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Review

Lymph node lymphatic endothelial cells as multifaceted gatekeepers in the immune system

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Single-cell technologies have recently allowed the identification of multiple lymphatic endothelial cell (LEC) subsets in subcapsular, paracortical, medullary, and other lymph node (LN) sinus systems in mice and humans. New analyses show that LECs serve key immunological functions in the LN stroma during immune responses. We discuss the roles of different LEC types in guiding leukocyte and cancer cell trafficking to and from the LN parenchyma, in capturing microbes, and in transporting, presenting, and storing lymph-borne antigens in distinct types of lymphatic sinuses. We underscore specific adaptations of human LECs and raise unanswered questions concerning LEC functions in human disease. Despite our limited understanding of human lymphatics – hampering clinical translation in inflammation and metastasis – we support the potential of LN LECs as putative targets for boosting/inhibiting immunoreactivity.

Highlights

Lymphatic endothelial cells (LECs) line all the complex sinus systems of mammalian lymph nodes (LNs).

Recent unbiased single-cell analyses have revealed unanticipated heterogeneity in LEC types within LN sinuses in mice and humans.

There are marked molecular species-specific differences between mouse and human LECs.

LECs from draining LNs can regulate the transport of lymph-borne antigens into the LN parenchyma.

Mammalian LN LECs can control the trafficking and sinus-residency of many leukocyte types.

The effects of pathological processes such as inflammation, infection, and tumorigenesis on LN LECs are starting to be understood.

Molecules expressed on LECs might be potentially targeted to control cell trafficking and immune responses.

Significance

Recent breakthroughs further define the molecular identity and function of LN lymphatics in mice and humans during homeostasis and disease. As a key LN stromal cell subset, LECs offer new targets for controlling antigen presentation, leukocyte migration, and cancer cell metastasis.

Unique characteristics of mammalian LN lymphatics

The mammalian lymphatic vasculature drains cells, molecules, and solutes from the periphery into the systemic circulation and undergoes remarkable organ-specific adaptations as lymph enters LNs. These serve as specialized meeting points for antigens and responding lymphocytes, and receive cellular input from the blood and lymphatic vasculature; by contrast, LN cellular output and antigen delivery to the LN stroma rely mainly on lymphatic vessels [1]. Peripheral LNs have incoming **afferent lymphatic** vessels [2], an elaborate intranodal lymphatic vasculature system [3], and an **efferent lymphatic** (see Glossary) vessel leading to the next LN along a chain of LNs. Lymphatics properly function with the contribution of other non-leukocytic stromal cell types such as blood vessel endothelial cells and **fibroblastic reticular cells**; their function is well established and of paramount importance in mounting appropriate immune responses in the LN [2,4–6].

Compared to lymphatic capillaries in the periphery and afferent lymphatic vessels [5], the lymphatic vasculature in LNs shows several differences in structure, phenotype, and function. Recent technological advances have allowed the analyses of LN LECs at an unprecedented level, proving that earlier knowledge obtained using easily accessible peripheral LECs cannot be generalized to LN LECs. In fact, unbiased single-cell analyses have allowed LN LEC characterization into several unique subtypes and provided mechanistic explanations for many LN LEC functions that modulate overall immune reactivity. We discuss exciting new insights regarding this specialized component of the lymphatic vasculature, focusing on cellular differentiation, leukocyte and cancer cell trafficking, antigen presentation, and the control of pathogen spread. New findings cement the *bona fide* role of this understudied stromal cell type in fine-tuning adaptive immune responses in LNs, and pinpoint new putative molecular targets for manipulating antigen presentation and harmful cell trafficking.

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Sinus systems in LNs

Each mammalian peripheral LN typically has several incoming afferent lymphatic vessels which penetrate the collagenous capsule of the LN at the paracortical side of the node [3,5]. The lymphatic vasculature then opens as a subcapsular sinus (SCS), which encircles the entire LN stroma, before changing into a branched medullary sinus system at the LN hilus (the depressed focal entry point of blood vessels) (Figure 1A).

All LN sinuses are lined with LECs. Fate-mapping experiments using cell type-specific fluorescent reporter mouse lines show that LECs in different LNs have distinct mesodermal origins [7]. Almost all subcapsular and medullary LECs in popliteal, inguinal, axillar, and brachial LNs derive from $Pax3^+$ paraxial progenitor cells. By contrast, $Hoxb6^+$ lateral plate mesoderm progenitor cells give rise to all LN LECs in mesenteric LNs. Of note, other stromal subsets, including blood endothelial cells, are derived from lateral plate mesoderm cells in all LNs, and this is interesting because it suggests that LECs in non-mesenteric LNs have unique developmental origins [7].

The SCS is lined by an LEC layer (**SCS ceiling LECs**) that faces the capsule, and by another LEC layer (**SCS floor LECs**) that overlies the lymphocyte-rich parenchyma (Figure 1A) [8]. The width of the SCS varies, but often equals one lymphocyte in diameter (~10 μm) in mice [8]. Moreover, the SCS is innervated by sensory neurons [9]. Optogenetic stimulation of LN-innervating sensory neurons in Nav1.8cre/+ × *Rosa26*$^{ChR2–eYFP/+}$ mice using blue light mostly affects SCS ceiling LECs in murine LNs, as evidenced by the alteration of gene expression patterns analyzed by Seq-Well sequencing. Several genes, including those involved in antigen processing and presentation and in the turnover of sphingosine-1-phosphate (S1P), were selectively altered in this LEC subset [9]. Moreover, the activation of LN-innervating sensory neurons resulted in downregulation of several genes involved in LEC development and **lymphatic patterning**, such as *Nrp2* and lymphatic vessel endothelial hyaluronan receptor 1 *(Lyve1)*, suggesting anti-**lymphangiogenic** modulation, although this remains to be rigorously demonstrated [8]. Numerous ***trans*-sinusoidal LEC**-covered connective tissue cords (pillars) traverse the SCS from the LN ceiling to the floor in both mouse and human LNs. They are presumed to provide structural support to the LN because the pillar core is formed by fibrillary collagen bundles which may keep the SCS ceiling and floor apart in the otherwise hollow SCS space [10,11]. The pillars also form a 3D sieve for filtering incoming cells and large particles, as demonstrated by two-photon imaging following intralymphatic injections of cells and latex beads in mouse models [8].

The lymphatic vasculature of a LN has some species-specific characteristics [3,5] (Figure 1A and Table 1, Key table). Specifically, mouse LNs have a clear paracortical sinus system; these sinuses originate from the paracortical lymphocyte-rich area of the LN parenchyma without forming physical contact with the SCS, and empty into the medullary sinus [12]. In humans, the SCS makes prominent radial invaginations (trabecular sinuses) into the LN stroma, whereas this sinus system is not found in mice [13]. The trabecular sinuses effectively divide the LN parenchyma of each LN into distinct, segmental compartments in humans. This may allow physical separation of simultaneous immune reactions in human LNs, but the functional significance of the more unified LN parenchyma in mice compared to humans during immune responses remains to be studied.

Molecular identity of LEC subtypes

LECs in different sinus systems have unique characteristics (Table 1). The gold standard for identifying LECs is the expression of Prox-1, a master transcription factor needed for LEC specification in mice and humans [14,15]. Using single-cell RNA sequencing (scRNA-seq), we and others showed that human and mouse LNs have at least six and five transcriptionally distinct LEC subsets, respectively [11,16,17]. Furthermore, both species share five Prox-1^+ subsets, including

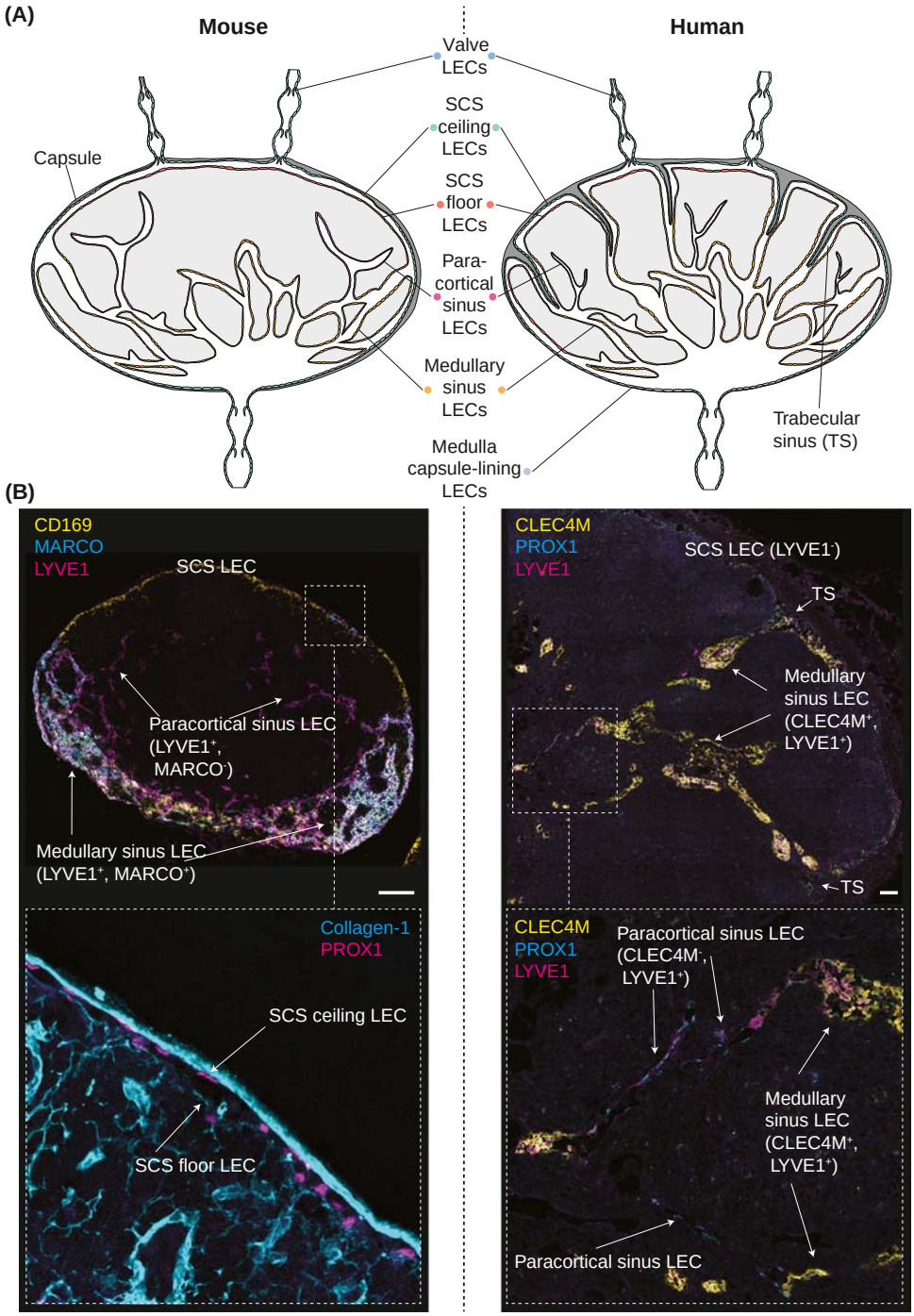

Figure 1. Lymph node (LN) lymphatic endothelial cell (LEC) subsets in mice and humans. (A) Mouse and human LNs have five and six LEC subsets, respectively. Valve LECs, subcapsular sinus (SCS) ceiling LECs, SCS floor LECs, paracortical sinus LECs, and medullary sinus LECs are found in both mice and humans, but medulla capsule-lining LECs (MFAP4$^+$) have been identified only in humans. (B) Representative micrographs of immunofluorescent staining showing LEC subsets at particular LN locations. (Left) In mice, paracortical and medullary sinus LECs highly express Lyve1

(Figure legend continued at the bottom of the next page.)

Glossary

Afferent lymphatics: lymphatic vessels which serve as channels for the delivery of cells and antigens from the periphery into lymph nodes (LNs).
Angiopoietin 2 (ANGPT2): one of the ligands for endothelial TEK receptor tyrosine kinase. The encoded protein affects angiogenesis during embryogenesis and tumorigenesis.
Atypical chemokine receptor 4 (ACKR4): this G protein-coupled receptor can bind the chemokines CCL19, CCL21, and CCL25 and scavenge them to generate a chemokine gradient.
Autophagy: a physiological cellular process that plays a major role in the maintenance of cell homeostasis through the degradation of protein aggregates and damaged organelles.
Chemoattractants: a group of small molecules such as chemokines that induce cells to migrate towards them.
Claudin 5 (CLDN5): a component of tight junction strands that is highly expressed by endothelial and epithelial cells.
Efferent lymphatics: lymphatic vessels by which cells (mostly lymphocytes) leave LNs.
Exosomes: extracellular vesicles produced in the endosomal compartment. They contain different materials such as proteins and RNA depending on their cellular origin; they can transfer molecules from one cell to another.
Fibroblastic reticular cells: fibroblasts in lymphoid organs that produce collagen-rich reticular fibers and form stromal networks and conduits.
Forkhead box protein C2 (FOXC2): the expression of this transcription factor is induced by lymphatic flow; it plays an important role in the formation of lymphatic valves.
Gap junctional protein α4 (GJA4): also known as connexin-37, GJA4 is expressed by lymphatic valves and its expression is regulated by lymphatic flow, PROX1, and FOXC2.
Lymphangiogenesis: the process of new lymphatic vessel formation; is often associated with tumor development.
Lymphatic patterning: highly structured lymphatic vascular network mainly generated during development.
Lymphatic valves: structures within larger lymphatics that prevent lymph backflow.

Key table

Table 1. Key characteristics of human and mouse lymph node LECs

Lymph node LEC subsets	Human markers	Mouse markers	Functional specialization
SCS ceiling LECs (LEC I, cLEC)	ACKR4, NT5E, CAV1, NTS, NUDT4	Ackr4, Cav1, CD9, Nudt4	Form a gradient for CCR7 ligands in SCS to control unidirectional dendritic cell (DC) migration into LN parenchyma [18]; scavenge acetylated low-density lipoprotein (LDL) [17]; may have inputs from sensory nerves around LNs [9]
SCS floor LECs (LEC II, fLEC)	TNFRSF9, CCL20, CXCL1–CXCL5, ACKR1	Madcam1, Ccl20, CD274	Regulate immune cell entry into LN parenchyma through the SCS [11,17]; maintain innate-like lymphocytes in the SCS [87]; scavenge oxidized LDL [17]
Medullary sinus LECs (LEC VI, Marco-LEC)	CLEC4M, CLEC4G, CD209, MARCO, LYVE1, ACKR1, CXCL1–CXCL3, CD206, STAB1, STAB2	Marco, Lyve1, CD274, Clec4g, Stab1, CD206	Recruit neutrophils via Lewis X–CD209 interactions (possibly to prevent the systemic spread of pathogens in humans) [16]; capture arboviruses via the scavenger receptor Marco to limit viral dissemination [88]
Paracortical sinus LECs (LEC IV, Ptx3-LEC)	PTX3, PDPN, NRP2, CCL21, LYVE1, ITIH3, CD36, FLT4, ITGB1	Ptx3, Pdpn, Nrp2, Ccl21a, Itih5, Flt4, Lyve1, CD206, Stab1, Stab2	Regulate lymphocyte egress from LNs [11,17]
Valve LECs (LEC V, valve)	CLDN11, ESAM	Cldn11, Esam Foxc2	Prevent lymph backflow [89]
Medullary capsule-lining LECs (LEC III, cLEC1)	MFAP4, LYVE1	Not present	Unknown

SCS floor LECs, SCS ceiling LECs, **medullary sinus LECs**, **paracortical sinus LECs**, and valve LECs. By contrast, human LNs only harbor **MFAP4$^+$ capsule-lining LECs** in the medulla [16]; however, it is currently unclear why human LNs harbor this unique LEC subset, a finding that may represent a fruitful area of future investigation. Species-specific differences between mouse and human are summarized in Box 1.

SCS ceiling LECs highly express **atypical chemokine receptor 4 (ACKR4)**, which regulates the gradient and concentration of CCR7 ligands in murine SCS [16,18]. In humans, SCS ceiling LECs additionally synthesize CD73 (also known as NT5E), generating anti-inflammatory adenosine [16]. Moreover, SCS ceiling LECs (rather than floor LECs) of draining LNs selectively scavenge acetylated low-density lipoproteins following intradermal injections in mice [17].

(magenta), whereas medullary sinus LECs, but not paracortical sinus LECs, express Marco (cyan). (Right) In humans, SCS floor and ceiling LECs lack LYVE1 (magenta), but paracortical and medullary sinus LECs express LYVE1. Medullary sinus LECs express the C-type lectin CLEC4M (yellow), which is absent in paracortical sinus LECs. Additional markers of each LEC subset are listed in Table 1. Mouse and human LNs were stained with antibodies against the indicated proteins. Microscope images were taken using a Zeiss LSM 880 confocal microscope with a 20× objective. They are not published and are shown here for illustrative purposes. Scale bars, 100 μm.

Macrophage receptor with collagenous structure (MARCO): a protein expressed by some types of macrophages and endothelial cells; may bind both Gram-negative and -positive bacteria via its scavenger receptor cysteine-rich domain.
Medullary sinus LECs: lymphatic endothelial cells (LECs) that line lymphatic sinuses in the LN medulla. Myeloid cells such as macrophages and neutrophils are present in the sinuses.
MFAP4$^+$ capsule-lining LECs: microfibril-associated protein 4-positive LECs in human LNs; these are located within the capsule of the LN medulla in humans.
Neuropilin 2 (NRP2): a transmembrane receptor for lymphangiogenic VEGF-C; important for generating lymphatic networks.
Paracortical sinus LECs: these line lymphatic sinuses in the paracortex or cortex of LNs. Lymphocytes exit LNs from these sinuses.
Plasmalemma vesicle-associated protein (PLVAP): essential for the formation of diaphragms in subcapsular sinus (SCS).
Proteinaceous diaphragms: sieve-like structures in the SCS which only allow low molecular weight antigen to flow into the reticular conduit network. This structure is composed of PLVAP.
Reticular conduits: tubular networks that allow low molecular weight antigens to flow into the LN parenchyma from the SCS. The conduits are composed of collagen bundles enwrapped by fibroblastic reticular cells.
Sentinel lymph node(s): the first LN or group of nodes which directly drain the tumor and are the primary sites reached by metastasizing cancer cells.
SCS ceiling LECs: LECs lining the ceiling (outer wall) of LN subcapsular sinus.
SCS floor LECs: LECs lining the floor (inner wall) of LN subcapsular sinus.
Transcytosis: a type of transcellular transport in which macromolecules are transported though the cell cytoplasm.
Transitional zone LECs: LECs that connect the SCS floor and medullary sinus.
***Trans*-sinusoidal LECs:** also known as bridge LECs, LECs that connect the SCS floor and ceiling.

Box 1. Differences between human and mouse lymph nodes (LNs)

The organogenesis of LNs depends on lymphatic endothelial cells (LECs) [4,81]. Mice have 44 LNs, which always develop at the same anatomical locations [82]. The normal number of LNs in humans remains to be determined, but it is known to vary and probably lies in the range of 400–600 per individual [83,84]. The locations of LNs in humans show anatomic variability. A typical LN in mice weighs ~2–5 mg and in humans ~0.5–1 g. LNs are organized as individual nodes along the lymphatic vasculature in mice, whereas they often appear in clusters in humans [83].

Trabecular sinuses are unique to human LNs [3,16]. They effectively divide a LN into several physically isolated compartments [85]. In mice, which lack trabecular sinuses, the whole parenchyma of a given LN apparently forms one physically uninterrupted entity. The functional significance of the LN compartmentalization remains unknown. The paracortical sinus system, by contrast, is more clearly developed in mice than in humans [12,85].

Distinct subsets of LN LECs display species-specific differences (see Table 1 in main text). The overall overlap in gene expression score is between 20 and 35% in the five conserved LEC clusters (ceiling, floor, medullary, paracortical, and valve), with the highest overlap being among the ceiling LEC subsets [11]. Three types of ceiling LECs can be identified in humans, but only one in mice, possibly reflecting the more complex sinus architecture (trabecular sinuses) and immune microenvironments in humans [11]. Human-specific ceiling LECs include medulla-overlying cells (MFAP4$^+$) and a putative activated subset (HEY1$^+$, CCL2$^+$, E-selectin$^+$) of ceiling LECs [11,16].

Some LEC subset marker genes lack orthologs in the other species. Examples include human CD209 and CLEC4M in medullary LECs, IL-32 in paracortical LECs, and mouse floor LEC marker Glycam1 [11,16]. In addition, the corresponding LEC clusters in humans and mice show several non-conserved gene expression patterns [11]. MAdCAM-1 and MSR-1 are canonical floor LEC markers in mice but are poorly expressed in adult human floor LECs, although MAdCAM-1 is detected on floor LECs in fetal mesenteric LNs [86]. By contrast, human floor LECs express ACKR1 and IL-6 which are absent or poorly expressed in a non-subset-selective manner in mice [11]. MMP2 and LOX are expressed in human but not mouse paracortical LECs [11]. Mouse-specific LEC markers include ApoE in ceiling and medullary LECs and RGS4 in floor LECs [11]. The absence of gene expression in RNA sequencing data needs to be interpreted with caution, but if validated in further studies, differences might point to functional changes and may shed light on the evolution of unique functions in human LN lymphatics.

SCS floor LECs are molecularly distinct from SCS ceiling LECs. They express multiple inflammatory chemokines, including CCL20, CXCL1, CXCL2, CXCL3, and CXCL5, highlighting their importance in immune cell trafficking [16]. It has been proposed that SCS floor LECs overlying B cell follicles and interfollicular areas are molecularly distinct in their expression of CD44, glycosylation-dependent cell adhesion molecule 1 (Glycam-1), and cochlin [17], but, based on original imaging of the interfollicular region, this area appears to be defined by the area between two nodes in murine inguinal LNs rather than by a domain between B cell follicles [17]. Therefore, further studies to clarify the extent of SCS floor LEC heterogeneity are needed.

In murine and human LNs, a LEC subset connecting the SCS floor and ceiling (*trans*-sinusoidal LECs; also called bridge LECs) has been reported [11]. This subset expresses an intermediate number of markers for SCS ceiling LECs (ACKR4 and caveolin 1, Cav1) and floor LECs (mucosal vascular addressin cell adhesion molecule, MAdCAM-1; and CD274) in mice and humans. *Trans*-sinusoidal LECs were also identified by another group who showed that bridge LECs highly express SERPINE1 at both the mRNA and protein levels in human LNs [19]. However, further studies are warranted to resolve whether the bridge LECs identified across these studies reflect identical subsets.

LECs at the medullary sinus are transcriptionally similar to SCS floor LECs; both express lysozyme (LYZ) and DARC (also known as ACKR1); the latter scavenges inflammatory chemokines such as CXCL2 and CCL2, and these medullary LECs also express multiple neutrophil **chemoattractants**, including CXCL1, CXCL2, and CXCL3, in humans [16]. However, medullary sinus LECs also present unique features, such as the expression of multiple C-type lectins, including CD206, CLEC4M, CLEC4G, and CD209, in humans [16]. We found that CD209 on medullary sinus LECs regulates neutrophil adhesion in human LN medulla, and this may prevent

pathogen dissemination through the LN [16]. This subset also highly expresses the **macrophage receptor with collagenous structure (MARCO)** scavenger in mice and humans [11].

Transitional zone LECs are located between the SCS floor and medullary sinus LECs, and were identified in mice but not in humans [11]. Mouse transitional zone LECs express intermediate markers for SCS floor LECs (such as MAdCAM-1 and CCL20) and medullary sinus LECs (e.g., MARCO, Lyve-1) [11]. However, because this subset does not bear any unique molecular markers, it remains unclear whether it is a genuinely distinct LEC population or it has unique functions.

Paracortical sinuses, which are the exit sites for lymphocytes in LNs, selectively express pentraxin 3 (PTX3) and strongly express podoplanin (PDPN), the CCR7 ligand CCL21, vascular endothelial growth factor receptor 3 (VEGFR3, also known as FLT4), sphingosine kinase 1 (SPHK1), and CD36 in both mice and humans [11,17]. The high expression of CCL21 and SPHK1 may locally control lymphocyte exit from LNs by recruiting $CCR7^+$ lymphocytes and generating a local S1P gradient around paracortical sinuses [11]. Paracortical sinuses also highly express other molecular signatures of sprouting LECs such as **neuropilin 2 (NRP2)** in mice and humans [20,21]. During development, LECs sprout from pre-existing lymphatics via NRP2, a transmembrane receptor for lymphangiogenic vascular endothelial growth factor C (VEGF-C), to form a complex lymphatic network in mice [22]. In humans, this LEC subset is abundant in head and neck LNs compared to axillary LNs, and was initially identified as a 'capillary-like' LEC (LEC subset IV) [16]. By comparing human and mouse LN datasets [11], we identified this subset to be more abundant in mice than in humans. However, it also represents a paracortical sinus LEC subset with a direct connection to medullary sinuses in both species, as evidenced by others [11].

Lymphatic valves typically express claudin-11 (CLDN11), endothelial cell adhesion molecule (ESAM), and **Forkhead box protein C2 (FOXC2)** in humans and mice [16,23,24]. Two types of $CLDN11^+$ LECs are recovered from the SCS of human LNs: one type highly expresses CD9 and CAV1, and another expresses **angiopoietin 2 (ANGPT2)**, **claudin 5 (CLDN5)**, and **gap junctional protein α4** (**GJA4**, also known as connexin-37) [16]. Based on immunohistochemical staining, these two subsets seem to correspond to the upstream and downstream sides of lymphatic valves, respectively [16].

A recent study of human LNs [19] classified $MARCO^+$ and $PTX3^+$ LECs as perifollicular and medullary sinus LECs, respectively, a categorization which is not in line with our identification [11,16,17]. Indeed, several studies have demonstrated MARCO expression in the LN medulla of both mice and humans [11,16,17,25] (Figure 1B). Because MARCO is expressed in medullary sinus and PTX mRNA expression was highly detected in paracortical regions of mouse LNs [11,17], we posit that $MARCO^+$ and $PTX3^+$ LECs are indeed medullary and paracortical sinus LEC, respectively, in both species. Because all immune cells and non-endothelial stromal cells in LNs are heterogeneous [4], recent single-cell technologies have allowed the identification of heterogeneous LEC subsets which are present at distinct locations in mouse and human LNs. Currently, we have limited knowledge of their functions and developmental origins, but this is certainly an interesting area for future studies.

Role of LN LECs in antigen transport and presentation

The principal immunological functions of LNs require efficient antigen display to large pools of lymphocytes. Migratory dendritic cells (DCs) represent the best-characterized antigen delivery route for T lymphocytes in LN parenchyma [2,5]; however, antigens from the periphery can also obtain entry to the LN by other means. Specifically, soluble, non-cell-bound antigens (i.e., free antigens) are readily carried in lymph [26]. In fact, even bacteria such as *Streptococcus*

pyogenes translocate freely in lymph to the draining LNs [27]. Studies analyzing the transport of subcutaneously injected, fluorescently labeled antigens in mice have shown that free antigens arriving at the SCS have at least four possible fates: (i) entry into the **reticular conduits** of the LNs [28,29], (ii) **transcytosis** through the SCS floor LECs [30], (iii) uptake by SCS macrophages and DC [31], or (iv) immediate delivery to the medulla (and then further on to the next LN) via the SCS and/or trabecular sinuses, without entering the parenchyma of the draining LN (Figure 2) [3,32]. Thus, SCS LECs are central to many of these antigen-sorting decisions.

Small lymph-borne antigens (<70 kDa) can enter collagen-based cables in the reticular conduit system of mouse LNs [33]. Antigen entry into conduits is regulated by LECs, which are in close contact with the conduit bundles at the SCS floor because conduit filling is compromised in Plvap-deficient (*Plvap*$^{-/-}$) mice [10]. **Proteinaceous diaphragms**, which are sieve-like filters of LECs formed by **plasmalemma vesicle-associated protein (PLVAP)** fibrils [10,34], separate the luminal content of sinuses from the tissue parenchyma and may physically or physicochemically limit the entrance of lymph-borne proteins and oligosaccharides from the SCS into the conduits [10]. Specifically, in Plvap-deficient mice, large proteins and oligosaccharides, which are normally excluded from conduits, gain entry to the conduit system [10]. PLVAP filters on LECs can also regulate the entry of viral particles into the conduit system; indeed, vaccinia virus particles are greatly enhanced in the conduits of conditionally Plvap-deficient mice (*Plvap*$^{fl/fl}$; *Prox1–creERT2*$^{tg/+}$) compared to wild-type controls [35].

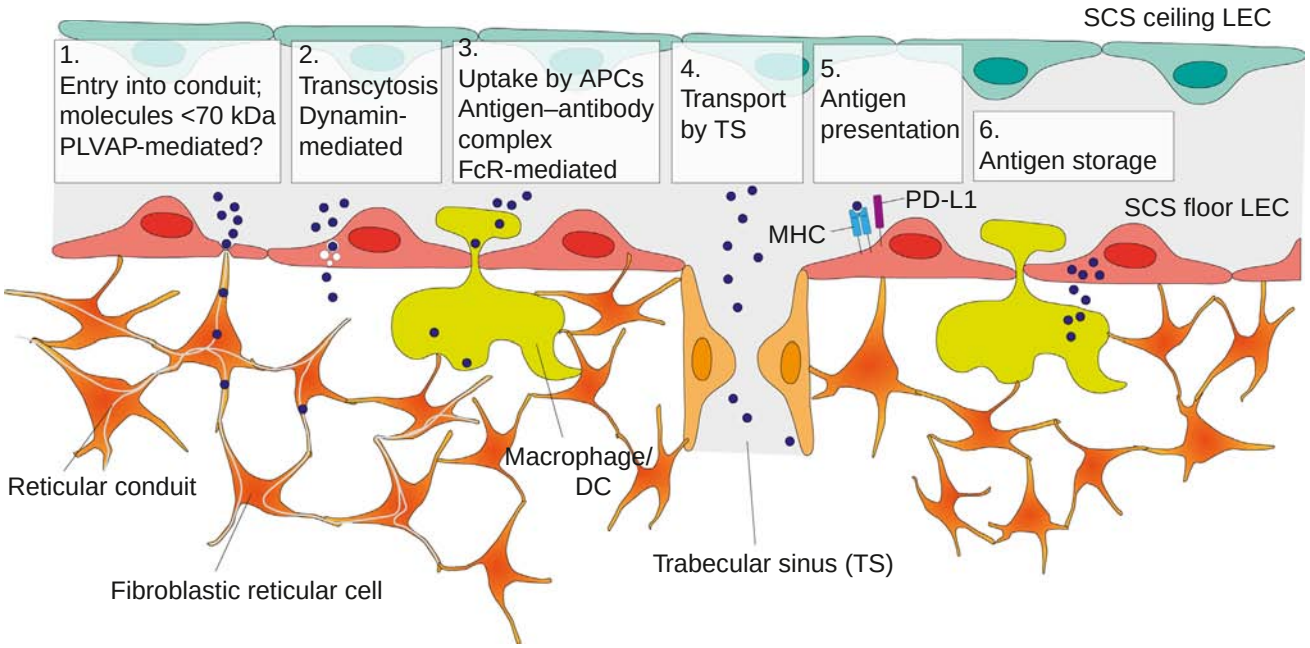

Figure 2. Lymph node (LN) lymphatic endothelial cells (LECs) present antigens and regulate the delivery of soluble antigens into the LN parenchyma. When lymph-borne soluble antigens (free antigens) arrive at the subcapsular sinus of a draining LN, they have one of at least four possible fates, partly depending on the properties of the antigen: (1) small antigens (<70 kDa) enter the reticular conduits of the LN, possibly through PLVAP^{+} diaphragms in the subcapsular sinus (SCS) floor LECs [10], (2) antigens (<500 kDa) are transferred through the SCS floor LECs by dynamin-mediated transcytosis [30], (3) antigen–antibody complexes are phagocytosed by Fc receptors (FcRs) on SCS macrophages or dendritic cells (DCs; antigen-presenting cells, APCs) [37], or (4) some antigens pass passively through trabecular sinuses (TS)/SCS to reach medullary sinuses and/or the next LN. (5) LN LECs can present antigens on MHC class I and II molecules [38], often in the context of immunosuppressive PD-L1 molecules [41], and (6) store (microbial) antigens for prolonged periods for slow release to professional APCs [44]. The evidence is mainly based on mouse studies.

In addition, soluble antigens can enter the LN parenchyma through SCS floor LECs outside the conduits [30,36]. In mice, antigen transcytosis takes place in a receptor-independent manner via endocytic LEC vesicles which shuttle the cargo from the SCS to the parenchyma [30]. The *trans*-sinusoidal transfer allows the entry of subcutaneously administered antigens (up to ~500 kDa) into the LN parenchyma within seconds [30]. Soluble antigen–antibody complexes entering the SCS are efficiently recognized by SCS macrophages that propel the immunocomplexes to follicular DCs [37] – an effect which has been demonstrated via two-photon microscopy imaging of the deposition of phycoerythrin- and anti-phycoerythrin immunocomplexes into mouse follicles [37]. Because floor LECs crucially control the positioning and survival of SCS macrophages, this mode of antigen transfer is also indirectly LEC-dependent [37].

In addition to sorting incoming antigens, LN LECs express self-antigens and can directly present these antigens on MHC class I and II molecules [38]. Because LN LECs are largely devoid of co-stimulatory molecules, and instead strongly express coinhibitory molecules such as PD-L1 [17,39,40], their main role is likely to maintain peripheral tolerance, although this remains to be rigorously demonstrated. For instance, on the one hand, PD-L1 expression in Prox-1$^+$ LECs (including both LN and peripheral LECs) can impair tumor-specific immune responses by triggering apoptosis of cytotoxic central memory CD8$^+$ T cells in MC38 and B16F10 melanoma orthotopic tumor mouse models [41]. On the other hand, the immunostimulatory function of LEC-mediated antigen presentation has also been implicated in peripheral tolerance, given that LN LECs can prime the generation of central memory and stem cell-like memory CD8$^+$ T cells from naïve T cells, as seen in mouse experiments with MHC I-deficient bone marrow chimeras in which CD8$^+$ T cell responsiveness to ovalbumin was assessed [42]. However, the presumed contribution of LN LECs versus peripheral LECs in tumor antigen cross-presentation and LEC antigen-specific killing, as well as in the inhibition of metastasis, remain to be dissected [43].

LN LECs can store lymph-borne antigens for prolonged periods of time [44]. In fact, in mice, LN LECs can harbor viral and vaccine antigens for at least 5 weeks after exposure [45]. Both SCS and medullary LECs can contribute to antigen storage in mice, based on single-cell analyses of LN LECs following subcutaneous injection of oligonucleotide-tagged antigens [44]. Antigen storage may allow slow intranodal release of antigens for resident dendritic and other antigen-presenting cells, even after the acute peripheral antigen exposure has subsided [44,45] – a mechanism which may be especially relevant for mounting long-term immunological responses to control microbial infections.

Thus, the lymphatic vasculature can modulate both antigen presentation and distribution in LNs. Based on the preferential expression of coinhibitory molecules (such as PD-L1) rather than co-stimulatory molecules (CD80 or CD86, see above), LN LECs presumably contribute to the induction of tolerance [41], but their potential involvement in T cell differentiation, infection control, and the induction of antitumor immunity and/or autoimmunity remains elusive and clearly warrants further investigation.

LN leukocyte entry and exit

A long-standing dogma is that several leukocyte types enter LNs via afferent lymphatic vessels, whereas only lymphocytes leave nodes under physiological conditions [46]. However, a recent mouse study demonstrated that neutrophils were also present in efferent lymph collected from the thoracic duct and cisterna chyli leaving the LN [47]. The numbers of neutrophils were low (0.04% of cells) under normal conditions compared to lymphocytes [47]. Leukocyte entry into LNs via LN LECs is guided by chemokines and adhesion molecules (Figure 3), such as CCL21, CXCL1–CXCL5, and CD209, which display many leukocyte-subtype selective features in

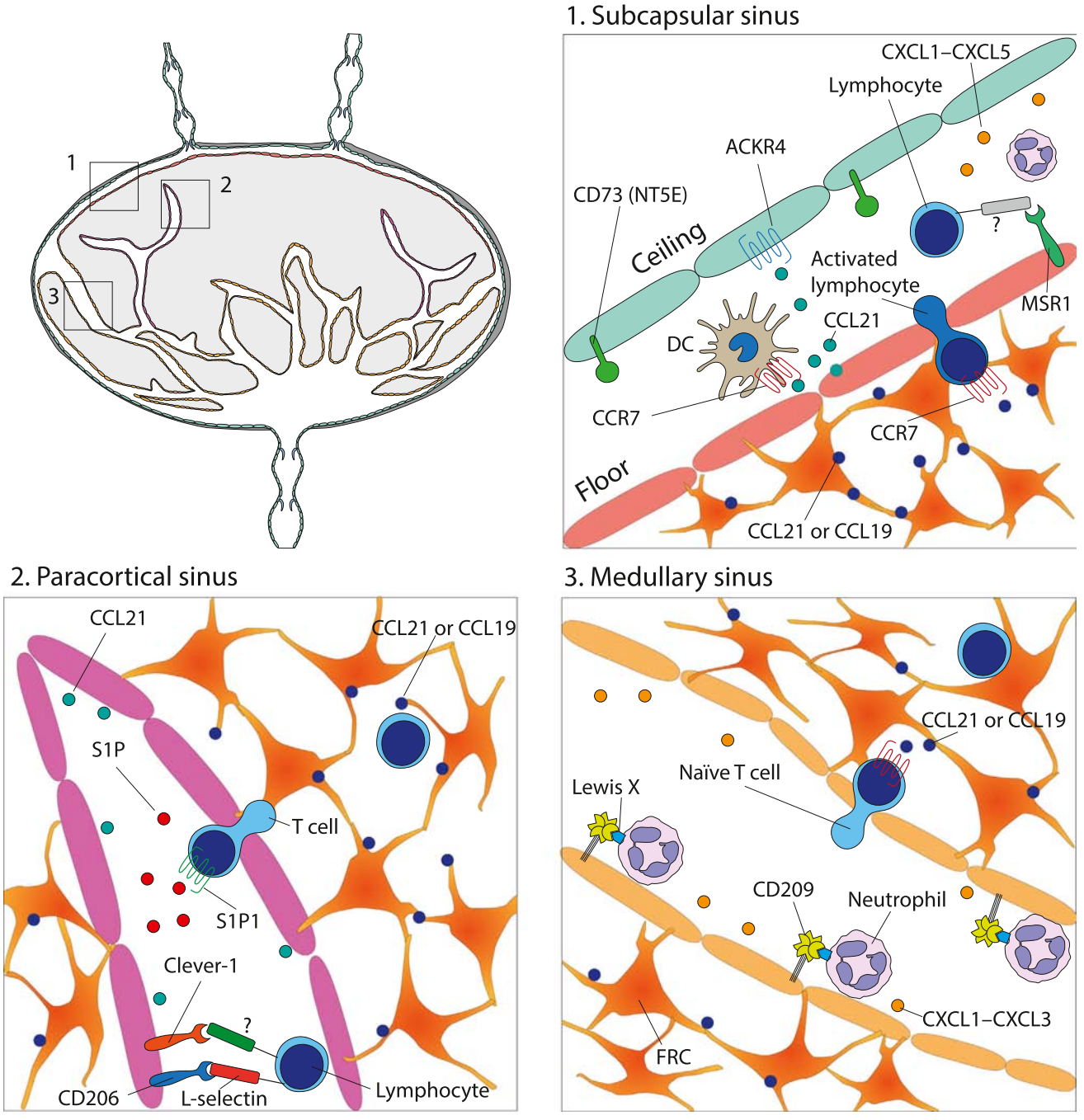

Figure 3. Leukocyte trafficking in subcapsular, paracortical, and medullary lymphatic sinuses. In the subcapsular sinus (SCS) (1), SCS ceiling lymphatic endothelial cells (LECs) express ACKR4, which creates a gradient of CCR7 ligands in the SCS and regulates the unidirectional dendritic cell (DC) migration through the SCS floor [18]. Activated lymphocytes migrate through the SCS floor in a CCR7-dependent manner [8]. SCS LECs express MSR1, which promotes lymphocyte migration into the LN parenchyma [3], and CD73, which delivers immunosuppressive signals to leukocytes [54]. SCS floor LECs also highly express neutrophil chemoattractants, including CXCL1–CXCL5, which may be involved in neutrophil recruitment and adhesion in SCS [16]. In the paracortical sinus (2), T cells exit LNs through the sinus LECs by following a S1P gradient [3]. Paracortical sinus LECs highly express CCL21, which may also be important for T cell exit [11]. Moreover, paracortical sinus LECs express CD206 and Clever-1, both of which are likely to

(Figure legend continued at the bottom of the next page.)

directing different cell types to their specific anatomical locations within nodes [3,48]. Lymphocyte and neutrophil egress from LNs is largely controlled by S1P – the main driver for overruling retention signals. The importance of S1P as an exit signal has been verified by its targeting in multiple sclerosis, which leads to the trapping of inflammatory cells within LNs, thus preventing their trafficking into the brain [49]. *Ex vivo* human studies and *in vivo* mouse studies have indicated that other multifunctional adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1), CD206, Clever-1, and roundabout guidance receptor 4 (Robo4), also contribute to egress [3,50]. Importantly, these molecules may serve as target candidates for drug development when leukocyte functions such as trafficking might need to be perturbed. Therefore, detailed analyses of clinical data from studies targeting adhesion molecules [e.g., VEGFR3 via lymphangiogenic VEGF-C156S [51] or via antibody-binding of Clever-1 (immunosuppressive) [52]] might hopefully bring new biological insight into the putative role of LN LECs in leukocyte and/or cancer cell trafficking in clinical settings.

DCs

Mouse studies have shown that, within LNs, the movement of incoming DCs is regulated by the decoy receptor ACKR4 (CCRL1) located on SCS ceiling LECs [2,18,53]. It binds to both full-length immobilized and cleaved soluble CCL21, and thus competes for binding to the CCL21 ligand CCR7 on DCs, thereby generating a chemokine gradient that guides DC entry into the node parenchyma [2,18,53]. When the gradient is not formed, DC entry into the LN is severely compromised, as seen in the diminished numbers of DCs in *Ackr4*$^{-/-}$ [18] and *Ackr4*$^{-/-}$ skin-grafted mice compared to wild-type controls [53]. Therefore, ACKR4 expressed in peripheral lymphatics may also be crucial for limiting the dissemination of soluble CCL21 produced in the periphery of the draining LN.

The activity of incoming DCs is essential for determining the extent of immune responses within nodes and is modulated by at least two immunosuppressive molecules on LECs, namely CD73 and Clever-1 [54,55]. CD73 is an ectoenzyme that produces anti-inflammatory adenosine [56]. Recent *ex vivo* human and *in vivo* mouse studies have shown that CD73-deficient LECs from *Nt5e*$^{-/-}$ mice and siRNA-treated human LECs induce MHC II expression on DCs relative to controls [54]. Similarly, although the number of DCs reaching draining LNs in Clever-1 knockout (*Stab*$^{-/-}$) mice is diminished in cell adoptive transfer and fluorescein isothiocyanate (FITC)-painting experiments in mice, incoming DCs express higher amounts of MHC II and trigger better antigen-specific T cell responses than those in the draining LN of wild-type control mice [55]. It is highly likely that the noted effects are at least mediated in part by LN LECs expressing these target molecules, which might be linked to their immunomodulatory potential.

Lymphocytes

Lymphocyte interactions with different LEC subtypes have been dissected in detail using intralymphatic immune cell transfers and live imaging in mice. When naïve lymphocytes are injected alone, they reach the parenchyma of draining LNs via the medullary sinus. However, when injected with DCs (mimicking the physiological situation) or are activated, they prefer the SCS as their entry site into nodes [8]. The incoming activated T cells are instantly arrested on the SCS floor in a process that probably involves passive mechanical sieving between the *trans*-sinusoidal pillars [8]. Activated T cells then migrate randomly on the sinus floor without the help of chemokines or integrins, but chemokine receptors, especially CCR7, are then needed

promote lymphocyte egress from LNs [3]. In the medullary sinus (3), lymph-borne naïve lymphocytes (in the absence of DCs) migrate to the LN parenchyma using CCR7 [90]. Medullary sinus LECs also express neutrophil-attracting chemokines and selectively express CD209, which mediates neutrophil adhesion to the human LN medulla [16]. Abbreviations: FRC, fibroblastic reticular cells; S1P, sphingosine-1-phosphate; S1P1, sphingosine-1 phosphate receptor 1.

to guide them from the SCS into the T cell zones in the parenchyma [8]. Moreover, lymphocyte transfer experiments into knockout (*Plvap*$^{-/-}$ and *Msr1*$^{-/-}$) mice have indicated that PLVAP diaphragms and macrophage scavenger receptor 1 (MSR1) expressed on SCS floor LECs control lymphocyte translocation from the SCS into the parenchyma [3].

Mouse studies have demonstrated that paracortical sinuses are the primary pathways for T lymphocytes leaving the LN [3]. B cell behavior in mouse LNs has been visualized by intravital imaging and advanced microscopy techniques. The results show that many memory B cells exit via the SCS [57]. In the SCS, B memory cells may take up antigens from SCS macrophages and reenter germinal centers using CCL21 gradients mediated by ACKR4 [57]. Alternatively, memory B cells drain out from the LN via the subcapsular and medullary sinuses [57]. The cell-specific differences in migration pathways suggest that T and B lymphocytes express different sets of traffic-guiding molecules.

Neutrophils

A unique subset of neutrophils that are distinct from blood-borne neutrophils is present in mouse and human LNs even under physiological conditions [58,59]. These neutrophils express MHC II and are located on the SCS lining and interfollicular areas [58]. Immunohistochemical analyses subsequent to intralymphatic injections of neutrophils and DCs in mice show that resting and *in vitro*-activated neutrophils mainly localize in the subcapsular and medullary sinuses of the draining LN, and cotransfer of DCs allows entry of resting neutrophils to the parenchyma via the SCS. By contrast, *in vivo*-activated neutrophils are able to migrate through the LEC lining into the interfollicular areas of the LN parenchyma [60].

The presence of neutrophil chemoattractants (CXCL1–CXCL5) on LECs of the subcapsular floor and medullary sinuses in humans most likely contributes to LN neutrophil migration [16]. Moreover, LECs in the human medullary sinus express CD209, which binds to its Lewis X (CD15) ligand on neutrophils [16]. Because neutrophils bind via a Lewis X–CD209 interaction to medullary LECs in *in vitro* adhesion assays, this interaction is presumably important in keeping neutrophils in this location to restrict the systemic spread of pathogens [16]. Of note, the absence of CD209 in mice suggests that distinct molecular mechanisms mediate neutrophil–LN LEC interactions in mice, although this remains to be assessed.

Macrophages

Sinusoidal LECs are crucial for promoting the differentiation of LN-resident macrophages [61–63]. Genetically engineered mice have been especially important for dissecting the roles of key molecules mediating LEC–macrophage interactions: conditional deletion of colony stimulating factor-1 (CSF-1) production by LECs (*Prox1*^{creERT2}*Rosa*^{tdTOM}*Csf1*$^{flox/flox}$ mice) leads to a marked reduction in the number of subcapsular and medullary sinus macrophages compared to wild-type controls, demonstrating the importance of CSF-1 in maintaining the macrophage niche in lymphatic sinuses [61]. CSF-1 may have a comparable function in humans given that single-cell analysis of human LN LECs revealed high expression of CSF-1 in the floor and medullary sinus LECs, although this warrants further investigation [16]. In addition, Rank deficiency in LECs (*Rank*$^{\Delta Prox1}$ mice) during normal embryogenesis as well as during vesicular stomatitis virus infection in adult mice reduces the number of sinus macrophages [62]. Moreover, interactions between sialoglycans, which are highly displayed on SCS floor LECs, and Siglec-1/CD169 on macrophages, are crucial for regulating macrophage localization, as well as the pro-inflammatory phenotype of mouse SCS LECs [63]. Thus, the molecular interactions between macrophages and LECs appear to be important for the proper anatomical localization and function of macrophages.

Sinusoidal LECs in cancer

Many cancer types such as breast cancers and melanoma preferentially spread via the lymphatics [64]. As an example, murine melanoma cells can upregulate several genes with immunomodulatory potential in the SCS floor LECs of the **sentinel LN** [65]. In fact, primary tumors can modify the lymphatic vessels and draining LNs in several ways to create pretumoral niches for dissemination [66–68]: they can induce lymphangiogenesis, co-opt existing vessels, incorporate myeloid cells into lymphatics, and deliver **exosomes** to draining LNs [69–72]. For instance, the exosomal molecule interferon regulatory factor 2 (IRF-2) induces the release of VEGF-C by macrophages and remodels the lymphatic network, as evidenced from morphometric analyses and by assessing LYVE-1-positive areas in sentinel LNs in a mouse model of CT26 colorectal cancer [73]. Of note, IRF-2 has also been reported to be highly expressed in exosomes derived from the serum of patients with colorectal cancer and LN metastases [73]. In patients with follicular lymphoma, LEC numbers decrease sharply, which is suggestive of widespread lymphatic damage, and their ceiling, bridging, and floor LECs show upregulation of CD74, HLA-DR, and heat-shock proteins in the affected LN [19]. *In silico* analyses have suggested increased TNFSF10-dependent cancer cell killing interaction potential between several LEC subsets and malignant B cells [19]. However, an important detail is that cell dissociation methods may result in significant loss of specific LN LEC types, potentially skewing the interpretation of results, as recently reported in preliminary analyses (A.J. Radtke *et al.*, unpublished) that compared scRNA-seq data and image-based cell atlases of normal and follicular lymphoma LNs, which will require further corroboration [74]. Moreover, the effects of numerous other cancer cell types on different LEC subtypes and vice versa in affected LNs remains to be rigorously studied.

LN LECs in inflammation and infection

Sheep have been widely used to measure lymphocyte trafficking into LNs during inflammation [75]. These studies have shown that lymphocyte migration via the afferent lymphatics can increase 30-fold from the periphery to draining LNs via the afferent lymphatics, resulting in enlargement of the nodes [75]. In addition, preventing lymphocyte exit from the nodes contributes to LN enlargement [75]. The inflammation-induced increase in LN LEC proliferation coincides with type 1 interferon-induced PD-L1 overexpression on human and mouse LECs [76], although the mechanistic relevance of this association remains to be studied. Of note, other disease processes such as age-dependent accumulation of adipocytes in human LNs might cause marked loss of medullary sinus LECs and their replacement by collecting lymphatic vessels [77]; indeed, the functional consequences of such events and their disease-specific contexts remain currently unknown.

Murine skin inflammation models have been used to dissect the responses of LEC subsets lining different LN sinuses by scRNA-seq. Specifically, mice were treated with imiquimod-containing skin cream (mimicking human psoriasis); among various LECs, SCS floor LECs exhibited the highest number of upregulated genes including *Ccl20*, *Glycam1*, *Fn1*, *Cd200*, and *Anxa2,* and downregulated genes (e.g., *Jam3*) [78]. In this study, altered transcription was partly model-dependent because *Anxa2* was upregulated after imiquimod treatment but not following oxazolone challenge, suggesting that different inflammation-inducing factors can trigger factor-specific responses in LECs [78]. By contrast, mouse studies showed that **autophagy** was essential for retaining cellular homeostasis control of the immunomodulatory functions of LN LECs under the inflammatory setting of collagen-induced arthritis (CIA) [79]. By deleting *Atg5* in conditional *Prox1–cre*^{ERT2} *Atg5*^{flox} mice (ATG is an autophagy protein indispensable for the early steps of autophagosome formation), the resulting impaired autophagy led to decreased proliferation of regulatory T cells and resulted in aggravated inflammation of CIA, based on

histology [79]. Additional experiments suggested that LN LEC autophagy was most likely mediated via MHC II expression and antigen presentation [79].

Human and mouse sinusoidal LECs express several scavenging and phagocytic receptors such as CD206, macrophage scavenger receptor-1 (MSR-1), MARCO, and Clever-1 [3]. Apart from their role in antigen storage during microbial infection and vaccination, LN LECs can directly regulate bacterial proliferation. For instance, in the case of *Mycobacterium tuberculosis*, the bacteria form extensive intracellular cords in human LN LECs, thereby allowing the bacteria to evade immune defense and persist within LECs [80]. Recently, following subcutaneous injections of arthritogenic alphavirus particles in mice, viral particles and viral RNA were shown to accumulate in MARCO$^+$ medullary LN LECs, as evidenced by immunostaining and scRNA-seq [25]. Therefore, in this model, medullary LECs rather than LN macrophages were crucial in limiting viral dissemination [25]. Thus, although the division of labor between sinus LECs and macrophages in controlling various other infections and inflammatory contexts remains to be established, it is clear that LECs play an important role in such processes.

Concluding remarks

New technologies have revealed that intranodal LECs undergo fine-tuned specialization at distinct anatomical compartments of the elaborate lymphatic sinus system of LNs. LN LECs undergo active engagement with other cell types – either permanently residing or traveling through nodes – and these can also directly and indirectly contribute to the distribution and presentation of antigens in LNs.

Although research on LN LECs has progressed swiftly, several fundamental issues remain unaddressed (see Outstanding questions). For instance, new experimental tools will be necessary to specifically target LN LECs separately from capillary LECs from other tissues, and preferably even in an LN LEC subset-specific manner. It is important to remember that most studies to date have been performed using mouse inguinal and popliteal LN LECs. We do not know whether LECs in LNs exposed to other types of antigens (e.g., mesenteric LNs, lung LNs) share the identified molecular and functional properties of skin-draining LNs. Moreover, LN LEC research in humans is still in its infancy. In particular, the interindividual heterogeneity of LN LECs under normal conditions as well as in patients with cancer (likely depending on the type of cancer), different types of infection (bacteria or viruses), and autoimmune diseases (organ-specific and systemic) remain to be studied. Moreover, LNs typically undergo dramatic expansion and contraction phases during immune responses, and LN LEC structure, phenotype, and functions are thus predicted to be highly dynamic. Therefore, increasing our knowledge of LN LEC biology can contribute to identifying new and exciting molecular candidate targets for ideally modulating leukocyte trafficking, the dissemination of malignant cells, and inflammatory and immune responses.

Outstanding questions

How functionally different are LN LEC subsets from LEC subsets in peripheral tissues? To address this issue, we will need new genetic and other tools to target specific LEC subsets. The identification of specific candidate markers in LN and peripheral tissue LECs could be best done by single-cell analysis.

Are LN LECs from anatomically distinct locations (e.g., skin-draining, brain-draining, gastrointestinal tract-draining, lung-draining, genitourinary tract-draining) molecularly and functionally distinct? Does the specific lymph composition at different anatomical areas (e.g., intestinal lipids draining into mesenteric LNs) alter LEC subtypes?

What are the functions of human LN LEC subsets? To date, most LN LEC analyses have been performed in mouse models, which may not accurately reflect LN LEC functions in humans owing to species-specific differences.

Why are LN LECs heterogeneous? LN LEC subtypes reside in distinct locations and interact with different cell types such as macrophages, fibroblastic reticular cells, and other stromal cell types. However, the molecular mechanisms regulating LEC heterogeneity and their significance remain unknown.

How much interindividual heterogeneity is present in LN LECs in healthy individuals? Do disease states such as cancer, infection, and autoimmunity dynamically change LN LECs molecularly and functionally? Cancer metastasis or bacteria/virus infections can dramatically remodel the structure of draining LNs, but how a disease state changes each LN LEC subset is unknown.

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Declaration of interests

S.J. and M.S. are cofounders of Faron Pharmaceuticals. A.T. declares no conflicts of interest.

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