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Biomarkers of Lymphatic Function and Disease

State of the Art and Future Directions

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Abstract

Substantial advances have accrued over the last decade in the identification of the processes that contribute to lymphatic vascular development in health and disease. Identification of distinct regulatory milestones, from a variety of genetic models, has led to a stepwise chronology of lymphatic development. Several molecular species have been identified as important tissue biomarkers of lymphatic development and function. At present, vascular endothelial growth-factor receptor (VEGFR)-3/VEGF-C/VEGF-D signaling has proven useful in the identification of clinical lymphatic metastatic potential and the assessment of cancer prognosis. Similar biomarkers, to be utilized as surrogates for the assessment of inherited and acquired diseases of the lymphatic circulation, are actively sought, and will represent a signal advance in biomedical investigation.

1. The Lymphatic System

As an adjunct to the closed blood circulatory system, the lymphatic vasculature maintains fluid and colloid homeostasis through an extensive network of open-ended vessels distributed throughout the body. Circulatory dynamics require that fluid and macromolecules exit the blood capillary lumen into the interstitial space; a substantial fraction of this extravasated extracellular fluid must return to the central circulation as the protein-rich fluid known as lymph. Driven by pressure gradients that emanate from the concerted action of musculoskeletal movement, respiration, smooth muscle contraction, and one-way valves, lymph is drawn from interstitial tissue spaces into an increasingly larger hierarchy of lymphatic vessels, ultimately reaching the venous circulation by way of the thoracic duct. Both anatomically and functionally, the lymphatic system participates in important physiological functions, including immune surveillance, and the absorption and transport of dietary fats. Moreover, the lymphatic vasculature constitutes the conduit system in which circulating dendritic cells and lymphocytes traverse the body, monitoring tissues for micro-

bial intrusion and forming the sites of antigen presentation and recognition in sentinel and regional lymph nodes, thereby comprising the infrastructure of acquired immunity.[1,2]

1.1 Lymphatic Anatomy

Lymphatic capillaries arborize as superficial plexuses lined with a single layer of unfenestrated lymphatic endothelial cells (LECs). Unlike blood vessels, LECs lack a continuous basement membrane, allowing lymph to follow osmotic and colloid gradients at overlapping cell junctions.[3] Fibrillin-containing filaments anchor the lymphatic capillaries to the interstitium, thereby facilitating the opening of LEC junctions at times of increased interstitial pressure.[4] Fluid, macromolecules, and cells passively flow into the capillary lumen and travel to collecting lymph vessels deeper within the dermal layers. These larger lymphatics actively propel lymph through the contraction of their vascular smooth muscle, aided by the presence of intraluminal unidirectional valves. These collecting lymphatics coalesce to create vessels of increasing caliber, culminating in the formation of the cisterna chyli and thoracic duct.

1.2 Lymphatic Dysfunction

Given the central role of the lymphatic system in circulatory, metabolic, and immune-related homeostasis, it is not surprising that lymphatic vascular dysfunction can manifest within various predictable sequelae, ranging from impaired immune responses[5] and metabolic status[6] to the appearance of a debilitating and disfiguring form of regional swelling known as lymphedema.[7] It is somewhat paradoxical that, historically, the central physiological and pathological importance of the lymphatic system has not stimulated a proportional degree of scientific exploration into the basis of lymphatic health and disease. As a consequence, the diagnosis and treatment of patients with lymphatic diseases has relied chiefly upon empirically derived insights, often with limited success.

More recently, however, scientific investigation of the lymphatic vasculature has benefited specifically from more focused scientific attention to the pathophysiology of cancer metastasis.[8-11] Tissue cultures of blood vascular endothelial cells and lymphatic endothelial cells derived from human skin have been characterized, allowing cell-based *in vitro* studies.[12-15] Moreover, animal models of lymphatic disease, unavailable until quite recently,[16] have been realized for distinct forms of congenital and acquired lymphedema[17-19] [table I]. The availability of suitable disease-specific animal models, along with a growing comprehension of lymphatic biology[10,20,21] and the more recent identification of lymphatic-specific biomarkers,[22,23] ensure the requisite insight and investigative tools to permit in-depth investigation and putative therapies of the broad array of lymphatic diseases.

1.3 Lymphatic Development

Although the lymphatic vasculature has been anatomically recognized for centuries[42] and its embryonic origins proposed more than one hundred years ago,[43] it is only recently that the development of the mammalian lymphatic system has been elucidated with convincing evidence. Identification of distinct regulatory milestones, from a variety of genetic models, has led to a stepwise chronology of lymphatic development. The non-disjunct physiological progression of lymphangiogenesis can be generalized into four distinct stages: (i) lymphatic competence; (ii) lymphatic commitment; (iii) lymphatic specification; and (iv) lymphatic vessel coalescence and maturation. Endothelial cells of embryonic veins are believed to be the source of lymphatic progenitors that develop under the coordinated influence of lymphatic-specific signals.[44] All endothelial cells of the cardinal vein possess the ability or competence to develop along the lymphatic-fate continuum, but the passive existence of the hemovasculature alone is not sufficient to induce lymphangiogenesis,[44] revealing a distinctive developmental divergence governing the lymphatic and blood vasculature lineages. Comparative microarray analyses of adult blood vascular endothelial cell (BEC)- and LEC-restricted transcriptomes have revealed that ≈98% of all genes identified are expressed at statistically indistinguishable levels within the two cell types.[15,45] Differences, however, do exist and these features have been recently reviewed.[21,24]

1.3.1 Competence

LEC competence represents the first step in the process of lymphatic commitment, within which all endothelial cells of the embryonic cardinal vein display an independent ability to respond to the (as yet, unidentified) initiating and instructive factor(s) of lymphatic development (figure 1). This process is initiated by mouse embryonic day 9–9.5.[44] The priming of LECs to initiate lymphatic development is likely dependent on a form of molecular signaling that is distinct from that found in blood vascular development, given the secondary but independent appearance of lymphatic vessels after blood vascular vasculogenesis is initiated. At present, LEC competence is defined by the surrogate expression of lymphatic vessel endothelial hyaluronan receptor-1 (LYVE1) and vascular endothelial growth factor receptor-3 (VEGFR3; also known as Flt-4).[24] Nevertheless, lymphatic competence is a dy-

Table I. Genetic mouse models of lymphatic disease (reproduced from Cueni and Detmar,[24] with permission from Macmillan Publishers Ltd)

Gene	Function	Model	Disease feature
ANG1[25]	Growth factor	Transgenic	Lymphatic hyperplasia
ANG2[26]	Growth factor	Knockout	Chylous ascites, abnormal lymphatic patterning, edema
EphB2[27]	Receptor ligand	Mutant	Defective lymphatic remodeling, lymphatic hyperplasia, chylothorax
FOXC2[28,29]	Transcription factor	Knockout	Lymphatic hyperplasia and dysfunction, abnormal pericytes, agenesis of valves
HGF[30]	Growth factor	Transgenic	Lymphatic growth and enlargement
ITGA9[31]	Adhesion receptor		Chylothorax, lymphedema
ELK3[32]	Transcription factor	Knockout	Dilated lymphatics, chylothorax
NRP2[33]	Endothelial receptor	Knockout	Defective lymphatic vascular development
PDPN[34]	Membrane glycoprotein	Knockout	Lymphedema, abnormal lymphatic pattern, lymphatic dilatation
PROX1[6,35]	Transcription factor	Knockout	Chylous ascites, absent lymphatics
SOX18[36]	Transcription factor	Spontaneous missense mutations	Edema, chylous accumulation, cardiovascular defects
SYK and *LCP2* (*SLP76*)[37]	Tyrosine kinase/adaptor protein	Knockout	Chylous ascites, abnormal lymphaticovascular connections
VEGFC[38]	Growth factor	Transgenic	Hyperplastic lymphatics
VEGFC[39]		Knockout	Lymphatic hypoplasia, delayed development, lymphedema
VEGFR3 (*Flt-4*)[40]	Endothelial receptor	Knockout	Cardiovascular failure, defective vascular remodeling
VEGFR3 (*Flt-4*)[41]		Inactivating mutation	Lymphedema

ANG1/2 = angiopoietin-1 and -2; **ELK3** = ETS-domain protein; **EphB2** = ephrin-B2; **FOXC2** = forkhead box C2; **HGF** = hepatocyte growth factor; **ITGA9** = integrin α9; **LCP2** = lymphocyte cytosolic protein-2 (SH2 domain-containing leukocyte protein, 76kD [SLP76]); **NRP2** = neuropilin-2; **PDPN** = podoplanin (T1α); **PROX1** = prospero-related homeobox 1; **SLP** = Src homology 2-domain containing leukocyte protein; **SOX18** = sex determining region Y-related high mobility group box 18; **SYK** = spleen tyrosine kinase; **VEGFC** = vascular endothelial growth factor-C, **VEGFR3** = vascular endothelial growth factor receptor-3 (FMS-like tyrosine kinase-4 [Flt-4]).

namic state, encompassing events limited to the venous vascular endothelium and distinct from the biology of arterial endothelium. While the physiological functions of the VEGFR3 receptor and its pro-angiogenic vascular endothelial growth factor (VEGF) family of ligands have benefited from intense investigation, the function of the LYVE1 receptor remains largely enigmatic, even though its identification as a lymphatic-specific cellular marker constituted a seminal advance in the study of lymphatic vascular biology.[46]

LYVE1 was initially cloned as a homolog of CD44.[47,48] CD44 serves as a transmembrane glycoprotein receptor for the extracellular matrix glycosaminoglycan, hyaluronan, but binds other molecules, including osteopontin, collagens, and matrix metalloproteinases that participate in a variety of cellular processes, including lymphocyte migration and activation, hematopoiesis, and tumor metastasis.[49] Although LYVE1 has been shown to be closely associated with the lymphatic vasculature throughout development and maturity, the precise function of the receptor remains unknown, beyond its presumed involvement in the process of hyaluronan metabolism.[46] The primary receptor for hyaluronic acid, CD44 is known to facilitate cell migration by the removal of pericellular matrix surrounding fibroblast and epithelial cells, and the suppression of intercellular adhesion in processes of wound healing, inflammation, and tumor progression.[21,50] Hence, LYVE1, beyond its utility as a lymphatic endothelial marker, may potentially play an active role in both pathologic and physiologic lymphangiogenesis, through its ability to transport hyaluronic acid across the lymphatic vessel wall. The recent characterization of phenotypically normal LYVE1$^{-/-}$ knockout mice, which possess no overt structural or functional lymphatic or immunological abnormality, suggests that LYVE1 does not exclusively control lymphatic development or integrity but, rather, that it exerts an influence of a much more restricted or compensated scope.[20,51]

LYVE1 is expressed early on BECs, during lymphatic competence.[35] Throughout development, and with few exceptions (most notably macrophages and hepatic sinusoidal blood capillaries after

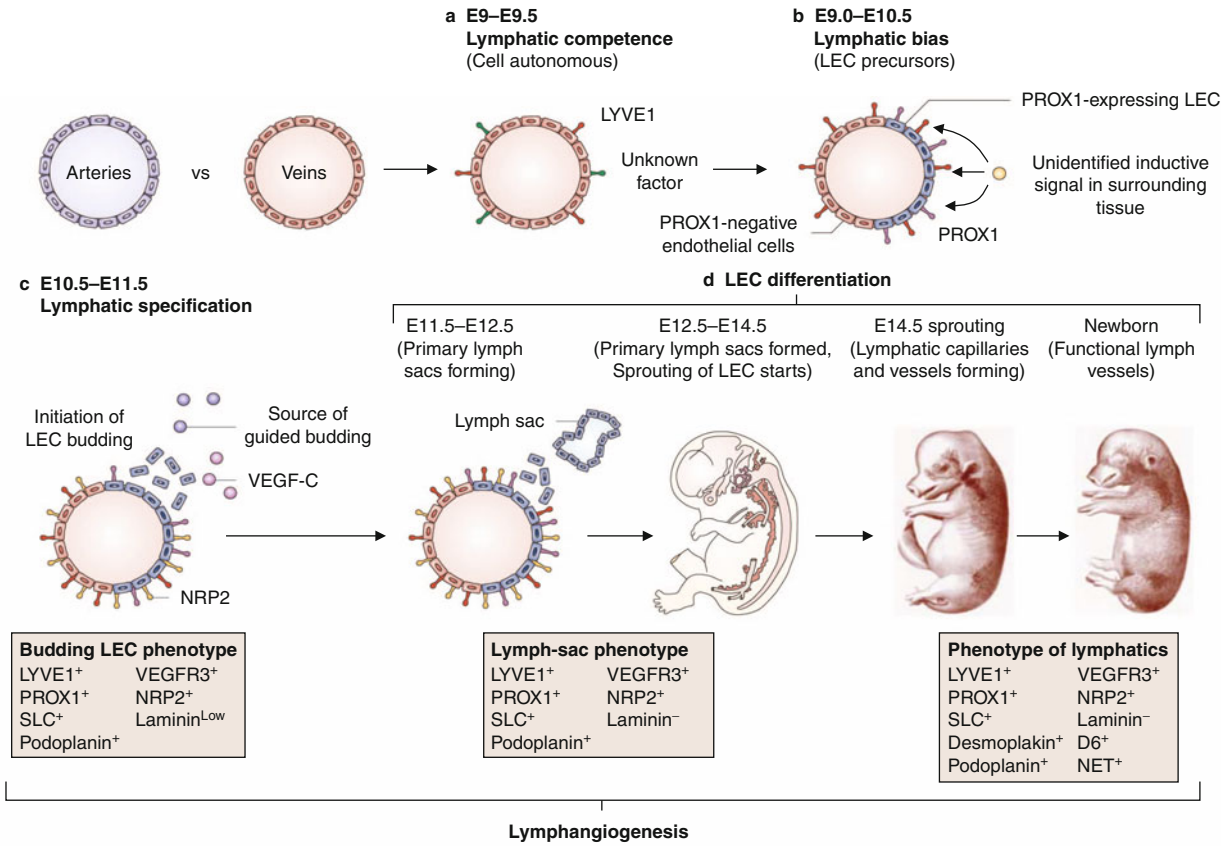

Fig. 1. Model for differentiation of the murine lymphatic vasculature. The figure summarizes a proposed four-step model for the formation of the lymphatic vasculature (reproduced from Oliver,[44] with permission from Macmillan Publishers Ltd). **D6** = B-chemokine receptor D6; **E** = embryonic day; **LEC** = lymphatic endothelial cell; **LYVE1** = lymphatic vessel endothelial hyaluronan receptor 1; **Net** = ETS-domain protein ELK3; **NRP2** = neuropilin 2; **PROX1** = prospero-related homeobox 1; **SLC** = secondary lymphoid chemokine; **VEGF-C** = vascular endothelial growth factor C; **VEGFR3** = vascular endothelial growth-factor receptor 3.

birth), LYVE1 is associated exclusively with vessel and lymph node endothelia to terminal differentiation,[47,52] except for the collecting lymphatic vessels of the mature vasculature where LYVE1 is downregulated.[27] Nearly exclusive localization to lymphatic endothelium, and technical manipulability, confers LYVE1 the ability to serve as a valuable molecular and histochemical marker. Identification of this marker has permitted much more detailed study of the lymphatic vasculature where, previously, discrimination from blood vessels was difficult or impossible.[44,53] Prior methods of lymphatic vascular identification were limited to histological assessment of gross lymphatic-specific features, such as their endoluminal valves and lack of basement membrane, clearly limited by imprecision and lack of resolution.

Prior to the identification of the LYVE1, substantial investigative effort was centered on the VEGFR3 receptor, given the functional importance of the VEGF family of ligands and the relevant receptor expressed on the lymphatic endothelium.

VEGF is a key regulator of the endothelial cell functions required for vasculogenesis and for physiological and pathological angiogenesis.[54,55] The VEGF family includes seven members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and placental growth factor (PlGF), and three receptors, VEGFR1 (also known as Fms-like tyrosine kinase, Flt-1), VEGFR2 (also known as fetal liver kinase, Flk-1), and VEGFR3 (Flt-4).[56] Of these, VEGF-C and VEGF-D and their cognate receptor, VEGFR3, represent the first and best studied of the lymphatic-specific signaling mechanisms.[57-62]

In contrast to LYVE1, VEGFR3 is broadly expressed on both early blood and lymphatic vessel endothelium, and plays a critical role in the development of both lineages of vasculature.[63] Targeted gene inactivation of VEGFR3 results in early cardiovascular collapse, demonstrating the development of the hemovasculature before the lymphatic system and, hence, precludes the ability to study the resulting dysfunction of lymphatic vessels.[40] In time, VEGFR3 expression becomes limited to LECs as expres-

sion is downregulated in BECs and, by the arrival of vascular maturity, VEGFR3 is largely restricted to the lymphatic endothelium,[57,64] further supporting the venous origin of the lymphatic system. However, VEGFR3 expression has also been detected on proliferating blood capillaries associated with tumor neovascularization or within wound granulation tissue;[60,65] thus, VEGFR3 alone may not possess sufficient specificity to serve as a lymphatic vascular marker, particularly under nonhomeostatic conditions. The identified ligands for VEGFR3, VEGF-C, and VEGF-D, have been characterized as pro-lymphangiogenic factors in a number of models of varying developmental and postnatal stages,[38,56,58,66-68] and with respect to both therapeutic potential[18,69,70] and cancer metastasis.[71] In conjunction with VEGF-C and VEGF-D, the angiopoietin signaling system participates in the formation of both blood and lymphatic vessels. Although considered antagonists with respect to angiogenesis,[72,73] angiopoietin 1 (ANG1) and angiopoietin 2 (ANG2) cooperatively induce lymphangiogenesis as an agonist of the endothelial-specific receptor tyrosine kinase TIE2.[26,73-75] Such signaling is responsible for lymphatic remodeling following differentiation, forming functionally mature lymphatics through lymphangiogenesis. Podoplanin (PDPN), also known as T1α, is an integral glycoprotein found in the plasma membrane of podocytes and co-localizes with VEGFR3 in early lymphatic structures by mouse embryonic day 11 in a number of tissues.[76] Unlike VEGFR3, however, T1α/podoplanin is not expressed either on adult hemovascular endothelium or tumor vasculature.[77]

1.3.2 Commitment

Lymphatic commitment is marked developmentally and functionally by the expression of prospero-related homeobox 1 (PROX1), a nuclear transcription factor that, unlike LYVE1 and VEGFR3, is expressed exclusively in cells of committed lymphatic lineage.[35] The PROX1-positive subpopulation of venous endothelial cells wholly constitutes the progenitors of the mature lymphatic system, arrayed in a polarized fashion along the cardinal vein.[64] The mechanism of this differential and ordered expression remains unknown; nevertheless, PROX1 is clearly necessary and sufficient for lymphatic commitment. The molecular milieu in which PROX1 operates is also not known; both the downstream initiating and regulatory factors and the other upstream requisite or supplemental events have yet to be identified. PROX1$^{-/-}$ knockout mice proceed to bud LECs from the cardinal vein but fail to commit to the lymphatic lineage and, subsequently, fail to express later lymphatic-specific markers including LYVE1, instead retaining blood vascular markers.[64] PROX1 thus shifts commitment of venous endothelial cells from a default blood vascular fate to that of lymphatic lineage.[53,64,78] Consistent with these impressions in mice, overexpression of PROX1 in tissue culture induces expression of lymphatic-specific genes. Hemovascular-specific genes in venous endothelial cells are reciprocally suppressed, while the expression of the lymphatic endothelial cell markers, podoplanin and VEGFR3, is upregulated.[79]

1.3.3 Specification and Maturation

A committed LEC eventually achieves complete autonomy from the local microenvironment of the cardinal vein and migrates peripherally during the lymphatic specification stage, even if adoptively isolated in culture.[13] As the LEC attains a higher level of differentiation, additional lymphatic-specific markers are expressed, while those that reflect putative blood vascular lineage are increasingly suppressed.[64] Developing LECs bud from the parental cardinal vein on mouse embryonic day 10.5, independently of further PROX1 influence, as the first morphologically concerted event of lymphangiogenesis.[53] Other factors, such as neuropilin-2 (NRP2), a mediator in the VEGFR-VEGF signaling pathways,[80,81] and T1α/podoplanin, a cell-surface glycoprotein,[34,77] are expressed by LECs as budding and migration seed the periphery throughout the embryo for the formation of nascent lymphatic structures known as primary lymphatic sacs. From these blind-ended constructs of specified LECs, secondary budding and migration characterize the final stage of lymphatic development. As these near terminally differentiated cells bud, they form capillaries in a centrifugal fashion to form lymphatic vasculature around tissues and organs.[24]

Maturation of LECs into differentiated lymphatic vessels establishes the foundation of the lymphatic vasculature by mouse embryonic day 14.5,[82] which continues to organize until the first few days of postnatal life.[24] Additional lymphatic markers, such as desmoplakin and β-chemokine receptor D6, continue to be expressed in this late stage of terminal differentiation, and are thought to be among the last markers expressed. The mature lymphatic network shortly after birth expresses the complete profile of lymphatic markers found in adulthood.[44]

2. Secondary Lymphangiogenesis

The role of secondary lymphangiogenesis, beyond embryonic development, has not been well studied, except in the context of tumor biology. Lymphangiogenesis likely occurs in parallel with angiogenesis in the setting of wound healing and inflammation in a similar, transient fashion.[65] The growth of these lymphatics occurs *de novo*, originating from pre-existing lymphatic capillaries.

Table II. Lymphatic features of human cancer and metastasis (reproduced from Ji,[21] with permission)

Tumor type	Peritumoral lymphatics	Intratumoral lymphatics	Lymph node metastasis	Features
Oropharyngeal/laryngeal	+	+	+ or ×	Unrelated to weak VEGF-C expression; ITL associated with regional recurrence
Uterine cervical squamous cell	+	×	+	VEGF-C expressing peritumoral macrophages relate to PTL growth
Papillary thyroid carcinoma	×	+	+	ITL associated with LNM
Head and neck squamous	+	+	+	LYVE1+ ITL can relate to prognosis; high correlation between PTL and LNM; lymphatic density may be prognostic
Breast cancer	+		+	LNM occurs via pre-existing PTLs; no association between VEGF-C expression and lymphatic density
Cutaneous melanoma	+	+	+ or ×	Low-level VEGF-C expression; lymphangiogenesis is prognostic; lymphatic vascular density correlates with LNM
Prostate cancer	+	+	+	Correlation between VEGFR3+ LECs and LNM
Pancreatic endocrine tumors	+	+	+	VEGF-C expression correlates with LNM
Lung/breast/colon tumor and melanoma	+	+	×	CD34/ LYVE1 coexpression in tumor-associated LECs
Endometrial carcinoma	+	+	+	VEGF-A, not -C or -D, correlates with lymphatic vascular density

ITL = intratumoral lymphatics; **LEC** = lymphatic endothelial cell; **LNM** = lymph node metastasis; **LYVE1** = lymphatic vessel endothelial hyaluronan receptor-1; **PTL** = peritumoral lymphatics; **VEGF** = vascular endothelial growth factor; **VEGFR3** = vascular endothelial growth-factor receptor-3; **+** denotes presence; × denotes absence.

Recent studies suggest that circulating lymphatic progenitor cells may, as well, play an important mechanistic role in secondary lymphangiogenesis.[83] The new vascular structures form in close proximity to, but separately from, blood vessels.[65,84] In diseases of known inflammatory etiology, such as psoriasis, as well as in chronic states of inflammation,[85] lesions exhibit exuberant changes of lymphangiogenesis. Whether these newly formed lymphatic vessels are functionally significant with persistence or resolution of chronic inflammation is not known.[24]

Much of the recent progress in understanding the process of lymphatic development has been made with respect to tumor biology, where metastatic spread through the lymphatic system is a prominent area of clinical interest. Dissemination of metastatic tumor cells to draining lymph nodes and seeding of secondary sites of tumorigenesis is a not uncommon phenomenon in many cancers. Staging of such spread confers substantial implication in patient prognosis.

Cancerous cells have classically been believed to gain access to the lymphatic circulation passively, invading local lymphatics randomly as the tumor expands. Recent evidence suggests a more active natural history of lymphangiogenic induction and metastatic transformation through tumor-mediated upregulation of both VEGFR3 and VEGF-C expression and signaling.[71,86,87] Blocking VEGFR3 suppresses cancerous spread.[88] Conversely, inhibition of lymphangiogenesis in transgenic mice expressing soluble VEGFR3 produces an acquired lymphedema phenotype.[89] Taken together, these observations suggest a central role for VEGFR3/VEGF-C signaling in proliferative lymphangiogenesis during tumor metastasis. Direct evidence has been adduced for cancers of the breast,[71] cervix,[90] pancreas,[86] prostate,[91] and others[3] (table II). Tumor-mediated expression of the primary VEGFR3 ligands, VEGF-C and VEGF-D, correlates with progression to metastasis, lending further support for the recruitment of lymphangiogenesis by aggressive tumors.[8] VEGF-C induces expression of the VEGFR3 receptor, leading to paracrine lymphangiogenic responses.[68]

A number of clinical studies underscore the prognostic value of VEGF-C, VEGF-D, and VEGFR3 expression profiles and resultant lymphatic hyperplasia with regard to tumor progression,[92] regional lymph node metastasis,[91] patient survival,[93] and mortality.[94] The utility of VEGF-C/D and VEGFR3, individually and as a panel of biomarkers for pre-cancerous and cancerous staging, warrants further study. Pre-existing lymphatic vessels seem unaffected morphologically and functionally by prolonged VEGFR3 blockade. Systemic administration of an anti-VEGFR3 antibody may, thus, reduce tumor-associated lymphangiogenesis and cancer metastasis without affecting homeostatic lymphatic flow.[88,95,96] VEGF-A has been found to mediate tumor-induced proliferation of VEGFR2-positive lymphatic vessels prior to metastasis,[97] perhaps representing an anticipatory component of sentinel lymph

node lymphangiogenesis and cancerous spread. This possible mechanism of increased metastatic potential has been extended recently to VEGF-C-expressing tumors,[98] and likewise presents an attractive target of intervention.

Despite the existence of circulating CD34+ endothelial progenitor cells (EPCs) into maturity,[99,100] the contribution of such a nidus of angiogenesis is controversial. Likewise, lymphatic endothelial progenitors have been discovered in the mature circulation but the degree to which they mediate secondary lymphangiogenesis in cancer is not yet understood. Incorporation of bone marrow-derived progenitor cells during cancer-associated lymphatic formation has not yet been documented,[96] although such association has been observed in other settings of lymphangiogenesis.[101,102]

3. Primary Lymphedema

The primary lymphedemas are developmental disorders in which the lymphatic vasculature fails to achieve a homeostatic capacity for fluid transport. These uncommon diseases most commonly arise at or following puberty in so-called Meige disease,[103] or hereditary lymphedema praecox. Lymphedema present at birth, or hereditary congenital lymphedema, carries the eponym of Milroy disease.[104] This autosomal-dominant inherited disorder is characterized, in many of the patient cohorts, by mutations in the VEGFR3 domain.[17,41,105] A murine model of Milroy disease, typified by a heterozygous inactivating mutation of the tyrosine kinase domain of VEGFR3, and poorly functional lymphatics, has been utilized to demonstrate a potential for curative gene therapy.[28] These so-called Chy mice (named for their propensity to develop chylous ascites) have hypoplastic lymphatic vessels. Ascites develops soon after birth, mirroring the disease of patients harboring the same mutation. Induction of functional lymphatic vessels in Chy mice has been observed through the overexpression of VEGFR3 ligands, supporting the sufficiency of VEGF-C/D signaling to promote lymphangiogenesis.[28] In the Chy animal model, lymphatic insufficiency and edema precedes dermal expansion and fibrosis[28] without apparent effect on the hemovascular system.[89]

Additional animal models have helped to identify possible genetic mechanisms to explain the presentation of primary lymphedema. Forkhead box C2 (FOXC2)$^{-/-}$-knockout mice[106] reproduce the causative and physiological genetic defect of the lymphedema-distichiasis syndrome, a hereditary lymphedema characterized by the presence of distichiasis at birth and the onset of bilateral lower limb lymphedema by, or following, puberty.[107] In contrast to Milroy disease, where lymphatic hypoplasia predominates, lymphedema-distichiasis syndrome is characterized by normal or hyperplastic lymphatic vessels, suggesting that FOXC2 participates in the functional integrity of lymphatic vessels, with a less prominent developmental role. Indeed, FOXC2 is a critical transcription factor in pathways of metabolism, perhaps linking lymphatic dysfunction to insulin resistance.

A role for the transcription factor SOX18 (sex determining region Y-related high mobility group box 18) was recently identified in recessive and dominant forms of the rare hypotrichosis-lymphedema-telangiectasia syndrome,[41] characterized by the distinctive presentation of childhood hypotrichosis and lymphedema with telangiectasia or vascular nevi on the palmar surfaces.

T1α/PDPN$^{-/-}$ knockout mice also exhibit congenital lymphedema, with profoundly lymphedematous limbs at birth. Comparative anatomical and histological analysis between wild-type, heterozygote, and knockout animals reveals graded hyperplasticity of lymphatic vessels but the near-absence of lymphatic capillary and plexus formation.[82] Consistent with observed expression of T1α/podoplanin during developmental lymphatic speciation *in vivo*, T1α/podoplanin appears to be responsible for outlining the superficial capillary beds that anastomose with deeper lymphatic networks in the subcutaneous tissues.[82] Absence of T1α/podoplanin, therefore, facilitates the development of nascent lymphatics that are dysplastic, lacking the functional patterning and capacity of wild-type lymphatic vasculature. For the developmental disorders of lymphatic insufficiency, known etiological characteristics can be employed, such as the absence of VEGFR3 or PROX1, as biomarkers. Identification of more desirable, positively expressed circulating surrogates, however, remains elusive.

4. Secondary Lymphedema

Secondary lymphedema is a consequence of lymphatic stasis acquired through a variety of insinuating insults, a spectrum that includes, trauma, infection, surgery, radiation damage, and malignancy.[7] Perhaps the most important causes are iatrogenic, a not uncommon result of cancer therapeutics that entail regional lymph node dissection and irradiation. With regard to breast cancer alone, up to one-third of breast cancer survivors develop post-treatment lymphatic dysfunction.[108]

A number of homeostatic and pathological processes underlie the clinical picture of lymphedema. In the setting of osmotic and hydrostatic gradients maintained by the blood vasculature, disruption of lymphatic transport impairs the clearance of interstitial fluid and macromolecules. Stagnation of protein-rich lymph fluid

within the local interstitium elevates tissue colloid pressure, thereby promoting further edematous fluid accumulation. Local ischemia ensues, and the affected tissues are primed for the persistent and progressive inflammatory changes that characterize chronic lymph insufficiency.[109] Hypercellularity of the lymphedematous tissue is primarily organized by fibroblasts, adipocytes, and keratinocytes, followed by infiltration of inflammatory cells, particularly polymorphonuclear neutrophils.[7,110,111] Macrophages and other mononuclear cells are recruited to the lymphedematous tissues, typifying an unrelenting inflammatory cascade that causes profound changes in dermal and subcutaneous architecture.[7,18,110-112] Subsequent tissue expansion and fibrotic changes, with matrix degeneration and concomitant adipose deposition, quickly ensue.[7] Gross tissue-level alterations present as chronic and debilitating swelling and pain of entire limbs, without a known cure or pharmacologic intervention.[7] The edema, initially pliable, with some lability, progresses to a rough and dense fibrotic mass of irreversible consequence.

Current treatments for lymphedema do not address the underlying pathophysiology and, thus, provide only modest delays in the establishment of end-stage sequelae, such as disfigurement and loss of function. Psychological distress often accompanies the physical features of lymphedema, further complicating management.[7,113-116] At present, the standard of care comprises compression therapy and manual massage to temporarily relieve the edema burden of affected limbs, but such physiotherapeutic intervention, while supportive, cannot prevent the natural progression of the disease.[7,107,117] The secondary development of co-morbidities, such as depression and chronic, recurring infection,[7] is common, underscoring the physiological importance of the lymphatic system; these complicating features must be actively recognized, diagnosed, and treated.

The biological attributes of experimentally induced lymphedema have been studied in the extremities and internal organs of various animals; until recently, the observations have been under-representative of our clinical understanding of lymphedema.[16,118-121] In animal models, ablation of the lymphatic vessels results in lymphedema amenable to therapeutic lymphangiogenesis; both direct administration of recombinant VEGF-C and plasmid-mediated gene therapy of VEGF-C demonstrated the ability to ameliorate the established pathology of acquired lymphedema.[70,122] Mouse models afford several advantages; in particular, the ready availability of tools to assess and manipulate murine genetics facilitates disease characterization with far greater clarity and power. Disruption of the lymphatic vasculature in a model of surgically induced lymph stasis of the murine tail displays marked acute inflammatory processes in the absence of a confounding inflammatory stimulus. Most recently, growth factor-mediated therapeutic lymphangiogenesis has been demonstrated in a murine tail model of acquired lymphedema.[122] Nevertheless, the hope for effective therapeutic lymphangiogenesis continues to inspire continued development for relevant models of acquired lymphatic insufficiency, notably towards reproducing the chronic state of the human disease.

5. Conclusions

The morphological, histochemical, and molecular changes observed in settings of lymphatic insufficiency have not, as yet, yielded a reliable circulating biomarker. Separation of the specific and direct tissue effects of lymph stasis from those resulting secondarily and indirectly must be accomplished. The expression of the anti-inflammatory cytokine interleukin-10, for example, is likely a physiological response to the inflammatory cascade initiated by lymph stasis, and not a part of the primary disease process. The role of wound repair mechanisms and activation of immune effectors is less certain and requires further experimentation to evaluate its relation to lymphatic vascular insufficiency. Microarray analysis of normal and lymphedematous animals reveals several hundred significant differences in gene expression which, when categorized by physiological function, include known pathways of acute inflammation, fibrosis, wound healing, angiogenesis, adipogenesis, activation of the Wnt pathway and complement system, cytoskeletal organization, and oxidative stress.[18] Certainly, the identification of these putative mediators requires further substantiation.

Recently characterized models of lymphatic development in the *Xenopus laevis* tadpole[123] and the zebra fish *Danio rerio*[124] may provide a means of efficiently screening candidates of functional and pathological importance. Insofar as the models may parallel the biology of human lymphedema, these investigative platforms afford the ability not only to identify the elements of disease development, but also to study the effects of putative pharmacotherapy to manipulate those mediators. In particular, the ability of therapeutic lymphangiogenesis with recombinant VEGF-C administration to surmount the pathological processes has been demonstrated recently.[122] The vascular endothelial growth factor receptor VEGFR3 and corresponding ligands, primarily VEGF-C, appear to be pivotal in both pathological and therapeutic pathways. Hence, the moderation of these factors may be instrumental in promoting homeostasis in the face of unremitting insult. The role of lymphangiogenesis in the setting of the

inflammatory processes observed in acquired lymphedema, however, remains an area of investigation.

Although the proliferation of lymphatic vessels during inflammation was described very early,[125] the mechanism of this observation has only recently been studied. In the context of inflammation, VEGF-C expression is provided, at least in part, by macrophages which, when activated, secrete the growth factor alongside several other angiogenic and lymphangiogenic factors.[71,126] Inflammation induces the expression of VEGF-C and VEGFR3 in dendritic cells, possibly through the intermediary of pro-inflammatory cytokines.[127] Pro-inflammatory cytokines induce transcription of VEGF-C mRNA in tissue culture,[128] presumably through nuclear factor kappaB (NF-κB)-mediated promoter activation. These cytokines may, thus, help regulate lymphatic vessel growth during inflammatory challenge.[129] One such cytokine, tumor necrosis factor-α (TNFα) is particularly interesting in this regard. TNFα is expressed at sites of inflammation and is a known angiogenic factor, albeit indirectly[130] and, thus, may help bridge the simultaneous observation of inflammation and lymphangiogenesis in the setting of lymphatic insufficiency. Macrophages and dendritic cells observed histologically in animal models of lymphedema, while orchestrating the destructive acute inflammatory processes that belie the acute disease, may participate in repair mechanisms that while insufficient pathologically, represent an area of possible attenuation by targeted treatment. Suppression of the underlying derangement of innate immune function may, therefore, be an effective therapeutic strategy for acquired lymphedema, alone or in concert with pro-lymphangiogenic factors.

Analogous to proposed angiogenic treatment strategies for diseases of the peripheral and coronary blood vasculature, molecular approaches may ultimately provide a therapeutic window to reverse the pathology of both primary and secondary lymphatic insufficiency.[25,54,131-133] Initial experimental observations indicate that gene- and growth factor-mediated therapeutic lymphangiogenesis with VEGF-C holds promise for the treatment of lymphatic insufficiency, harnessing the regenerative capacity of the lymphatic vascular system.[25,28,70,122] Amelioration of other self-perpetuating components of the disease process, such as acute and chronic inflammation, may, however, also be necessary to attain complete amelioration of disease.

In summary, within the last decade, substantial advances have accrued in the identification of the processes that contribute to lymphatic vascular development in health and disease. The relationship of these processes to the identification and pathogenesis of heritable disorders of the lymphatic vasculature, acquired lymphatic vascular insufficiency, and the propagation of malignancy through lymphatic channels, suggests that much can be accomplished to mitigate the impact of disease. In all of these endeavors, the identification of biomarkers of the disease and its responsiveness to therapeutic intervention will represent a signal advance.

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