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The dual role of tumor lymphatic vessels in dissemination of metastases and immune response development

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ABSTRACT

Lymphatic vasculature plays a crucial role in the immune response, enabling transport of dendritic cells (DCs) and antigens (Ags) into the lymph nodes. Unfortunately, the lymphatic system has also a negative role in the progression of cancer diseases, by facilitating the metastatic spread of many carcinomas to the draining lymph nodes. The lymphatics can promote antitumor immune response as well as tumor tolerance. Here, we review the role of lymphatic endothelial cells (LECs) in tumor progression and immunity and mechanism of action in the newest anti-lymphatic therapies, including photodynamic therapy (PDT).

Abbreviations: Ags, antigens; AKT, protein kinase B; AMD, age macular degeneration; Ang1/2, angiopoietin-1/2; APCs, antigen-presenting cells; CCL5/19/21, chemokine (C–C motif) ligand 5/19/21; CCR4/5/l7, C–C chemokine receptor type 4/5/7; COX-2, cyclooxygenase-2; CD, cluster of differentiation; DCs, dendritic cells; ERK1/2, extracellular-signal-regulated kinases; HIF-1, hypoxia-inducible transcription factor; ICAM-1, intercellular adhesion molecule 1; IRES, internal ribosome entry site; LECs, lymphatic endothelial cells; LEN, lenalidomide; MDSCs, myeloid-derived suppressor cells; MHC, major histocompatibility complex; mTOR, mammalian target of rapamycin kinase; PDT, photodynamic therapy; PGE2, prostaglandin E2; PROX-1, prospero homeobox 1; SMCs, smooth muscle cells; SOX18, SRY-related HMG-box; STAT3, signal transducer and activator of transcription 3; TAMs, tumor-associated macrophages; TCM, tumor-conditioned media; Tie2, angiopoietin receptors; TGFBlp, transforming growth factor-β-induced protein; TGF-β, transforming growth factor β; TNF, tumor necrosis factor; TNFR1, tumor necrosis factor receptor 1; VEGF-A/VEGF-C/VEGF-D, vascular endothelial growth factor C/D; VEGFR, vascular endothelial growth factor receptor

Introduction

One of the main causes of cancer evasion is the ability of tumor cells to spread to local and distant tissues and organs. Therefore, for several decades, a major focus of cancer research was to understand the mechanisms which unlock the ability of tumor cells to form metastases. Many studies showed that the lymphatic development can be re-activated during tumor lymphangiogenesis and, as it was widely reviewed before, the tumor lymphangiogenesis strongly depends on vascular endothelial growth factor C/D (VEGF-C/D) and vascular endothelial growth factor receptor 3 (VEGFR-3) pathway.[1] In lymphangiogenesis, the pivotal role is also played by the SRY-related HMG-box (SOX18), which through activation of prospero homeobox 1 (Prox-1) transcription, induces expression of lymphatic-specific genes, e.g. podoplanin.[2]

Recent research has been focused on uncovering the active role of lymphatic endothelium in tumor cells transport and modulation of antitumor immune response.[3] The communication between immune system and tumor is initiated with blind-ended capillaries of afferent lymphatics that merge into larger collecting vessels and connect tissue with lymph nodes. This lymphatic link between tumor and the distant microenvironment is associated with flow of tumor antigens (Ags), cytokines and enzymes but is also crucial for the progression and dissemination of tumor cells. In face of the discovery of several key lymphatic-specific molecular markers, the number of studies on lymphatic biology has been recently augmented. Therefore, we review the role of lymphatic endothelial cells (LECs) in tumor progression and immunity as well as the requirements for tumor cells to enter initial afferent lymphatic vessels. We discuss the newest achievements in anti-lymphatic therapies underlying some innovative treatments such as photodynamic therapy (PDT).

Structure and function of lymphatic vessels

Normal tissue fluid homeostasis in the human body is dependent on mutual reinforcing functions of blood and lymph

vessels system. In contrast to blood vessels responsible for delivery of oxygen, nutrients, hormones and cells to the body tissues, lymphatic vessels are specialized in uptake of tissue fluid with macromolecules, microbes and other substances from interstitial space.[4]

The lymphatic vessels are present in tissues that frequently come in contact with foreign Ags, such as the skin and mucous membranes. Moreover, lymphatic vessels are found in all vascularized tissues, including recently discovered central nervous system lymphatic vasculature, with notable exception of bone marrow.[5] Tissue fluid first enters lymphatic capillaries, comprised of LECs organized in highly permeable single-layered, blind-ended and thin-walled sacs. LECs are attached to the extracellular matrix through elastic anchoring filaments. These fibrillin-stranded structures protect vessels from collapse and stretch under high-interstitial pressure. This leads to the opening of endothelial flaps, allowing fluid and macromolecules to enter the vessel's lumen.[6] The lymph is further transferred to larger pre-collector vessels that contain occasional valves, a basement membrane and sparse coverage of smooth muscle cells (SMCs). The pre-collectors converge into the lymphatic collecting vessels, with LECs forming continuous "zipper-like" junctions, surrounded by a basement membrane and supported by incessant layer of SMCs. The lymph flow is achieved by SMCs contractility, vasomotion and the activity of surrounding skeletal muscles. The bileaflet valves in collecting vessels prevent the backflow of lymph.[7] The collectors pass the lymph to the lymph nodes and further to lymphatic trunks and right lymphatic and thoracic ducts, where lymph is eventually drained back into the venous circulation at the venous angles.[1] Therefore, the central function of lymphatic vascular system is to sustain tissue-fluid homeostasis. Additionally, lacteal lymphatic vessels absorb and transport fat soluble vitamins and dietary fat, such as chylomicrons, from the small intestine, bypassing the liver that normally clear hydrophilic substances collected by blood from the intestine. In addition, the lymphatic system is essential for proper functioning of the immune system. Its role is especially invaluable in the immune response as lymphatic vessels enable leukocytes trafficking and transport of Ags and antigen-presenting cells (APCs) from the interstitium to the lymph nodes, where they communicate with naive lymphocytes. Until recently, the role of lymphatic endothelium in immune response modulation was underestimated. It was thought that LECs' function was restricted only to the passive transport of immune cells and Ags.[8] Conversely, it was shown that interstitial pressure and fluid flow can activate LECs, leading to increase of fluid and solute permeability, uptake and expression of adhesion molecules required for immune cell migration.[9] Furthermore, current data suggest that LECs suppress dendritic cells (DCs) maturation and further priming of CD8+ T cells, express components of antigen-presenting machinery, major histocompatibility complex (MHC) class I and II molecules, and may be significant contributors to peripheral tolerance.[10] Lately, Swartz group provided data suggesting that LECs can constantly uptake and cross-present exogenous Ags to CD8+ T cells, under normal conditions, implying the contribution of LECs in sustaining of CD8+ T-cell tolerance to exogenous Ags present in the lymph.[8]

The lymphatic system plays an important role not only in physiological but also in pathological conditions. The fluid production reaches up to two-thirds of the total volume of interstitial fluid every day.[11] Hence, any dysregulation of extracellular fluid balance, caused by insufficient lymphatic vessel function, leads to interstitial accumulation of fluid and to lymphedema. Additionally, a high-protein edema fluid triggers an inflammatory reaction, a subsequent fibrosis, an adipose tissue augmentation, an impaired immune response and wound healing.[12] Moreover, during the inflammation, the gene expression profile of LECs is changed and the enhancement of C-C chemokine receptor type 7 (CCR7)-positive DCs migration to lymphatic vessels is mainly induced by increased secretion of a CCL21 chemokine.[13]

The tumor lymphatic vessels development

Recent research has defined a tumor lymphangiogenesis as a multifactorial process occurring due to the interactions between tumor, endothelial and immune cells. These cells are the source of protein factors that lead to LECs proliferation, migration and vessels development. Due to a high-interstitial fluid pressure of most solid tumors, the lymphatic vessels in the tumor mass are collapsed and have a limited functionality.[9] Thus, they are unable to transport cancer cells to distant organs and their role has remained unclear, whereas lymphatics at the tumor margin (peritumoral) facilitate the spread of cancer cells (Fig. 1A).[14]

Tumor cells and tumor-infiltrating myeloid cells stimulate direct formation of peritumoral lymphatic vessels via secretion of IL-10, VEGF, TGF-β and PGE2 (Fig. 1B).[15] However, the central role in the embryonic and postnatal lymphatic formation is played by VEGFR-3, that is phosphorylated through the interaction with secreted proteins: VEGF-C and VEGF-D. Nevertheless, the lymphangiogenesis can be additionally initiated by the activation of multiple types of receptor tyrosine kinases such as VEGFR-2, insulin-like growth factor receptor, fibroblast growth factor receptor 3 and angiopoietin receptors (Tie1 and Tie2). However, in view of the fact that tumor cells as well as tumor-associated cells (fibroblasts, immune cells) can overexpress VEGF-C, the VEGF-C-VEGFR-3 pathway has been so far best described.[16] The overexpression of VEGF genes seems to be related directly to conditions of tumor environment. The physiological environment of even microscopic tumors can be characterized by high-interstitial pressure and hypoxia that promote tumor growth and metastatic dissemination. The correlation between hypoxia-inducible transcription factor (HIF-1) and lymphangiogenesis was extensively studied.[17] HIF-1 was shown to stimulate transcription of important lymphangiogenic factors: platelet derived growth factor B, Prox-1 and SOX18.[18,19]

However, it seems that hypoxia can augment the VEGF-C protein levels also via a HIF-1 independent effect on VEGF-C IRES-dependent initiation of translation. Moreover, this hypoxia-induced switching from cap-dependent to IRES-dependent VEGF-C translation was even higher in tumor cells that had metastasized to the lymph nodes compared to tumor cells
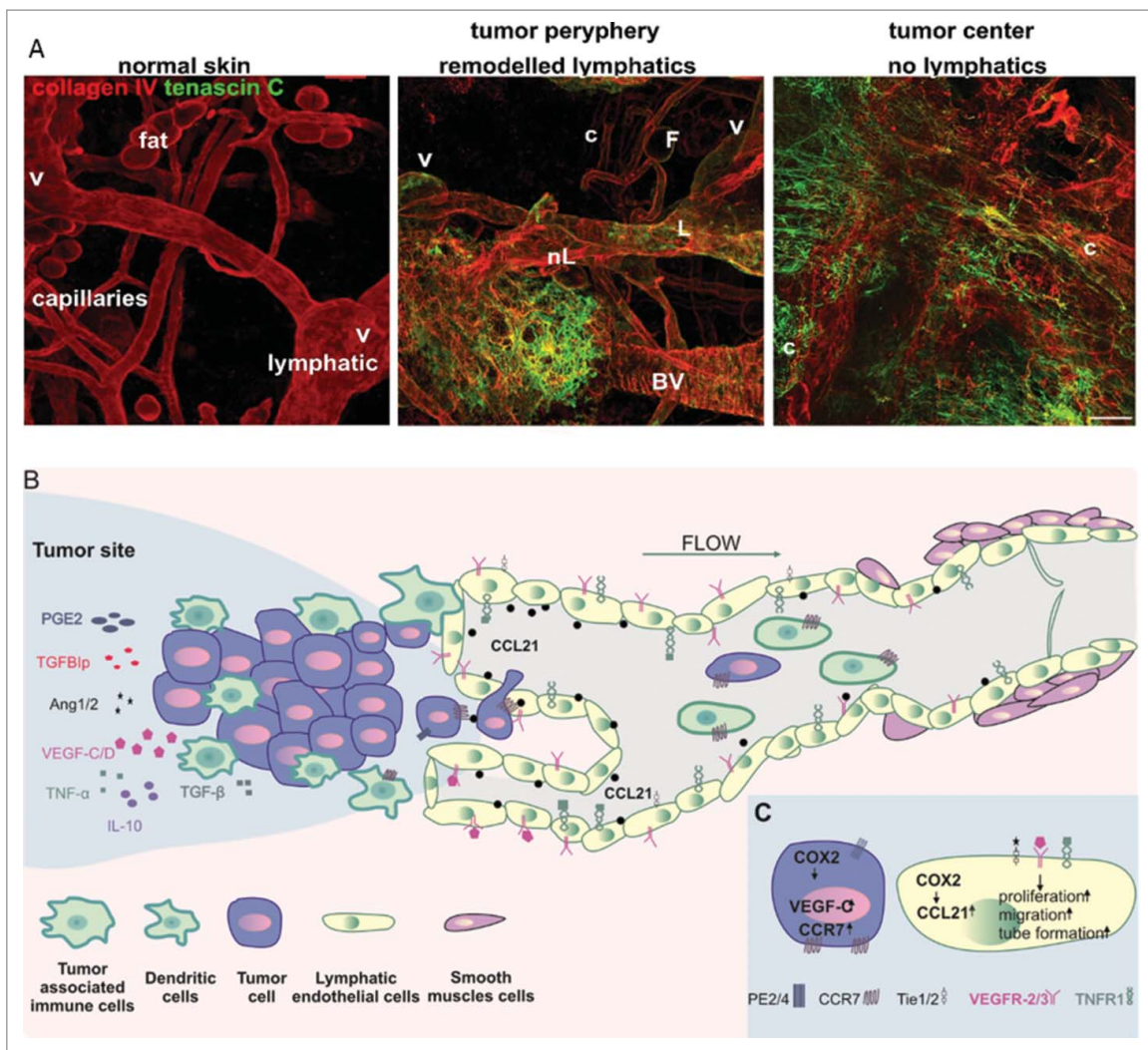
Figure 1. (A) The lymphatic vessel in normal and mouse melanoma tumor tissue. Left. In normal skin, basement membrane-supported lymphatic vessels (L) can be morphologically distinguished after identification of intravascular valves (V) from blood capillaries (c) and adipocytes (F). Normal tissue is also free from tenascin C, extracellular matrix characteristic for tumor remodeled tissue. Middle. At the tumor margin, new extracellular matrix is deposited and pre-existing lymphatic (L) and blood vessels (BV) are remodeled. Also, new, poorly organized vessels are formed (nL). Right. In the center of the tumor, deposition of new matrix paralleled the loss of organized tissue architecture with large tortuous granulation tissue-like blood (c) vessels and a collapse of lymphatics. (B) The overview of LECs, tumor and tumor-associated immune cells' interactions during tumor lymphangiogenesis and lymphatic vessels enlargement. Secretion of a variety of cytokines and growth factors mobilize tumor cells as well as dendritic cells to get inside the initial lymphatic vessels. (C) COX-2 increases level of prostaglandin receptor (EP2) and enhances expression of VEGF-C, CCR7 as well as CCL21. Binding of VEGFR-3, Tie1/2 and TNFR1 ligands induces LECs proliferation and capability of tube formation.

that were present in the primary tumor.[20] These results are in line with the pre-clinical studies on melanoma xenografts showing that lymph nodes metastases correlate with the size of hypoxic tissue fraction in the center of tumor as well as microvascular density in the tumor periphery.[21] Interestingly, VEGFR-3 and VEGF-C expressed by metastatic tumor cells, acts as autocrine stimulation mechanisms that may induce tumor cells proliferation and invasiveness.[22] Therefore, much focus has been placed lately on VEGF-C implication in the lymphangiogenesis and tumorigenesis. In addition, there are data indicating the role of pro-inflammatory TNF-α-TNFR1 signaling pathway in the VEGF-C-induced lymphangiogenesis. TNF-α stimulates LECs migration and morphological changes in a VEGFR-3-independent manner. The downregulation of TNF-α expression in ovarian tumors restrains lymphangiogenesis and lymphatic metastasis. However, the blocking of VEGFR-3 still suppresses TNF-α-induced lymphatic metastasis.[23] Another, newly described secreted protein, involved in the

process of tumor lymphangiogenesis is transforming growth factor-β-induced protein (TGFBIp). Tumors expressing TGFBIp develop more metastases via induction of LECs migration and tube formation as well as increase of lymphatic vessels permeability.[24] Interestingly, the TGFBIp-induced effect on tumor dissemination can be abolished by lithium treatment, that suppress the metastatic potential of colon cancer without affecting the growth rate of tumor cells.[25]

The tumor-generated unique conditions of draining lymph nodes, promoting metastases

It has been evident that cancer cells coordinate the pre-metastatic niche formation through the secretion of a variety of cytokines, enzymes and growth factors [26] Pre-treatment of animals with tumor-conditioned media (TCM) not only increases lymphangiogenesis in draining lymph nodes and peripheral areas of tumors but also accelerates metastasis of MDA-MB-231 and

SUM-149 in mammary fat pad xenografts models.[27] Moreover, it is suggested that in lymph nodes lymphangiogenesis occurs even before the arrival of tumor cells and leads to the formation of the pre-metastatic niche. Interestingly, tumor cells may influence the gene expression profile of LECs. In rat model of gastric cancer, the significant differences between expression profile of LECs, from control and metastatic tumor, were detected in over 800 of genes.[28] In LECs from pre-metastatic organs, treated with TCM, expression of CCL5 and VEGF-induced tumor cell recruitment and colonization, is increased. Additionally, these changes in LECs expression profile were observed as a result of tumor-cell-derived-IL-6 action through the phosphorylation of STAT3.[29] Although, the role of IL-6 in cancer development has been still controversial, multiple studies have shown that elevated level of IL-6 correlates with a poor clinical prognosis.[30] Furthermore, high-tumor expression of IL-6 in human mammary tumors results in mobilization of tumor-associated suppressive myeloid cells and accelerates formation of metastases.[31,32] Importantly, induction of IL-6 expression in non-metastatic mouse mammary tumor EMT6 cell line causes recruitment of MDSCs and tumor cell spreads, comparable to that triggered by metastasizing 4T1 cancer cells.[33]

The VEGF-C-stimulated tumor lymphangiogenesis establishes and increases the efficacy of communication between tumor and lymph nodes. Moreover, initial studies suggest that VEGF-C together with enhanced lymph flow of tumor Ags may promote a suppressive function of LECs, leading to direct inhibition of activated CD8$^+$ T cells and induction of immune tolerance to tumor Ags.[3] Although further studies are needed to confirm these data, all these observations can indicate that tumor cells as well as tumor-associated cells influence LECs, which next may actively participate in formation of metastases.

Lymphatic vessels' role in antitumor immune response and metastases formation

Recent research has highlighted that lymphatic vessels can actively contribute to the disease progression through management of leukocyte accumulation and retention within inflamed tissue. Moreover, it seems that lymphatic vessels actively participate in tumor spread as well as induction of immune tolerance. Although, secretion of VEGF-C and VEGF-D by tumor has been correlated with lymphatic metastases both in humans and in animal models, the mechanism of tumor cell escape into lymphatic vessels has still remained unclear.[34] Thus, a great deal of interest has been focused on the elucidation of mechanism of tumor cells interaction with LECs (Table 1).

CCR7 was one of the first described molecules, overexpressed by tumor cells that increases their lymphatic-dependent metastatic potential. It is suggested that CCR7 is upregulated in many cancer cells and guide them up lymphatic-secreted CCL21 gradients.[35] In breast cancer, the expression of CCR7 was reported to be increased by cyclooxygenase-2 (COX-2) activity via prostaglandin E2 and E4 receptors (Fig. 1C).[36] High expression of this enzyme was correlated with lymph node metastasis, poor prognosis and short survival.[37] The anti-CCR7 antibody as well as knockdown of CCR7 in COX-2-overexpressing MDAMB-231 cells significantly attenuates the effect of migration and invasion of breast cancer cells.[36] Additionally, COX-2 enhances secretion of VEGF-C, that in turn increases expression of CCR7 ligand, and CCL21 by LECs as well as development of new lymphatic vessels.[38] Tumor cells also secrete CCR7 ligands to generate autologous gradients of CCL19/21 chemokines that promote their migration toward functional lymphatics.[39] The selective presentation of CCR7 ligands on LECs can be explained by the expression of non-typical chemokine receptor D6. Due to the expression of D6, only cells that are CCR7 positive (e.g., mature DCs) are able to migrate to lymph nodes.[40] However, D6 expressed by tumor cells, was shown to inhibit the proliferation of tumor cells and was negatively correlated with prognosis in breast cancer.[41]

Another chemokine receptor involved in tumor cells spread through lymphatic vessels is CCR4. CXCL12 through CCR4 leads to actin polymerization and pseudopodia formation of breast tumor cells and have a significant influence on metastasis to regional lymph nodes and lungs.[42]

Table 1. Molecules potentially involved in tumor cells trafficking through the lymphatic vessels.

Molecule involved in cell migration	Role in leucocytes trafficking by lymphatic vessels	Expression on tumor cells	Influence on tumor progression
CCR7	Upregulated CCR7 during activation and maturation of DCs to respond to lymphatic-secreted CCL21 and elicits directional migration[62]	Melanoma, colorectal, mammary, gastric, non-small cell lung, head and neck cancers, thyroid and squama cell carcinomas[42,63]	Expression is correlated with lymph node metastasis.
CCR4	Selectively expressed on Th2 cells and regulatory T cells	Breast, lung, gastric cancer [64]	Expression is associated with lung and lymph nodes metastasis. In breast cancer, correlated with HER2 positive tumors and poor prognosis
ICAM-1	ICAM-1 knockout mice have defects in lymph node recruitment of DCs[43]	Melanoma, breast cancer, gastric cancer, esophageal cancer, colorectal carcinoma[65]	The results are controversial. In some cases ICAM-1 plays a major role in invasion of cancerous cells while in others decreased expression inhibits formation of metastases[66]
COX-2	COX-2 plays an important role in leucocytes migration and adhesion, likely by modulating p110g PI3K–mediated cell signaling[67]	Breast cancer, esophageal cancer pancreatic cancer, various colorectal tumors, adenocarcinoma, prostate and bladder cancers[68]	Upregulates CCR7 via EP2/EP4 receptor signaling pathways and enhances lymphatic metastasis
CD99	Involved in transmigration of monocytes, neutrophils, lymphocytes and DCs	Ewing sarcoma, synovial sarcoma and low-grade fibromyxoid sarcoma[69]	Knocking down CD99 in Ewing sarcoma reduces tumor ability to form metastases

Table 2. Overview of lymphangiogenesis inhibitors investigated in pre-clinical studies.

Drug name	Molecular target	Treatment	Mechanism of action	Outcome
Soluble VEGFR-3[70]	VEGF-C	Pre-clinical studies in endometrial cancer model	Inhibits lymphatic endothelial cell growth *in vitro*	Suppresses *in vivo* lymph node and lung metastasis
Canstatin[71]	Ang1/Ang2	Pre-clinical studies in colon carcinoma model	Reduces tumor blood and lymphatic vessel densities	Reduces final volume and weight of tumors
Endostatin[72]	VEGFR-3	Pre-clinical	Causes inhibition of bFGF-induced corneal angiogenesis and lymphangiogenesis	—
16K hPRL[73]	VEGFR-3	Pre-clinical studies in melanoma model	Induces apoptosis and inhibits proliferation, migration and tube formation of human dermal lymphatic microvascular endothelial cells	Prevents lymphatic metastasis
SAR131675[74]	VEGFR-3	Pre-clinical studies in breast cancer model	Reduces TAM infiltration	Reduces lymph node and lung metastasis
cVE-199[75]	VEGF-D	Pre-clinical studies in neuroblastoma model	Inhibits lymphangiogenesis	Prevents lymphatic metastasis of neuroblastoma
Nrp2[76]	Semaphorin	Pre-clinical studies in breast cancer model	Inhibits VEGF-C-induced phosphorylation of VEGFR-3, ERK1/2, and AKT	Tumor cells expressing sema3C contained a lower concentration of lymph vessels and form lymph nodes metastasis much less effectively
Biomimetic peptide SP2012[53]	c-MET	Pre-clinical studies in breast cancer model	Inhibits blood and lymphatic endothelial cell viability, migration, adhesion and tube formation	Inhibits lymphangiogenesis in primary tumors
Rapamycin[77]	mTOR	Pre-clinical studies in head and neck cancer model	Inhibits lymphangiogenesis	Prevents dissemination to the cervical lymph nodes

Although the chemokine-dependent migration mechanism of tumor cells appears to be similar to those used by DCs, this similarity to DCs cannot be established as a rule for other molecules. For example ICAM-1 promotes the exit of leukocytes from tissue to lymphatics.[43] However, in case of tumor, the role of adhesion molecules in metastatic dissemination has not been yet fully understood. In melanoma, the high expression of ICAM-1 correlates with the risk of metastasis, while breast cancer patients with CAMs-positive tumors have a better prognosis.[44] Nevertheless, it is probable that some molecules like CD99 may be involved in migration of leucocytes as well as tumor cells. CD99 has been described as a transmigration mediator of monocytes, neutrophils, lymphocytes and DCs.[45] The results with anti-CD99 antibodies suggest a homophilic interaction between CD99 on the leukocytes and CD99 on the endothelial cells.[46] Interestingly, the truncated isoform of CD99 enhances migration and metastasis ability of cancer cells, while the full length of CD99 acts as an onco-suppressor in a wider group of tumors.[47] However, the direct mechanism of tumor cells and LECs interaction mediated by CD99 has not been yet investigated.

The anti-lymphangiogenic therapies

Monoclonal antibodies and recombinant proteins

Angiogenesis and lymphangiogenesis play a pivotal role in tumor cells growth and dissemination, therefore, factors involved in these processes are potential targets in antitumor therapies. Since VEGF-C/VEGF-D-VEGFR-3 signaling axis is the most prominent in lymphangiogenesis, neutralization of this pathway in metastatic disease is provided with either anti-VEGF or anti-VEGFR antibodies or soluble form of VEGF receptor—sVEGFR-3. Interestingly, VEGFR-3 and VEGFR-2 together promote proliferation and migration of LECs, therefore simultaneously blocking both of the receptors seems to be more effective in inhibition of peritumoral lymphangiogenesis

than single anti-VEGFR treatment (Table 2). Bevacizumab, an anti-VEGF antibody, is applied in the treatment of colon, non-small cell lung, kidney and ovarian cancer. Moreover, there are some ongoing clinical trials using bevacizumab in combination with different drugs, such as dexamethasone or VGX-100. VGX-100 is an anti-VEGF-C antibody, which is currently in phase I of clinical trials.[48] However, in the mentioned combined therapies, VEGF-D is still active and may induce lymphatic vessels formation, therefore these therapies only partially block peritumoral lymphangiogenesis. Another agent with promising effect in pre-clinical studies is AMG-386 (Trebananib) an Angiopoietin-1/2-neutralizing peptibody, consisting of Ang-binding sequence fused with Fc region of antibody. It prevents Ang1/2 binding to Tie-2 receptor, therefore inhibits the growth of tumor in mouse xenograft models and currently is in phase III of clinical trials in ovarian cancer treatment.[49] Moreover, AMG-386 is under current investigation in glioblastoma therapy combined with bevacizumab, however this study has not been completed yet.[50] There are some interesting results indicating that sVEGFR-3 poses soluble extracellular ligand-binding domain, able to trap VEGF-C and leads to its inactivation, what results in inhibition of lymphangiogenesis. Also, ramucirumab, the fully human monoclonal antibody that binds to VEGFR-2 inhibits not only angiogenesis but may affect lymphangiogenesis. [51]

Chemotherapy

Another group of antitumor lymphangiogenesis drugs are small molecule receptor tyrosine kinase inhibitors targeting VEGFR-3 including regorafenib used in the treatment of metastatic colorectal cancer and gastrointestinal stromal tumors and axitinib applied in renal cell carcinoma therapy. A promising choice for anti-lymphangiogenic therapy is lenalidomide (LEN). This immunomodulatory agent currently is used in the treatment of multiple myeloma, transfusion-dependent

Table 3. Overview of lymphangiogenesis inhibitors investigated in clinical studies.

Drug name	Molecular target	As a monotherapy	As a combined therapy
VGX-100	VEGF-C	—	With bevacizumab – Phase I ongoing (NCT01514123) in treatment of advanced solid tumors
Lenalidomide	VEGF-C	Lenalidomide is used in pre-clinical studies to inhibit growth of peritumoral lymphatic vessels	
Bevacizumab	VEGF	Bevacizumab is an inhibitor of angiogenesis studied in various pre-clinical trials as anti-lymphangiogenic drug. Approved for various treatment: breast, lung, colorectal, renal and brain cancer	
AMG-386 (Trebananib)	Ang1/Ang2	Treatment of endometrial adenocarcinoma – Phase II ongoing (NCT01210222).	Used in the treatment of renal cell carcinoma with sorafenib – Phase II completed (NCT00467025). No data have been published so far
		Treatment of advanced solid tumors – Phase I completed (NCT00102830). No data have been published so far	Used in the treatment of renal cell carcinoma with sunitinib – Phase II ongoing (NCT00853372)
MEDI3617	Ang1/Ang2	—	Used in treatment of melanoma with tremelimumab – Phase I ongoing (NCT02141542).
			Used in the treatment of advanced solid tumors with bevacizumab/paclitaxel/carboplatin/gemcitabine – Phase I completed (NCT01248949). No data have been published so far
CVX-060	Ang1/Ang2	Treatment of advanced solid tumors – Phase I completed (NCT00879684). 0.3, 1, 3, 6, 12, 15 mg/kg of b.w. intravenous infusion in Stage 1 and 15 mg/kg of b.w. intravenous infusion in Stage 2, administered once-weekly in a 4-week cycle	—
Sorafenib	VEGFR-3	Sorafenib inhibits VEGFR-2 and VEGFR-3, blocks proliferation of different tumor cells and inhibits tumor lymphangiogenesis.[78] Was approved for renal cell carcinoma and hepatocellular carcinoma treatment.	
Sunitinib	VEGFR-3	In pre-clinical studies, sunitinib blocked VEGFR-2 and VEGFR-3 phosphorylation induced by VEGF-C or VEGF-D and inhibited LECs proliferation and migration.[79] Was clinically approved for renal cell carcinoma and gastrointestinal stromal tumor treatment	
Axitinib	VEGFR-3	In pre-clinical studies used as VEGFR-3 inhibitor.[55] Approved for renal cell carcinoma treatment	
Regorafenib	VEGFR-3	Regorafenib is used in pre-clinical studies to inhibit VEGFR-2 and VEGFR-3 autophosphorylation, VEGFR-3 intracellular signaling and to block LECs migration.[80]	
Pazopanib	VEGFR-2/ VEGFR-3	Pazopanib exert anti-angiogenic and anti-lymphangiogenic potential in pre-clinical studies as VEGFR-2 and VEGFR-3 inhibitor.[55] In clinical studies is use in combination with bevacizumab. Approved for renal cell carcinoma treatment	
IMC-3C5	VEGFR-3	Treatment of neoplasma – Phase I completed (NCT01288989). No data have been published so far	—

myelodysplastic syndrome and mantle cell lymphoma. In LECs, LEN is shown to reduce levels of PROX-1 factor, podoplanin and VEGFR-3. Several studies indicate that LEN affects not only LECs but also tumor-associated macrophages (TAMs), which are primarily responsible for the secretion of VEGF-C. Additionally, LEN triggers various effects on the immune system, which may contribute to its therapeutic outcome. It stimulates CD4$^+$ and CD8$^+$ T lymphocytes and also increases the expression of IL-2 and IFNγ.[52]

Novel anti-lymphatic agent, collagen IV biomimetic peptide (SP2012), inhibits metastases to lungs in breast cancer tumor xenograft model and leads to LECs apoptosis.[53] Moreover, other well-known kinase inhibitors including sorafenib, sunitinib and pazopanib are already approved for the treatment of various cancer by Food and Drug Administration (Table 3). These drugs, well known for their anti-angiogegenic action, also prevent phosphorylation of VEGFR-3, leading to lymphangiogenesis inhibition.[54,55]

All these experimental and clinical studies highlighted the critical role of lymphatic vasculature in tumor metastatic spreading and point them as antitumor therapies target. Although lymphatic vessels are an important element of human immune system function, little is known so far about the impact of lymphatic destruction on immune response to cancer cells.

Photodynamic therapy as a new anti-lymphatic approach

Recently described approach used to damage lymphatic vessels is PDT. PDT is a light-based therapeutic modality approved for the treatment of various solid tumors as well as non-oncological conditions such as age macular degeneration (AMD). In the clinical settings, PDT procedure requires administration of a photosensitizing drug, that selectively accumulates in the tumor tissue, and irradiation of the lesion with a visible light of an appropriate wavelength.[56] Light-excited photosensitizer transfers its energy to the molecular oxygen, leading to formation of reactive oxygen species. Antitumor effects of PDT result from direct tumor damage, collapse of tumor vasculature and induction of antitumor immune response.[57] Antivascular effect of PDT has been extensively studied during the past decades, whereas anti-lymphatic action of PDT is a recently described phenomenon. While pre-existing lymphatic vessels cannot be eradicated with anti-lymphangiogenic agents, PDT can be applied for the destruction of even pre-existing tumor lymphatic vessels. Tammela et al. have been the first to publish that verteporfin-PDT can damage tumor-associated lymphatic vessels in mice and pigs. Moreover, PDT of tumor lymphatic vasculature led to the eradication of intra-lymphatic tumor cells and prevented metastasis of mouse melanoma cells and subsequent recurrence. Interestingly, Tammela et al. also have shown that combination treatment of PDT and AdVEGFR-3-Ig reduces the surface area of peritumoral lymphatic capillaries when compared with single treatment.[58] Furthermore, as PDT is an established procedure in various ophthalmological diseases such as AMD, Bucher et al. have used PDT to induce regression of corneal lymphatic vessels without affecting blood vessels in mouse model.

In our previous study, we have identified the optimal conditions to selectively close lymphatic collecting vessels without

injuring the blood vasculature in a mouse ear dermis.[59] We have shown that PDT selectively ablates lymphatic vessels and subsequently leads to closure of lymphatic drainage in a particular region. According to our recent results, both apoptosis and autophagy are involved in cell death induced by verteporfin-PDT in LECs.[60] In addition to Tammela studies presenting that PDT-damaged lymphatic vessels regrowth after stimulation with VEGF-C, we have shown that lymphatic vessels eventually regenerate, without any additional treatment, by recanalization of blocked collectors leading to restoration of lymphatic drainage.[58,59] Therefore, in order to avoid tumor metastasis and further relapse, treatment combining PDT with anti-lymphangiogenic agents seems to be rational. Such combination therapy would be a promising approach as, according to current literature, there are several known pharmacological inhibitors of lymphangiogenesis as well as it is possible to obtain recombinant proteins able to suppress formation of lymphatic vasculature.

However, we would like to emphasize an undiscussed problem concerning anti-lymphangiogenic therapies' influence on development of specific antitumor immune response. Since Burnet and Thomas extended "immune surveillance hypothesis," suggesting that cancer cells are recognized as "foreign" by the immune system, various immune therapies have been extensively studied. Therefore, new effective therapies, stimulating immune system and overcoming tumor suppression, become available including, exciting recent developments, immune checkpoint blockade.[61] PDT is also thought to be an immune therapy as it can induce strong local inflammatory reaction that under some unique circumstances and in combination with immune-modulating drugs can lead to the development of systemic antitumor immune response and the formation of memory responses.[57] A prolonged damage of tumor lymphatic vasculature may totally abrogate immune cell trafficking from the tumor to the draining lymph nodes and can subsequently lead to impaired development of antitumor immune response (unpublished results). Thus, we speculate that, even though anti-lymphangiogenic therapies can overcome tumor tolerance and prevent metastasis, it may affect antitumor immune response leading to poor PDT outcome.

Conclusions

In the light of the latest findings, the lymphatic vessels are not only passive channels but also can participate in the development of antitumor immune response. It is becoming clear that the process of cells' entry into the lymphatic vessels requires active and highly complex interactions with LECs. However, in contrast to blood vessels the mechanisms of tumor cell intravasation has not been well described. Hence, it is not known what type of junctions are preferred between tumor cell interactions and whether the initial or/and collecting vessels are the gates for metastatic cells entry. Therefore, to better understand the molecular control of immune and tumor cells interaction with LECs, further research is warranted.

The latest discoveries have demonstrated the potential of novel therapeutic strategies to prevent formation of tumor metastases. As we discussed here, the VEGF-C-stimulated tumor lymphangiogenesis may have important bearing on

formation of pre-metastatic niche in draining lymph nodes, and promotes progression and dissemination of tumor cells. Nevertheless, the lymphatic vessels' role in the process of antigen presentation and antitumor adaptive immune response development should not be diminished, especially in the light of recently described effective immunotherapies. Thus, the currently generated inhibitors of VEGF-C-VEGFR-3 pathway are probably just the beginning of therapies modulating lymphatic function and hopefully the nearest future will bring great advances in this field.

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