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Lymphatics in Lung Disease

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Abstract

The lymphatic circulation appears to be a vital component in lung biology in health and in disease. Animal models have established the role of the lymphatic circulation in neoplastic and inflammatory diseases of the lung, such as asthma and cancer, and allowed for the understanding of the molecular controls of lymphangiogenesis in normal lung development. Understanding the role of lymphatics in human lung disease appears likely to contribute to the understanding of the pathogenesis of disease and the development of novel therapeutic targets.

Keywords

lymphangiogenesis; metastasis; lymphangiectasis; lymphangioleiomyomatosis; VEGF-D; VEGF-C

The lymphatic circulation has received little attention in pulmonary research. Fluid homeostasis and host defense are two critical roles for the lung, in addition to gas exchange.¹ The main function of the lymphatic circulation is to drain fluid from tissues and return it to the vascular circulatory system.² The lymphatic circulation is also involved in the immune system of the body, since lymphocytes and dendritic cells move through the lymphatic system to reach the lymphoid organs.² Therefore, the lymphatic circulation must be a vital component in lung biology in health and in disease.

The lymphatic system comprises a vasculature consisting of thin-walled capillaries as well as larger vessels that are lined by a layer of endothelial cells. Lymphatics structures are distinguished from those of the arterial and venous circulations by the absence of pericytes.³ Identification of unique lymphatic markers that differentiate its vessels from the arterial and venous circulations enabled a more informed study of the lymphatic circulation.

Among the most important markers are:

1. Prox1, which is found exclusively in the lymphatic endothelium, and is a transcription factor required for programming the phenotype of the lymphatic endothelial cell (LEC)⁴;
2. LYVE-1, a CD44 homologue, and an LEC hyaluronan receptor, which is present exclusively on LECs and macrophages⁵; and

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3. VEGFR-3 (vascular endothelial growth factor receptor 3), a receptor for VEGF (vascular endothelial growth factor)-C and VEGF-D, which is not detected on blood vascular endothelial cells, and is present on those in the lymphatics.⁶

The discovery of such genes, growth factors, and receptors involved in lymphatic development and function, in addition to the production of transgenic mice and improvement in imaging techniques has enabled a better understanding of the lymphatic circulation. Many of the human diseases associated with lymphatic abnormalities are, however, uncommon and remain poorly characterized.

The best described of numerous pathways that regulate lymphangiogenesis involve VEGF-C and VEGF-D.⁷ These proteins promote lymphangiogenesis by activating VEGFR-3, a cell-surface tyrosine kinase receptor, leading to initiation of a downstream signaling cascade. In addition, VEGF-C and VEGF-D bind to neuropilin-2 (Nrp2), a semaphorin receptor, which is also expressed in lymphatic capillaries.⁷ Knockout mice for Nrp2 exhibit lymphatic hypoplasia.⁸

During human gestation, lymph sacs appear at 6–7 weeks. Experimental data from mice support the Sabin hypothesis that lymphatics arise by sprouting from embryonic veins.⁹ The nuclear transcription factor Prox1¹⁰ and endothelial receptor-ligand VEGF-C are integral to these events. A complete absence of lymphatic vasculature was observed in Prox1-knockout mice, whereas overexpression of Prox1 in human blood vascular endothelial cells suppressed many blood vascular-specific genes and upregulated LEC transcripts.¹⁰ Signals leading to the expression of Prox1 and its target genes in LECs, however, are not known.

VEGF-C deletion leads to a complete absence of lymphatics in mouse embryos.¹¹ Although LECs initially differentiate, they fail to migrate and form lymphatic sacs. VEGF-D deletion does not affect lymphatic development,¹² although exogenous VEGF-D rescues the phenotype of VEGF-C knockout mice.¹¹ VEGFR-3 deletion leads to defects in blood vessel remodeling and embryonic death.¹³

Lymphangiogenesis during Lung Organogenesis

VEGF-D mRNA is not detectable during early development of the mouse lung (E12.5). It was first seen at E13.5, the pseudoglandular stage, and by E14.5, it was diffusely abundant throughout the mesenchyme and visible by immunohistochemical study. It remained elevated until birth and was not detected in the postnatal lung.¹⁴ Levels of VEGF-D mRNA were higher in the distal than proximal mesenchyme. VEGF-D was associated with cadherin-11-positive fibroblasts,¹⁵ but not endothelial cells, pericytes, or smooth muscle cells. VEGF-D was not necessary for lymphatic development, as VEGF-D knockout mice did not show evidence of lymphatic abnormalities.¹² Although lungs of VEGF-D-null mice appeared to develop normally, the numbers of VEGFR-3-positive vessels adjacent to the muscular surface of bronchioles in the lungs of knockout mice were significantly lower than those of wild-type mice. This difference did not translate into a difference in the weights of wet and dry lungs, suggesting that knockout mice had a functional lymphatic system.

VEGF-C is critical for the development of the lymphatic system *in utero*, and VEGF-C knockout mice died at E15.5 from accumulation of fluids in their tissues.¹¹ During normal embryogenesis, there was extensive lymphatic vessel sprouting from embryonic veins and the developing lung was a site of VEGF-C and VEGFR-3 expression.¹⁶

VEGF (VEGF-A) is a key angiogenic growth factor, with loss of even one allele leading to death *in utero* at E8.5–E9.5 from severe vascular malformations.^{17,18} Recently published evidence suggests that the overexpression of VEGF during a late stage of lung organogenesis

resulted in increased numbers of lymphatic vessels distally. The increase was mediated by an increase in VEGFR-3 through an unknown mechanism, with associated increased sensitivity to VEGF-C and VEGF-D.¹⁹

Podoplanin, a transmembrane mucin-type glycoprotein is highly expressed in LECs.⁹ In mice, podoplanin deficiency led to abnormal lung development and perinatal mortality. Type I cell differentiation was blocked, as indicated by smaller airspaces, fewer type I cells and reduced levels of aquaporin-5, a type I-cell water channel.²⁰ Moreover, podoplanin knockout mice had defects in lymphatic, but not blood vessel pattern formation.²¹ These defects were associated with diminished lymphatic transport, congenital lymphedema, and dilation of lymphatic vessels.²¹

Net, a member of the Ets-domain transcription factors, is a strong inhibitor of transcription when MAP kinases are not activated. Net mutant mice²² have lymphangiectasia specifically in the thoracic wall, leading to chylothorax in the early postnatal phase, resulting in respiratory failure and death. Lymphangiectasia precedes the appearance of chylothorax, suggesting that lymphatic drainage is impaired. Net is located on chromosome 12q, and there is one case report of partial trisomy of 12q associated with chylothorax in humans.²³

Mice lacking integrin $\alpha 9$ developed chylous pleural effusions and die shortly after birth (2 days). In $\alpha 9$ -null mice, the anatomy of the thoracic duct was normal except for edema and perivascular lymphocytes surrounding the thoracic duct.²⁴ Possible mechanisms include interaction of the $\alpha 9$ subunit with $\beta 1$ integrins, which can induce tyrosine phosphorylation of VEGFR-3. Activation of both $\beta 1$ and VEGFR-3 is necessary for LEC migration.²⁵ Alternatively, it is now known that Prox-1 induces LEC differentiation through integrin $\alpha 9$.²⁶

EphrinB2 is a transmembrane growth factor.⁹ Mice with a mutated carboxy-terminal PDZ interaction site of *ephrinB2* die with a chylothorax during the first 3 weeks after birth.²⁷ Although these mutant mice have a normal blood vasculature, there is an abnormal postnatal remodeling of the lymphatic vasculature, leading to hyperplasia of the collecting lymphatic vessels, lack of luminal valve formation, and a failure to remodel the primary lymphatic plexus.²⁷

In humans, the role of VEGF in the maturation and alveolarization of the developing lung has been well established,²⁸ but the role of lymphatics and the lymphangiogenic growth factors remain poorly understood. Bronchopulmonary dysplasia is a disease characterized by maturation arrest of the lung.²⁹ A recent study examined the role of lymphangiogenesis and lymphangiogenic growth factors VEGF-D and VEGF-C in the maturation process.³⁰ Although the role of VEGF-D remains more difficult to determine since VEGF-D levels in tracheal aspirate fluids were too low to detect, VEGF-C seems to play a prominent role in the development of human lung. VEGF-C levels in tracheal fluid declines in the first postnatal week from a high in the first 2 postnatal days. Infants born prematurely may suffer from this decline in VEGF-C with a lack of development of their lymphatic systems, leading to abnormal lung fluid homeostasis. Prenatal injection of corticosteroids increased VEGF-C levels, which may help the maturation process. VEGF-C was present in the bronchial epithelium of fetuses and infants, but only fetuses showed alveolar staining. VEGF-C was seen in alveolar macrophages in infants suffering from respiratory distress syndrome or late bronchopulmonary dysplasia, indicating that inflammatory cells have a major role to play in the lymphangiogenic process.

Lymphangiogenesis and Lung Cancer

Small Cell Lung Cancer (SCLC) and Non–Small Cell Lung Cancer (NSCLC)

It was originally thought that lymphatic spread by tumors occurred by means of preexisting peritumoral lymphatics, without intratumoral lymphangiogenesis.³¹ In a lung cancer model, there is now direct evidence of the role of high levels of VEGF-C in promoting both tumor lymphangiogenesis and tumor spread to the regional lymph nodes.³² These effects can be suppressed by blocking VEGFR-3 signaling.³³

Results of human clinicopathologic studies show that in SCLC, VEGF-C, and VEGF-D transcripts and protein levels are elevated.³⁴ In one NSCLC study, serum VEGF-C levels were used as markers for lymph node metastasis, and their use with computed tomography scans to stage lymph node invasion was proposed.³⁵ In NSCLC, a lower survival rate is associated with increased expression of the lymphangiogenic growth factors VEGF-C and VEGF-D and their receptor VEGFR-3.³⁶ Moreover, angiogenesis in NSCLC is associated with concomitant lymphangiogenesis. In the absence of angiogenesis, NSCLC cells invade host lymphatics during tumor development.³⁶ Although lymphangiogenesis occurs, the newly formed lymphatic vessels do not survive.³⁷ Lymph node metastasis occurs via existing lymphatic vessels or newly created shunts by active angiogenesis and lymphangiogenesis. Metastatic tumor spread to lymph nodes plays a major role in staging lung cancer. Strategies that have been found to be useful in animal models to stop lymphatic spread of tumor cells, such as VEGFR-3-blocking antibodies, or small molecule kinase inhibitors, represent new possibilities for the treatment of human cancers.³⁸

Kaposi's Sarcoma

Kaposi's sarcoma (KS) is the tumor most often seen in patients suffering from HIV/AIDS. KS most frequently affects the skin,³⁹ and pulmonary KS carries a poor prognosis.⁴⁰

The causative agent of KS, human herpesvirus-8, was originally thought to infect LECs, since tumor cells express VEGFR-3 and podoplanin.³⁹ Subsequently, it was shown that blood vascular endothelial cells infected with the virus are re-programmed into LECs that express Prox-1, the master regulator of lymphatic lineage, with downregulation of blood vascular endothelial cell genes.^{41,42}

Lymphangiogenesis and Asthma

Patients with chronic asthma can develop chronic airflow obstruction that is fixed or only partially reversible, as well as persistent bronchial hyperreactivity.⁴³ The pathogenesis of chronic airflow obstruction involves remodeling of the airway wall secondary to inflammatory cell infiltration, myocyte and myofibroblast hyperplasia, mucus metaplasia, subepithelial fibrosis, and edema formation.⁴³ The lymphatic system, via its ability to facilitate the clearance of edematous fluid, has recently been identified as playing an important role in attenuating airway wall remodeling in asthma.

The pathogenesis of airway lymphatic vessel hyperplasia has been investigated using a murine model of *Mycoplasma pulmonis* pulmonary infection, with evidence of chronic inflammation manifested by angiogenesis and microvascular remodeling.^{44,45} This model is relevant for asthma, as mycoplasma infection can initiate or exacerbate disease in humans and animal models.⁴⁵⁻⁴⁷ In an elegant study by McDonald and colleagues, *M. pulmonis* respiratory infection was shown to induce airway lymphangiogenesis that was dependent upon signaling by VEGF-C and VEGF-D through VEGFR-3.⁴⁴ In animals treated with a soluble VEGFR-3-Ig, which binds and sequesters VEGF-C and VEGF-D, lymphatic growth (as measured by the number of lymphatic sprouts) was reduced by 96%, without effects on blood vessel remodeling.

Treatment with antibodies directed against VEGFR-1 or VEGFR-2 did not inhibit lymphatic vessel growth, whereas an anti-VEGFR-3 antibody markedly reduced lymphangiogenesis. Although antibiotic treatment of infected animals was associated with a return of remodeled blood vessels to their normal state within 1 month, the extensive network of new lymphatic vessels had not regressed after 12 weeks of treatment. Airway epithelial and inflammatory cells were sources of VEGF-C, whereas VEGF-D was produced by airway neutrophils, macrophages, airway smooth muscle cells, and lymphatics.

To define better the VEGFR-3 ligands that mediate lymphangiogenesis, mice were inoculated with adenoviral constructs that generated VEGF-C or VEGF-D.⁴⁵ Overexpression of VEGF-C in airway epithelial cells was associated with lymphangiogenesis similar to that seen in *M. pulmonis* infection, whereas overexpression of VEGF-D induced an exaggerated proliferation of lymphatic vessels. Overexpression of VEGF-C or VEGF-D was specific for lymphatic proliferation, as neither induced angiogenesis. Functional effects of impaired lymphangiogenesis on mucosal edema were also assessed in this model system.⁴⁵ Inhibition of lymphangiogenesis by adenoviral delivery of a soluble VEGFR-3-Ig construct prior to *M. pulmonis* infection was associated with significantly increased bronchial lymphedema. In addition, blockade of lymphangiogenesis reduced infection-induced hypertrophy of draining bronchial lymph nodes. This study shows that new lymphatic vessels provide a mechanism for clearance of extravasated fluid from the abnormal leaky blood vessels in inflamed airways.

The murine model of *M. pulmonis* pulmonary infection was also utilized to assess potential roles in lymphangiogenesis of innate and acquired immune responses in host defense. Mast cell-deficient (Kit^{W-sh}/Kit^{W-sh}) mice were used to evaluate the contribution of mast cells, which participate in host defense in septic peritonitis, in contracting *M. pulmonis* infection.⁴⁷ Mast cell-deficient mice had a higher mycoplasma burden and mortality rate than wild-type mice, consistent with the conclusion that mast cells play an important role in innate immune responses to pulmonary mycoplasma infection. Furthermore, there was a striking increase in lymphangiogenesis in mast cell-deficient mice that exceeded the angiogenic response at 28 days and persisted despite resolution of the acute infection. This finding supports the notion that lymphangiogenesis reverses more slowly than angiogenic remodeling after *M. pulmonis* infection. In a separate study, lymphatic remodeling after *M. pulmonis* infection was substantially reduced in the airways of lymphocyte-deficient *Rag1*^{-/-} mice and B cell-deficient (*Igμ*^{MT}) mice.⁴⁸ Remodeling of blood and lymphatic vessels was significantly enhanced by transfer of serum, which contained immune complexes, from infected wild-type mice to B cell-deficient mice. Thus, *M. pulmonis*-induced chronic lymphangiogenesis and angiogenesis were dependent upon immune complex-mediated inflammation.

Taken together, these studies showed that airway inflammation induces a lymphangiogenic response via VEGF-C and VEGF-D, which are produced primarily by inflammatory cells, signaling through VEGFR-3 in LECs. Newly formed lymphatic networks persisted for extended periods of time despite the resolution of inflammation and provided a pathway for removal of extravasated plasma that occurs secondary to endothelial gap formation and microvascular leakage.⁴⁹ Thus, with impaired lymphangiogenesis, accumulation of extravasated fluid may result in bronchial lymphedema and contribute to airway remodeling with persistent airflow obstruction in asthmatic lungs.

Lung Transplantation

Obliterative bronchiolitis (OB) is a major cause of late failure of lung transplantation, since it is present in 70% of transplanted lungs by 5 years post transplant.⁵⁰ OB is characterized histologically by a fibromyxoid reaction originating from the airway walls that obliterates both small and large airways. This translates physiologically into a progressive decrease in forced

expiratory volume in 1 second (FEV₁), leading to dyspnea, infections, and loss of the grafted lung. The pathogenesis is not completely understood, but most of the evidence points toward a major role for adaptive immunity in the etiology of OB.⁵¹

In a rat model of tracheal transplant,⁵² transfer of the VEGF gene resulted in the doubling of the number of LYVE-1+ lymphatic vessels in allograft airway walls. Conversely, treatment with PTK 787, a broad VEGFR protein tyrosine kinase inhibitor, resulted not only in a 50% reduction in the number of LYVE-1+ lymphatic vessels, but also in decreased numbers of CD4 and CD8 T cells in airway walls. These observations suggest that lymphangiogenesis enhances antigen presentation to the host immune system, thereby increasing alloimmune sensitization. In agreement with other studies, the effects of VEGF could be due to the recruitment of inflammatory cells such as macrophages that produce VEGF-C and VEGF-D.^{45,53} In this model, lymphangiogenesis may lead to continuous presentation of donor tissue to lymph nodes, with persistent alloimmune response, and resulting rejection or graft injury.⁵²

The role of lymphangiogenesis in human allograft rejection of solid organs has been well documented in kidney^{54,55} and heart transplantation.⁵⁶ In human lung transplant recipients, the role of angiogenesis is increasingly recognized⁵⁷⁻⁶⁰ in the pathogenesis of airway obstruction, leading eventually to OB, but to date, there are no published reports regarding lymphangiogenesis in lung transplantation. Total body lymphoid irradiation has been proven safe and efficacious in slowing the decline of lung function in transplant recipients suffering from OB.⁶¹

Pulmonary Lymphangiectasia

Lymphangiectasia refers to the dilation of normal lymphatic vessels, most often secondary to more proximal obstruction or to development of lymphatic valvular incompetence. Primary lymphangiectasia is a rare disorder that most commonly affects the pulmonary and the intestinal lymphatic vasculature.⁶² Predisposing factors are unknown. Classically, pulmonary lymphangiectasia presents as respiratory distress of the newborn, resulting in death. Historical series had a mortality of 100%, but advances in neonatal respiratory care have improved prognosis.⁶² In the pre-natal period, the disease often results in stillborn infants. The immediate neonatal presentation involves cyanosis and respiratory distress. Postneonatal or late presentation, which can occur after months without respiratory symptoms, typically includes cough and wheezing. Symptoms in a few cases, did not appear until late childhood or adolescence.⁶² Pleural effusions were described in 15% of cases,⁶³ and typically were chylous exudates, with a high lymphocyte content.⁶⁴ In the neonatal presentation, the chest X-ray reveals diffuse interstitial infiltrates. Later in infancy and childhood, reticulonodular infiltrates and hyperinflation were seen,⁶⁵ and sometimes pleural effusions. On computerized tomography of the chest there was ground-glass appearance with interstitial densities. Lymphangiography showed an interstitial pattern of pulmonary lymphatics with opacification and formation of collateral lymphatic vessels.⁶²

Primary pulmonary lymphangiectasia can be part of generalized lymphangiectasia involving the intestines, or limited to the lungs. It also includes syndromic forms of primary pulmonary lymphangiectasia, where it is associated with unrelated congenital anomalies, including genetic syndromes.⁶² Secondary pulmonary lymphangiectasia can be divided into lymphatic and cardiovascular obstructive forms. Thoracic duct agenesis causing lymphatic obstruction has been reported. Among the cardiovascular forms are hypoplastic left heart syndrome, anomalous pulmonary venous return, pulmonary vein atresia, and congenital mitral stenosis.⁶²

Autopsy data show that the incidence of pulmonary lymphangiectasia is 0.5–1% of all stillborn or neonatal infant deaths.⁶² There was a male predominance of the primary forms. Most cases

are sporadic, with a few familial cases of pulmonary lymphangiectasia demonstrating an autosomal-recessive inheritance pattern.⁶⁶

Chylous Pleural Effusions

Chylomicrons and very-low-density lipoproteins produced from dietary fat are delivered to the cisterna chyli, from which one major lymphatic vessel, the thoracic duct, returns them to the vascular circulation.⁶⁷ The thoracic duct originates in the cisterna chyli at the level of the second lumbar vertebra, where it crosses into the thoracic cavity through the esophageal hiatus. In the thoracic cavity, the thoracic duct runs extrapleurally in the posterior mediastinum, close to the esophagus and the pericardium, on the right side of the anterior surface of the vertebral column and continues toward the superior mediastinum after crossing to the left at the level of the 4th–6th thoracic vertebrae.⁶⁷ The thoracic duct then arches 3–5 cm above the clavicle, passing anterior to the subclavian artery, vertebral artery, and thyrocervical trunk to terminate in the region of left subclavian and jugular veins.^{68,69} Depending on diet, 1500–2500 mL of chyle per day are transported through the thoracic duct.⁶⁸ The protein content of chyle is about 3g/dL and the electrolyte composition is similar to that of serum. The cellular population is predominantly lymphocytes, (400–6800/mm³) with most of them T lymphocytes.⁷⁰

A chylothorax is formed when chyle enters the pleural space. Four causes of chylothorax are recognized. In adults, tumors are responsible for more than 50% chylothoraces, and, among those, lymphoma is the cause of about 75% of them.⁶⁷ The second most frequent cause of chylothorax is trauma, most often thoracic surgery that requires the mobilization of the left subclavian artery.⁷¹

Idiopathic chylothorax including congenital chylothorax is the third most common in the neonate, and is the most frequent cause of pleural effusion.⁷² Congenital chylothorax is more frequent in males (2:1) and on the right side of the thorax. Thoracotomies have shown that the thoracic duct is normal in most of the afflicted babies, and occasionally generalized oozing is described.^{64,73} It may be isolated or part of a larger group of lymphatic abnormalities and it has also been associated with Turner's, Down and the Noonan syndromes.⁶⁴ Familial congenital chylothorax has been reported in both males⁷² and females.⁷⁴ The fourth chylothorax category is “other disorders” (e.g., lymphangioleiomyomatosis, tuberculosis, sarcoidosis, KS).

Idiopathic Pulmonary Fibrosis

Information regarding the role of lymphatics in the pathogenesis of idiopathic pulmonary fibrosis (IPF) is scant. Bleomycin is commonly used in animal models of pulmonary fibrosis.⁷⁵ In a rat bleomycin model,⁷⁶ deposition of collagen IV and collagen I was observed with time after bleomycin injury. The results showed that collagen type IV accumulation specific for the angiogenic process⁷⁷ was followed by collagen type I deposition, indicating the presence of lymphangiogenesis.

There are no studies reported of *de novo* lymphangiogenesis in human IPF or other interstitial pneumonias; however, multiple studies^{78,79} have found an increased prevalence (40–60%) of enlarged mediastinal lymph nodes. Although one study showed no correlation with severity of disease,⁷⁸ another showed a correlation over time between lymph node enlargement and evolution of the disease⁷⁹ in the absence of infection or malignancy.

Tuberculosis

In guinea pigs, inflammation of the pulmonary lymphatics was one of the earliest lung lesions to develop after infection with *M. tuberculosis*.⁸⁰ Lymphangitis developed in the stroma of the

connective tissue of major lymphatic vessels and in the alveolar spaces 5 – 15 days after infection. Lymphatics surrounding bronchi and vessels were delineated with granulomatous lesions, resulting from inflammation in the lymphatic walls and expanding into the lumen. Lesions in both lung parenchyma and lymphatics progressed similarly, leading to central necrosis and granulocyte infiltration. After the resolution of acute inflammation, chronic stages were characterized by the infiltration of fibroblasts, fibroplasias, and dystrophic mineralization. In humans, the Gohn primary complex represents lung and lymph node lesions. In children and immunocompromised hosts, such as patients with HIV, the lymph nodes represent the first site of extrapulmonary disease.

Despite the increasing evidence from animal models supporting the involvement of lymphatics in lung disease, thus far, studies in humans are few. Understanding the role of lymphatics in human lung disease appears likely to contribute to the understanding of the pathogenesis of disease and the development of novel therapeutic targets.

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