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The lymphatic vasculature: An active and dynamic player in cancer progression

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Abstract

The lymphatic vasculature has been widely described and explored for its key functions in fluid homeostasis and in the organization and modulation of the immune response. Besides transporting immune cells, lymphatic vessels play relevant roles in tumor growth and tumor cell dissemination. Cancer cells that have invaded into afferent lymphatics are propagated to tumor-draining lymph nodes (LNs), which represent an important hub for metastatic cell arrest and growth, immune modulation, and secondary dissemination to distant sites. In recent years many studies have reported new mechanisms by which the lymphatic vasculature affects cancer progression, ranging from induction of lymphangiogenesis to metastatic niche preconditioning or immune modulation. In this review, we provide an up-to-date description of lymphatic organization and function in peripheral tissues and in LNs and the

Abbreviations: ANG, angiopoietin; COX, cyclooxygenase; DC, dendritic cell; DT, diphtheria toxin; ECM, extracellular matrix; FGF, fibroblast growth factor; FOXC, forkhead transcription factor; FRC, fibroblast reticular cell; HEV, high endothelial venule; HGF, hepatocyte growth factor; HIF, hypoxia-inducible factor; IDO, indoleamine 2,3-dioxygenase; IFP, interstitial fluid pressure; LEC, lymphatic endothelial cells; LMC, lymphatic muscle cells; LN, lymph node; lncRNA, long noncoding RNA; LVD, lymphatic vessel density; LYVE, lymphatic vessel endothelial hyaluronan receptor; MDK, midkine; MHC, major histocompatibility complex; miRNA, microRNA; NOS, nitric oxide synthase; NRP, neuropilin; OVA, ovalbumin; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; PDPN, podoplanin; PDT, photodynamic therapy; PD-1, programmed cell death protein 1; PD-L1, programmed death ligand 1; PROX, prospero-related homeobox; S1P, sphingosine-1-phosphate; S1P1, sphingosine-1-phosphate receptor 1; SCS, subcapsular sinus; SEM, semaphorin; SOX, SRY-box transcription factor; TAM, tumor associated macrophages; TGF, transforming growth factor; Treg, regulatory T cell; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; YAP, Yes-associated protein.

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changes induced to this system by tumor growth and progression. We will specifically focus on the reported interactions that occur between tumor cells and lymphatic endothelial cells (LECs), as well as on interactions between immune cells and LECs, both in the tumor microenvironment and in tumor-draining LNs. Moreover, the most recent prognostic and therapeutic implications of lymphatics in cancer will be reported and discussed in light of the new immune-modulatory roles that have been ascribed to LECs.

KEYWORDS
cancer, immune modulation, lymphangiogenesis, lymphatic vessels, metastasis

1 | GENERAL STRUCTURE AND FUNCTION OF THE LYMPHATIC SYSTEM

The lymphatic system comprises an extensive network of lymphatic vessels and lymphoid organs and tissues, such as the bone marrow, the spleen, and ~500–600 lymph nodes (LNs).[1] While lymphatic vessels have originally been regarded as passive conduits for fluids and immune cells, it is nowadays clear that this highly specialized vascular network plays vital roles in controlling fluid homeostasis, immune surveillance, and lipid absorption.[2,3] Moreover, research on the lymphatic vascular system has been facilitated over the last two decades by the discovery of lymphatic-specific markers (see Box 1), and in recent years lymphatic vessels have emerged as central players in disease, particularly in the context of cancer and inflammation.[4–8]

Different from the blood vasculature, which is organized in a closed, circular system, the lymphatic vascular system consists of an open, hierarchically organized vascular network, which begins in virtually all vascularized tissues of the body (see Box 2 for information on its developmental origin). Lymphatic vessels initiate in the peripheral tissue as blind-ended lymphatic capillaries that merge into larger collecting vessels and eventually deliver

Box 1: LEC-specific markers

Although the expression of these molecular markers is not entirely specific to LECs, their use, in combination with pan-endothelial markers such as VE-cadherin or PECAM-1 (CD31), has enabled the unambiguous molecular distinction between blood and lymphatic vasculature in tissues. LEC markers include the transcription factor prospero-related homeobox 1 (PROX1), which acts as a master regulator factor of LEC identity (see Box 2),[9,10] the tyrosine kinase vascular endothelial growth factor (VEGF) receptor 3 (VEGFR3), that binds to the lymphatic growth factor VEGF-C,[11,12] and the mucin-type glycoprotein podoplanin (PDPN).[13] LECs of initial lymphatic capillaries can be further distinguished from lymphatic collectors by high expression of the lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1)[14,15] and by high expression of the chemokine (C-C motif) ligand 21 (CCL21).[16] The latter forms a perilymphatic gradient that attracts CCR7-expressing dendritic cells and other leukocytes into lymphatic capillaries.[17]

Box 2: Lymphatic vasculature development

The formation of the lymphatic vasculature occurs in a similar manner in all vertebrates during embryonic development. Lineage tracing experiments suggest that a substantial part of the lymphatic vasculature originates from differentiation of lymphatic progenitor cells from embryonic veins.[19,20] However, in recent years, more evidence in support of alternative origins of tissue-specific lymphatic vessels has emerged.[21] Specifically, in the mesentery and skin, non-venous single progenitor cells expressing vascular endothelial growth factor (VEGF) receptor 3 (VEGFR3), prospero-related homeobox 1 (PROX1), and neuropilin 2 (NRP2) were identified to contribute to the formation of these local tissue-specific lymphatic vessel networks.[22,23]

In mice, lymphatic development starts around embryonic day (E)9-9.5, after the blood vascular circulation is established. At this time-point, SRY-box transcription factor 18 (Sox18) and orphan nuclear receptor chicken ovalbumin upstream promoter transcription factor (COUP-TFII) act in concert to induce the expression of PROX1 in a subset of endothelial cells in the wall of the cardinal vein.[19,24,25] PROX-1, as a master transcription factor of lymphatic identity, in turn, downregulates blood vessel marker genes and drives expression of VEGFR3 in the lymphatic progenitor cells.[26] $PROX1^+$ progenitor lymphatic endothelial cells (LECs) next elongate, bud off from the cardinal and intersomitic veins around E10,[27,28] and start migrating away from the cardinal vein, in a process dependent on a mesodermal cell-derived gradient of VEGF-C. Subsequently, LECs upregulate other LEC-specific markers, such as PDPN[29] and various adhesion molecules that are required to interconnect with other migrating LECs. Around E11.5, progenitor LECs migrate alongside the anterior and posterior axes of the embryo in capillary structures that eventually assemble to form the lymphatic sacs.[27,28] At this stage, initial lymphatic vessels start to form and diverge into two types of lymphatic vessels: capillary lymphatic vessels and collecting lymphatic vessels. Analyzing the development of lymphatic collectors in the mesentery, forkhead transcription factor 2 (Foxc2) was shown to be particularly important for the formation and maturation of lymphatic collectors.[30] Specifically, small clusters of cells in newly formed collectors start to upregulate *Prox1* and *Foxc2* expression around E16, thus initiating lymphatic valve formation via mechanosensitive Connexin37 (Cx37) and calcineurin/NFAT signalling.[31]

their content, that is, the lymph, into the bloodstream (Figure 1). Generally, lymphatic vessels that lead toward an LN are designated as *afferent* lymphatic vessels, while lymphatic collectors leading away from an LN are designated as *efferent* lymphatic vessels.[18] Considering that LNs are arranged in a sequential manner along the lymphatic vasculature, efferent lymphatic vessels can simultaneously be afferent lymphatic vessels for downstream LNs.

1.1 | Lymphatic vessels in peripheral tissues

In comparison to capillaries of blood vessels, lymphatic capillaries are larger in diameter (approximately 50–60 μm, as compared to the 5–10 μm wide blood capillaries)[32,33] and consist of oak leaf-shaped lymphatic endothelial cells (LECs), which are interconnected by discontinuous, button-like junctions.[33] Capillary LECs are loosely connected by anchoring filaments to a discontinuous basement membrane[34]—a setup that prevents lymphatic capillaries from collapsing when tissue fluid pressure increases, for example, during an inflammatory response (Figure 1B). Rather, once the interstitial pressure increases, this causes the opening of the lymphatic "flaps," which represent gaps of 2–3 μm between overlapping LECs. This, in turn, allows the entry of all components of lymph, that is, interstitial

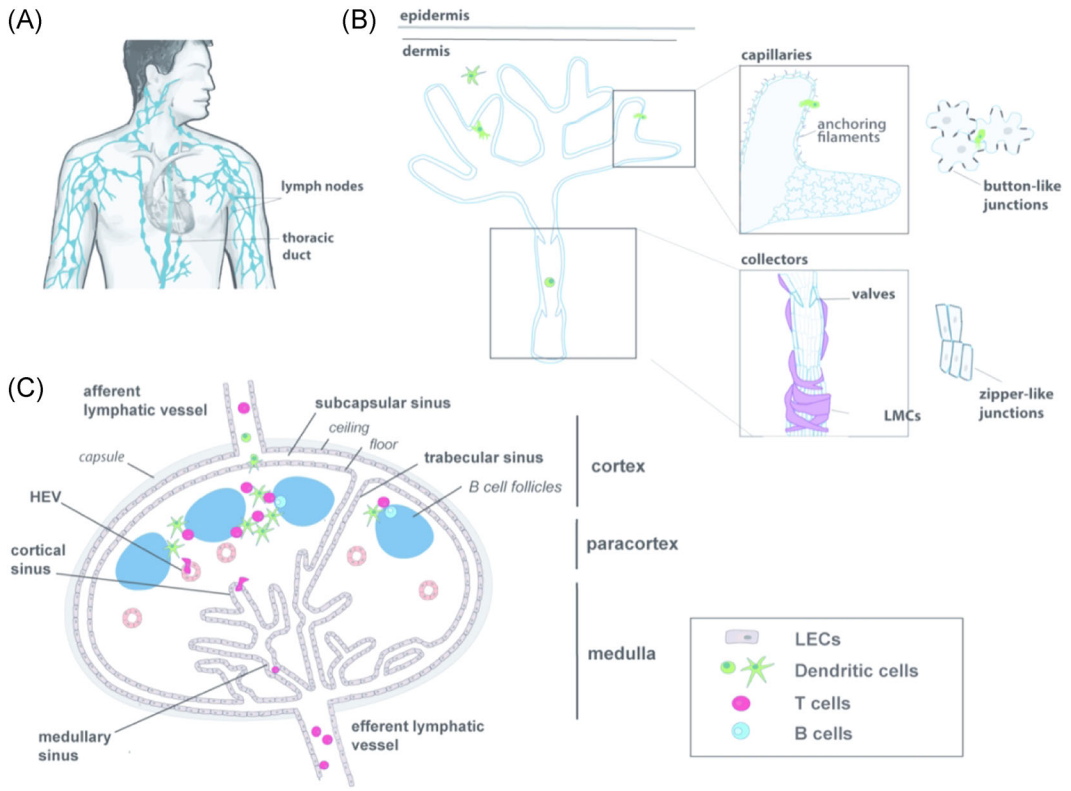

FIGURE 1 Structure of the lymphatic network. (A) The lymphatic network is made up of vessels that originate in peripheral tissues and run through lymph nodes (LNs). Lymphatic vessels eventually converge in the central body region into two lymph ducts (i.e., the thoracic duct and the right lymph duct), which fuse with the blood circulation at the level of the subclavian veins. (B) In peripheral tissues like the skin, lymphatic vessels begin as blind-ended capillaries, which merge into collecting vessels. Capillaries (upper insert) are surrounded by a thin basement membrane and contain lymphatic endothelial cells (LECs) connected by button-like junctions. This arrangement generates open flaps, which represent the main entry point for leukocytes. Flap opening is regulated by filaments that connect the flap with the basement membrane. Collecting vessels (lower insert) have a thicker basement membrane and are surrounded by contractile lymphatic muscle cells. LECs forming the collector wall are tightly joined by continuous, zipper-like cell–cell junctions. Moreover, collectors contain valves to facilitate fluid propagation. Leukocytes within collecting vessels are passively transported with the lymph flow. (C) At the level of the LN, afferent lymphatic collectors connect with the LN subcapsular sinus. The latter surrounds the entire LN parenchyma and is the site of entry for leukocytes arriving via afferent lymphatics. The LN parenchyma is divided into the outer cortex containing the B cell follicles, an inner paracortex containing the T cell area, and HEVs as well as the medulla. The entire parenchyma is interspersed by a network of trabecular and cortical sinuses, which fuse with the medullary sinus located in the region, where the efferent lymphatic vessel exits the LN. Leukocytes exiting from the LN transmigrate through cortical and medullary sinuses to access the efferent lymphatic vessel [Color figure can be viewed at wileyonlinelibrary.com]

fluid, immune cells, and macromolecules, into the capillary lumen. Capillaries first merge into pre-collecting vessels, an intermediate vessel type with characteristics of both capillaries and collectors,[35] and subsequently into collectors, which represent the second main type of lymphatic vessels in the body.

Lymphatic collectors transport lymph collected from capillaries and pre-collectors through a series of sequential LNs, allowing the lymph to eventually be passaged back toward the blood circulation (Figure 1A–C). Different from LECs in capillaries, LECs in lymphatic collectors have an elongated shape and are tightly connected by continuous

zipper-like junctions. Moreover, collecting vessels are surrounded by a continuous basement membrane to prevent leakage of lymph back into the interstitium.[33,36] In addition, lymphatic collectors are lined by lymphatic muscle cells (LMCs) that undergo phasic contractions to generate lymphatic flow and propagate lymph.[37,38] To ensure unidirectional flow upon contraction, lymphatic collectors contain intraluminal valves that separate vessels into sequential segments, so-called lymphangions, and prevent backflow from one segment into the previous one.[39] The last steps in the lymph's journey toward the blood vascular system occur through the largest lymphatic vessels in the body, namely the thoracic and lymphatic ducts, which join the blood vascular system at the level of the subclavian veins.[21,40]

While the above-described structure of the lymphatic network is characteristic for lymphatics present in the skin[36] or, for example, in the trachea,[33] there are clear differences in the architecture of the lymphatic vessel network in other organs. For example, the lung,[41] the intestine,[42,43] the heart,[4,44,45] and the central nervous system[46,47] have lymphatic networks with unique structures and functions. New insights into organ-specific networks have recently been extensively reviewed elsewhere.[21,46,48]

1.2 | Organization of the lymph node and its lymphatic sinus system

LNs provide a confined and highly specialized environment for the initiation of an adaptive immune response, continuous immunosurveillance, and propagation of immune tolerance. LNs can be structurally divided into a cortex, a paracortex, and a medulla. Immune cells derived from peripheral tissues (mostly antigen-presenting dendritic cells (DCs) and antigen-experienced T cells), together with soluble antigens and immune mediators reach the LN via afferent lymphatic vessels (Figure 1C).[9] The latter enter into the subcapsular sinus (SCS), which is found beneath the collagen-rich LN capsule and forms part of a complex sinus system surrounding the LN parenchyma (Figure 1C). The SCS overlays the LN cortex, that is, the outer region in the LN parenchyma, which harbors B cell follicles and interfollicular T cell zones. To enter from the SCS lumen into the LN parenchyma, DCs and activated T cells require the CCR7 chemokine receptor to migrate into the CCL21-rich T cell zone.[10] By contrast, naive T cells gain access to the LN parenchyma via the medullary sinus system.[10] Besides CCL21, certain DCs reportedly also require the chemokine CCL8 for exit from the SCS.[49] In comparison to the cortex, the paracortex is more deeply located within the LN parenchyma and contains the T cell zones and high endothelial venules (HEVs). The latter represent the entry points for the majority of T and B cells that enter the LN from the blood circulation.[50] Besides a specialized vascular network, the LN also contains a so-called conduit system formed by extracellular matrix (ECM) components that are ensheathed by fibroblastic reticular cells (FRCs). The conduit system starts from the SCS and serves to transport low-molecular-weight macromolecules, including antigens, but also for antibodies (IgM) and virions between different LN compartments.[51–53] The organization of the LN into specialized compartments allows antigen-presenting cells, tissue-derived antigens, and soluble immune mediators to efficiently come into close contact with naïve B and T lymphocytes and recirculating central memory T cells.[54] After a few hours of scanning antigen-presenting cells in the T cell zone for the presence of cognate antigen, most T cells will exit the LN again by exiting via the efferent lymphatic vessel. By contrast, if a T cell encounters its cognate antigen on an antigen-presenting cell, it will become activated, proliferate, and differentiate, to give rise to millions of antigen-specific effector cells, which after several days will also leave the LN.[50,55–57] Both naïve and antigen-specific lymphocytes exit the LN parenchyma through blind-ended lymphatic cortical sinuses that emerge adjacent to HEVs in the paracortex. The cortical sinuses merge into medullary sinuses, which further connect with the efferent lymphatic vessel leaving the LN.

1.2.1 | Lymphatic endothelial cells of the lymph node

LECs present in LNs have several unique properties in comparison to LECs of peripheral lymphatic vessels. According to recent single-cell RNA sequencing studies, murine and human LN LECs cluster into different subtypes

with differential gene expression: ceiling LECs, floor LECs, (para-) cortical and medullary LECs.[58–61] Ceiling LECs and floor LECs line and form the SCS. To facilitate DC and T cell entry, ceiling LECs express the atypical chemokine receptor 4 (ACKR4), a scavenging receptor for the chemokine CCL21 expressed by floor LECs. The resulting CCL21 gradient guides the transmigration of DCs across the SCS to enter the LN parenchyma.[62] Floor LECs are interspersed by a layer of $CD169^+$ macrophages and a few sinus-resident DCs that capture soluble antigens.[63,64] Moreover, floor LECs were shown to directly transcytose macromolecules, such as subcutaneously administered IgG and IgA antibodies,[65] to the LN parenchyma. In addition, floor LECs express plasmalemma vesicle-associated protein (PLVAP), which forms permeable diaphragm-like filters (*fenestrae*) both on their luminal and abluminal side and controls the entry of macromolecules into the LN conduit system.[66] Generally, sinusoidal LN LECs exhibit a high endocytic capacity[67] and can directly present antigens to immune cells.[68,69] LN LECs express major histocompatibility complex (MHC) Class I and II molecules,[70,71] and further MHC-II molecules can be acquired from migratory DCs.[72] However, the expression of the typical costimulatory molecules (e.g., B7 family), which are needed for T cell priming, is low or absent in LN LECs in steady state.[68,70,73] Instead, LN LECs reportedly express co-inhibitory molecules including programmed death-ligand 1 (PD-L1; Figure 2).[70,73] Medullary and cortical LECs were additionally shown to contribute to clonal deletion of autoreactive $CD8^+$ T cells by expressing self-antigens such as melanocyte-specific tyrosinase, intestinal epithelial protein A33, and intestinal or pancreatic polypeptide (PPY) in an autoimmune regulator (AIRE)-independent manner.[71] Besides affecting $CD8^+$ T cells, LN LECs have also been implicated in the induction $CD4^+$ T cell anergy. Specifically, LECs were shown to transfer endogenous antigens to anergy-inducing DCs,[74] or to directly induce $CD4^+$ T cell anergy by peptide-loaded MHC-II complexes that they acquired from DCs.[72] A recent study by Nadafi et al.[75] further demonstrated that LN stromal cells, including LECs, can generate antigen-specific regulatory T cells (Tregs) by conversion of naive $CD4^+$ T cells into Tregs through MHC-II expression and self-antigen presentation.[75] Taken together, these observations suggest that LN LECs rather promote tolerance than immune-stimulatory reactions in steady-state conditions. On the other hand, a recent study demonstrated that antigen presentation by LN LECs can selectively induce the generation of long-lived antigen-specific $CD8^+$ memory T cells that have the ability to rapidly differentiate into effector T cells upon rechallenge.[76] In addition to their direct involvement in antigen presentation, proliferating SCS LECs have been

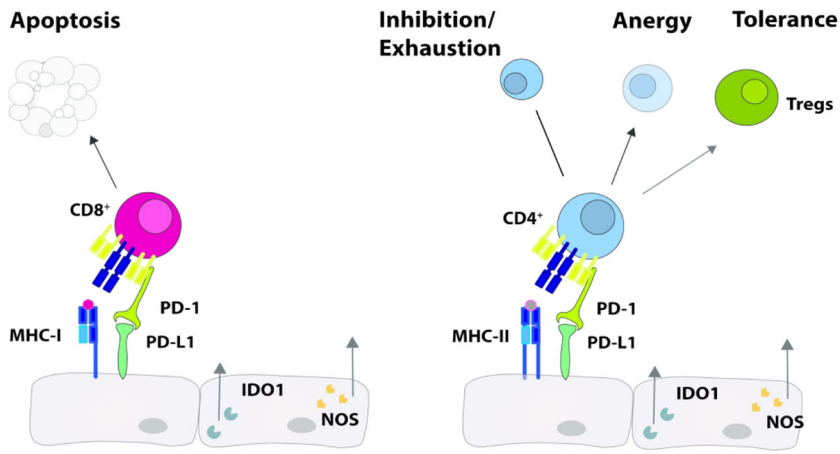

FIGURE 2 Roles of LECs in immune modulation of $CD4^+$ and $CD8^+$ T cells. Antigen presentation of $CD4^+$ and $CD8^+$ T cells in absence of costimulation, combined with programmed cell death protein 1 (PD-1)/PDL-1 signaling can have various outcomes on T cell fate and function. Immunosuppressive signals are further enhanced by inducible nitric oxide synthase (NOS)- and indoleamine 2,3-dioxygenase (IDO)-mediated production of NO and tryptophan metabolites. Of note: with the exception of the PD-1/PD-L1 interaction, the depicted functions have primarily been studied in LN LECs[83,84][Color figure can be viewed at wileyonlinelibrary.com]

shown to simply capture and "archive" viral antigen during viral infections. Although these antigens were not directly presented to $CD8^+$ T cells by LECs, they persisted in LECs for subsequent transfer to antigen-presenting cells. In this way, LECs were shown to enhance the effector functions and protective capacity of circulating memory $CD8^+$ T cells.[77,78] Finally, LN LECs have also been shown to actively control the egress of lymphocytes from the LN. LN LECs produce sphingosine-1-phosphate (S1P),[79] a chemotactic sphingolipid that promotes lymphocyte egress from the LN by binding to S1P receptor 1 (S1P1) expressed on exiting T cells.[80] Mechanisms that determine lymphocyte retention in the LN parenchyma include the downregulation or internalization of S1P1 in a CD69-dependent manner after MHC molecule engagement and activation.[81,82]

2 | MEDIATORS OF TUMOR-ASSOCIATED LYMPHANGIOGENESIS

Historically considered as a passive route for tumor cell dissemination, experimental evidence, and clinical studies have shown that the lymphatic vasculature plays an active role in primary tumor dissemination and in the metastasis process (Figure 3). Similar to angiogenesis, lymphangiogenesis is a multistep process in which activated LECs proliferate and migrate in response to specific stimuli to form new vessels. Lymphangiogenesis involves the interaction of different cell types and ECM components and is regulated by a variety of lymphangiogenic growth

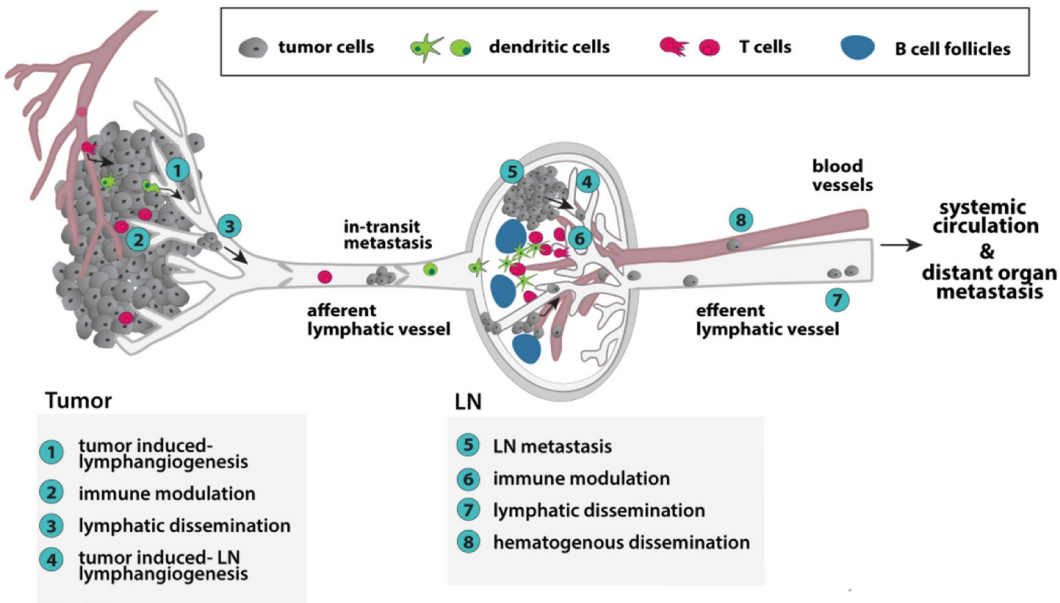

FIGURE 3 Schematic illustration of the principal routes of lymphatic metastasis and roles of lymphatic vessels in tumor progression. Lymphangiogenesis is induced by the primary tumor at the site of tumor growth and in draining LNs. Lymphangiogenesis and remodeling in the primary tumor create more surface area for tumor cell–LEC interaction and facilitates lymphatic spread. In the draining LN, it prepares the niche for subsequent colonization with tumor cells. Metastatic foci can occasionally form in transit or—more frequently—in draining LNs. From the LN, metastatic cells can further spread by accessing the systemic circulation via nodal blood vessels, or by entering into efferent lymphatics. The latter may lead to metastasis in subsequent draining LNs and eventually also allows for systemic spread. Besides tumor cells, also antigen-presenting DCs, T cells, and other leukocytes use lymphatics to migrate to the tumor-draining LNs, where antitumor immunity may be induced. Lymphatic endothelial cells within both the tumor and in tumor-draining LNs actively participate in immune modulation, especially in the dampening of antitumor immunity [Color figure can be viewed at wileyonlinelibrary.com]

factors and other mediators, including cell adhesion molecules and noncoding RNAs. The most important factors supporting tumor-associated lymphangiogenesis will be discussed in the following.

2.1 | Soluble mediators

Tumor-associated lymphangiogenesis depends on the presence of various soluble mediators (Table 1) mainly produced by cancer cells themselves, but also by macrophages, mast cells, T cells, activated platelets, as well as stromal cells within the tumor microenvironment.[85–90] A selection of the most important soluble lymphangiogenic mediators is provided below.

TABLE 1 Soluble mediators involved in tumor lymphangiogenesis

Soluble mediator	Category	Role	References
ACTIVIN A	Growth factor	Anti-lymphangiogenic	[91]
AM	Hormone	Pro-lymphangiogenic	[92]
ANGs	Growth factor	Pro-lymphangiogenic	[93,94]
EGF	Growth factor	Pro-lymphangiogenic	[95]
EPO	Growth factor	Pro-lymphangiogenic	[96]
FGF2	Growth factor	Pro-lymphangiogenic	[97,98]
HGF	Growth factor	Pro-lymphangiogenic	[99–101]
IGF	Growth factor	Pro-lymphangiogenic	[102,103]
IL6	Cytokine	Pro-lymphangiogenic	[104,105]
MDK	Growth factor	Pro-lymphangiogenic	[106]
OPN	Matrix protein	Pro-lymphangiogenic	[107]
PDGFs	Growth factor	Pro-lymphangiogenic	[108–111]
S1P	Sphingolipid	Pro-lymphangiogenic	[112,113]
SLIT2	Matrix protein	Pro-lymphangiogenic	[114]
TGFβ	Growth factor	Pro-lymphangiogenic	[115–117]
		Anti-lymphangiogenic	[118,119]
TNF-α	Cytokine	Pro-lymphangiogenic	[120,121]
VEGF-C	Growth factor	Pro-lymphangiogenic	[122–125]
VEGF-D	Growth factor	Pro-lymphangiogenic	[126,127]
WNT1	Secreted glycoprotein	Anti-lymphangiogenic	[128]
WNT5B	Secreted glycoprotein	Pro-lymphangiogenic	[129]

Abbreviations: AM, adrenomedullin; ANG, angiopoietin; EGF, epidermal growth factor; EPO, erythropoietin; FGF2, basic fibroblast growth factor; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; IL, interleukin; MDK, midkine; OPN, osteopontin; PDGF, platelet-derived growth factor; S1P, sphingosine 1 phosphate; TGFβ, transforming growth factor β; TNF-α, tumor necrosis factor α; VEGF, vascular endothelial growth factor; WNT, wingless-type MMTV integration site family.

2.1.1 | Vascular endothelial growth factors

Similar to physiological conditions, the major recognized drivers of tumor lymphangiogenesis are vascular endothelial growth factor (VEGF)-C and VEGF-D. Both mainly act through the tyrosine kinase VEGF receptor 3 (VEGFR3) in association with its coreceptor neuropilin 2 (NRP2), which are both expressed on the surface of LECs (see 87,89,90 and references therein). VEGF-C and VEGF-D are produced as pre-pro-polypeptides and undergo a step-by-step process of proteolysis to achieve their mature/active structure.[130] Once VEGF-C and VEGF-D bind to VEGFR3, this activates protein kinase C/ERK signaling and triggers AKT phosphorylation, thereby promoting LEC migration and proliferation.[131] When assessed in murine experimental models, tumor overexpression of VEGF-C, VEGF-D, VEGFR3, and/or NRP2 correlated with increased lymphatic growth and tumor cell metastases to LNs and distant organs.[122,123,126,132–135] Accordingly, blockade of VEGF/VEGFR signaling using neutralizing anti-VEGFR3 or anti-NRP2 monoclonal antibodies, soluble forms of VEGFR3, inhibitors of the receptor kinase activity, or *sVEGFR3* gene therapy has been shown to reduce lymphangiogenesis in the primary tumors as well as metastasis to LNs and distant sites.[87,90,136,137]

Several mediators have been described to upregulate VEGF-C and VEGF-D expression within the tumor microenvironment (see 85 and references therein). A pivotal role in modulating the expression of lymphangiogenic VEGFs is played by the hypoxic tumor environment.[138] Indeed, hypoxia-inducible factor 1α (HIF-1α) induces the expression of several growth factors and other molecules stimulating LEC activation, proliferation, and migration. Accordingly, high HIF-1α levels have been found to correlate with VEGF-C expression and increased peritumoral lymphangiogenesis in several types of cancers (see 139 and references therein). In addition, the distribution and the expression of lymphangiogenic factors within the tumor mass may be orchestrated by interstitial flow, a transport mechanism that plays a pivotal role in organizing the development of new lymphatic vessels.[140,141] Indeed, it has been shown that interstitial fluid flow influenced VEGF-C spatial and temporal distribution promoting lymphangiogenesis not only inside the tumor mass, but also in the peritumoral normal tissue.[141–143] Accordingly, results obtained in vitro in a model of osteosarcoma cells cultured under high-pressure conditions revealed an increased expression of VEGF-C, thus suggesting that fluid flow may directly modulate the production of lymphangiogenic factors.[144]

Thus far, VEGF-C and/or VEGF-D levels were found upregulated in several human tumors (see 85,90 and references therein). Moreover, in the majority of clinical studies high VEGF-C and VEGF-D expression was found to correlate with increased tumoral lymphangiogenesis, lymphatic metastasis, and reduced patient survival.[90,122,124,145–147] Other studies, however, did not confirm this correlation,[148,149] which suggests that additional biological mechanisms may be involved in the regulation of lymphatic metastasis, such as the presence of other growth factors able to directly modulate lymphangiogenesis. For example, VEGF-A, one of the main mediators involved in blood vessel formation and angiogenesis, also promotes the proliferation and migration of LECs in vitro.[150,151] In a cutaneous squamous cell carcinoma model, overexpression of VEGF-A was found to strongly induce tumor lymphangiogenesis and LN metastasis.[152] Accordingly, inhibition of VEGF-A/VEGFR1 and VEGF-A/VEGFR2 signaling pathways with antiangiogenic compounds inhibited VEGF-A-mediated lymphangiogenesis and sentinel LN metastasis in xenograft mouse models.[153,154]

2.1.2 | Platelet-derived growth factor

Platelet-derived growth factor B (PDGF-BB) directly stimulates lymphangiogenesis and vessel remodeling through the binding to PDGF receptor α (PDGFRα) and PDGFRβ[108,109] expressed on LEC surface. Even though in human cancers PDGF-BB overexpression is often associated with VEGF-C upregulation,[110] PDGF-BB activity on LECs seems to be independent of VEGF-C signaling, since treatment with an anti-VEGFR3 antibody did not affect its pro-lymphangiogenic activity.[108,111] Interestingly, surface expression of PDGFRβ by LECs is dependent on the transcription factor prospero-related homeobox 1 (PROX1).[155]

2.1.3 | Hepatocyte growth factor

Hepatocyte growth factor (HGF) may stimulate lymphangiogenesis either directly or through indirect mechanisms of action. HGF was found to promote lymphangiogenesis in vivo[99,100] by interacting with its cognate receptor c-Met expressed in tumor-associated lymphatics.[156] In line with this evidence, it has been recently reported that the pro-lymphangiogenic SRY-box transcription factor 18 (SOX18)[157] promoted tumor progression by activating HGF and epidermal growth factor pathways in a clear cell renal cell carcinoma mouse model.[158] On the other hand, HGF may indirectly exert its lymphangiogenic activity by cooperating with VEGF-C[159] or via induction of VEGF-C/VEGF-D expression.[101]

2.1.4 | Fibroblast growth factor

The pro-tumoral/proangiogenic basic fibroblast growth factor (bFGF/FGF2)[160] modulates lymphangiogenesis by binding to FGF receptor 3 (FGFR3) expressed by LECs.[161] The interaction between FGF2 and the lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) on the LEC surface appears to play a fundamental role in mediating FGF2-dependent lymphangiogenic activity, possibly by participating in FGF2 internalization.[97] Relevant to tumor progression, blockade of FGF2 by a neutralizing antibody reduced the density of lymphatic vessels in a xenograft model of lung cancer.[162] In addition to the direct stimulation of LEC-expressed FGFR3 described above, FGF2 may also cooperate with VEGF-C and foster tumor lymphangiogenesis and metastasis through FGFR1/VEGFR3-dependent pathways.[98]

2.1.5 | Angiopoietins

Angiopoietins (ANGs) participate in lymphangiogenesis through binding to LEC-expressed TIE receptors.[163] Overexpression of ANG1 and ANG2 in malignant pancreatic β cells was found to result in augmented lymphatic vasculature in the tumor mass.[93] Moreover, inhibition of ANG2 impaired tumor lymphangiogenesis and affected LN and lung metastasis formation in human lung carcinoma xenograft mouse models.[164] These results are in agreement with clinical evidence showing that in patients affected by papillary thyroid carcinoma ANG1 overexpression correlated with lymphovascular invasion and LN metastasis.[94] Notwithstanding, ANG1 expression has been found to correlate with decreased risk of LN metastasis in early stage cervical cancer patients,[165] suggesting that ANG signaling may exert different functions in different tumor settings.

2.1.6 | Transforming growth factor β

The effect of transforming growth factor β (TGFβ) on tumor lymphangiogenesis is still controversial. Depending on the experimental tumor model and the involvement of other mediators, TGFβ may exert both pro- or anti-lymphangiogenic activities. For instance, TGFβ increased the expression of VEGF-C by cooperating with sine oculis homeobox homolog 1 (SIX1), a transcription factor whose overexpression correlates with poor clinical prognosis in numerous malignancies,[115] and enhanced the expression of lymphangiogenic genes in KRAS-mutated pancreatic carcinoma models.[116] Recent work by Evans et al.[117] on a triple-negative breast cancer model found that TGFβ1 produced and released by tumor-associated macrophages (TAMs) adherent to LECs activated RhoA cascade signaling in LECs, inducing cell contraction in vitro and increasing permeability in vivo. Conversely, TGFβ may also exert inhibitory effects on the lymphatic vasculature. Specifically, TGFβ was found to inhibit the expression of collagen and calcium-binding EGF domain-1 (CCBE1),[118] a molecule involved in the proteolytic activation and

maturation of VEGF-C.[166] Accordingly, it has been shown that the blockade of endogenous TGFβ signaling resulted in enhanced VEGF-C induced lymphangiogenesis in xenograft mouse models.[119]

2.1.7 | S1P

S1P is a sphingolipid involved in various physiological and pathological conditions.[112] During cancer progression, S1P reportedly stimulates tumor cell migration and invasion, inflammatory cell recruitment, angiogenesis, and lymphangiogenesis.[167] In accordance, inhibition of S1P production was found to reduce lymphangiogenesis in a murine model of breast cancer.[113] Even though S1P alone is not able to stimulate LEC sprouting in vitro, the combination of S1P, FGF2, and VEGF-A was found to induce a strong lymphangiogenic response, which could be exploited for the screening of inhibitory compounds in vitro and in vivo.[168]

2.2 | Other mediators

In addition to the above-described soluble mediators, also other molecules, such as adhesion molecules, microRNAs (miRNAs), and long noncoding RNAs (lncRNAs), have been reported to play a relevant role in tumor lymphangiogenesis.

2.2.1 | Integrins

Several integrins have been described to orchestrate angiogenesis during cancer progression.[169,170] Comparably little is known about how integrins participate in tumor lymphangiogenesis, the majority of reports focusing on the role of β1 integrin (also known as CD29).[170] For instance, activation of β1 integrin signaling by semaphorin 7a (SEM7A), a glycophosphatidylinositol membrane-anchored protein frequently overexpressed in mammary tumors, stimulated lymphangiogenesis and tumor cell spreading in a breast cancer model.[171] In addition, α4β1 integrin was found to be upregulated in spontaneous and experimental tumor-associated LECs, where its activity was crucial for VEGF-C-mediated lymphangiogenic stimulation both in vitro and in vivo.[172] Accordingly, antagonists of α4β1 were shown to inhibit tumor lymphangiogenesis and LN metastasis formation in experimental murine tumor models.[172] Finally, β1 integrin expression by the mucin-type glycoprotein podoplanin (PDPN)$^+$ TAMs was recently found to play a key role in mediating TAM chemotactic migration and adhesion to LECs, thereby contributing to TAM-mediated lymphangiogenesis.[173] Along this line, a similar role has also been ascribed to LEC-expressed integrin β4, which was shown to retain a subpopulation of TAMs on the LEC surface, thereby supporting the growth of tumor lymphatic vessels.[117]

2.2.2 | Other adhesion molecules

Besides integrins, other cell adhesion molecules reportedly play a role in tumor lymphangiogenesis. For instance, loss of neural cell adhesion molecule (CD56) in a transgenic mouse model of pancreatic β cell carcinogenesis was associated with VEGF-C and VEGF-D upregulation and with increased tumor lymphangiogenesis and LN metastasis.[174] A further example is melanoma cell adhesion molecule (CD146), a transmembrane protein upregulated in almost all cancer types. CD146 was found to function as cell surface receptor for a variety of different ligands, including VEGF-C. VEGF-C-mediated activation of CD146 resulted in lymphatic vessel sprouting via p38 and ERK pathways.[175] In addition, CD146 can interact also with PDGFRβ, contributing to vessel stabilization (as reviewed in 176).

2.2.3 | Noncoding RNAs

In the last years, considerable attention has been paid to miRNA and lncRNA role in tumor progression, including their impact on lymphangiogenesis and lymphatic metastasis. Several miRNAs/lncRNAs have been found to be expressed in LECs and to promote or inhibit lymphatic vessel formation in different types of cancer (recently reviewed in 177). A detailed list of these miRNAs and lncRNAs, and their roles in tumor lymphangiogenesis is included in Table 2 and Table 3, respectively.

3 | LYMPHATICS IN THE PRIMARY TUMOR

In the early stages of tumor progression, the lymphatic network undergoes active modifications, including the formation of novel lymphatics from pre-existing ones, both inside and around the tumor mass, and the dilation of vessel lumen, thus facilitating cancer cell entrance into the lymphatic circulation.[7,90] These changes contribute to a substantial remodeling of vessels, resulting in an increased flow rate, and thus also facilitate the spreading of cancer cells to tumor-draining LNs.[90,207,208] In addition, emerging pieces of literature underline that newly formed lymphatic vessels play key, and sometimes contradictory roles in the anti- and pro-tumor immune responses taking place in the tumor microenvironment. These roles will be introduced in the following.

3.1 | Cancer cell-lymphatic interplay in the primary tumor

In response to the lymphangiogenic stimuli produced during tumor growth, lymphatics undergo a dynamic process of vascular remodeling (Figure 3). There are few transcriptional studies that have thus far been published on tumor-derived lymphatic collectors,[207] tumor-associated LECs expanded in vitro,[209] and recently on LECs directly isolated from the primary tumor of an orthotopic triple-negative breast cancer.[210] Taken together, these studies revealed the modulation of genes and pathways involved in cell migration, inflammation, and immune-related mechanisms in tumor LECs when compared to control LECs. These findings strongly suggest that cancer lymphatic vessels possess a specific molecular profile reflecting the plasticity of LECs and their capacity to adapt in response to tumor microenvironment. The exact mechanisms underlying the lymphatic invasion of tumor cells have not been fully elucidated. The interplay between tumor cells and the lymphatic system occurs at different levels as outlined in the following.

3.1.1 | Tumor cell attraction and entrance into lymphatics

Even though the exact mechanisms of cancer cell entrance and dissemination through lymphatics have not yet been fully elucidated, the capacity of tumor cells to exploit immune-specific chemokine signaling pathways has become evident (recently reviewed in 88). For example, the CCL21/CCR7 signaling, that normally mediates the trafficking of immune cells through $CCL21^+$ lymphatic vessels, has been described to promote lymphogenous spreading of $CCR7^+$ cancer cells.[211,212] CCL1, on the other hand, appears to be primarily expressed by SCS LECs and to specifically facilitate the entry of $CCR8^+$ metastatic tumor cells to the tumor-draining LN. Its receptor CCR8 is expressed in human and murine metastatic melanoma. In murine melanoma, treatment with a CCR8 antagonist was able to reduce LN metastasis, resulting in the retention of tumor cells in collecting vessels at the junction with the subcapsular sinus.[213] In addition, other chemokines expressed by LECs, such as CXCL10, CXCL12, CXCL1, and CCL5, were found to induce migration of CXCR3-, CXCR4-, CXCR2-, or CCR5- expressing tumor cells and to be involved in cancer cell invasion and LN metastasis (reviewed in 88). On the other hand, chemokines not only

TABLE 2 miRNAs involved in tumor lymphangiogenesis

miRNA	miRNA tumor levels	Lymphangiogenesis-related miRNA target	Tumor model	References
miR-182-5p	Downregulated	VEGF-C	Colon cancer	[178]
miR-186			Chondrosarcoma	[179]
miR-624-3p				[180]
miR-381				[181]
miR-27b				[182,183]
miR-507				[184]
miR-195-3p			Oral squamous cell carcinoma	[185]
miR-300				[186]
miR-128			Non-small cell lung cancer	[187]
miR-206	Downregulated	KRAS ANXA2 VEGF-C	Pancreatic ductal adenocarcinoma	[188]
miR-503-5p	Downregulated	VEGF-A	Colon cancer	[189]
miR-126			Oral squamous cell carcinoma	[190]
miR-4306	Downregulated	SIX1 CDC42 VEGF-A	Breast cancer	[191]
miR-486-5p	Downregulated	NRP2	Colorectal carcinoma	[192]
miR-93	Downregulated	ANG2	Lung adenocarcinoma	[193]
miR-129-5p	Downregulated	ZIC2	Nasopharyngeal carcinoma	[194]
miR-7	Downregulated	NF-κB	Gastric cancer	[195]
miR-526b	Upregulated	CPEB2A	Breast cancer	[145]
miR-655		PTEN		
miR-19a	Upregulated	TSP-1	Colorectal cancer	[196]
miR-155	Upregulated	BRG1	Natural Killer/T cell lymphoma	[197]
miR-221-3p	Upregulated	VASH1	Cervical squamous cell carcinoma	[198]
miR-548k	Upregulated	ADAMTS1	Esophageal squamous cell carcinoma	[199]

Abbreviations: ADAMTS1, a disintegrin and metalloproteinase with thrombospondin motifs 1; ANG2, angiopoietin 2; ANXA2, annexin A2; BRG1, Brahma-related gene 1; CDC42, cell division control protein 42; CPEB2A, cytosolic polyadenylation element-binding 2A; HIF-2α, hypoxia-induced factor 2α; KRAS, Kirsten rat sarcoma virus; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NRP2, neuropilin 2; PTEN, phosphatase and tensin homolog; SIX1, sineoculis homeobox homolog 1; TSP-1, thrombospondin-1; VASH1, vasohibin-1; VEGF, vascular endothelial growth factor; ZIC2, zinc finger protein ZIC2.

TABLE 3 lncRNAs involved in tumor lymphangiogenesis

lncRNA	lncRNA tumor levels	Effect on cancer lymphangiogenesis	Tumor model	References
ANRIL	Upregulated	Increased lymphangiogenesis	Colorectal cancer	[200]
ASLN-C07322	Upregulated	Sponging of miR-128-3p and upregulation of VEGF-C	Metastatic colon cancer	[201]
BLACAT2	Upregulated	Upregulation of VEGF-C	Bladder cancer	[202]
HNF1A-AS1	Upregulated	Sponging of miR-30b-3p and upregulation of PI3K/AKT signaling pathways	Gastric cancer	[203]
HUMT	Upregulated	FOXK1 activation and upregulation of VEGF-C	Triple-negative breast cancer	[204]
LNMAT1	Upregulated	Upregulation of MCP1 and macrophage recruitment	Bladder cancer	[205]
LNMAT2	Upregulated	Upregulation of PROX1	Bladder cancer	[206]

Abbreviations: AKT, protein kinase B; ANRIL, antisense noncoding RNA in the INK4 locus; BLACAT2, bladder cancer-associated transcript 2; FOXK1, forkhead box K1; HNF1A-AS1, hepatocyte nuclear factor 1 homeobox A – antisense RNA 1; HUMT, highly upregulated in metastatic triple-negative breast cancer; LNMAT1, lymph node metastasis associated transcript 1; LNMAT2, lymph node metastasis associated transcript 2; MCP1, monocyte chemoattractant protein 1; PI3K, phosphoinositide 3-kinase; VEGF, vascular endothelial growth factor.

mediate the attraction of tumor cells toward lymphatics, but may also act in an opposite manner. Specifically, the chemokines CXCL5, CXCL12, and CCL27 or CCL28 produced by tumor cells were found to induce a directed migration in LECs expressing the respective chemokine receptors (CXCR2, CXCR4, and CCR10), thereby contributing to lymphangiogenesis and metastasis in vivo (see 88,214 and references therein).

In addition to the role of integrins in lymphangiogenesis (see Section 2.2), cancer cell-expressed integrins are also involved in tumor cell metastasis. For example, the trans-activation of β1 integrin, expressed on cancer cells, by SEM7A, expressed on neighboring cells, was found to promote invasion into tumor lymphatics.[171] In line with these results, inhibition of the α4β1 ligand vascular cell adhesion molecule 1 (VCAM-1) decreased lymphatic vessel permeability and tumor cell intravasation.[210]

3.1.2 | Changes in lymphatic vessel integrity

In the primary tumor, formation of lymphatic vessels may occur both within and/or around the tumor mass. Since most of the intratumoral vessels appear collapsed and squeezed due to the high interstitial fluid pressure (IFP) and the solid pressure exerted by tumor cells, these vessels are thought to be functionally compromised.[215–217] By contrast, peritumoral vessels reportedly represent the principal route of escape for cancer cells from the primary tumor.[218–220] Different mechanisms mediate vessel dilation, alter endothelial integrity, and facilitate the entry of cancer cells into peritumoral lymphatics. For instance, enlargement of peritumoral capillaries and collecting lymphatic vessels has been shown to be influenced by prostaglandin stimulation of LECs.[221] Prostaglandins may be secreted by LECs themselves as a consequence of VEGF-D stimulation,[207] or by TAMs through VEGF-C-mediated upregulation of cyclooxygenase 2 (COX2).[222] Alteration/destruction of lymphatic endothelium has been reported in the presence of tumor-derived arachidonic acid metabolite 12S-HETE, produced by 15-lipoxygenase-1 (ALOX15) catalysis, which favored endothelial cell retraction and transiently reduced VE-cadherin expression.[223] When compared to normal dermal LECs, LECs isolated from a T-241/VEGF-C fibrosarcoma mouse model presented an altered expression of molecules involved in the maintenance of the integrity of cell junctions and the subendothelial matrix.[209] Moreover, it has been shown that high levels of lymphangiogenic signaling by VEGF-C resulted in altered LMC coverage of lymphatic collectors[224] and reduced the integrity of endothelial cell junctions,[122] contributing to an increase of lymphatic permeability.

3.1.3 | Fluid flow into/through tumor lymphatics

Tumor cell intravasation and delivery to the LN are also dependent on fluid flow.[225] At the primary tumor site a high IFP is observed, as a consequence of the uncontrolled flow and of the abnormal permeability of pathological/cancer blood vessels.[226] In addition, the intratumoral lymphatics are often collapsed and unable to properly clear the accumulated fluids.[227] As a consequence, fluid drains from the center to the tumor periphery,[225] thus creating an IFP gradient that promotes cancer cell invasion and/or ameboid migration toward the peritumoral lymphatic vessels.[228,229] It has been reported that chemokines produced and secreted by tumor cells may distribute along the IFP gradient and regulate autologous chemotaxis of chemokine receptor-expressing tumor cells toward the lymphatics.[230] Interestingly, the IFP may also affect tumor macrophages inducing their polarization to an M2 phenotype.[231] Polarized macrophages, in turn, orchestrate the chemotactic migration and the escape of tumor cells through the lymphatic drainage.[231,232]

Compared to the blood circulation, once entered in the lymphatic vasculature, circulating tumor cells experience less shear stress and lower flow velocities what favor their survival and arrest within lymphatics or in the LN SCS (as reviewed in 225). Interestingly, in vitro evidence have shown that lymphatic-like, but not vascular-like, fluid shear stress may activate Yes-associated protein 1 (YAP1) and a transcriptional co-activator with PDZ-binding motif

(TAZ) signaling pathways in circulating tumor cells, resulting in increased cancer cell migration and proliferation, respectively.[233,234] Whether shear stress is able to orchestrate such intracellular signaling also in vivo remains to be determined.

3.1.4 | Adaptation of tumor cells to survive in lymphatics and LNs

Once they have entered into the circulation, cancer cells are exposed to biological and mechanical stress that might complicate their journey to target organs and result in cell death. This process has been widely explored in the case of blood circulation, where the association with blood components such as platelets, may confer mechanical protection to circulating tumor cells and mechano-adaptive molecular mechanisms—that is, rearrangement of cytoskeleton via RhoA/actomyosin activation in response to fluid shear stress—contribute to cancer cell dissemination.[235,236] Little experimental evidence is available concerning the resistance or cancer cell trafficking within lymphatic vessels. The lower lymph flow velocities result in reduced fluid shear stress in lymphatic vessels (less than 1 dyne/cm^2) in comparison to blood vessels (where it can rise up to 1000 dynes/cm^2 in turbulent vessels),[225,237] what should favor tumor cell survival.[225] In addition to a more favorable mechanical context, it has recently been reported that the exposure of melanoma cells to the lymph environment, characterized by low levels of free iron and high levels of glutathione and oleic acid, reduced oxidative stress and ferroptosis in circulating tumor cells and explained their ability to survive and give rise to subsequent metastasis.[238] Additionally, S1P produced by LECs has been hypothesized to support the survival of tumor cells once they have entered the lymphatic circulation.[167]

Similar to blood circulation, cohesive migration of tumor cells has also been described in lymphatic vessels. For example, experimental evidence has shown that breast cancer cells initially entered lymphatic vessels as single cells and later were found as cell aggregates within the vessel.[239] Giampieri et al.[240] demonstrated that TGFβ1 can mediate this switch of cancer cells from cohesive to single-cell motility, and that blockade of TGFβ signaling prevented single-cell movement, but not collective one, in vivo and that these cell clusters were capable of lymphatic invasion. Interestingly, it has been suggested that the perturbation/blockage of the free flow in the lymphatic system by tumor cell clusters may lead to abnormal flow stagnation, favoring the accumulation and the growth of tumor cells at lymph vessel junctions.[241,242]

Finally, it has been shown that metastatic tumor cells may require a metabolic rewiring to adapt and colonize LNs. Indeed, in a murine model, melanoma cells were found to upregulate genes of the fatty acid oxidation (FAO) pathway and to shift their metabolism in this direction via the activation of the transcriptional factor YAP.[243] In the fatty acid-rich LN microenvironment, metastasizing cells activated this YAP-dependent metabolic pathway and maintained it through autocrine production of YAP-activating bile acids. Notably, this activation of YAP was observed also in a cohort of LN-metastatic melanoma patients where this adaptive behavior of tumor cells correlated with further dissemination and reduced survival.[243]

3.1.5 | Contribution of lymphatics to cancer stem cell niche

In the primary tumor, the interaction between cancer cells and stroma generates an active microenvironment where tumor cells with stem-like traits reside. This "cancer stem cell niche" is believed to support cancer progression, resistance, and outgrowth of metastasis.[244] Indeed, cancer stem cells have a greater potential in inducing angiogenesis and lymphangiogenesis, and can favor the formation of new blood and lymphatic vessels through the production of different factors/mediators, as reported for stem cell-like glioma,[245] glioblastoma,[246] and serous ovarian cancer.[247] Moreover, in this context a population of cancer stem cells can directly enter the flow or undergo epithelial-to-mesenchymal transition acquiring a motile phenotype which eventually results in the metastasis of tumor cells. Finally, it is worth mentioning that, beyond the primary tumor, LECs may promote the formation of a

lymphovascular niche in the LN as a milieu for the recruitment and maintenance of cancer stem-like cells, which represent the most resistant and durable candidates for metastasis and tumor relapse. In this regard, $CD133^+$ tumor cells were found associated with tumor lymphatic vessels in metastatic LNs and organs, and it has been shown that LECs promote the migration of a $CD133^+/CXCR4^+$ cell subset to target organs producing CXCL12.[248]

3.2 | Immunomodulation in the tumor microenvironment

Lymphatic vessels have been described as active players in the creation and maintenance of the immunosuppressive tumor microenvironment (Figures 2 and 3). This may at a first glance appear counterintuitive, considering that tumor-associated lymphatic vessels naturally have essential functions for the initiation of antitumor immune responses, by transporting free antigen or tumor antigen-bearing DCs to the tumor-draining LN. The requirement of lymphatic vessels for mounting antitumor immune responses has been described in several tumor studies using animal models with impaired lymphatic vessel growth or lymphatic vessel dysfunction.[249–251] For example, intradermal implantation of B16F10 melanoma into K14-VEGFR3-Ig mice, which are devoid of lymphatic vessels in the dermis, resulted in less distant (lung) metastasis, a marked reduction in leukocyte infiltration, and an impaired antitumor immunity in response to dermal vaccine delivery.[251] Moreover, a recent study performed in mice lymphatic-specific expression of the *Diphtheria toxin* (DT) receptor, what allows for a DT-mediated depletion of LECs, found that local ablation of lymphatic vessels around subcutaneously implanted melanoma and breast cancer tumors resulted in increased peritumoral edema, enhanced inflammatory cell accumulation, increased tumor PD-L1 expression, and decreased accumulation of cytotoxic T cells in the tumor microenvironment.[250] In a glioblastoma setting, Song et al.[252] recently described that ectopic expression of VEGF-C enhanced $CD8^+$ T cell priming in the draining cervical LNs and promoted intratumoral accumulation of $CD8^+$ T cells and rapid cancer clearance, presumably due to enhanced lymphatic drainage of antigens to the LNs.[252] Taken together, these observations suggest that peritumoral lymphatic vessels, in principle can contribute to inhibition of tumor growth by facilitating the egress of activated immune cells from the tumor to initiate anti-immune response and by modulating the tumor environment. However, at the same time, numerous reports have demonstrated that an increased tumor-associated lymphatic vascular density correlates with poorer outcomes in many cancer types.[7,90,123] This apparent discrepancy between pro-immune functions of tumor-associated lymphatic vessels and clinical outcomes highlights the dual role of the lymphatic vasculature during tumor progression. While CCR7/CCL21-dependent migration through lymphatic vessels allows immune cell trafficking from the tumor to the tumor-draining LN and priming of $CD8^+$ cytotoxic T lymphocyte responses, this pathway can, on the other hand, also be hijacked by cancer cells to enhance LN metastasis. Previous studies have established that CCR7-expressing cancer cells can utilize VEGF-C-mediated induction of local CCL21 expression in tumor-associated lymphatic vessels to escape from the primary tumor to the tumor-draining LN.[212,253]

Besides favoring tumor cell metastases, increasing evidence also suggests that tumor-associated lymphatic vessels adopt an immunosuppressive phenotype characterized by the expression of immune-inhibitory molecules (Figure 2). The establishment of an immunosuppressive environment in the primary tumor is a known mechanism of tumor escape, as it can cause T cell dysfunction and exhaustion.[254,255] PD-L1, a checkpoint inhibitor, was one of the immunosuppressive molecules upregulated in tumor-associated lymphatic vessels.[256,257] PD-L1 blockade on LECs was shown to increase antigen-specific T cell activation in vitro, suggesting that LEC-presented PD-L1 can dampen $CD8^+$ T cell responses in the tumor microenvironment.[257] Lane et al.[256] concluded that antigen-specific $CD8^+$ T cells were the drivers of PD-L1 expression on LECs in an interferon-γ-dependent manner in a mouse model of melanoma. This, in turn, caused reduced $CD8^+$ T cell accumulation at the primary tumor side, indicative of a negative feedback loop.[256] Besides PD-L1, also the inducible nitric oxide synthase (iNOS) and indoleamine 2,3-dioxygenase (IDO) were upregulated in tumor-associated lymphatic vessels of metastatic LNs.[83] iNOS catalyzes the production of NO, which is known to have suppressive functions on T cell proliferation,[84] while IDO is a rate-

limiting enzyme involved in tryptophan degradation, resulting in metabolites that are involved in Treg differentiation and inhibit effector T cell proliferation.[258] Thus, it appears that tumor-associated LECs contribute to the local immunosuppressive tumor environment, which limits the action of tumor-infiltrating lymphocytes and causes an exhausted or tolerogenic phenotype (Figure 2). Altogether these pleiotropic and contradictory roles played by the lymphatics, by at the same time facilitating LN-tumor trafficking as well as immune modulation, open new questions regarding the suitability to target lymphatic vessels or lymphangiogenesis for preventing tumor dissemination.

4 | LYMPH NODE METASTASES

LNs represent the first site of metastasis in several solid tumors (Figure 3). This crucial step of initial dissemination from primary tumor requires a complex reorganization and the activation of several adaptive pathways both in the lymphatics and in the tumor cells. It has been reported that in tumor-draining LNs a number of alterations in the lymphatic vessels and in the vasculature occur even before the arrival of cancer cells. Indeed, tumor cells rewire the lymphatics and the tumor-draining LNs in many ways to cause the remodeling, the expansion of the lymphatic vascular network, and the formation of pre-metastatic niches.[89,259] In the cancer settings, the enlargement of tumor-draining LNs goes along with increased recruitment or proliferation of lymphocytes, significant expansion of stromal cells, including the remodeling and proliferative expansion of the lymphatic network.[86,87,89,90,260]

4.1 | Lymphatics in the lymph node pre-metastatic niche

The formation of the metastatic niche in the tumor-draining LN has been reported to involve different pro-lymphangiogenic stimuli. Tumors release a plethora of soluble factors, as well as extracellular vesicles[261,262] or circulating tumor cells that enter and traffic through the blood and the lymphatic vessels.[225] These tumor-derived "messengers" trigger lymphangiogenesis in the LN as a key mechanism to create new lymphatics in sentinel LNs and favor metastatic spread.[263] Canonical pro-lymphangiogenic factors such as VEGF-C and VEGF-A have been widely investigated and implicated in LN lymphangiogenesis. In a mouse model of chemically-induced skin carcinogenesis, specific skin-restricted overexpression of VEGF-A was associated with increased primary tumor growth, proliferation of VEGFR2$^+$ tumor-associated lymphatic vessels, and also with LN lymphangiogenesis accompanied by metastasis in draining and distant LNs.[99] In a similar model, skin-specific overexpression of VEGF-C induced a significant expansion of lymphatic networks in the sentinel LN and increased the impact of LN and distant metastases, with no significant effect on primary tumor growth.[264]

Interestingly, it has been shown in different tumor models that VEGF-C activates PI3K in LECs and this promoted the remodeling of the lymphatic network in the draining LN, and the activation of the integrin α4β1, thus facilitating the binding of VCAM-1$^+$ metastatic tumor cells.[265] Tumor-induced lymphangiogenesis in metastasis-free sentinel LNs has also been reported from murine melanoma models after footpad[266] and ear-sponge implantation.[267] Moreover, in a murine model of nasopharyngeal carcinoma, the establishment of metastasis in the sentinel LN was preceded by enrichment of blood and lymph vessels, and the expansion of the lymph sinuses correlated with the weight of the primary tumor.[268] More recently, using a transgenic mouse model where a *Vegfr3*-driven reporter revealed sites of LEC activation (Vegfr3Luc nu/nu mice), Olmeda et al.[106] have confirmed that increased lymphatic vessel density (LVD) in the LN is required to obtain efficient nodal metastasis. In this model, regardless of the genetic alterations (i.e., BRAF, NRAS, PTEN, and p53 mutations) or of VEGF-C levels, subcutaneously grafted human melanoma cell lines caused LN and visceral dissemination only after a detectable local, distal, or systemic activation of the lymphatic network.[106]

In addition to VEGF family members, other mediators have been implicated in LN lymphangiogenesis. For example, erythropoietin was found to exert a potent lymphangiogenic effect facilitating LN lymphangiogenesis and

nodal metastases in murine models of breast cancer and melanoma.[96] In addition, in a murine model of lung carcinoma metastasis, the preparation of a pre-metastatic niche in the LN was triggered by DCs via activation of the COX-2-derived prostaglandin E2 that induced CXCL12 in the subcapsular regions of the LN.[269] Recently, the heparin-binding factor midkine (MDK), thus far implicated in the acquisition of critical hallmarks of cancer such as cell growth, survival, metastasis, migration, and angiogenesis,[270] has been identified as a mediator of LN lymphangiogenesis.[106] MDK is produced by melanoma cells (and other tumor types) as a secreted factor or as an exosome cargo and is able to activate the mTOR pathway in LECs,[106] a well-characterized player in lymphangiogenesis.[271] Interestingly, MDK accumulated in the lymphatic vessels of LNs and of visceral organs before tumor colonization in murine melanoma, and this was observed also in a cohort of melanoma patients.[106]

4.1.1 | Tumor-induced gene expression changes in LN LECs

Transcriptomics studies have shown that LECs adapt their gene expression signature in presence of the primary tumor—not only at the site of tumor growth, but also in tumor-draining LNs. Indeed, RNA sequencing of tumor-draining LNs of breast and melanoma models revealed that LEC sprouting and proliferative pathways are activated as early as 4 days after primary tumor implantation and before tumor cell seeding to the LN. In agreement with the presence of an active lymphangiogenic process, genes responsible for cell division, immune modulation, and cell adhesion were upregulated. Moreover, strong alterations in the expression levels of genes involved in cell–cell and cell–matrix adhesion were observed.[260] In particular, integrin αIIb was found to be significantly overexpressed in terms of mRNA and protein levels in activated LECs of tumor-draining LNs, and to co-localize with fibrinogen, which is increased and accumulated around lymphatic sinuses in these LNs. These observations, and the fact that integrin αIIb mediates adhesion of LECs to fibrinogen in vitro,[260] suggest that fibrinogen deposition via integrin αIIb might be one mechanism driving the formation of the pre-metastatic niche in draining LNs. In addition, other adhesive mediators have been found to be differentially expressed. For instance, the downregulation of *Jam3*, which encodes for the junctional adhesion molecule C (JAM-C), resulted in reduced vessel permeability and leukocyte trafficking in the LN.[260]

4.2 | Evasion of immune surveillance in the LN

Besides serving as a route for tumor dissemination to the draining LN and further systemic metastasis, lymphatic vessels were shown to actively propagate immune tolerance not only in the primary tumor but also in the tumor-draining LN. Before infiltrating the tumor and exerting an antitumor response, antigen-specific $CD8^+$ T cells need to be activated in the tumor-draining LN by antigen-presenting cells such as conventional DCs that present tumor-derived antigen to antigen-specific T cells. However, tumor cells can often evade immune recognition. This is on one hand due to typically diminished antigenicity of transformed cells compared to, for example, pathogens encountered in the context of an infection. Tumor cells also frequently lose MHC expression, rendering them invisible to antigen-specific cytotoxic T lymphocytes. Moreover, in contrast to infections that are also recognized by the innate immune system through pathogen-associated molecular patterns (PAMPs), growing tumors induce less activation of innate immunity.[272,273] As a consequence, tumor-antigen presenting DCs, that travel to the tumor-draining LN, often fail to express costimulatory molecules required for T cell activation.[274] In the worst case, this might not only prevent the mounting of an antitumor immune response but cause tolerance against the tumor antigens, by inducing the generation of Tregs or T cell dysfunction (anergy and exhaustion). In uninflamed, steady-state conditions, LN represents an immunosuppressive environment maintained by LN stromal cells such as FRCs, blood endothelial cells, and LECs.[275] Stromal cells, including LECs, produce immunosuppressive molecules that can profoundly affect the survival, fate, and activation of naïve T cells and other lymphocyte subsets to prevent

autoimmune reactions. Examples of such molecules are the immune checkpoint molecules PD-L1, NOS2/NO, which can promote the induction of Tregs from $CD4^+$ $CD25^-$ T cell and inhibit T cell proliferation,[75,84,276] or IDO, which inhibits naïve T cell proliferation.[277] Moreover, FRCs and LECs in steady-state LNs have been shown to present peripheral tissue antigens and induce T cell anergy or clonal deletion in a programmed cell death protein 1 (PD-1)/PD-L1 dependent manner or alternatively by induction of Tregs (Figure 2).[68,70,71,73,75] At this point, there are only a few experimental studies that have closely investigated the immunological consequences of tumor antigen uptake by LN LECs during tumor progression, and whether antigen presentation by LN LECs also exerts immunogenic or exclusively immunosuppressive activity. In support of the latter, Lund et al.[69] reported that in a B16F10 melanoma model expressing the foreign antigen ovalbumin (OVA), LECs in the tumor-draining LN were found to cross-present OVA on MHC Class I molecules. Subsequently, cocultured OVA-specific $CD8^+$ T cells with tumor antigen-presenting LN LECs caused abnormal proliferation and apoptosis of $CD8^+$ T cells ex vivo, suggesting that LN LECs might actively suppress the activation of tumor-specific $CD8^+$ T cells in tumor-draining LNs. Besides directly presenting antigen, it is perceivable that LN LECs could also impact adaptive immunity by taking up tumor-derived antigen and passing it on to other antigen-presenting cells, in analogy to the antigen archiving function described for medullary sinus LECs in the context of viral infection and vaccination.[77,78] Specifically, it was shown that LECs "pass on" antigen to cross-presenting DCs, which induce potent $CD8^+$ T cells responses. It is interesting to note that in the tumor context, similar mechanisms of antigen transfer have been reported for migratory and resident DCs. While migratory DCs were shown to capture and transport tumor antigen-containing vesicles to draining LNs, the induction of antitumor cytotoxic T cell responses depended on vesicle transfer and Ag presentation by resident DCs.[278,279] Future studies are expected to shed more light on the mechanisms of antigen presentation by LECs or antigen transfer between LECs and other antigen-presenting cells in tumor-draining LNs.

4.3 | Further dissemination from tumor-draining LNs

Long distance and visceral metastases represent the real life-threatening aspects of metastatic cancer. Formation of these types of metastases is always preceded by extravasation of cancer cells from the blood circulation into the target organ, demonstrating the importance of blood vessels in this process. Nevertheless, in most solid tumors the process of lymphatic metastasis precedes dissemination by blood vessels. Lymphatic metastasis is driven by multiple mechanisms, and a recent report suggests that cells that metastasize through the lymphatics are different from those spreading through the blood circulation.[280]

Metastatic cells from the tumor-draining LN can further colonize distal nodes or be a source of cancer cells that give rise to distant metastases (Figure 3). Entry of metastatic cells into the blood circulation may either occur by dissemination through efferent lymphatic vessels and the thoracic ducts into the subclavian vein or, as recently demonstrated, directly via the LN blood vasculature.[281,282] The latter possibility of direct lymph-to-blood vessel passage has been hypothesized for a long time until recent experimental evidence. Brown et al.[281] demonstrated that intra-lymphatic microinfusion of metastatic murine breast cancer cells into the SCS of popliteal LNs resulted in rapid infiltration of the LN parenchyma, invasion of blood vessels, and consequent seeding to the lungs, with no involvement of the thoracic duct. Interestingly, after entering the SCS, tumor cells were limited in their migration to the medullary sinus, and this prevented or slowed down further dissemination via the efferent lymphatics. In this avascular milieu tumor cells activated invasive programs and took contact with and wrapped around HEVs, which represent the main sites of leukocytes extravasation from blood into the LN.[281] Pereira et al.,[282] on the other hand, performed photoconversion experiments and established that, after orthotopic implantation of melanoma and breast cancer cells and sentinel LN colonization, the long-distance lung metastases where composed preferentially (around 70%) by cancer cells derived from the LN. Even though it remains to be established if tumor cell entry into nodal blood vessels also occurs in patients, these findings reveal that LN-resident tumor cells represent great contributors to the systemic dissemination of neoplastic cells.

This evidence further underlies the fact that LN metastases are not the final step of the metastatic process but just on stepping stone in systemic cancer spread. This has also been revealed by clonal genetic evolution studies of primary tumors, LNs, and distant metastases in human colorectal cancer, revealing a scenario where LN metastases and distant metastases often share a common origin.[283] In this complex panorama, also the metastatic sites may actively contribute to further expand systemic colonization. Indeed, lymphangiogenesis also occurs in metastases and strongly contributes to further dissemination to other organs generating an intra-metastasis spreading, as reported in prostate cancer patients.[284] This has been shown in a transgenic model where inducible lung overexpression of VEGF-C was accompanied by increased density of lymphatic vessels at metastatic sites as well as higher number of metastasis not only in the lungs, but also in other organs.[134] Importantly, despite the central role of LN metastases in terms of prognosis, various clinical trials have demonstrated that removal of regional LNs has no impact on the survival rate in different types of metastatic cancers, including melanoma.[285,286] These observations confirm that the tumor cells present in regional LNs at the time of resection are not the only ones responsible for the overall tumor diffusion in the body. In fact, further diffusion to distant LNs, intravasation into blood vessels, or micro-colonization of distant organs might have already occurred and secondary lesions might appear at distant sites after a short or long lifespan.

5 | PROGNOSTIC VALUE OF TUMOR-INFILTRATING LYMPHATIC VESSELS

Even though the architecture and functionality of tumor-infiltrating lymphatics may vary depending on tumor type, an increasing body of evidence indicates that a strong correlation may exist between tumor lymphangiogenesis and patient outcomes. Indeed, various parameters, such as overexpression of lymphangiogenic factors and their receptors, intratumoral and peritumoral LVD, enlargement, and remodeling of lymphatics, or tumor cell invasion of lymphatic vessels, have been associated with metastasis occurrence and reduced overall survival in different cancers (see 86,87,90 and references therein). For example, metastatic cutaneous melanomas were characterized by higher intratumoral and peritumoral LVD when compared to non-metastatic tumors.[287] This is in line with the observation that the overexpression of *VEGF-C* and *ANG2* correlated with a poorer prognosis in melanoma patients.[288,289] In addition, a multivariate risk analysis conducted on primary tumors and sentinel LN biopsies from 45 melanoma patients revealed that the lymphatic vascular area of primary melanomas was an extremely sensitive and specific prognostic marker for the occurrence of tumor-draining LN metastasis and could predict the metastatic phenotype more accurately than measurements of tumor thickness measuring.[290] Similar findings have been reported for head and neck, breast, lung, colorectal, and bladder carcinomas, where the peritumoral and/or intratumoral LVD have been found to associate with LN metastasis and shorter disease-free/overall survival in the majority of the clinical studies (see 86,90 and references therein).

Interestingly, the link between lymphangiogenesis and patient prognosis has also been reported in tumor-draining LN and distant metastases.[134,263] For example, a study conducted on 65 breast cancer patients showed that the proliferation index of LECs in tumor-draining LN metastases significantly correlated with the presence of metastasis-positive non-sentinel LN and further metastatic spread.[291] In addition, a retrospective study of 266 melanoma patients with lung metastases showed that the lymphatic area and the LVD around tumor metastases correlated with a poorer prognosis and a reduced survival.[134] Finally, it was recently reported that lymphatic exudates collected from metastatic melanoma patients that underwent lymphadenectomy were highly enriched in cancer biomarkers (e.g., extracellular vesicles containing tumor-derived proteins and miRNAs).[259,261] These results may open a new field of research based on tumor-draining lymph fluids as “liquid biopsy” to be utilized for the discovery of novel biomarkers, as well as for the tumor staging and patient outcome prediction, including risk of relapse.

6 | THERAPEUTIC PERSPECTIVES

As already discussed, lymphatic vessels mediate the spread of tumor cells to draining LNs and more distant sites in the body and at the same time may serve as reservoir for in-transit metastatic cells. Moreover, they are emerging as important players in tumoral immunosuppression. Colonization of LNs by tumor cells represents a recognized prognostic factor of tumor aggressiveness and patient survival.[87,90,286,292–295] However, even though the presence of LN metastasis correlates with poorer outcomes, removal of LNs does not always appear to be beneficial.[296–298] Nevertheless, given the multiple and key roles of lymphatics in cancer progression, a significant interest exists to therapeutically target lymphangiogenesis or to exploit the lymphatic vasculature for drug delivery.

6.1 | Lymphangiogenesis as a therapeutic target

Direct targeting of the lymphatic vasculature could allow to interfere with tumor cell dissemination to draining LNs and beyond, but might also represent a strategy for modulating tumoral immunosuppression. Considering that VEGFR3 has been identified as the main driver of lymphangiogenesis, it is not surprising that the majority of studies and drug development strategies have thus far focused on targeting the VEGF-C/VEGF-D/VEGFR3 axis. In fact, this is somewhat similar to the field of tumor angiogenesis, where numerous drugs targeting the VEGF-A/VEGFR1/VEGFR2 axis have been developed and approved over the last two decades. Well-known examples of the latter are monoclonal antibodies directed against VEGF-A (i.e., bevacizumab and ranibizumab) or VEGFR2 (i.e., ramuricumab), as well as other tyrosine kinase inhibitors (i.e., axitinib, tivozanib, sunitinib, pazopanib, cabozantinib, and cediranib) targeting also VEGF receptors.[299,300] Likewise, drugs that more specifically target components of the VEGF-C/VEGFR3 signaling pathway are being evaluated for clinical development (reviewed in 86,301; Figure 4). However, thus far, only a small number of antibodies, receptor traps, and small molecules that specifically target VEGF-C or VEGFR3 have entered clinical development.[302,303] A first Phase I clinical trial that assessed the tolerability of a humanized IgG1 antibody blocking VEGFR3 (LY3022856/IMC-3C5, developed by Eli Lilly and Company), was conducted in patients with advanced solid tumors and colorectal cancer. The trial was completed in 2014 with the conclusion that the treatment was well tolerated but had limited antitumor activity.[302] Similarly, results from a Phase I clinical study evaluating the effects of a human VEGF-C neutralizing monoclonal antibody (VGX-100), developed by Circadian Technologies) in patients with advanced solid tumors found this treatment to be well tolerated.[303] A Phase I study (NCT01514123) is still ongoing with the aim to evaluate the tolerability and possible synergisms of VGX-100 in combination with bevacizumab in adult subjects with advanced or metastatic solid tumors. However, for all investigational drugs, apparently, no follow-up or Phase II studies have been initiated so far, what may reflect the poor primary outcome on tumor growth and the need for additional insights on the actual impact of targeting lymphangiogenesis in advanced solid tumors. On the other hand, several molecules that inhibit signaling pathways involved in, but not exclusively occurring in lymphangiogenesis, such ANG1/ANG2, c-Met, or HGF, are currently under clinical development as anticancer treatments (reviewed in 86). The underrepresentation of drugs in clinical development that specifically targets lymphangiogenesis in cancer might be due to several reasons. On one hand, this might be caused by the only relatively recent recognition of the importance of lymphatic vessels in tumor dissemination, combined with the generally lengthy duration of drug development. On the other hand, despite overwhelming evidence of the involvement of lymphatic vessels in cancer spread, experience from clinical studies with VEGF-A/VEGFR1/VEGFR2-targeting drugs might suggest that the benefit of such monotherapies could fall short of the expectations raised by the preclinical studies. Bevacizumab, for example, turned out to have a modest impact on patient survival when given as a monotherapy and is therefore mostly used in combination therapies.[304–306] Furthermore, in contrast to antiangiogenic drugs, which reduce the growth of tumor lesions, anti-lymphangiogenic treatment is expected to primarily impact tumor cell dissemination and overall

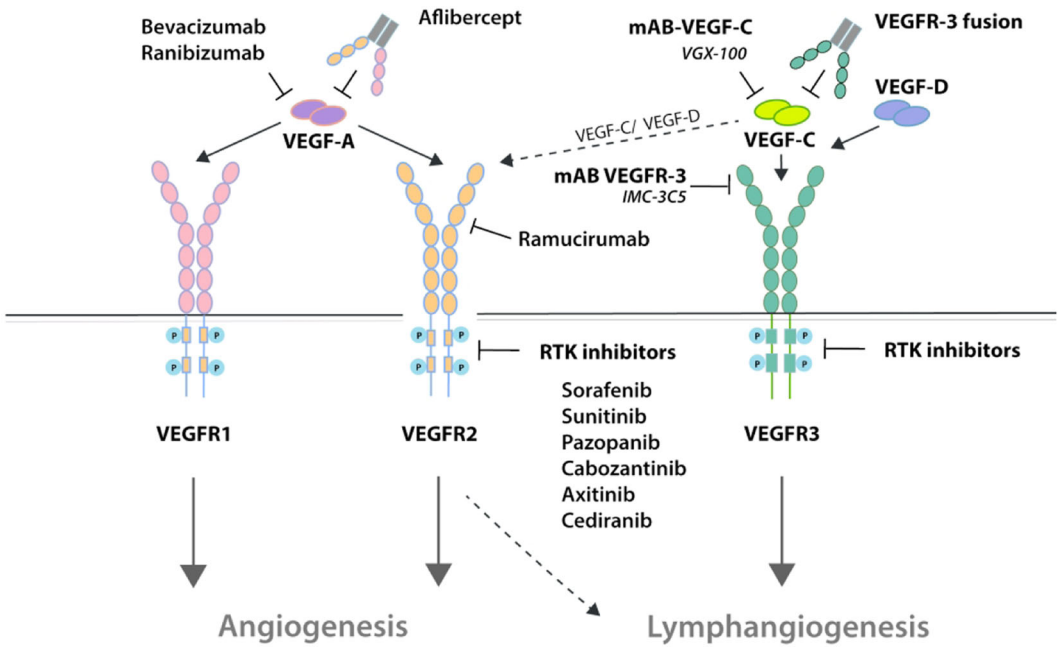

FIGURE 4 Drug targets in the vascular endothelial growth factor (VEGF)/VEGF receptor (VEGFR) signaling pathways. Graphic depiction of the currently approved drugs or drugs in development that target either VEGF-A/VEGFR2 or VEGF-C/VEGF-D/VEGFR3 signaling for inhibition of (lymph)angiogenesis. Drug formats comprise monoclonal antibodies, receptor tyrosine kinase (RTKs) inhibitors, and receptor traps, which represent the extracellular portions of the receptors fused to an antibody Fc moiety [Color figure can be viewed at wileyonlinelibrary.com]

survival, that is, parameters that typically can only be evaluated in lengthy and Phase II/III clinical trials involving high patient numbers.

It is worth mentioning that, besides anti-lymphangiogenic drugs, also other approaches have been proposed in the attempt to "hit" the lymphatic vasculature. For example, since LECs proliferate much less compared to tumor cells or other cells in the tumor microenvironment, they have been proposed to be insensitive to radiation therapy.[307] Combining radiotherapy with anti-lymphangiogenic drugs might therefore act synergistically to reduce the risk of lymphatic metastasis.[242] A potential caveat, however, is that radiation reportedly induces secondary dysfunctions of lymphatics, as the vessels lose their contractile capacity and ability to drain fluids when the surrounding irradiated tissue becomes fibrotic.[308] Combination of anti-lymphangiogenic drugs and radiotherapy might therefore be particularly problematic in patients with post-surgery lymphedema.

Another treatment that may display synergism with anti-lymphangiogenic therapy is photodynamic therapy (PDT). PDT is based in the uptake of photosensitizer molecules which, upon excitation by light of a particular wavelength, react with oxygen and generate reactive oxygen species in target tissues causing cell death.[309] To date, PDT has been clinically approved for the treatment of a variety of solid cancers (e.g., lung, bladder, skin, and pancreatic tumors).[310] Moreover, blood vascular-targeted PDT has been approved in Europe for the treatment of low-risk prostate cancer patients[311] and a number of Phase I/II/III clinical trials are ongoing to evaluate its efficacy for urologic, prostatic, and esophagogastric cancers (reviewed in 312). Interestingly, the potential of lymphatic-targeted PDT is currently under preclinical evaluation and is based on the use of intra- or peritumoral injection of phototoxic compounds, such as verteporfin, that under liposomal formulation preferentially accumulate in lymphatics, and once activated react with the surrounding tissues, destroy lymphatic vessels and, if present, tumor cells

within them (i.e., in transit lymphatic metastases).[313–315] However, even though the combination of PDT with anti-lymphangiogenic drugs may have the advantage to prevent the regeneration of novel lymphatic vessels,[314] both approaches reportedly suppress the induction of the antitumor immune response, by inhibiting the immunologic communication—, that is, DC trafficking and antigen drainage—with the draining LN.[316]

Adding to these difficulties in development might be the emerging complex and the dual role that lymphatic vessels appear to play in tumor progression/dissemination. Although lymphangiogenesis is generally associated with cancer spread and poorer patient prognosis,[86,87,90,317] recent evidence indicates that the presence of intratumoral and peritumoral lymphatic vessels and (high) VEGF-C expression at the same time may be beneficial, by enhancing the efficacy of cancer immunotherapy. Specifically, a study by Fankhauser et al.[149] reported that VEGF-C expression and high LVD correlated with a good cancer immunotherapy response. Although the study initially found that in murine melanoma models, blocking VEGFR3 signaling was associated with decreased infiltration of immunosuppressive Tregs, unexpectedly, VEGF-C signaling also potentiated the responsiveness of melanoma to immunotherapy. This effect was attributed to increased recruitment and local activation of $CCR7^+$ T cells in lymphangiogenic (VEGF-C expressing B16-OVA/VC) tumors. Similarly, in human metastatic melanoma patients, serum VEGF-C levels correlated with T cell activation and expansion after peptide vaccination and clinical response to checkpoint blockade.[149] Along the same line, Bordry et al.[83] reported that increased tumor LVD positively correlated with an immunosuppressive phenotype and increased $CD8^+$ T cell infiltration in patients with cutaneous melanoma. Taken together, it is likely that the success of therapies targeting tumor-associated lymphangiogenesis might depend on several factors including the tumor type, stage, and possibly the combination with immunotherapy. Therefore, it might be necessary to evaluate the potential benefits and risks of anti-lymphangiogenic drugs, depending on defined prognostic criteria.

6.2 | Lymphatic vessels as drug delivery routes

Most chemotherapeutic drugs are administered by the oral or intravenous route. Typically, these drugs display poor tumor uptake and distribute to and accumulate in normal organs and tissues, leading to substantial side effects. In addition, due to their small size, they are rapidly cleared from tissues by reabsorption into blood vessels, rather than entering lymphatic vessels.[318] Therefore, it is a unique challenge to achieve sufficient accumulation and retention of these types of drugs at the tumor or metastatic site and/or in lymphoid tissues.[133,319–321] In the case of LN metastases or in-transit metastases, one option for reaching higher therapeutic drug concentrations at the site of disease could consist of using the lymphatic vasculature as a delivery route. Due to the unique structure of initial lymphatic capillaries, uptake of compounds from the injection site in the interstitium into lymphatics as compared to the blood vessels is dependent on several factors, such as the size of the molecule/particle, as well as its charge or hydrophobicity.[85] Lymphatic uptake and LN accumulation are most efficient for macromolecules, including large peptides and proteins, ranging from 20 to 50 nm and for particles between 10 and 100 nm.[318,322] Particles larger than 100 nm cannot easily diffuse through the interstitium, and most of them remain at the injection site until being cleared by phagocytes.[323] By contrast, molecules smaller than 5–10 nm, such as most low molecular weight drug molecules, can cross blood vessel wall and therefore directly enter the blood circulation.[324,325]

Novel approaches aiming at delivering subcutaneously, intradermally, or intramuscularly injected low molecular weight therapeutics via lymphatic vessels to draining LNs take advantage of associating or packaging these drugs in or with macromolecular carriers, such as nanoparticles, capsules, polymers, micelles, liposomes, and dendrimers (extensively reviewed in 85,326,327). As an example, liposomes containing doxorubicin have been tested in clinical trials of patients with gastric carcinoma. In this study, injection of liposomal-doxorubicin into the gastric submucosa allowed to reach significantly higher concentrations of doxorubicin in the primary and secondary draining LNs in comparison to intravenously injected liposomal–doxorubicin or free doxorubicin injected both intravenously or also into the gastric submucosa.[328] Similarly, subcutaneous injection of depot forming cancer vaccines (i.e., specific

tumor antigens and adjuvant) exploit lymphatic delivery to LNs, and might be further enhanced by direct lymphatic targeting, local modulation of lymphatic drainage, or by recruitment of antigen-presenting cells.[327] An alternative, recently published approach made use of microneedles to enhance the lymphatic uptake and delivery of a CTLA-4 blocking antibody (i.e., a checkpoint inhibitor) to the tumor-draining LN in a murine orthotopic mammary carcinoma model.[329] Compared to systemic (intravenous) treatment with the checkpoint inhibitor, the intradermal delivery to the tumor-draining LN resulted in more effective tumor growth inhibition, increased tumor-infiltrating lymphocytes, and decreased metastasis, presumably due to a better targeting of the tumor-draining LN and consequently more efficient activation of tumor-specific T cells.[329] The therapeutic benefits of targeting immune checkpoint inhibitors to tumor-draining LNs was recently confirmed by another study and extended to the use of PD-1 inhibitors.[330]

Likewise, but less efficaciously than subcutaneous administration, intramuscular drug injection was demonstrated to increase lymphatic uptake of liposome-entrapped methotrexate[331] and mitomycin C conjugated with dextran.[332] As a further refinement of this principle, another recent study described the use of sonoporation using acoustic liposomes and ultrasound as a method to enhance the uptake of intra-lymphatically delivered doxorubicin to metastasis-bearing LNs growing in the SCS. In this case, intralymphatic delivery and release of doxorubicin were shown to induce tumor necrosis and prevented further tumor invasion into the LN parenchyma.[333] Another strategy would be to exploit peptides and molecules that bind to receptors expressed by (tumor-associated) lymphatic vessels to achieve lymphatic trafficking/homing. An interesting example is the synthetic nonapeptide Ly-1P, which was shown to target tumor cells, tumor-associated lymphatics, and macrophages.[334,335] Ly-1P has been synthetically linked/conjugated to polymeric micelles,[336] PEGylated, doxorubicin-containing liposomes,[337] or nanoparticles.[338] Subcutaneous injection of PEGylated, doxorubicin-containing liposomes, for example, resulted in increased accumulation of LyP-1-conjugated PEGylated liposomes compared to unconjugated liposomes in metastatic LNs and suppressed LN metastasis in vivo.[337,339] However as Ly-P1 also exhibits cytotoxicity toward tumor cells and gets internalized by tumor cells as well as LECs, safety, and efficacy of Ly-P1 would need to be further evaluated.[335,336] Moreover, a general drawback of the above-mentioned approaches might be that they will only allow to target metastatic disease in LNs draining tissues that are accessible by injection from outside the body, such as via the skin. Future studies will be required to elucidate which of these experimental approaches can be successfully translated to the clinics.

7 | CONCLUSIONS

Over the last decades, it has become clear that the lymphatic vasculature plays crucial roles both in the maintenance of tissue homeostasis as well as in disease. In addition to its contribution to immune-related and inflammatory disorders, an overwhelming scientific literature now documents the importance of lymphatic vessels in cancer biology and its relevance for the prognosis of cancer patients. While the lymphatic vasculature undoubtedly represents an active and dynamic player in cancer progression, it is also clear that further investigations will be required to increase our understanding of the complex interplay occurring among cancer cells, LECs, and the immune system, and for ultimately translating these new insights into therapies.

In the era of "immuno-oncology", more and more studies are reporting the key role of the lymphatic vasculature in modulating the antitumor immune response. More knowledge of both the tumor cell- LEC as well as the LEC-immune cell crosstalk will help to better understand the two-fold role that lymphatics appear to play in either promoting or dampening tumor spread. Already now it is apparent that no "one-fits all" explanation will be found, but that the role of lymphatics will likely depend on various aspects, such as the tumor type (e.g., aggressiveness, mutation load) and tissue localization. It is clear that LECs respond to the tumor environment, yet only a few studies have thus far explored this cellular plasticity in the tumor context. In view of recent advances in single-cell sequencing, it is likely that we will soon know much more about tumor-specific LEC signatures. This knowledge will be instrumental for the identification of new prognostic markers as well as potential therapeutic targets. In addition,

it will provide a better understanding of the lymphatic-tumor cell crosstalk and how it evolves during the different phases of cancer onset, progression, and dissemination.

Considering that lymphangiogenesis or immune-modulation by lymphatics appears to occur both locally in the tumor and in tumor-draining LNs, it is at present not possible to unambiguously assign the relative importance of a process taking place at either site for tumor progression. The availability of tools to specifically target lymphatic vessels in a particular tissue or in LNs, for example, with specific antibodies or mouse models allowing for genetic deletion of a gene in lymphatics at either site, would allow for great advances in this field. It is likely that new insights, for example, gained from emerging transcriptomics analyses into organ-specific differences in the lymphatic vasculature,[21] will open up new opportunities in the future.

From a therapeutic perspective, it is expected and desirable that more anti-lymphangiogenic approaches will soon reach the stage of clinical development. However, it remains to be carefully evaluated where and when anti-lymphangiogenic therapy can be applied to "mechanically" reduce the dissemination of tumor cells, without potentially impacting the endogenous antitumor immune response or the efficacy of immunotherapy. Also, in this regard it is likely that a case-by-case evaluation of the tumor type and disease burden will be required, considering not only the lymphatic involvement, but also immune-related parameters like tumor immunogenicity and responsiveness to tumor immunotherapy. Last but not least, further studies investigating how lymphatics may be used for the delivery of therapeutic drugs to metastatic LNs or sites of antitumor immune induction will likely reveal new avenues for exploiting the lymphatic vasculature in oncology.

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