymph drains via LN LVs, whereas a proportion of small solutes is absorbed from lymph via specialized conduits, a network of collagen fibrils, surrounded by follicular reticular cells. Molecular diaphragms formed by plasmalemma vesicle-associated protein restrict access of larger substances into conduits. Similar to fLECs, medullary sinus LECs are in close contact with macrophages, which clear pathogens and antigens. S1P produced by medullary and cortical sinuses LECs induces egress of lymphocytes into efferent LVs and promotes T cell survival. **(B)** Specialized functions of LN LECs. LN LECs contribute to maintenance of tolerance against self-antigens through the expression of peripheral tissue antigens and deletion of self-reactive CD8⁺ T cells. LN LECs can tolerize CD4⁺ cells either by transferring peptide–MHC-II complexes to DCs or acquiring them from DCs. LN LECs also produce immunosuppressive nitric oxide (NO) and indoleamine-2,3-dioxygenase (IDO1), which restrains proliferation of activated T cells. During acute viral infection, proliferating LN LECs uptake and store viral antigens. During the LN contraction stage, such antigens are transferred by dying LECs to DCs, which cross-present them to T cells to promote CD8⁺ T cell memory responses. Ag, antigen; FRC, follicular reticular cell; PLVAP, plasmalemma vesicle-associated protein.

A substantial degree of specialization is also present in the SCS LECs, where LECs facing the LN capsule (or “ceiling” LECs) express high levels of a CCL21/CCL19 decoy chemokine receptor CCRL1. Polarized CCRL1 expression creates a CCL21 gradient, allowing the directional DC migration into the LN paracortex to reach T cells (Ulvmar et al., 2014). Ceiling LECs are LYVE1 negative and also produce CCL1 chemokine (Das et al., 2013), whereas floor LEC are LYVE1 positive. The floor LEC layer is interspersed with CD169⁺ SCS macrophages, which take up antigens and pathogens (Fig. 4 A). Interestingly, although DCs are able to transmigrate directly through the floor of the SCS, naive T cells use the medullary sinus to reach LN parenchyma; however, transmigrating DCs also induce

structural alterations in the SCS floor, which allows subsequent homing of T cells through the SCS (Braun et al., 2011). Onder et al. (2017) recently highlighted a new role of LECs in LN formation. Different from the prevailing LN formation model (Mebius, 2003; van de Pavert and Mebius, 2010), this study reports that that initiation of LN development requires lymphoid tissue inducer cell-mediated activation of LECs and that the engagement of mesenchymal stromal cells is a subsequent event. Mechanistically, LN initiation is mediated mainly through receptor activator of the NF- κ B signaling–noncanonical NF- κ B pathway in LVs and is driven by CCL21 and S1P receptor–dependent retention of lymphoid tissue inducer cells in the LN anlage (Onder et al., 2017).

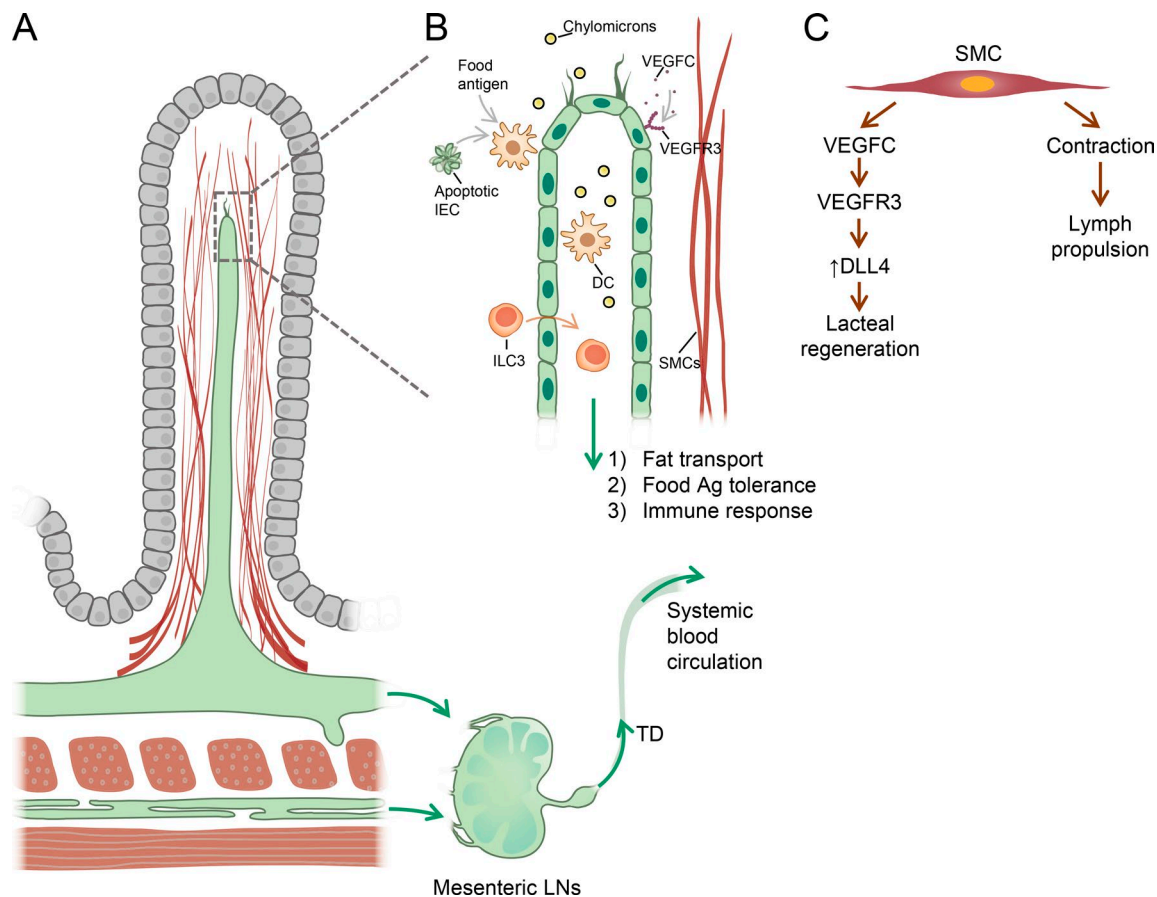


Figure 5. **LVs of the small intestine.** **(A)** Intestinal lacteals are positioned in the middle of intestinal villi. Smooth muscle cells in the villi are closely associated with lacteals, and their contractions promote lymph uptake and transport. Another lymphatic vascular plexus is located in the intestinal muscular layer. Lymph from both plexuses is drained to mesenteric collecting vessels, mesenteric LNs, and the thoracic duct and returned to the blood circulation. **(B)** Intestinal lacteals transport chylomicrons, cholesterol, gut hormones, and immune cells. Lymphatic trafficking of CD103⁺ DCs carrying food antigens and apoptotic intestinal epithelial cells to mesenteric LN drives the development of T reg cells and tolerance. The role of LVs trafficking in intestinal ILC3 function is not understood. **(C)** Role of villus SMCs in the maintenance of intestinal LVs. VEGF-C, produced by villus SMCs, and VEGF-C-dependent DLL4 signaling in LECs fuel continuous lacteal regeneration. Periodic contraction of villus SMCs, controlled by the autonomic nervous system, promotes lymph transport. Ag, antigen; IEC, intestinal epithelial cell; TD, thoracic duct.

Lacteals and lymphatic vasculature in the small intestine

In addition to the common task of all LVs in transporting interstitial fluid and immune cells, the intestinal lymphatic vasculature plays a major role in the uptake and transport of dietary fat. In mammals, dietary lipids are repackaged in enterocytes into large (200–1,000 nm) triglyceride-loaded particles or “chylomicrons,” which are secreted basally into intestinal stroma. In addition to triglycerides, chylomicrons can also incorporate fat-soluble vitamins and drugs, as well as some microbiota components, such as bacterial lipopolysaccharides (Bernier-Latmani and Petrova, 2017). The single lymphatic capillary located at the center of each small intestinal villus, called a lacteal, takes up chylomicrons and other interstitial fluid components from villi and transports them to the submucosal and mesenteric collecting LVs (Fig. 5, A and B). The lymphatic vascular plexus, located in the intestinal muscular layer, drains independently to the mesenteric

collecting LVs. Intestinal lymph is transported to the mesenteric LNs and thoracic duct and is subsequently released into the blood circulation (Fig. 5 A). Through these transport processes, lipid-soluble drugs can directly access the target organs while bypassing the liver, where most of drugs are degraded (Trevaskis et al., 2015). The mechanism and selectivity of chylomicron uptake into lacteals are not entirely clear and may involve both intercellular transport through LEC junctions and intracellular transport across LECs in vesicles (Bernier-Latmani and Petrova, 2017). Periodic squeezing of lacteals, which is mediated by the contraction of surrounding longitudinal smooth muscle cells in intestinal villi controlled by the autonomic nervous system, allows efficient drainage of absorbed lipids into the collecting vessel network (Choe et al., 2015).

Although the majority of adult LVs are quiescent, intestinal lacteals exhibit low but detectable proliferation under

steady-state conditions and frequently harbor filopodia, indicating an ongoing lymphangiogenic response (Bernier-Latmani et al., 2015; Bernier-Latmani and Petrova, 2016). Mechanistically, maintenance of lacteal integrity and fat transport function requires continuous Notch and VEGF-C-VEGFR-3 signaling, with VEGF-C being supplied by the surrounding smooth muscle cells (Fig. 5 C; Bernier-Latmani et al., 2015; Nurmi et al., 2015). Lacteals are characterized by a mix of both continuous zipper junctions and discontinuous button-like junctions, and loss of Notch signaling impairs the formation of mature button-like junctions (Bernier-Latmani et al., 2015). Optimal junctional organization and transport function of lacteals also require adrenomedullin-calcitonin receptor signaling; lymphatic-specific inactivation of calcitonin receptor results in intestinal lymphangiectasia and protein-losing enteropathy (Davis et al., 2017). Identification of the specific lacteal transcriptome will be important to further understand the molecular mechanisms underlying the functional specialization of lacteals.

The intestine harbors a large population of immune cells whose task is to maintain and fine-tune the balance between immune tolerance to the myriad of commensal intestinal bacteria and ingested harmless antigens and responses against potential pathogens. Similar to other tissues, CCR7-expressing DCs migrate into intestinal lymphatics in response to the CCL21 gradient generated by LECs. The transport of food antigens by CD103⁺ DCs to mesenteric LNs appears to be a key step in the establishment of oral tolerance through the induction of mesenteric LN T reg cells (Pabst and Mowat, 2012). Production of such T reg cells is also maintained by lymphatic trafficking of DCs after ingestion of apoptotic intestinal epithelial cells, which are continuously produced as a result of rapid intestinal epithelium turnover (Cummings et al., 2016). The CCR7⁺Rorγt⁺ subset of intestinal innate lymphoid cells also egresses via intestinal LVs to mesenteric LNs, but the functional significance of such transport requires further investigation (Fig. 5 B; Mackley et al., 2015). Given the importance of T cell trafficking via dermal LVs, it would also be interesting to characterize lymphatic transport of intestinal T cell subsets.

The role of LVs in intestinal pathological states, for example in inflammatory bowel disease (IBD), is attracting increasing attention (von der Weid et al., 2011; Bernier-Latmani and Petrova, 2017). Increased lymphangiogenesis and lymphatic vascular dysfunction, such as lymphangiectasia and intralymphatic lymphocyte stasis, have long been described as pathological features of Crohn's disease (von der Weid et al., 2011). Data from animal models suggest that damage to lymphatic vasculatures and its unproductive expansion may be contributing or perhaps even initiating factors in IBD (Bernier-Latmani and Petrova, 2017). Diphtheria toxin-mediated conditional ablation of LVs in the intestine leads to rapid animal demise because of extreme disruption to the intestinal mucosal barrier and septic shock (Jang et al., 2013). Furthermore, impaired intestinal lymphatic vascular func-

tion or lymphangiogenic response exacerbates intestinal inflammation and worsens symptoms in experimental models of colitis (von der Weid et al., 2011; D'Alessio et al., 2014; Davis et al., 2017). Mice deficient in D6 decoy chemokine receptor, which is highly expressed by LECs and is necessary to restrict aberrant inflammatory leukocyte adhesion to LVs, develop more severe colitis, further underscoring a major role of LVs in resolution of intestinal inflammation (Vetrano et al., 2010; McKimmie et al., 2013). Conversely, even transient inflammation during acute intestinal infection has been shown to induce a long-lasting damage of LV function and gut immunity, suggesting that cumulative immunological scarring of intestinal LVs could underlie the development of IBD (Fonseca et al., 2015). In contrast, prolymphangiogenic therapy using adenoviral delivery of VEGF-C markedly reduces disease severity (D'Alessio et al., 2014). Further studies of molecular characteristics of intestinal lymphatic endothelium in homeostasis and diseases, together with identification of immune cell subsets that travel via LVs, will be important to better understand the mechanisms behind responses against pathological insults.

Perspectives and open questions

The discovery of lymphatic-specific molecular markers, growth factors and their receptors, and transcription factors transformed the field of lymphatic vascular biology and laid the foundations for further conceptual advances in understanding the mechanisms and functions of organotypic lymphatic vasculatures. Many questions are emerging in this rapidly developing field, some of which are outlined below.

Efficient in vitro LEC fate programming for tissue engineering. All tissue regeneration procedures, from wound healing to transplantation of engineered organs, demand adequate vascularization by both BVs and LVs. Therefore, reliable methods for the in vitro production of LECs will likely improve the outcomes. Prox1 overexpression partially reprograms mature blood ECs toward the LEC lineage, and several stimuli, such as retinoic acid, WNT, or constitutively active ERK signaling, facilitate acquisition of LEC phenotype (Marino et al., 2011; Deng et al., 2013; Bowles et al., 2014; Nicenboim et al., 2015). Furthermore, generation of fully differentiated LECs from human pluripotent stem cells is feasible (Lee et al., 2015). However, unlike for blood ECs (Orlova et al., 2014), questions regarding the best combination of transcription factors, extracellular inputs, and intracellular signaling cascades for driving LEC fate commitment still require definitive answers.

Degree and functional significance of LEC heterogeneity. The discovery of the developmental heterogeneity of LECs has raised several further questions. What are the origins of LECs in other organs, and what is the reason for the existence of multiple LEC sources? Can LECs of different origins be distinguished in mature vessels, and do such LECs participate

equally in the growth and expansion of LVs during inflammation and regeneration? Single-cell sequencing approaches have already provided a host of important insights into the heterogeneity and population dynamics of immune and cancer cells (Papalexi and Satija, 2017); therefore, it is anticipated that the application of this methodology to LECs will open new perspectives for high-resolution mapping of LEC subpopulations in different organs and associated phenotypes.

Organ-specific lymphangiocrine signaling. BVs not only deliver oxygen and nutrients to tissues but also produce tissue-specific molecules that participate in organ repair and regeneration (Rafii et al., 2016; Augustin and Koh, 2017; Potente and Mäkinen, 2017). LECs are also capable of secreting distinguishable molecules, including growth factors, cytokines, and chemokines, which are defined as “lymphangiocrine” molecules. Most of such lymphangiocrine signals to date have been linked to the regulation of immune responses, especially in LNs (e.g., S1P, which promotes migration and survival of T cells). Identifying lymphangiocrine molecules in other organs and understanding how they contribute to the organ-specific function are essential future tasks to accomplish.

Maintenance of organ-specific differentiation. The transcriptional profiles of cultured human intestinal LECs differ from those of dermal LECs, indicating that some organ-specific features are conserved in vitro (Norrmén et al., 2010). However, standard cell culture conditions introduce significant biases when analyzing the specialized phenotypes of ECs. Analyses and comparisons of various “omics” of LECs isolated (1) from different organs, (2) at different stages of development, and (3) during tissue regeneration or in pathological conditions will undoubtedly be useful when characterizing their organotypic properties and identifying tissue-specific markers of LVs and the mechanisms for their maintenance. The current boom in the development of “organ-on-chip” devices will provide an important new opportunity for modeling and studying heterotypic interactions of LECs with other tissue components, including various epithelia, immune cell populations, and microbiota products.

ACKNOWLEDGMENTS

We apologize for not being able to cite all of the original research articles and related references due to space limitations. We thank Choong-kun Lee for figures and Ben Hogan, Intae Park, and Jeremiah Bernier-Latmani for reading the manuscript and useful discussions.

The work in the T.V. Petrova's laboratory is supported by the Swiss National Science Foundation (31003A-156266 and CR32I3_166326), MEDIC Foundation, the Emma Muschamp Foundation, Fondation Leenaards, the TheraLymph ERA-NET E-Rare Research Program (FNS 31ER30_160674), the Commission for Technology and Innovation, and the Swiss Cancer League (KLS 3406-02-2016). The work in the G.Y. Koh laboratory is supported by the Human Frontier Science Program (RGP0034/2016), and the Institute for Basic Science funded by the Ministry of Science and Information and Communications Technology, Republic of Korea (grant IBS-R025-D1).

The authors declare no competing financial interests.

Submitted: 12 October 2017

Revised: 27 November 2017

Accepted: 28 November 2017

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