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Lessons of Vascular Specialization From Secondary Lymphoid Organ Lymphatic Endothelial Cells

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ABSTRACT: Secondary lymphoid organs, such as lymph nodes, harbor highly specialized and compartmentalized niches. These niches are optimized to facilitate the encounter of naive lymphocytes with antigens and antigen-presenting cells, enabling optimal generation of adaptive immune responses. Lymphatic vessels of lymphoid organs are uniquely specialized to perform a staggering variety of tasks. These include antigen presentation, directing the trafficking of immune cells but also modulating immune cell activation and providing factors for their survival. Recent studies have provided insights into the molecular basis of such specialization, opening avenues for better understanding the mechanisms of immune-vascular interactions and their applications. Such knowledge is essential for designing better treatments for human diseases given the central role of the immune system in infection, aging, tissue regeneration and repair. In addition, principles established in studies of lymphoid organ lymphatic vessel functions and organization may be applied to guide our understanding of specialization of vascular beds in other organs.

Key Words: immune response ■ lymphatic endothelial cells ■ lymphatic system ■ lymph node ■ lymphoid tissue ■ Peyer's patches ■ spleen

Blood and lymphatic vascular systems play complementary roles in tissue homeostasis in vertebrates. The blood vascular circulatory system is composed of a central pump, the heart, and a closed system of blood vessels, including capillaries, veins and arteries. It is critical for transport of oxygen, nutrients, cellular waste products and immune cells across the body. The lymphatic vascular system lacks a central pump and is characterized by unidirectional flow of lymph from the tissues into the blood circulation through connections between the thoracic duct and jugular veins. Besides the lymphatic vessels' crucial role in maintaining fluid balance in the organism, in recent years an increasing body of knowledge has established the roles of lymphatic vessels in organ-specific functions and immunosurveillance.[1–3]

The lymphatic system is present in almost every organ and comprises specialized vessels and lymphoid organs/tissues. Blind-ended lymphatic capillaries, also called initial lymphatics, take up the interstitial fluid, macromolecules and immune cells. This process is facilitated by the presence of button-like junctions between lymphatic endothelial cells (LECs).[4] Lymphatic capillaries channel into larger collecting lymphatic vessels, characterized by their largely impermeable zipper-like junctions and their coverage by smooth muscle cells that drive flow of lymph to the draining lymph nodes (LNs). Intraluminal valves are another unique feature of collecting vessels, important for unidirectional flow of lymph. Lymph flow, cell surface mechanosensors, such as Piezo1, VE-Cadherin

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Nonstandard Abbreviations and Acronyms

APC	antigen-presenting cell
BEC	blood endothelial cell
cLEC	ceiling lymphatic endothelial cell
DC	dendritic cell
ECM	extracellular matrix
fLEC	floor lymphatic endothelial cell
FRC	fibroblastic reticular cell
HEV	high endothelial venule
LEC	lymphatic endothelial cell
LN	lymph node
LTi	lymphoid tissue inducer
LTo	lymphoid tissue organizer
LTßR	lymphotoxin beta receptor
MARCO	macrophage receptor with collagenous structure
MedRC	medullary reticular cell
MRC	marginal reticular cell
MS	medullary sinus
MS MF	medullary sinus macrophage
PD-L1	programmed death-ligand 1
PP	Peyer's patch
PTX3	pentraxin 3
S1P	sphingosine-1
SCS	subcapsular sinus
SLO	secondary lymphoid organ

(vascular endothelial cadherin) and PECAM-1 (platelet and endothelial cell adhesion molecule 1), and mechanosensitive transcription factors, such as FOXC2 (Forkhead box protein C2), GATA2 (GATA-binding factor 2) and NFATC1 (nuclear factor of activated T cells 1) are critical for the development and function of lymphatic valves.[5,6]

Primary lymphoid organs, such as bone marrow and thymus, are sites where B and T cells are produced from hematopoietic progenitors, whereas secondary lymphoid organs (SLOs) function as gathering hubs where naive lymphocytes encounter antigens and antigen-presenting cells (APCs), drained from peripheral tissues.[7] SLOs include LNs, spleen and mucosal-associated lymphoid tissues, such as tonsils, and bronchial, nasal and gut-associated lymphoid tissues, the latter including Peyer's patches (PPs) (Figure 1). SLOs are strategically positioned throughout the body (Figure 1) and continuously screen the body for foreign intruders.[7] The immune responses in both health and disease rely on trafficking of immune cells through the lymphatic vessels. Leukocytes cross the endothelial barriers on their way in and out of tissues,[8] making lymphatic (LECs) and blood endothelial

cells (BECs) essential nonhematopoietic components of the immune system.[1,2,9]

Organ-specific vascular specialization plays an essential role in organ functions.[2,10,11] SLOs with their densely populated and highly organized immune cell compartments, with unique subsets of fibroblasts and highly dynamic immune cell trafficking, feature some of the most striking examples of vascular organ-specific specialization.[12] Better knowledge of its mechanisms is key for improving success of traditional immunology applications such as development of vaccines, cancer immunotherapies, and treatment of autoimmune diseases. In addition, a growing body of knowledge implicates various immune cell subsets in maintenance of tissue-specific stem cells[13,14] and tissue regeneration after injury or damage during aging.[15,16] These recent progresses highlight the need for better understanding of immune-vascular interactions in health and disease. Understanding the molecular mechanisms of vascular specialization in lymphoid organs as well as the consequence of its disruption during aging and in pathological states is thus one of the crucial current challenges at the intersection of vascular biology and immunology with important implications for the entire organism homeostasis.

LN LYMPHATIC VESSELS

The LNs are small, encapsulated organs that in human can range from a few millimeters to several centimeter in size depending on the immune activation status. More than 95% of the LN consists of immune cells that are organized into the segregated B-cell zones (cortex), and T-cell zones (paracortex) and a less cell dense medulla, containing populations of stationary macrophages and plasma cells (Figure 2).[17] The LN vasculature together with highly diverse populations of mesenchymal stromal cells[18,19] plays a central role in maintaining this structure. The LN fibroblasts are often referred to as fibroblastic reticular cells (FRCs) but can be subdivided into 9 subsets of cells based on single-cell analysis of mouse LN stromal cells.[18] They form the structural framework of the organ and express chemotactic cues that guide immune cell positioning and can influence both immune cell activation and survival[19] (Figure 2). The lymphatic vasculature connects the LNs to the peripheral tissues, allowing surveillance of tissue antigens and recruitment of tissue-derived APCs, while specialized blood vessels, known as high endothelial venules (HEVs),[20,21] allow recruitment of T and Bcells from the blood into the LNs (Figure 2). The LN hence allows interaction between blood-derived naive T and B cells and tissue-derived APCs, which is a premise for not only induction of effective adaptive immune responses but also maintenance of tolerance.[19]
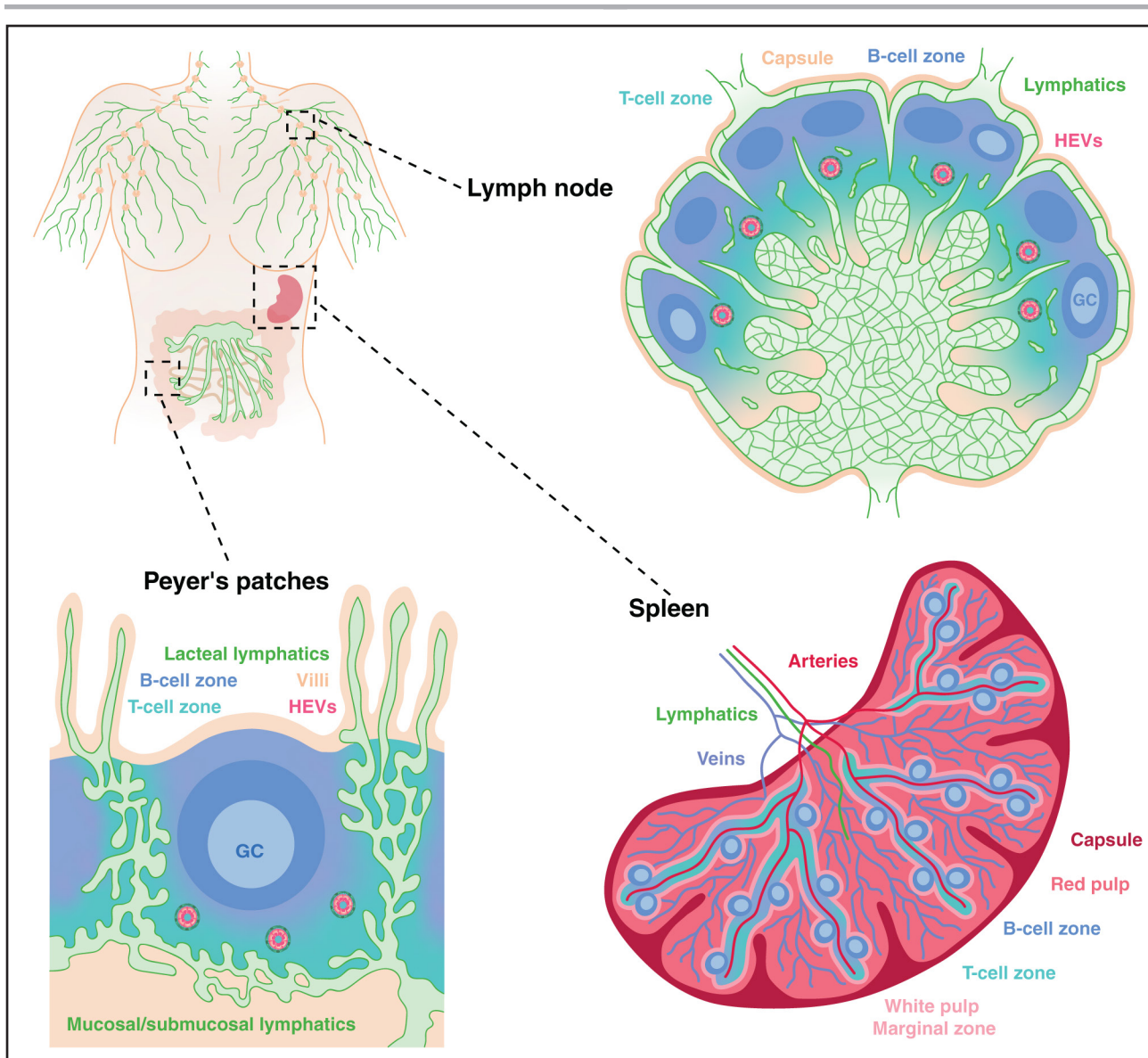
Figure 1. Secondary lymphoid organs (SLOs).
SLOs are the sites of the body where the naive lymphocytes encounter antigens and antigen-presenting cells to initiate adaptive immune responses. The figure illustrates 3 of the human body's SLOs including the axillary lymph node, the spleen and the Peyer's patches. All 3 SLOs are highly organized structures with segregated B-cell and T-cell zones to optimize the induction of adaptive immune responses. GC indicates germinal centers; and HEV, high endothelial venules.

Structure of the LN Sinusoidal Lymphatic Vascular Bed

The organization of lymphatic vasculature of the LN allows coordination of fluid flow, antigen exchange and a constantly ongoing immune cell trafficking in and out of the organ[17] (Figure 2). The incoming lymph is transported by ≥1 afferent collecting lymphatic vessels. It is then emptied into a subcapsular sinus (SCS), which consists of 2 layers of LECs forming the ceiling (cLECs) and the floor (fLECs) (top inset, Figure 2). These 2 layers are connected by perpendicular, bridging LECs, traversing from the floor to the ceiling. Together with collagenous fibers, which are part of the conduit system, LEC-bridges form a sieve-like structure.[22–26]

In mouse LNs, the average SCS height is around 14 μm wide with ≈16 μm transverse distance between strands,[26] emphasizing the dense structural complexity. The SCS floor controls size-restricted entry of lymph-derived factors into the LN.[23,27] Lymph-derived factors <70 kDa are transported through a network of reticular fibers, produced by the FRCs that descends from the SCS deep into the LN, forming the conduit system.[23,27] The conduit system also enables direct contact between lymph-derived factors, such as cytokines and chemokines, and the HEVs, providing a remote-control mechanism for tissue communication with the LN blood vasculature.[28] LN resident dendritic cells (DCs) in the T-cell zone are closely associated with the conduits allowing sampling of smaller antigens.[29]
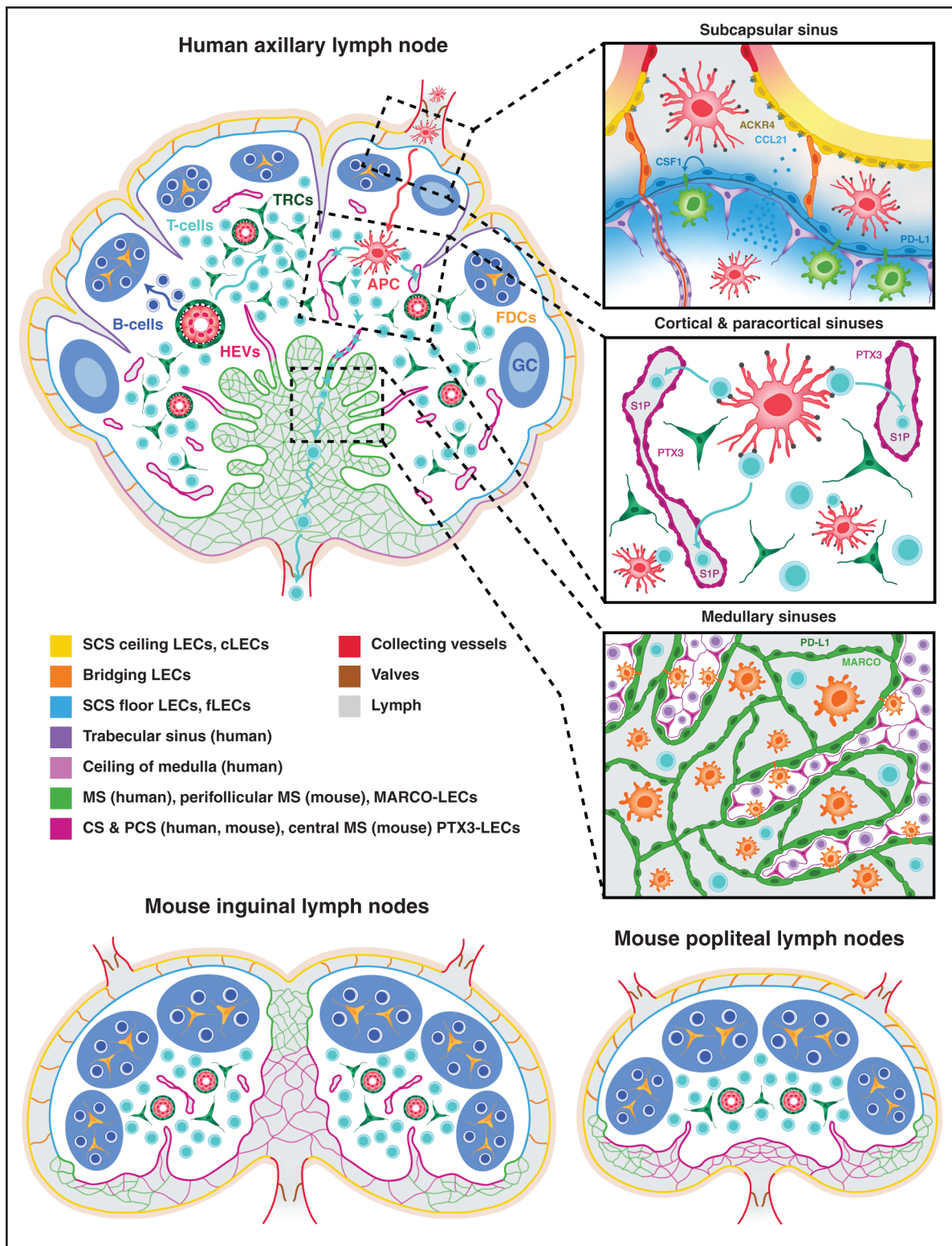
Figure 2. The lymph node (LN) lymphatic vasculature and stroma.
Localization of the lymphatic endothelial cell (LEC) subsets in a human axillary LN, in a mouse inguinal (2 lobes) LN and a mouse popliteal (1 lobe) LN. Each LEC subset is color-coded. High endothelial venules (HEV, red) provide routes for migration of blood-derived naive B and T cells in the LN. The B cells in the B-cell zone (cortex) are organized into follicles (blue round areas) guided by CXCL13+ (C-X-C motif chemokine ligand 13) follicular dendritic cells (FDCs, yellow) that together with specialized T follicular helper cells (not shown), allow efficient B-cell responses with formation of substructures called germinal centers (GC). CCL21+CCL19+ (C-C motif chemokine ligand 21 and 19) T-cell zone reticular cells (TRCs, dark green) guide blood-derived T cells and lymph-derived antigen-presenting cells (APCs), including dendritic cells (DCs), into the T-cell zone (paracortex) and also provide cytokine support for T-cell activation and survival. Selected key genes/functions (*Continued*)

The ceiling of the SCS is closely attached to the overlying collagenous capsule surrounding the LN and is relatively inert for immune cell transmigration (top left inset, Figure 2). In contrast, the SCS floor constantly interacts with immune cells and with underlying stromal cells. These include not only the incoming migrating immune cells, such as DCs and lymphocytes en route to the LN,[20] but also immune cells that cycle between the SCS and the LN parenchyma,[30,31] and more stationary cells, such as the specialized SCS macrophages,[32,33] which form a dense layer beneath the SCS. A subset of fibroblasts named marginal reticular cells (MRCs) line the SCS floor and cooperates with LN LECs in controlling the macrophage niche.[34] Different from mouse LNs, the capsule of the human LN, and other larger species, forms invaginations called trabeculae, which can extend deep into the LN. The sinuses associated with the trabeculae, called trabecular or transverse sinuses,[17] are continuous with the SCS (Figure 2).

From the SCS and the transverse sinuses, the lymph is transported into a network of sinuses called medullary sinuses (MSs), converging into one efferent collecting lymphatic vessel (Figure 2). Like the SCS, the MSs are closely associated with specialized macrophages (medullary sinus macrophage [MS MF]),[32,33] and long-lived plasma cells[35,36] (lower inset, Figure 2). Together, SCS and MS MF, surveille the incoming lymph and act as filters, preventing spread of lymph-borne pathogens and tumor cells to the blood circulation. Similar to fLECs, MSs are surrounded by a network of specialized FRCs called medullary reticular cells (MedRCs), which also line the sinuses.[37] Extensions from the MSs form blind-ended paracortical and cortical sprout-like sinuses,[17,38] that act as the exit routes for both recirculating naive and activated lymphocytes.[38–40]

New Molecular Mapping of the LN LEC Infrastructure Through Single-Cell Analysis

The first single-cell RNA sequencing (scRNAseq) analysis[41] of LN LECs analyzed LECs from metastasis-free axillary human LNs. It confirmed and expanded previous knowledge[42–44] about the heterogeneity and niche-specific specialization of the LN lymphatic endothelium. Subsequent scRNAseq analysis of mouse LN LECs,[25,45] combined with cross-mapping of human and mouse LN LECs,[25] identified 5 conserved subsets, which have been named after their localization within the LN or key distinguishing genes that defines each subset (Figure 2).

The main LN LEC populations include: cLECs and fLECs forming the ceiling and the floor of the SCS; the cortical and paracortical LECs (which in addition map to the central medulla in mouse LNs[25]), also named PTX3 (pentraxin 3)-LECs[25] or exit sinuses[45]; the medullary LECs (in mouse perifollicular medulla[25]) also named MARCO (macrophage receptor with collagenous structure)-LECs,[25] and the valve LECs (Figure 2). Besides these 5 subsets, in humans, the ceiling of the medulla, on the efferent side of the LN in connection to the hilus is built up by a LEC subset unique for man and is defined by the expression of the ECM (extracellular matrix)-associated gene, *MFAP4*.[41] Subpopulations of activated cLECs in human were identified based on higher levels of genes such as the chemokine *CCL2* (*C-C motif chemokine ligand 2*) and *E-Selectin* (endothelial selectin), in 3 out of 6 analyzed human samples.[25]

Similar to the blood vascular system,[46] there are areas of zonation between different types of lymphatic sinuses. Bridge LECs, connecting the fLEC and cLEC layers, were identified as one of such putative transitional populations in both mouse and man[25] (Figure 2). Molecularly, mouse bridge LECs expressed low levels of the cLEC marker *Bmp4* and intermediate expression of the fLEC marker *Bmp2*.[25] A recent scRNAseq analysis also assigned bridge LECs in human LNs as characterized by high expression of *SERPINE1*,[47] encoding PAI-1 (plasminogen activator inhibitor-1). In addition to bridge LECs, a population of transitional zone LECs, which in mouse are bridging fLECs to MARCO-LECs (perifollicular MSs), has been defined.[25]

Figure 2 Continued. for each subset are indicated in insets. In mice PTX3 (pentraxin 3)[high] LECs form paracortical (PCS) and cortical sinuses (CS) while PTX3[intermed] LECs form central medullary sinuses (MS). Transitions between PTX3[intermed] LECs and MARCO (macrophage receptor with collagenous structure)-LECs are seen in connection to the MSs in the human LNs, while PTX3^{high} LECs, like in mice, form paracortical and cortical sinuses. In mouse, MARCO-LECs in the MS are close to B-cell follicles, and hence called perifollicular MSs. Trabecular sinuses, which are formed by invaginations of the collagenous capsule that surrounds the LN, are only found in human, and display overlapping gene expression with the 2 layers of LECs forming the subcapsular sinus (SCS), that is, ceiling LECs (cLECs) and floor lymphatic endothelial cells (fLECs). **Top** inset, Lymph and tissue-derived immune cells arrive through afferent lymphatic vessels (red) and are emptied into the SCS consisting of the cLECs (yellow) and the fLECs (blue). The floor of the SCS is lined by a basement membrane (dark blue), specialized fibroblasts called marginal reticular cells (MRCs, purple) and a dense layer of SCS macrophages (SCS MFs, green). The latter are maintained by fLEC-derived CSF1. The conduit system (purple), which is built up by the LN reticular stromal cells, transports small antigens and cytokines directly, to the HEVs. In the SCS, bridging LECs (orange) and reticular fibers (dark orange) form a sieve-like structure. Lymph-derived APCs, enter through fLECs guided by adhesion molecules expressed by the fLECs, and CCL21 gradients created by ACKR4 (atypical chemokine receptor 4)-expressing cLECs. Constitutive expression of the immune check point molecule PD-L1 (programmed death-ligand 1) contribute to immune tolerizing functions of fLECs in antigen presentation. **Mid** inset, Recirculating and activated B and T cells exit the LN through the blind-ended paracortical or cortical sinuses lined by PTX3-LECs (pink) guided by LEC-derived S1P gradient. **Lower** inset, After entering the sinuses, the immune cells travel through the MSs built up of MARCO-LECs (green) and exit through the efferent collecting vessel. The MSs are surrounded by medullary cords containing stationary plasma cells (purple), MFs (orange) and medullary reticular cells (pink). The medullary sinus also contains a specialized subset of MFs called MS MFs (orange). S1P indicates sphingosine-1.

Conserved Versus Species-Specific Molecular Features of LN LEC Subsets

Examples of genes expressed in the LEC subsets of mouse and human LNs are listed in the Table, as also recently discussed by Takeda et al.[12] Mouse and human cLECs show the highest degree of molecular conservation.[25] The latter may be explained by the structural role of cLECs compared to other subsets with conserved expression of genes for ECM regulation, like *MMRN1*, and for mural cell interactions, such as *PDGFB* and *EDN1*. Mouse and human cLECs also share expression of *ACKR4* (atypical chemokine receptor 4; also known as CCRL1 [chemokine (C-C motif) receptor-like 1])[25,41,62] and mechanosensitive transcripts, such as transcription factor *FOXC2,* a feature shared with valve LECs.[25,41] fLECs in both mouse and man share expression of the immune check point molecule Programmed death-ligand 1 (*PD-L1,* also known as *CD274),* the invariant chain *CD74* and MHC (major histocompatibility complex) class II *(HLA-DRA/HLA-DRB1* human*, H2-Ab1* mouse)[25,41] consistent with their role in tolerance and antigen presentation.[55,56,63] Other conserved fLEC genes include the chemokine *CCL20*[25,41] and genes involved in leukocyte interactions. MAdCAM-1 (mucosal vascular addressin cell adhesion molecule 1) and LYVE-1 (lymphatic vessel endothelial receptor 1) are constitutively expressed on fLECs in mouse LNs, but their expression is region-specific in human LNs. While LYVE-1 is very low/undetectable in axillary LN fLECs,[25] the fLECs in pancreatic LNs LNs can display abundant expression of LYVE-1,[25] (M.H. Ulvmar, unpublished). MAdCAM-1 is, also not detectable in human fLECs from adult human axillary LNs,[25,41] but may be expressed at other sites or developmental stages.

The expression of the scavenger receptor MARCO in MARCO-LECs of MSs and PTX3 in PTX3-LECs, forming paracortical and central MSs in mouse, is also shared between human and mouse. MARCO and PTX3 amino acid sequences are highly conserved between human and mouse (92% PTX3[64] and 69% MARCO[65]), indicating conserved functions. High expression of several other scavenger receptors,[25,41–43,45] and enrichment in lysosomal transcripts,[25] suggests that MSs MARCO-LECs complement the roles of LN macrophages. Indeed, mouse LN MARCO-LECs have been shown to limit dissemination of arthritogenic alphavirus,[59] confirming filter-like function of this LN LEC subset. Human MARCO-LECs also highly express *CD209* and *CLEC4M*, which lack orthologues in mouse.[41] CD209 binds CD15 (LewisX) on myeloid cells and was proposed to retain or recruit neutrophils at this site for effective defenses in infections.[41]

Although MARCO-LECs share expression of PD-L1 (programmed death-ligand 1) with fLECs, both mouse and human PTX3-LECs lack PD-L1. Instead, this subset is characterized by high expression of lymphangiogenesis-related genes, such as VEGFR3 (vascular endothelial growth factor receptor 3), also known as FLT4 (fms related receptor tyrosine kinase 4), and its coreceptor NRP2 (neuropilin 2), indicating that PTX3-LECs are more sensitive to lymphangiogenic signals. In line with this hypothesis, PTX3 was upregulated in hyperproliferative dermal lymphatic vessels in response to constitutive

Table. Examples of Genes Expressed in Different Subsets of LN LECs and Described Functions

Location	LEC subset	Functions	Human[25,41]	Mouse[25,45]
Lymphatic valves	vLECs	Prevent backflow of lymph[48–50]	*FOXC2*high *CLDN11*high	*Foxc2*high, *Cldn11*high*
Subcapsular Sinus (+ trabecular sinuses in human)	cLECs	Structural role, chemokine gradient formation,[51] neuronal input sensory neurons[52]	*ACKR4, MMRN1, FOXC2, PDGFB, EDN1, CAV1, RANK (TNFRSF11A), NT5E*	*Ackr4, Mmrn1, Foxc2, Pdgfb, Edn1, Cav1, Rank (Tnfrsf11a), Cd36*high
	fLECs	Immune cell trafficking (entry and shuttling[25,41,45]), maintain MF niche,[34,53,54] Ag-presentation (tolerance[55,56]), antigen archiving[57,58]	*CCL20, CD74, MHC-II: (HLA-DRA/HLA-DRB1), PDL1 (CD274), CSF1, LYVE1*+/–*, MADCAM1*+/–*, ACKR1*	*Ccl20, Cd74, MHC-II: (H2-Ab1), Pdl1(Cd274), Csf1, Lyve1, Glycam1, Itga2b, Madcam1*
Paracortical sinuses (+ central MSs in mouse)	PTX3-LECs	Exit routes for lymphocytes,[40] proliferation and expansion in LN hypertrophy[25,41,45]	*PTX3, ITIH3, LYVE1, FLT4 (VEGFR3)*high*, NRP2*high*, PDPN*high*, CD36*high	*Ptx3, Itih5, Lyve1, Flt4 (Vegfr3)*high*, Nrp2*high
Medullary sinuses (perifollicular MSs in mouse)	MARCO-LECs	Maintain MF niche,[34,53] scavenging of virus,[59] antigen archiving,[57,58] recruitment neutrophils (human[41])	*MARCO, PDL1 (CD274), CSF1, LYVE1, ACKR1, CD209, CLEC4M*	*Marco, Pdl1(Cd274), Csf1, Lyve1, Itga2b*
Ceiling of the medulla (human)	MFAP4+ LECs	Structural role?[41]	*MFAP4*	*Not present*

All LECs share the expression of PDPN (podoplanin), VEGFR3 (vascular endothelial growth factor receptor 3) and NRP2, (neuropilin 2), but levels may differ with some subsets indicated as high. For further details online-published databases for mouse and human LN LECs: https://cellxgene.cziscience.com/collections/9c8808ce-1138-4dbe-818c-171cff10e650 (Butcher/Ulvmar labs).[25] cLEC indicates ceiling LEC; fLEC, floor LEC; LEC, lymphatic endothelial cell; LN, lymph node; MARCO, macrophage receptor with collagenous structure; MF, macrophage; MFAP4, microfibril associated protein 4; PTX3, pentraxin 3; scRNAseq, single-cell RNA sequencing; and vLEC, valve LEC.

*In contrast to the periphery where CLDN11 (claudin 11) is confined to valves in the mesentery and skin collecting vessel,[60,61] all mouse LN LECs express high levels of Cldn11. It thus cannot be used alone as a marker to distinguish valves within the mouse LN by scRNAseq.

activation of PI3 (phosphoinositide 3) kinase-signaling.[66] Such PTX3^{high} LECs expressed monocyte chemoattractant CCL2 and recruited VEGF-C+ (vascular endothelial growth factor c) monocytes,[66] although the role of PTX3 in this process is still unknown. In line with these findings, while LN PTX3-LECs do not produce CCL2 under homeostatic conditions, they upregulate this chemokine in response to oxazolone-induced inflammation.[25] The functions of PTX3 in LECs may also be linked to its well-established role as secreted pattern recognition molecule mediating opsonization and complement activation.[64] In addition, PTX3 may play role in organization of the ECM as it forms a complex with proteins of the Inter-alpha-trypsin inhibitor (ITIH) family and interacts with the hyaluronic acid−rich matrix during oocyte fertilization.[64] Interestingly, mouse LN PTX3-LECs express *Itih5* while human LN PTX3-LECs instead display high levels of *ITIH3*.[25] Human LN PTX3-LECs also express the ECM genes *MMP2* and *LOX,* indicating a possible role in ECM modulation.[67,68]

PTX3-LECs line cortical and paracortical sinuses, which act as exit routes for lymphocytes from the LN (mid inset, Figure 2). In line with their role in lymphocyte migration, both mouse and human PTX3-LECs have a gene profile reminiscent of initial capillary LECs.[25] In addition, human PTX3-LECs express high levels of *SPHK1* (*sphingosine kinase 1*), encoding an enzyme involved in lipid-signaling molecule S1P (sphingosine-1) production and lymphocyte exit from the LN.[40] In human, but not in mouse, PTX3-LECs are distinguished from other subsets by higher expression of PDPN (podoplanin) and the fatty acid translocase CD36, markers that together can identify this subset in human LNs.[25,37] In mouse LN, CD36 is expressed at highest levels in cLECs, where it was shown to scavenge lymph-derived modified low-density lipoprotein.[45] Inactivation of CD36 disrupt intestinal lymphatic integrity and causes visceral inflammation and obesity.[69] CD36 expression in LN LECs may therefore reflect a need to maintain vascular integrity of ceiling and the paracortical sinuses, as well as distinct metabolic requirements of LN LEC subpopulations.

Atypical chemokine receptors play an important role in modulating availability and gradients of chemokines in tissues. They are highly expressed in distinct lymphatic vascular compartments, highlighting essential roles of lymphatic vessels for optimizing immune cell trafficking within LNs. As mentioned, ACKR4 is produced by cLECs and in parts of collecting vessels,[25,51] while ACKR2 (also known as D6) is present in lymphatic capillaries.[60,70] The latter expression pattern is shared with both PTX3-LECs and cLECs in mouse, while in human LN ACKR2 is restricted to cLECs.[25] Human LNs also produce ACKR1 (also known as DARC [duffy antigen receptor for chemokines] or Duffy) in fLECs and MARCO-LECs,[25] whereas in mouse, ACKR1 is mainly detected in venules, with only sparse and low expression in LECs.[71] ACKR1 shuttles

chemokines across endothelial cell layers[72] and may function either as a scavenger or transporter of chemokines across the LN lymphatic endothelium in humans. The ACKR1 chemokine ligands CXCL3 (C-X-C chemokine ligand 3) and CXCL5 are expressed by human fLECs and MARCO-LECs, but they are absent in mouse LN LECs under homeostatic conditions.[25]

Although key conserved transcripts and pathways are likely to represent core functions within each subset of LECs, differences between mouse and human provide information about the adaptation to different immunologic, structural, or metabolic needs in these species. However, these data should not be over interpreted, as current analysis relies only on differentially expressed gene overlap scores across species. Where orthologues are missing, other genes may perform the same function, and this will require more in-depth analysis. Differences may also reflect more protected pathogen-free environment of mouse housing versus continuous environmental exposures of human LNs. Indeed, expression of inflammation-induced IL-6 in human but not mouse fLECs, MARCO-LECs and cLECs[25] support this possibility. In addition, human LNs frequently feature age-related degenerative changes caused by lipomatosis, which are rarely observed in inbred strains of mice.[37] Collectively, such changes may affect the LN transcriptome and affect proportions of different subsets of LECs (ie, reduction in MARCO-LECs) in human LNs with high variability from person to person and even across LNs from the same person.[37]

Regulation of LN LEC Phenotypes in Homeostasis

An outstanding current question of the field is the identity of environmental cues that control the structure and the distinct niche-specific molecular phenotypes of LN LECs. The LN microenvironment is characterized by unique cytokine- and chemokine-profiles expressed by specialized stromal and immune cell niches,[19] presence of areas with lower pH[73] and high levels of fatty acids.[74] The structural organization of sinusoidal vascular beds likely also give rise to unique patterns of flow and shear stress, which in turn may contribute to regional patterning of LEC transcriptomes.[75]

Part of the signals shaping the organization of LN lymphatics emanates from lymphocytes and specialized fibroblast subsets. The T-cell zone (paracortex) has low density of lymphatic vessels. Absence of T cells and T-cell−derived IFNγ (interferon γ) increased lymphatic vessel density,[76] suggesting an IFNγ-driven mechanism restricting LEC ingrowth into this area. T-cell deficiency upregulates MAdCAM-1 in MSs, a normally fLEC-confined marker in mice.[63] In contrast, B-cell deficiency downregulates MAdCAM-1 on fLECs and reduces PD-L1 expression on medullary LECs.[63]

Systemic LTßR (lymphotoxin beta receptor) blockade but not lymphatic-specific deletion of LTßR, recapitulated the phenotype,[63] indicating indirect contribution of LTßR-signaling to LN LEC specialization. Deletion of the Tumor necrosis factor receptor family member, RANKL (receptor activator of nuclear factor κB ligand), in LN stromal cells induced upregulation of the cLEC marker *Ackr4* and downregulated several fLEC markers based on analysis of bulk sorted LECs.[34] The latter indicates a role of RANK (receptor activator of nuclear factor κB)-signaling in establishing or maintaining LEC SCS phenotypes in cross-talk with the closely located MRCs lining the SCS.[34]

LN LECs are also highly responsive to signals from incoming specialized sensory neurons.[52] Such neurons connect to the capsule/SCS and to the perivascular space of the medulla and are also found in contact with blood vessels and the MSs. Such connections increase in inflammation[52] indicating that LN-innervating sensory neurons contribute to the control of immune response. Based on scRNAseq analysis, optogenetic neuronal activation elicited strongest transcriptional changes in a LEC cluster that showed features consistent with cLECs.[52] Notably, LEC transcripts associated with lymphangiogenesis or general vascular functions (eg, *Reln, Lyve1, Nrp2*), with neuronal synapses (eg, *Gata6, Ets2*) and transcripts linked to S1P-mediated pathway for lymphocyte egress (*Acer2, Asah2*), were downregulated.[52]

Further understanding of the regulation of the molecular phenotypes of LN LECs will require detailed analysis of LEC at different sites of the body, in response to environmental changes within the LN, and in relation to changes in stromal cell subsets and immune cells. Such knowledge will shed light to our understanding of changes in peripheral LECs in disease and organ-specific molecular adaptation.

Role of LECs in LN Macrophage Niches

LNs harbor distinct populations of macrophages according to their location, identity markers and functions. Broadly, macrophages reside in the sinuses of the LN and in the parenchyma, in the T- and B-cell zones.[33] SCS macrophages lining the SCS are in constant contact with incoming lymph (Top inset, Figure 2), whereas MS MF residing in the medullary part of the LN filter outgoing lymph (lower inset, Figure 2). Although SCS and MS MF are phenotypically different, both express CD169 (also known as Siglec-1), and are capable of catching and retaining pathogens, functioning as an additional level of physical protection to help fighting infections.[33]

fLECs and MS-LECs express high levels of CSF-1 (colony stimulating factor 1), a key regulator of survival, proliferation, differentiation and function of mononuclear phagocytes.[33] Consistently, LEC-specific ablation of *Csf1* reduced the network of both SCS and MS MF

with no perturbation of the parenchyma T-cell zone macrophages in LN or myeloid cells in other organs.[53] Expression of CSF-1 in fLECs and MSs LECs (MARCO-LECs) is shared between human and mouse[25] further supporting the essential role of these LEC subset in organizing macrophage niches in the LN.

In addition to serving as a source of soluble growth factors, LN LECs sustain peri-lymphatic niches for macrophages by promoting their adhesion to the endothelium. Analyses of surface glycomes of endothelial cells revealed exceptionally high density of α2-3-siaylated/3-O-sulfated O-glycans/glycolipids in fLECs and showed that such specialized glycocalyx provides anchorage for SCS macrophages via interaction with CD169 (Siglec-1). LNs of *Siglec1*[W2QR97A] mice, in which CD169 lacks the ability to recognize sialoglycans, harbored reduced number of LN SCS macrophages, which were also less proliferative and activated.[54] In addition to its role in macrophage adhesion, specialized glycocalyx of LN fLECs may also play a role in storage and release of chemokines and growth factors. The cell surface glycoprotein PDPN, which is expressed by all LECs, contribute to adhesion of macrophages to MSs, as lymphatic-specific loss of PDPN reduced density of macrophages in these locations, while SCS macrophages were not affected.[77] The same tendency was observed in the case of tumor-draining LNs.[77]

RANK-signaling is another pathway implicated in establishment of SCS and MS MF niches. LEC-specific RANK ablation during the first 2 weeks of postnatal development, but not in adult mice, reduced SCS and MS MF number without affecting other LN macrophages.[34] Furthermore, LEC activation by RANKL, expressed by the MRCs lining the SCS, and by MedRCs lining the MS,[18] was required to restore the niche for SCS macrophages recovery after disruption by viral infection.[34] Thus, LN LEC activation by RANKL is especially important for SCS and MS MF recruitment to their peri-lymphatic location during LN development or niche repopulation after inflammatory insult. The exact mechanisms linking RANK activation in LECs and perivascular macrophage recruitment remain elusive. In summary, LN LECs play multiple and functionally diverse roles in development and maintenance of specialized macrophage niches within the LN.

Immune Cell Entry and Exit Driven by LECs in LNs

The LN vasculature is constantly interacting with incoming and exiting immune cells. These routes need to be carefully controlled and adapted to the very different needs in homeostasis, tolerance, and in active inflammation. Here we will discuss this from the perspective of the different arms of the LN lymphatic vasculature: that is, the SCS, the paracortical/cortical sinuses and the MSs; and in reference to the molecular specialization of the LECs building these sinuses.

Subcapsular Sinus LEC Driven Entry and Shuttling of Immune Cells

Migratory DCs enter peripheral initial lymphatic vessels and exit through the SCS floor to reach the LN parenchyma (top inset, Figure 2). The interstitial and migratory steps into initial lymphatic vessels,[78,79] as well as the initial intravascular migration[80] and exit through the SCS floor,[81] occur in a chemokine receptor-dependent manner.[78–81] LECs in initial capillaries constitutively express CCL21 and CCL19, which are ligands of the chemokine receptor CCR7 (c-c chemokine receptor 7). However, their expression is virtually absent in LN LECs,[25] with only low levels detected in the PTX3-LECs of the paracortical sinuses both in mouse and human.[25] Instead, CCR7-ligands are highly and constitutively expressed by T-cell zone reticular cells, whereas soluble CCR7 ligands are transported with the lymph from peripheral tissues. In mouse, but not in human,[82] CCL21 is in addition highly expressed by the HEVs.

Efficient CCR7-driven directional DC migration requires chemokine gradients.[83] In the SCS such gradient is created through the polarized expression of ACKR4 in cLECs.[51] By scavenging the CCR7-ligand CCL21, ACKR4 forms gradients of CCL21 from the SCS floor into the interfollicular area[51] (top inset, Figure 2). Like *Ackr4* deficient mice, *Plt Ackr4* double mutants, which lacks stromal cell expression of CCL21 in the LN, but still have expression of CCL21 in peripheral lymphatics, accumulate migratory DCs inside the SCS.[51] Footpad injection of CCL21 in wild-type mice leads to its uptake in cLECs, whereas such uptake is abolished in *Ackr4* deficient mice, leading to deposition of lymph-borne CCL21 on cells inside the SCS and in the LN parenchyma. These data emphasize the role of cLEC-mediated restriction of lymph-derived transport of CCL21 into the LN as critical for CCR7-driven entry of DCs across the SCS.[51] ACKR4 is also expressed in the afferent collecting vessels closest to the LN and in areas of high flow in peripheral collecting lymphatic vessels, where it facilitates detachment of tissue-derived T cells from lumen wall and promotes their transport to the LN in inflammation.[62] ACKR4 in collecting lymphatic vessels, and in epithelial and peripheral stromal cells[62,84] likely cooperates with ACKR4 in cLECs in limiting dissemination of tissue-derived soluble CCR7 ligands into the LN[51,62,85] and thus allows effective immune cell recruitment.

In addition to its role in promoting migration of DCs, ACKR4 is also important for recycling of B cells from the SCS.[31] B cells migrate into the SCS as an exit route from germinal centers in reactive LNs. Some of these B cells leave the LN through further transport with the lymph back to the blood but some are recruited back into the LN in a CCR7-dependent manner that was inhibited in absence of ACKR4.[31] Functionally, this shuttling behavior was linked to interaction between B cells and SCS macrophages and is important for adaption to antigenic drift in B-cell memory responses.[31]

Other immune cells shuttling in and out of the SCS are the IL7R^{high}CCR6^{+} cells,[30] residing in or close to the SCS. These include Lymphoid tissue inducer (LTi)-like RORγt[+] (RAR-related orphan receptor gamma) innate lymphoid cells and γδ T cells, which likely contribute to IL-17−driven early responses to pathogens.[30] Similar to the germinal center-emigrating B cells,[31] their exit from the parenchyma to the SCS is driven by S1PRs (S1P receptors), while their return depends on CCR6 and integrins (eg, LFA1 [lymphocyte function-associated antigen 1][30]) whose ligand/receptor CCL20 and ICAM-1 (intercellular adhesion molecule 1) respectively, are expressed by fLECs.[25,41] in situ protein analysis indicates that fLECs are the main source of CCL20 in the SCS in homeostasis.[30]

As mentioned earlier, the SCS display a sieve-like structure of bridge LECs and reticular fibers.[22–26] Whether and how this structure affects immune cell migration has not been well understood. Intralymphatically injected activated T cells arrest in this network, independent of adhesive molecules, suggesting that the mechanical sieve effect retains arriving cells.[26] In contrast, further migration of the activated T cells into the LN from the SCS was CCR7-dependent and facilitated by integrins with the T cells displaying reduced ability to home in *Ackr4* deficient mice.[26] A slow and confined migration pattern associated with interaction to the conduit collagenous network, descending from the capsule, was also reported for Natural killer (NK) cells homing to the LN in infection.[86] PLVAP (plasmalemma vesicle associated protein) and CAV1 (caveolin 1) have been directly linked to sieve functions of the SCS.[23] Both *Plvap*-deficiency and injection of unconjugated MECA (mouse endothelial cell antigen)-32 antibodies, targeting PLVAP in vivo, affected lymphocyte homing into the LN after subcutaneous injection, increasing and decreasing it, respectively.[23] Enigmatically, MECA-32 antibody binding to PLVAP did not affect T-cell entry when T cells were injected intralymphatically.[26] Gaps of 0.1 to 1.0 μm in the SCS floor were proposed to be sites permissive for T-cell transmigration.[26] How these gaps are controlled molecularly is still not known. Besides CCR7, chemokine receptors CCR5 and CCR8 affect transmigration of activated T cells across the SCS.[26] CCR5 and CCR8 ligands are very low in resting LN LECs, but they are upregulated in fLECs in response to inflammation.[25,87]

Activation status of immune cells affects their homing via LN SCS. Although activated T cells enter directly through the SCS, naive T cells injected into afferent lymphatics preferentially enter into the parenchyma from the MSs.[81] Similarly, naive neutrophils do not enter through the SCS, while activation-licensed neutrophils do enter through the SCS.[88] However, presence of DCs may shift the routes of migration of other immune cells. Coinjection of DCs enables naive T cells to enter at sites of DC transmigration through the SCS floor.[81] Similarly,

lymph-derived resting neutrophils to a large extent remain inside the sinuses, but enter the LN when co-injected with DCs.[88] The underlying mechanisms and whether if DCs interact with fLECs or other stromal cells in the SCS niche, in regulating this process, remain to be determined.

It is likely that many additional molecules assist immune cells homing through the SCS. LYVE-1 on initial lymphatic vessels interacts with hyaluronan-coated leucocytes and facilitates the entry of DCs through the lymphatic vessels.[89] As LYVE-1 is highly expressed on fLECs but not cLECs, a similar mechanism may facilitate DC exit from the SCS.

Paracortical and Cortical Sinuses—Routes of Exit From the LN

The blind-ended sprout-like paracortical and cortical sinuses are the main route for exit of activated and naive recirculating lymphocytes from the LN[38] (mid inset, Figure 2). In the thymus, spleen and LNs, the exit of lymphocytes depends on the S1PR1-S1P-signaling pathway.[38,90] S1P is secreted through the transporter SPNS2.[91,92] *Lyve1-Cre* mediated deletion of *Spns2*[93] or the S1P producing kinase *Sphk1*, in a *Sphk2* null background,[40] inhibited lymphocyte egress,[40,93] and reduced T-cell survival in the LN.[93] Loss of S1P-signaling also affected lymphatic vascular patterning in the periphery,[40] and in the case of *Sphk2* null mice, reduce the number of LECs and the MSs in the LN.[92] The latter phenotype may be related to the LEC-autonomous roles of S1P-signaling.[94] Wild-type bone marrow chimeras did not rescue the effects of the *Lyve1-Cre* mediated deletion, indicating that LECs are a major source of S1P,[40,93] although contribution from other radio resistant cells cannot be fully excluded. ITGA9 that is highly expressed in medullary and paracortical sinuses also contributes to lymphocyte egress in inflammation, through the regulation of S1P secretion.[95]

scRNAseq analysis of LN LECs has confirmed expression of *Spns2* in all mouse LN LEC subsets, with lower expression in fLECs[25] (and database https://cellxgene.cziscience.com/collections/9c8808ce-1138-4dbe-818c-71cff10e650) and significantly higher levels in PTX3-LECs in one study.[45] *Sphk1* and *Sphk2* are detected in all mouse LEC subsets and are enriched in human PTX3-LECs of paracortical sinuses.[25] LN LECs also express the S1P degrading enzyme S1P-lyase that regulates S1P and lymphocyte egress.[96] Differential expression S1P-regulating factors across the LN lymphatic network are likely important for stringent control of immune cell navigation.

The role of other regulators of lymphocyte migration, expressed by the paracortical sinuses, still needs to be evaluated. Both mouse and human paracortical sinuses share similarities with capillary initial lymphatic vessels, including expression of LYVE-1 and MRC1. They also display very low, but detectable, levels of CCL21, at least on the mRNA level. In humans, CCL21 expression in PTX3-LECs is more evident than in mouse LECs.[25,41] CCL21 is highly expressed in initial capillary vessels, essential for recruitment of APCs and circulation of T cells in and out of the LN. Although LYVE-1 is expressed by all LN parenchymal LECs, MRC1 show very low expression in fLECs but is high in PTX3-LECs and in MARCO-LECs, with highest expression in the PTX3-LECs. In peripheral lymphatic vessels, MRC1 has been shown to be a receptor for CD44 supporting lymphocyte migration,[97] and may similarly affect egress out of the LN.

Medullary Sinuses—Retention Balanced With an Ability to Convey Exiting Cells

Cells entering through the SCS but not transmigrating into the LN parenchyma, or cells exiting from the paracortical sinuses, travel through the complex network of MSs before leaving the LN. These sinuses are therefore typically filled with immune cells. As discussed above, LEC-derived S1P regulates this trafficking.[40] Beside this, S1P has also been linked to a mis-localization of NK-cells, from medullary cords to the T-cell zone.[98] Using reporters that allow tracing of the S1P-signaling,[98] a gradient of S1P activity was also demonstrated, inside sinuses and in the medullary cords (parenchyma lining the MSs), whereas S1P activity was low in the paracortex.[98] Flattening of S1P gradient in the absence of the transporter *Spns2*, reduced production of IFNγ by the NK-cells and their protective function in bacterial infection.[98] As discussed earlier, MSs, like the SCS, maintain specialized subsets of macrophages,[32,99] which are controlled by LECs together with stromal cells.[34] The medulla is also a home of the resident long-lived plasma cells,[35,36] whose maintenance depends on the specialized medullary fibroblasts (MedRCs) that populate this area.[100] It is not known if LECs also have a role in maintaining a niche for plasma cells in the medulla.

In conclusion, the molecular and structural specialization of SCS, MSs, paracortical and cortical sinuses LECs is essential to allow entry, shuttling, and exit from the LN. We have likely only scratched the surface of understanding the molecular specialization that allows for this strict coordination of immune cell trafficking.

Role of LECs in Antigen Archiving and Presentation

The LN continuously surveille the status of peripheral tissues through incoming lymph, which delivers a complex mixture of peptides, lipids, antigens, and immune cells.[101] Upon infection or inflammation the lymph composition changes and either soluble exogenous antigens or APCs carrying antigens, enter the LN.[1,57,102] The lymph-borne

lymphocytes are drained into the SCS of the LN from where they transmigrate into the parenchyma. The mode of delivery of soluble antigens into the LN is size dependent. Small antigens and molecules with a molecular weight of <70 kDa rapidly reach the LN follicles taking advantage of the reticular conduit system.[29,103] Together with the SCS LECs the conduit network acts as a nonclosed filtering system that has been shown to be dependent on PLVAP[23] since in *Plvap*-deficient mice large antigens are also found in the conduit system. Large antigens, on the other hand, are usually either absorbed by SCS macrophages[104–106] or transcytosed through the fLECs via a dynamin-dependent transport system.[107] The size restriction to enter the conduit system apply to incoming lymph-derived factors. Large pentameric molecules of IgM that are produced inside the LN can be transported through the conduit for rapid secretion into the blood circulation.[108]

Once in the LN, antigens are presented to lymphocytes eliciting an adaptive immune response. This response is dependent on the capacity of T cells to recognize cognate antigens presented by APCs through MHC. Briefly, CD4+ T cells, specialized in providing help during specific immune responses, recognize epitopes of proteins presented by MHC-II, whereas cytotoxic CD8+ T cells, directly involved in killer functions, recognize endogenous antigens presented by MHC-I cell holders.[109]

LN LECs, besides being structural components and paths for lymph and lymphocyte transport, also contribute to controlling immune responses directly via antigen archiving and presentation.[57,102,110] Here we highlight some key findings. For further details, please we refer to these comprehensive reviews.[57,102,110]

Antigen retention in LN is a function normally associated with follicular DCs that can store antigen for many months and even years after the immunologic insult has been resolved.[111] LECs are also capable to archive antigens under specific conditions, such as inflammatory stimulus (eg, TLR [Toll-like receptor]-stimulation) and T-cell expansion within the lymphoid tissue, which drive LEC proliferation.[58,59] LECs' archiving ability lasts for ≈1 month, complementing the functions of follicular DCs in retaining antigens, and acting as a reservoir in early stages of the immune response. The mechanisms of how LECs retain unprocessed antigens is not clear, but may involve caveosomes.[112]

LECs' archived antigens can be transferred to other cells directly, for example, to migratory CD11c+MHC-II^high DCs,[113] or released when LECs die in the process of LN contraction, and subsequently transferred to *Batf3*-dependent migratory DCs.[114] These APCs can then cross-present such antigens for MHC-II to circulating T cells inducing, for instance, CD4 anergy in a LEC-dependent but indirect fashion.[113] Others have demonstrated that LECs are indeed also capable of direct cross-presenting exogenous antigens to CD8+ T cells directly, through MHC-I, under homeostatic conditions.[56]

Importantly, LECs also express MHC-II themselves,[55,113] and mouse LN LECs express MHC-II at higher levels than peripheral LECs.[113] Presence of MHC-II is an important feature of professional APCs,[102] nevertheless LECs lack the ability to fully process and present antigen, since they lack H2-M,[113] a key enzyme that is required to free MHC-II for peptide binding.[102] LN LECs can also acquire MHC-II complexes from hematopoietic cells, as demonstrated by using MHC-II−/− mice as recipients of bone marrow chimeras, inducing CD4+ T-cell dysfunction[115] and supporting regulatory T-cell homeostasis.[116] In line with the role of LN LECs sustaining self-tolerance, loss of MHC-II in LN stromal cells, including LN LECs, leads to dysregulated immunity and production of autoantibodies in aged mice.[116] The tolerogenic role of LN LECs can also be coopted by tumors to suppress tumor-antigen-specific CD8+ T-cell responses.[117,118]

Activation of T cells requires both recognition of the MHC-I, or II molecule and a second signal, delivered by a co-stimulatory molecule, such as CD40, CD80 (B7.1), and CD86 (B7.2), by APCs.[119] LN LECs express very low levels of CD40 and negligible levels of CD80 and CD86.[55] In contrast, LN LECs produce multiple inhibitory receptors that dampen the T-cell activation, such as PD-L1 (CD274) that has been linked to their roles in immune tolerance.[55,56,63] Deletion of PD-L1 in LECs restricts the responses of memory CD8+ T cells within tumor-draining LNs in experimental models of cancer.[120] LEC-specific deletion of PD-L1 alone did not affect tumor growth, but increased the response to adoptive T-cell therapy.[120] In peripheral dermal LECs PD-L1 is upregulated in response to IFNγ, which limits CD8+ T-cell accumulation in tumor microenvironments.[121] The latter result illustrates the ability of peripheral LECs to acquire LN LEC-specific functions.

LN LECs have also been suggested to serve as a source of some self-peripheral tissue antigens,[117,122–125] allowing induction of peripheral tolerance and complementing the known role of the thymus in the deletion of autoreactive T cells.[123,124] However, many of such potential self-peripheral tissue antigens have been shown more recently to represent bona fide LEC transcripts[25,41,61] illustrating the challenges of identifying true self-peripheral tissue antigens.

In summary, LECs both archive and present antigens, functioning as a nonhematopoietic, nonprofessional APCs. This endogenous or foreign antigen presentation occurs directly or indirectly, through cooperation with APCs and through MHC-I or II molecules. LN LECs can drive either effector (indirectly through antigen archiving) or tolerogenic immunologic responses (through antigen presentation) and thus influence the quality of immune response. LN LECs share antigen storage and presentation capacities with other LN stromal cells,[102] adding another layer of complexity. New tools have recently

been developed to elucidate antigen uptake and presentation by LECs in vivo,[112] which will help to explore this process at a cellular resolution.

RESPONSES OF LN LYMPHATIC VESSELS

LEC Expansion and Contraction in Inflammation

Acute inflammation-induced LN hypertrophy involves proliferative responses of both the blood and lymphatic vasculatures. As inflammation resolves, pruning and cell death allows restoration of LN size and homeostasis.[87,126,127] In the context of tumor-induced or chronic inflammation, the LN remains enlarged over extended periods of time. Following acute inflammatory stimuli, the LN LECs' numbers begin to increase by day 3 to 4 and peak at day 5 to 15 depending on the inducing stimulus.[87,127–129] Thereafter, the LN LEC population, along with LN BECs and FRCs, starts to decline, unless the inflammatory stimulus or infection is not resolved.

LECs' proliferation is primarily seen in the central medulla and the cortical/paracortical sinuses of the mouse LN,[128,130] which is consistent with the involvement of prolymphangiogenic PTX3-LECs.[25] Accordingly, the proliferation in response to synthetic double stranded RNA and the TLR3 ligand polyinosinic–polycytidylic acid sodium (Poly [I:C]), and anti-CD40 therapy, was mainly confined to PD-L1^{low} LN LECs,[127] which include PTX3-LECs and cLECs but not PD-1^{high} fLECs and MARCO-LECs.[25] Lineage-tracing studies proposed that expansion of the LN blood vasculature relies on clonal proliferation of the high endothelial cells of the HEVs, which give rise to both capillaries and new HEVs.[126] More recently, Apelin$^+$ capillary resident precursors that displays stem cell and migratory signatures, have been shown to contribute to the homeostatic LN blood endothelium turnover and neogenesis of HEVs after immunization.[131] Identification of markers of LN LEC subsets opens many possibilities for exploring the LECs reorganization during LN vasculature remodeling through similar lineage-tracing studies. Following resolution of inflammation, the contraction of blood vasculature is achieved by the stochastic death of supernumerary BECs,[126] while the spatial dynamics of LECs' contraction has not yet been examined.

Various stromal and immune cell populations cooperate to induce LEC proliferation during inflammation. The initial stages of LN LEC proliferation are orchestrated by innate immune cells such as incoming DCs and macrophages. The direct contact of CD11$^+$ DCs and LECs, as well as DC-dependent upregulation of VEGF-A in FRCs, contribute in part to the initiation of lymphatic vascular expansion.[132–134] In addition, in a model of acute skin inflammation, production of VEGF-A, -C and -D by CD11b$^+$Gr-1$^+$ macrophages at the site of inflammation and in the corresponding draining LN promote intranodal lymphangiogenesis, which was attenuated upon VEGFR3 or VEGFR2 ligand blockade or macrophage depletion.[135]

Expansion of lymphatic vessels at the peak of infection/inflammation is further sustained by the adaptive immune cells.[136] Absence of B and T cells nearly abrogates LN expansion at later time points during immunization.[133] Consistently, *Rag*-deficient mice, which lack mature B and T cells, failed to expand LN stromal cells following herpes simplex virus 1 (HSV1) infection.[87] T cells or B cells were sufficient to initiate early expansion, however B cells were needed to sustain the stromal reactions.[87] In an acute immunization model, LN lymphangiogenesis was highly dependent on the presence of B cells within the LN and was partially blocked by inhibition of VEGFR3 or VEGFR2. The spatial localization of VEGF-A in B cell follicles in activated LNs, and the ability of activated B cells to secrete VEGF-A in vitro, suggest that B cell–derived VEGF-A contributes to LN lymphangiogenesis.[137] The significant role of VEGF-A, and presumably its main receptor VEGFR2, in LN lymphangiogenesis is surprising, given the dominant role of VEGF-C/VEGFR3-signaling in LEC proliferation in most other settings.[138] However, LEC-specific ablation of VEGFR2 prevented tumor-associated lymphangiogenesis,[139] hinting at the significant contribution of this receptor to lymphangiogenesis in pathological or immune-rich environments.

LN swelling in inflammation involves changes in mechano-induced circuits with initial relaxation of the FRC network to allow LN expansion.[140,141] The cell surface glycoprotein PDPN mediates these FRC responses through interaction with CLEC-2$^+$ DCs. Genetic inactivation of *Pdpn* in FRCs using CRE recombinase under the promotor of *Pdgfra,* inhibited LN expansion in acute inflammation.[141] Interestingly, *Pdpn* deletion in FRCs also reduced LEC proliferation, whereas BEC proliferative responses were unaffected.[141] This observation suggests that mechanical stretching contributes to LN LEC proliferation, as it was demonstrated during embryonic lymphangiogenesis.[142] PDPN is also constitutively expressed by all LECs, and is upregulated both in models of inflammation and in cancer.[77,87] Whether it can play similar mechano-signaling roles in LECs as in FRCs is not known. In a model of tumor-induced chronic LN hypertrophy, where PDPN was upregulated specifically on fLECs, deletion of PDPN in LECs did not affect LN swelling based on LN weight.[77]

An important open question concerns the existence and role of imprinting of LN stromal subsets in response to repeated immunologic exposures. Analysis of LN LECs', FRCs' and BECs' responses[87] show that following acute HSV1-infection, LN stromal subsets returned to homeostatic levels without '"memory"' signature at least at a population transcriptional level. However, it is tempting to speculate that the LN LECs display immunologic

memory, as demonstrated in peripheral LECs during intestinal infection.[143]

Molecular Plasticity of LN LECs in Inflammation

In addition to inducing proliferation, inflammation activates transcriptional programs for immune regulation in LN stromal cells.[87,127,144] Such reactions of LN stroma play critical roles in fostering productive immune responses (for review[145,146]). A full understanding of inflammation-induced molecular, structural and functional changes in the LN lymphatic vessels is still lacking. We will therefore only discuss a few examples of inflammation-dependent responses of LN LECs, with focus on similarities and differences between different models and in relation to other stromal cells in the LN.

Early responses to type 1 IFN inducing agents, like viruses or oxazolone treatment for 48-72 hours, induce prototypical IFN transcriptional targets, including *Cxcl9*, *Cxcl10* and *Irf7*, in LN LECs.[25,87] Such responses are evident also after 12 hours systemically induced oval-bumin specific T-cell responses.[144] In addition to LECs, *Cxcl9* and *Cxcl10* are coregulated also in BECs and FRCs.[87] Stromal cells likely cooperate with innate and adaptive immune cells harboring the CXCL9 and CXCL10 chemokine receptor CXCR3 (C-X-C chemokine receptor 3), such as NK-cells, effector T cells,[147] and monocyte subsets,[148] in forming niches for immune activation/modulation cues within the LN. At 48 hours postoxazolone-induced inflammation, downregulation of gene expression in LN LECs dominate over upregulation.[25] This is consistent with a principal type 1 IFN response at this time point,[25] known to induce transient inhibition of transcription as a defense against virus replication.[149]

ScRNAseq analysis of LN LECs has also revealed niche-confined responses to inflammation. fLECs, and to a lesser degree PTX3-LECs, were the main LEC LN subsets responding during early stages of oxazolone-induced inflammation.[25] Although all LN LECs express the different receptor subunits for type 1 and type 2 IFN, fLECs display higher expression of *Ifnar2* and *Ifngr2*[25] compared to other subsets. fLECs and PTX3-LECs are the sites for cell entry and egress, respectively; it is thus possible that high responsiveness of these LEC subsets to inflammatory stimuli reflects a need to coordinate these processes in inflammation. While both *Cxcl9* and *Cxcl10* were upregulated in inflamed fLECs and PTX3-LECs, other early responses diverged. For example, the chemokine *Ccl5* was induced in fLECs, while *Ccl2* was induced in PTX3-LEC. CCL5 acts upon many types of lymphocytes and innate leucocytes, while CCL2 is important for monocyte recruitment. CCL5, but not CCL2, was also upregulated in LN LECs and FRCs after HSV1-infection.[87] Neither of these cytokines are detected in these LEC subsets in the resting mouse LN.[25,45] However, CCL2 has a high basal expression in multiple subsets of FRCs.[18,150] The exact mechanisms driving differential chemokine expression in fLECs and cLECs is not understood, but it may include inflammation-related changes in vascular permeability. Indeed, increase of vascular permeability in response to VE-cadherin blockade induced CCL2 in both FRCs and LECs,[150] conversely, Angiopoietin-1, which reduces vascular permeability, reduced CCL2 in FRCs but not LECs.[150] Functionally, CCL2 expression by T-cell zone reticular cells, with possible contribution from LECs, promoted accumulation and survival of LN plasma cells/plasma blasts through CCL2-driven recruitment of monocytes.[150]

ITGA2B (integrin subunit alpha 2b), also known as CD41, is highly expressed in mouse but not human fLECs and MARCO-LECs.[25] It is further upregulated in mouse LN LECs in experimental tumor-induced inflammation, where it was proposed to interact with LN fibrinogen,[129] and upon HSV1-infection.[87] ITGA2B upregulation was reduced by systemic blockade of IFNγ in the context of tumor-induced inflammation and hypertrophy,[129] indicating that ITGA2B may be a general marker of inflammatory response of mouse LN LECs.

PD-L1 is another gene upregulated in LN LECs in different models of inflammation,[25,87,127] together with genes associated with antigen presentation.[87] It also belongs to one of the genes coregulated in both BECs and LECs in HSV1-infection.[87] Upregulation can be mediated by type 1 IFN[127] while the basal homeostatic expression of PD-L1 in LN LEC is not affected by deletion of *Ifnar1*.[127]

Many LN LEC subset-specific genes retain their original pattern in inflammation; however, some changes have been documented. In chronic inflammation induced by topical application of the Aldara cream, which induces both a TLR7-dependent and TLR7-independent inflammatory responses,[151] cLEC marker *Anxa2* was induced in fLECs.[152] Upregulation of *Anxa2* in bulk sorted LECs was also observed in response to HSV1-infection[87] but not in early oxazolone- or tumor-induced inflammation.[25] The functional significance of *Anxa2* upregulation is not known, but may be linked to its ability to interact with calcium binding protein S100A10 (also expressed by cLECs), which in turn regulates the activation of plasmin.[153] Like for several other mentioned examples, *Anxa2* is not a LEC-specific gene but is expressed by both LN BECs and FRCs.

Taken together, current data support active roles of LN LECs and their inherent plasticity in response to different inflammatory cues. Future research needs to integrate data from endothelial, mesenchymal, and immune cells to fully understand the formation of regulatory niches in the LN and their impact on the immune responses, as well as on structural regulation in LN hypertrophy.

Emerging Role of Circadian Rhythms in Lymphatic Vessels and Lymphoid Organ Function

Within the last 2 decades an increasing body of knowledge has showed that circadian rhythms impact different aspects of the biology of an organism.[154] This circadian clock can be divided in different types, the primary or central and the peripheral. The first consists of a group of neurons from the suprachiasmatic nucleus of the hypothalamus that are regulated mainly by light. The latter can be found in all tissues and respond to synchronizers, such as food intake and exercise, and that are themselves synchronized by the central clock.[154–156]

The impact of such rhythms have been widely studied in the immunology field, where it has been shown that the number of circulating leukocytes changes during a day due to cyclic fluctuations of exit of cells from BM and recruitment of immune cells to tissues.[155,156] By screening multiple organs, He *et al.* have shown that endothelial cells are also under circadian governance since they observed rhythmic expression of different adhesion molecules in various organs, such as VCAM-1 (vascular cell adhesion molecule 1), ICAM-1, and MAdCAM-1 in the LN. They also show that by combining an endothelial specific ablation of *Bmal1*, a transcription factor central for circadian clock function, with adoptive transfer of normal immune cells, there is a loss of oscillations of immune cells, both in blood and tissues.[157] This demonstrates that rhythmicity in the microenvironment is key for dictating oscillatory patterns in the recruitment of leukocytes.[157] In addition, Holtkamp et al[158] demonstrated that migration of DCs into skin lymphatics follows a circadian pattern, peaking during resting phase in mice. This appears to be governed by both LECs and DCs since *Ccl21*, *Ccr7* and *Lyve1* genes are under direct control of BMAL1 (brain and muscle ARNT-like 1) and by genetically ablating rhythms, in either type of cells, this led to an abrogation of circadian trafficking.[158]

Since recruitment of immune cells is a vital step for adaptive immune responses, future work exploring rhythmicity in immune and endothelial cells, both blood and lymphatics cells, in vaccination or in immunotherapeutic treatments may prove to be beneficial for clinical applications.[159]

ROLE OF LYMPHATIC VESSELS IN LN DEVELOPMENT

Mice harbor up to 38 discrete LNs,[160,161] most of which arise during embryonic development in 1 of 16 stereotypic anatomic locations near the tripod-like intersections of blood vessels and in proximity of lymphatics (Figure 3). Mesenteric LN anlagen is initiated around E9.0, whereas the development of peripheral LNs proceeds from E11 to E12 in anterior-posterior fashion.[162,163] Developmentally,

inguinal, axillary, brachial, and popliteal peripheral LN LECs originate from paraxial mesoderm, whereas LECs in the mesenteric LNs are derived from the HOXB6+ (homeobox B6) lateral plate mesoderm.[164] The functional significance of distinct developmental origins of LN LECs remains to be investigated. Humans harbor 500 to 800 LNs, organized in chains, which develop between 8 and 11 weeks of gestation and concentrated in the neck, axilla, thorax, abdomen, and groin areas.

LN development is a multistep process relying on a cross-talk of hematopoietic LTi cells with different stromal cell populations, including mesenchymal lymphoid tissue–organizer cells (LTo) cells, and BECs and LECs, referred to as endothelial LTo cells.[19] The LTβ-dependent and chemokine-mediated clustering of extravasated (pre) LTi and LTo cells is essential for LN anlagen initiation and is described in detail elsewhere.[19,165] Based on the analyses of mice with defective vascular smooth muscle cell development and 3D imaging of embryonic inguinal LNs, transmigration of LTi cells via the gaps in the immature smooth muscle cell coverage at the sites of blood vessel branching represents the earliest step of LN development.[24]

Although lymphatic vessels were proposed to determine LN initiation,[166,167] the initial extravasation of LTi cells from blood vessels likely proceeds independently of closely associated lymphatic vessels, as it has been documented in mice lacking lymphatics.[168,169] However, functionally competent lymphatic vessels are indispensable for early LN anlagen expansion, and their absence or dysfunction precludes further LN development.[24,166,168,170] Embryonic lymphatic vessels contribute to LN development in multiple ways. They transport additional LTi cells from the periphery to the site of LN initiation[24,166] (Figure 3). Peri-nodal lymphatics generate interstitial flow, which potentiates the production of the chemokine CXCL13 in LTos, important for retention of LTi cells.[24] Finally, lymphatic vessels are remodeled and expanded around the LN anlagen to form the LN capsule, a key step in the formation of a mature LN (Figure 3).

The LN capsule formation relies on prolymphangiogenic VEGF-C/VEGFR3-signaling,[24,170] likely driven by high expression of VEGF-C in LTo cells in response to persistent LTβR activation.[171,172] However, the process itself is morphologically different from sprouting lymphangiogenesis. During the latter, VEGF-C gradient drives formation of filopodia-bearing tip cells, followed by proliferating stalk cells, which ultimately form a mesh-like lymphatic vasculature with blind-ended capillaries.[173] In contrast, LN capsule formation is better described as ensheathment process, in which a collecting lymphatic vessel forms a double-walled cup-like structure, which expands to completely engulf the developing LN (Figure 3). Functionally, the LN engulfment mechanism preserves peri-nodal lymphatic vessel
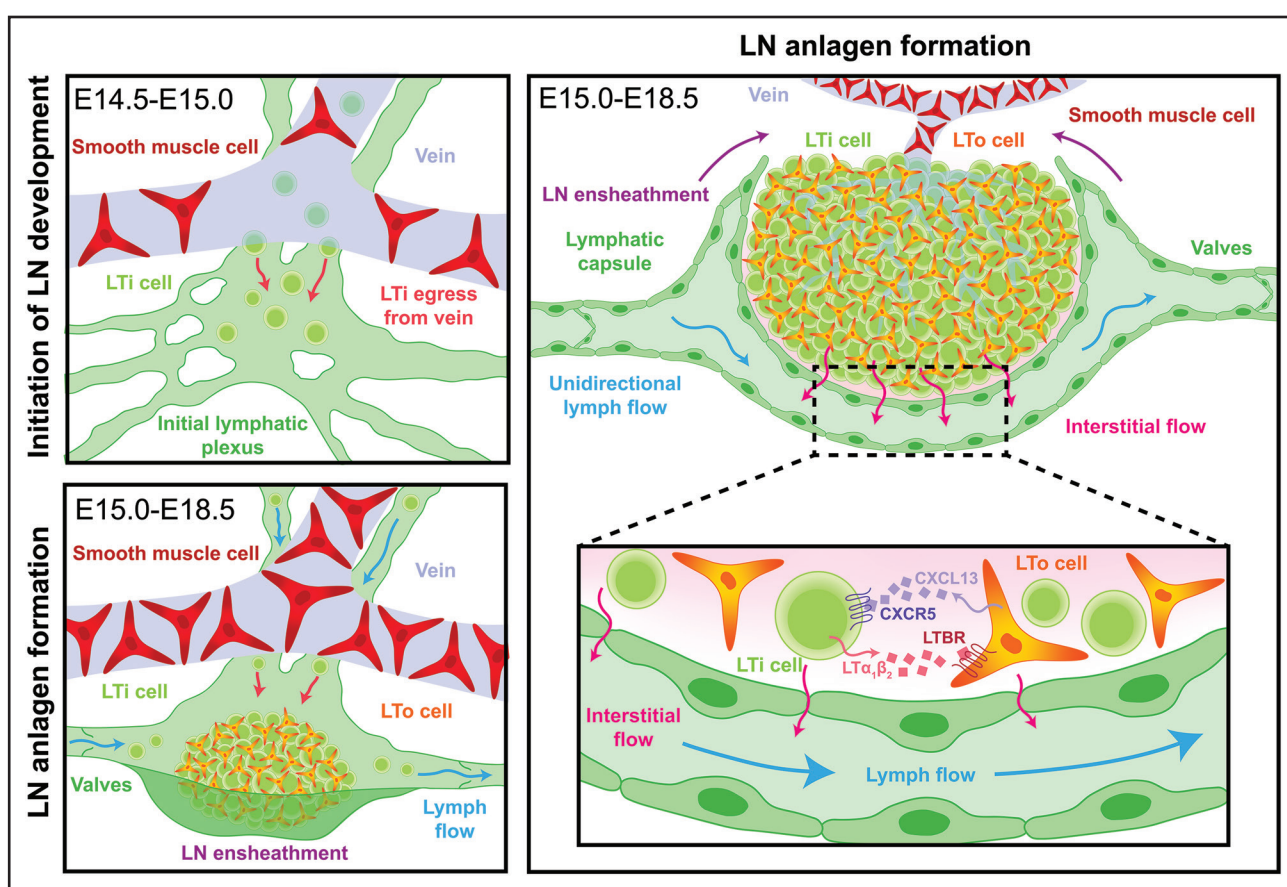
Figure 3. Lymphatic vessels in lymph node (LN) development.
Initiation of LN development (**top left**): Hematopoietic lymphoid tissue inducer (LTi) cells (green) extravasate from immature veins (purple) at bifurcation sites of large blood vessels. The process is facilitated by incomplete smooth muscle cell (red) coverage at this stage of development. LN anlagen formation (**low left**): Developing collecting lymphatic vessels reorganize at the site of LN initiation and expand to form a LN capsule. lymphatic vessels also bring additional LTi cells to the LN anlagen contributing to their expansion. This expansion is potentiated by fibroblastic lymphoid tissue organizer (LTo) cells (orange). LN anlagen formation (**right**): Transversal cut showing collecting vessels reorganization giving rise to LN ensheathment. Lymph flow in the subcapsular sinus together with interstitial fluid flow, generated by interstitial fluid absorption by lymphatic vessels, potentiates the expansion of the developing LNs. Inset of LN anlagen formation (**low right**): interstitial fluid flow enhances CXCL13 (C-X-C motif chemokine ligand 13) expression by LTo cells, which retain CXCR5+ (C-X-C chemokine receptor 5) LTi cells. Cross-talk between $LT\alpha_1\beta_2$ produced by LTis and LTβR+ (lymphotoxin-β receptor) LTo cells leads to further signal amplification, resulting in LTi cell maturation and accumulation.

integrity and transport function, allowing continuous delivery of LTi cells for LN expansion.

Despite VEGF-C/VEGFR3−dependent collective migration and proliferation of LECs, only rare sprouting LECs are observed at the top of converging endothelial sheets during LN capsule formation. The underlying mechanism is not clear; it is possible that the uniformly high levels of VEGF-C in the LN anlagen preclude tip cell selection. In addition, in contrast to peripheral embryonic LECs, LN LECs express low levels of guidance molecule NRP2, important for LEC spouting behavior.[174,175] Reduced LEC proliferation and involution of mesenteric and inguinal LNs occurs during late gestation in *Clec1b*-deficient embryos, implicating this endogenous PDPN ligand present on platelets, inflammatory DCs, and possibly other hematopoietic cells, in LN development.[176] LEC-specific

loss of LTβR or especially of RANK interrupts LN development,[24] highlighting the need to better understand the role of these pathways in LN-associated lymphatics.

Embryonic SCS LECs display polarized expression of cLEC and fLEC markers and recruitment of CD169+ macrophages, indicating that signals for the SCS LN LEC differentiation and for positioning of specialized LN cells are present before birth. Such prenatal specialization ensures functionality of LNs immediately from birth. However, prenatal LNs lack medullary and cortical lymphatic sinuses, which only start to develop from P3[170] in parallel with the maturation of HEVs and postnatal influx of B and T cells. LNs of *Vegfc*+/- postnatal mice are small and lack medullary lymphatics, indicating that postnatal LN lymphangiogenesis is also VEGF-C/VEGFR3 dependent.[170]

NEW KIDS ON THE BLOCK: LYMPHATIC VESSELS OF THE SPLEEN AND PPs

Although LN lymphatic vessels have been intensely studied, information on lymphatic vessels of other SLOs, such as PP and especially the spleen, is scarce.

Spleen

Splenic functions include filtration of blood for pathogens and antigens, maintenance of erythrocyte homeostasis, iron metabolism, and extramedullary hematopoiesis.[177] Since the spleen lack afferent lymphatic vessels, antigens, and immune cells need to enter the tissue via blood vessels,[177,178] which makes this organ an extraordinary sentinel for blood borne pathogens, compared with the LNs that are sentinels for tissue-derived antigens. Pellas and Weiss[179] showed over 30 years ago the presence and the arrangement of deep lymphatic vessels that originated within the proximal periarterial lymphatic sheaths of white pulp (Figure 1). They further demonstrated that both T and B cells exit the white pulp via these lymphatic vessels. A more recent study confirms the presence of LYVE-1+ lymphatic vessels in mice, running along the central arteries in the white pulp and trabecular arteries and exiting the spleen from the hilum.[180] To date, little information is available about specialization and function of spleen lymphatics, although lymphatic vascular lesions of spleen, such as lymphangioma, have been described.[181]

Peyer's Patches

PP, named after Swiss anatomist Johann Conrad Peyer,[182,183] are most abundant in the ileum, the end portions of the small intestine (Figure 1). Mice harbor 6 to 12 PPs, while humans may have several hundreds of PPs.[183,184] PPs are composed of B-cell follicles, separated by T-cell zones, covered by the subepithelial dome which is then enveloped by the follicle-associated intestinal epithelium.[182,183] PPs are nonencapsulated and devoided of afferent lymphatics.[182,183] Antigen entry in PPs takes place mainly via M-cells, a specialized type of epithelial cell that take up a wide range of types of particles and antigens, including bacteria, virus and fungi, while lymphocytes enter PPs through HEVs, similar to LN.[183]

PPs are the main location for the B-cell antibody class switch to IgA and thus are the major supplier of intestinal IgA, highly present in the mucosa.[183] In line with their role in B-cell dependent immune responses, PPs contain proportionally more B cells (80%) in comparison to LNs (up to 20%)([183] S. Arroz-Madeira, unpublished, 2021). The lymphatic vessels of PPs are the major route for B- and T-cell egress from the intestine. Similar to LNs, the egress of lymphocytes from PPs strongly relies on LEC-derived S1P-signaling.[40] In addition, CXCL12 expression by peri-lymphatic fibroblasts is important for counteracting CXCL13-mediated signal for B-cell retention in PPs and promoting egress of CXCR4+ B cells from PPs.[185] Of interest, CXCL12 display a different expression pattern in LNs, where it is produced in the proximity of HEVs.[186] In photoconversion experiments, B-cell residency time in mouse PPs was estimated to be 10 hours, which is shorter than the 16 hours residency time for LNs.[185] Such decreased residency may be facilitated by both the existence of a promigratory CXCL12 gradient, and by the close proximity of perifollicular lymphatic vessels and the PPs HEVs, revealed by scanning electron microscopy.[187] PPs lymphatic vessels express high level of guidance receptor ROBO4, which selectively promotes egress of naive B but not T cells through the regulation B-cell adhesion to the efferent lymphatic vasculature.[188] Overall, the studies to date provided a glance into similarities but also fascinating differences regulating lymphocyte trafficking via PPs lymphatic vessels as compared to LNs.

ROLE OF LYMPHATIC VESSELS IN AGING OF LYMPHOID ORGANS

The function of immune system declines with age. It manifests as a reduced number of immune cells, alterations in proportions of different immune subtypes and a decline in their specific functions.[189] Immunosenescence is a term commonly used to describe the age-induced immune deficiency, including effects on both the innate and adaptive immunity.[190] The ability of immune cells to sustain their functions is not only dependent on cell intrinsic changes, but is also linked in the body's ability to retain their functional homes, that is, the lymphoid organs.[191–193] A reduced formation of new hematopoietic cells stems from a decline in the bone marrow ability to support stem cell niches[191] and from the involution of the thymus.[194,195] Increasing evidence suggests that aging of SLOs also affects immune cell survival and activation. The latter includes reduced numbers and function of PPs[196,197] and disorganization of the microarchitecture of the spleen.[198] It is being increasingly recognized that various immune cell subsets participate in maintenance of tissue-specific stem cells[13,14] and promote tissue regeneration after injury.[15,16] Therefore, better understanding of age-related degenerative changes of lymphoid organs is needed for designing treatments of multiple human diseases.

The aged mouse LNs have a reduced ability to support survival and homeostatic proliferation of naive T cells.[192,193] This was attributed to multiple stromal microenvironmental changes in the LN that impaired the entry and the survival of the T cells.[192,193] In mice, fibrosis accompanied by a loss of the specialized functions of the LN FRCs, is a major age-related change,[199,200] with skin-draining LNs affected more than internal LNs.[201] Thickening of the capsule and fibrosis are also observed

in aging human LNs[202,203] where a specific type of fibrosis (hyalinization) also has been reported[204] (Figure 4).

Similar to mice, degenerative human LN features differ according to the LN position in the body. Fibrosis dominates age-induced pathological changes within LNs of body cavities, such as mesenteric LN.[202,203] However, in skin-draining human LN, such as the axillary LNs, lipomatosis, a pathological replacement of the normal LN parenchyma by adipose tissue,[202,205] is more frequent[37] (Figure 4). On a macroscopic level, LNs with extensive lipomatosis appear transparent since the normal tissue is replaced with fat.[206] While it is a common phenomenon,[205] the underlying mechanisms for this pathology and the consequences for the LN functions have only recently started to be studied.

Analysis of human LNs showed that lipomatosis starts from the medullary regions.[37] Coexpression of fibroblast markers in LN adipocytes suggests that lipomatosis is initiated by transdifferentiation of MedRCs into adipocytes. Accordingly, cultured mouse MedRCs display a higher adipogenic capacity in comparison with BP3+ populations which includes T-cell zone reticular cells.[37]

Lipomatosis was associated with a downregulation of LTß expression in the surrounding tissue.[37] LTß counteracts adipogenic differentiation of mouse embryonic LN mesenchymal LTo precursor cells.[207] Therefore, declining expression of LTß in aging may be a contributing factor to the development of human LN lipomatosis.

The effect of lipomatosis on the LN vasculature includes a gradual loss of the MSs.[37] It is possible that adipocytic transdifferentiation of MedRCs deprives MS-LECs of both essential structural support and key survival factor(s), leading to LEC apoptosis. Along with the loss of MSs, compensatory expansion of LECs with the molecular features of collecting vessels was observed.[37] LN lipomatosis also leads to dedifferentiation of nearby HEVs, which display dilation, loss of their normal cuboidal endothelial morphology, and partial loss of their expression of PNAd (peripheral node addressin).[37] Such dedifferentiated HEVs also display reduced density of surrounding TCF1/7 (T cell factor 1/7) naive T cells.[37] The mechanism leading to the replacement of MSs with collecting-like lymphatics and the remodeling of HEVs in LNs with
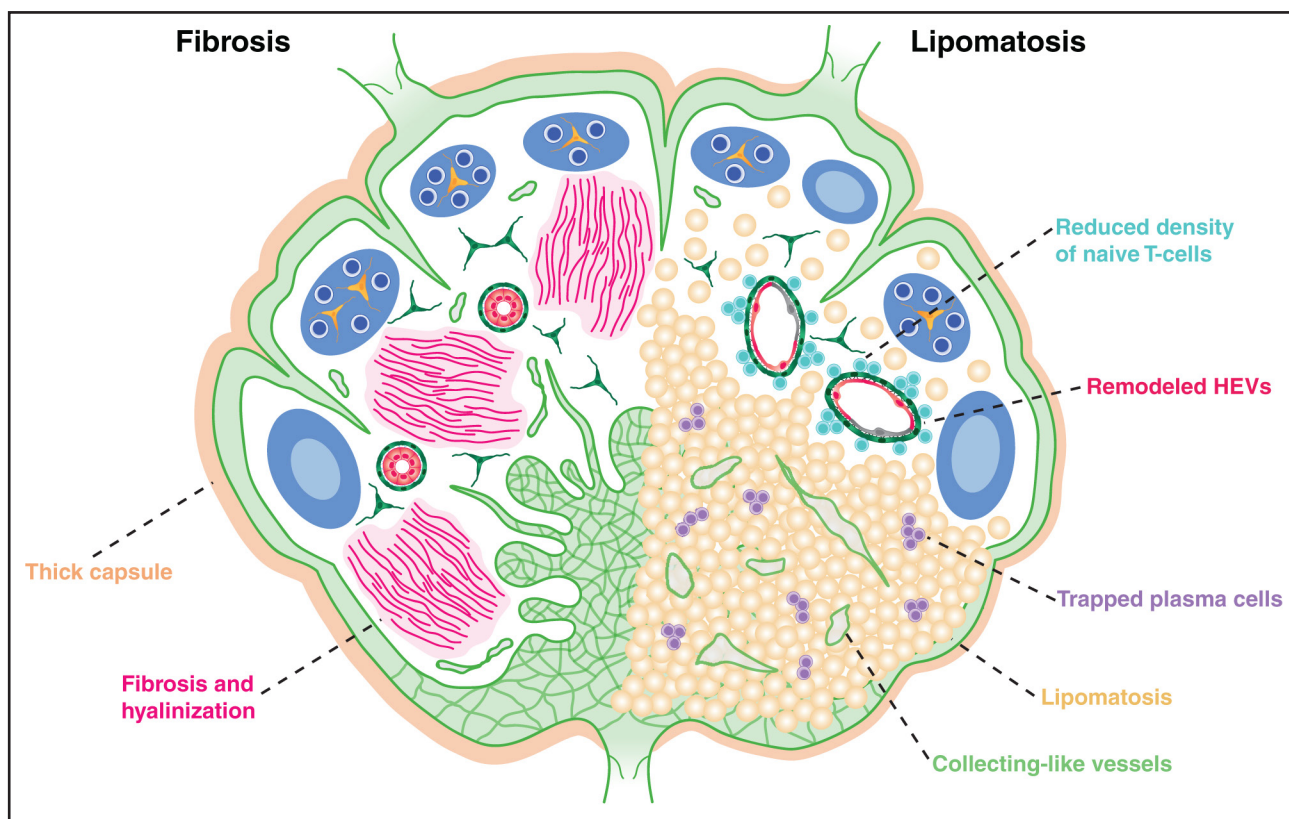

Figure 4. Aging pathologies of the human lymph node (LN).
The 2 main aging pathologies of the human LN are fibrosis (**left**) and lipomatosis (**right**). In fibrosis, the capsule (beige) is getting thicker and fibrosis (pink fibers) with or without hyalinization (light pink) is formed in the LN parenchyma. In lipomatosis, the medullary reticular cells transdifferentiate into adipocytes (round beige structures). As lipomatosis progress, lymphatic endothelial cells (LECs) of medullary sinuses (MSs) are lost; and replaced by collecting-like LECs, normally not present inside the LN, in the expanding intra-LN adipose tissue. The stationary plasma cells (purple) that normally are found in the medullary cords are trapped in the adipose tissue. Lipomatosis also induces dilation and dedifferentiation of nearby high endothelial venules (HEVs, red/gray), manifesting as endothelial thinning and partial loss of PNAd (peripheral node addressin: red, loss of: gray). This leads to a reduced density of naive T cells (turquoise) around HEVs. More color coding: lymphatics (green), B-cell follicles (dark blue) with germinal centers (light blue), follicular dendritic cells (yellow), T-cell zone reticular cells (dark green).

lipomatosis remains to be investigated. Adipocytes produce many different factors that could potentially affect the vascular homeostasis, including VEGF-A.[208]

Besides the LN age-related vascular changes, peripheral lymphatic vessel function also declines with age as evidenced by reduced density of the initial capillary vessels, and impaired pumping function of collecting vessels due to vessel dilation and loss of smooth muscle cell coverage (for review, see González-Loyola et al[6]). The resulting reduction in incoming lymph flow into the LN might indirectly affect LN functions. Indeed, experimental lymphatic vessel occlusion leads to impairment of LN stromal functions including dedifferentiation of the HEVs,[209,210] in turn reducing homing of naive T cells into the LN. Maintenance of HEVs depends on migratory CCR7+ DCs, which serve as important source of LTβ.[211] Thus it is likely that aging of peripheral lymphatic vasculature amplifies age-induced degeneration of the LN vasculature and stroma. Additionally, LN lipomatosis may reduce LN flow to downstream LNs in the chain, at least transiently before compensatory vessels have been established to replace the destroyed MSs.

Spontaneous LN lipomatosis is rare in inbred strain of mice.[199,200] However, it can be induced experimentally by the combined deletion of the transcriptional effectors of Hippo pathway YAP (yes-associated protein) and TAZ (transcriptional co-activator with PDZ-binding motif) and LTβR in LN FRCs[212] even in young animals. The effects of the combined deletion indicate a cooperation of the mechanosensitive YAP/TAZ and the lymphotoxin pathways in restricting adipocyte fate in LN fibroblasts. Mouse and human LN fibroblast share a similar pattern of expression of these pathways in LN FRCs.[37] However, the mechanistic reasons underpinning development of LN fibrosis versus LN lipomatosis in these 2 species are not understood.

LN fibrosis and lipomatosis in aging, with dysregulation of both the blood and lymphatic vasculartures, are likely to interfere with the body's ability to mount immune responses. This has major implications for not only vaccination and the response against new infections but also protection against malignancies. LNs are the earliest metastatic sites in human cancer. Adipocytes and accumulation of fat are already known to contribute to a premetastatic niche in for example the bone marrow.[213] LN lipomatosis can, therefore, both contribute to a premetastatic niche in the context of LN metastasis and impair the ability to mount productive antitumor immunity.

CONCLUSIONS

Studies of LN lymphatic vasculature considerably broadened the spectrum of known functions of LECs. In addition to their general functions as lymph conduits, LN LECs establish chemokine gradients for migration and trafficking of immune cells, serve as a paracrine source of factors

for maintenance of LN immune cell niches, and play active roles in antigen storage and presentation. Further functions glanced from the single-cell profiling studies include roles of LECs as scavengers, complementing the function of LN macrophages, and source and organizers of the ECM. It will be important to establish now whether and how peripheral LECs of different organs highjack LN LEC-specific functions, such as, for example, antigen storage and presentation, or maintenance of niches for immune and other cells. Such knowledge will open exciting new avenues for development of novel therapies for many common diseases based on a deeper understanding of the molecular mechanisms underlying LN stromal cell differentiation and function.

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