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Methods for studying pulmonary lymphatics

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Take home message:

The pulmonary lymphatics are critical for proper lung function, yet seldom considered in lung pathology. Newer and more convenient methods for studying lung lymphatics are now available and might create new diagnosis and treatment opportunities.

Introduction

The pulmonary lymphatic system comprises a vast network of lymph vessels (LVs) and lymph nodes that unite the interstitial space with the vascular system, serving an essential role in fluid balance and immune response [1]. Although ubiquitous in the lung parenchyma and mandatory for normal physiology, pulmonary LVs are often not considered when describing the pathophysiology of pulmonary diseases [2]. In the past, the study of pulmonary lymphatics represented a difficult challenge due to their small size, thin walls, variable routes, complex interconnections and the lack of reliable identification techniques. Advances in many areas now allow for more precise and less complicated identification of LVs in the pulmonary parenchyma. Consequently, they permit the study of the role of LVs in pathological states and their potential role as a therapeutic target or route to deliver therapeutics to manage respiratory diseases. This review aims to outline a few key methods that have been used to study pulmonary LVs.

Particle inhalation/injection

The earliest experiments on pulmonary lymphatic anatomy were conducted by the inhalation of fine particulates [3] (such as India ink, white lead dust, potassium iodide) in dogs, direct injection of these particulates into the trachea of rabbits and by inhalation of dye (India ink) and carbon particles in anesthetised dogs. These experiments, performed in the early 20th Century, demonstrated the inhaled substances' lymphatic uptake within 1 hour of inhalation. New technologies such as near-infrared fluorescent optical imaging with indocyanine green [4] have allowed selective uptake of particles into the lymphatic vasculature to remain current. The inhalation of lipid nanoparticles [5] has also been used to map the draining patterns of pulmonary lymph. Future applications of these techniques could include lymphatic delivery of therapeutics [6] and more detailed cancer staging systems.

Histology techniques

The corrosion cast technique (Figure 1) can provide detailed structural information of pulmonary lymphatics using both light and electron microscopy, down to 10 µm scales [7]. The use of methyl methacrylate provides finely detailed moulds of the LVs [8]. Though an appropriately large vein, blood is flushed out through a heparinised solution, and then methyl methacrylate is infused, and the resin hardens for 1 hour afterwards. These moulds are then extracted by corrosion of the histological sections with sodium hydroxide to obtain the casts. The limitation of this method is the requirement for fixation and its use in *ex vivo* study. More widespread adoption of this technique could lead to a more detailed three-dimensional anatomical description of lymphatic in disease states.

The study of lymphangiogenesis and characterisation of LV networks is possible with fluorescent-labelled antibodies that bind to specific lymphatic endothelial cell (LEC) markers. The analysis of ultrastructure using antibodies and the understanding of gene expression patterns of LVs have permitted the differentiation between LVs and blood vessels [9]. This approach's challenge is that the markers are not consistently expressed and are often dependent on the developmental stage, tissue-type and inflammatory status [10]. Several markers must be used concurrently to identify LVs when imaged by fluorescent microscopy because of this variation (Figure 2).

The key LEC markers in the lung are:

Lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) is a CD44 homologue hyaluronan receptor found on LECs, macrophages, and blood endothelial cells [11].

Podoplanin belongs to the family of type-1 transmembrane sialomucin-like glycoproteins. Podoplanin and LYVE-1 are commonly used markers for lymphatic identification in various tissues throughout the body. However, in the lung, they can label other cell types, making unequivocal identification of LVs challenging [9, 12]. Podoplanin is present in lung alveolar type I cells [13] and can also be expressed by different types of tumour cells such as cancer-associated fibroblasts, malignant mesothelioma, squamous lung carcinoma, lung adenocarcinoma and pleomorphic carcinoma of the lung [14]. Clone D2-40 anti-podoplanin monoclonal antibodies have been useful in demonstrating the presence of LVs in interalveolar walls and seems to be selective to the form of podoplanin expressed on pulmonary LECs [15].

Prospero Homeobox Protein-1 (Prox-1) is a transcription factor involved in the differentiation of the LECs from veins [16]. Prox-1 is used in the lung to identify lymphatic capillaries [17], but neuroendocrine cells can also express it [18]. Mice with Prox-1-driven Enhanced Green Fluorescent Protein (EGFP) expression enabled a detailed view of lymphatic networks *in vivo [16]*.

Cluster of Differentiation 90 (CD90 or Thy-1) is expressed on lung LV endothelium and can reliably detect LVs in lung sections, even in the presence of inflammation from allergen exposure [19]. Axonal processes of mature neurons, mesenchymal stem cells, hematopoietic stem cells, NK cells, fibroblasts and myofibroblasts also express CD90[19].

Vascular endothelial growth factor receptor 3 (VEGFR-3) binds with VEGF C and VEGFC D [20]. VEFGR-3 expression is present in lymphatic vessels in developing mice [21]. In humans, VEGFR-3 can be found on LECs but also on blood vessels [21].

Further optimisation of the protocols used to identify LECs in histology will permit the routine identification of lymphatics in lung biopsies and their inclusion in diagnostic criteria.

Tissue-based techniques

LECs can be extracted from tissue samples by collagen digestion and posterior labelling and purification [22]. These techniques allow for experimental intervention *in vitro*. This approach has been used to study the role of lymphatics in asthma [22], COPD [23], transplantation [24] and cancer [25] as well as an avenue to develop therapeutics [26] that modulate lymphangiogenesis. Cells obtained from this method can be studied using various tissue-based techniques, including proliferation assays, chemotaxis evaluation, tube formation assays, protein expression assays, electric cell-substrate impedance sensing, and flow cytometry, among others.

Radiological techniques

New lymphatic imaging and interventional techniques have facilitated a resurgence of interest in lymphatic anatomy in the lung and other regions. In vivo imaging techniques are currently resolution-limited, and as of this publication, there are no described imaging methods capable of detecting intraparenchymal lung lymphatics in most pulmonary pathologies. In some pathologies with dilated conducting lymphatics, radiological techniques can offer diagnostic and therapeutic options. The more commonly used techniques in these cases are:

Pedal lymphangiography (PL) is the most widely used *in vivo* imaging technique for studying LVs, including pulmonary lymphatics. It involves the cannulation of lymphatic ducts in the dorsum of the foot or the web spaces between toes. An injection of oily contrast media such as Ethiodol (Savage Laboratories, Melville, NY) or Lipiodol ultra-fluid (Guerbet Laboratories, Bloomington, Indiana) can be tracked by fluoroscopy up the legs and into the central lymphatics including the pulmonary lymphatics [17]. It provides a detailed view of the lymphatic system thanks to its compatibility with advanced CT and MRI scanners. However, PL is invasive, time-consuming, and challenging, making it a significant barrier for most practitioners. Also, complications can include wound infections, contrast embolisation, intravascular injection and allergic reactions to the contrast agent [27]. A new technique involving the interstitial injection of a small amount of gadolinium-based contrast agent (GBCA) has been recently published [28]. A small amount of GBCA is injected in the web spaces of the toes (0.5-1 mL per webspace). The patients are then instructed to walk or perform knee-bends for 5 minutes after GBCA injection. Imaging is obtained within 50 minutes of the injection. It has a reported 92% success rate in identifying the retroperitoneal nodes, cisterna chyli and TD. This success rate is comparable to dynamic contrast magnetic resonance imaging.

Intranodal lymphangiography (INL) (Figure 3) is an alternative to the conventional PL and is less technically challenging and more reliable [29]. Contrast agents such as Lipiodol or Ethiodol are injected into a node, most often an inguinal node, using ultrasound guidance. If successful, immediate opacification of the LVs is evident under fluoroscopy. The procedure can be performed under local anaesthesia or intravenous sedation. After injection of the contrast agent, saline solution can be injected to obtain a more distal distribution of the contrast [29]. This technique also allows for the insertion of needles/guidewires/catheters into the central LVs for interventions. This advantage is used for embolisation in the treatment of plastic bronchitis [30].

Non-contrast T2-weighted magnetic resonance lymphangiography is a non-invasive technique that can image central and peripheral lymphatic systems, including pulmonary LVs, with reasonable spatial resolution. It is sensitive enough to identify the site of postoperative chyle leaks on patients undergoing thoracic surgery in 67% of the cases [31]. This technique's main limitation is the lack of dynamic flow visualisation and necessary resolution to visualise lymphatic ducts under 0.8 mm of diameter [32].

Dynamic contrast magnetic resonance lymphatic imaging techniques (Figure 3) involve using INL to inject an adequate MR contrast agent. Sequential MR scans are made for about 15 minutes [33]. The result is an image that shows both static images as well as flow. The disadvantages of this technique are the requirement of an interventional radiologist capable of cannulating inguinal lymph nodes and the need to move the patient from the interventional radiology suite to the MR room, leading to accidental removal of the lymphatic cannulation.

Advances in the image resolution of these techniques will allow for routine evaluation of LVs in the clinical setting, allowing for more precise diagnosis and staging of diverse lung pathologies.

Nuclear medicine techniques:

Lymphoscintigraphy involves injecting a radiolabelled tracer in the periphery and estimating the subsequent uptake into the regional lymph nodes by nuclear imaging [34] employing a gamma camera. It has been used as a technique to visualise the lymphatic flow and abnormal lymphatic perfusion. This dynamic flow information is useful for the differential diagnosis of congenital abnormalities of the TD, including congenital pulmonary lymphatic valvular incompetence [35]. Lymphoscintigraphy is limited to two-dimensional images.

Single-photon emission computed tomography (SPECT) combines the principles of lymphoscintigraphy and computer tomography. It produces accurate three-dimensional images which can be combined with conventional CT scans to provide precise anatomical data [36]. The nuclear medicine techniques carry the risk of an allergic reaction to the tracer and local infection.

Conclusion

The study of lung lymphatics, once a problematic undertaking, is now accessible to both clinicians and researchers with many options and different techniques. These methods will undoubtedly help advance our understanding of the role that lymphatics play in pulmonary health and disease. Novel diagnostic and treatment strategies derived from a better understanding of pulmonary LV's are currently being researched and might enter everyday clinical practice in the coming years.

Technique	Definition	Compatible with:	Future direction:	Main drawbacks:
Particle injection/inhalation	Administration of fine particulates to track their absorption into LV's.	Light or electron microscopy Near-infrared microscopy X-rays, CT, MR	Delivery method for therapeutics Systems for cancer staging	Mucocilliary action can affect results
Corrosion casts	Moulding of lymphatic vessels	Light or electron microscopy	Anatomy under pathological conditions	Cannot be used *in vivo*
LEC markers	Fluorescent-labelled antibodies that target LECs	Light or electron microscopy	Diagnosis/staging of pathology according to LV's LV's as therapeutic targets Therapeutic regulation of lymphangiogenesis	Requires use of multiple markers *In vivo* use feasible only in animal models
Pedal lymphography	Lymphatic cannulation in the foot with subsequent contrast medium administration and imaging	X-rays Fluoroscopy CT, MR	Diagnostic tools and scales based on LVs morphology	Complicated and time-consuming procedure Contrasts agent allergies
Lymphoscintigraphy	Injection of radiolabelled tracer and subsequent imaging	Nuclear imaging	Dynamic studies of lymphatic flow	Contrast agent allergies Sub-optimal resolution
Intra-nodal lymphangiography	Lymphatic cannulation in an inguinal LN with subsequent contrast medium administration and imaging	X-rays Fluoroscopy CT, MR	IR treatment options for pulmonary lymphatics	Requires an IR suite and specialists Contrast agent allergies
Non-contrast T2-weighted MR lymphangiography	MR sequence specific for central lymphatics including pulmonary lymphatics	MR	Non-invasive imaging of congenital pathologies with LVs involvement	Non-dynamic Sub-optimal resolution
Dynamic contrast magnetic resonance	Combination of INL and MRI	MR	Diagnostic tools and scales based on LVs morphology Dynamic studies of lymphatic flow IR treatment options for pulmonary lymphatics	Requires an IR suite and specialists Contrast agent allergies

Table 1: Summary of techniques sued to study pulmonary lymphatic vessels. LVs = lymphatic vessels, CT = computed tomography, MR = magnetic resonance, LEC = lymphatic endothelial cell, LN = lymphatic node, IR = interventional radiology.

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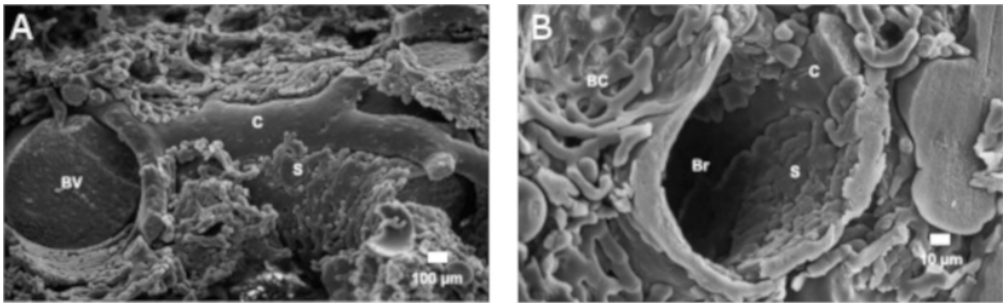

Figure 1: Electronic microscopy images of rat lung lymphatics using the cast and corrosion technique. Image A shows a collector lymphatic (C) adjacent to a blood vessel (BV) and initial lymphatics (S). Image B shows the initial (S) and collector lymphatics (C) around a bronchus (Br) that has been digested. Blood capillaries (BC) can also be identified. Image modified from Schraufnagel D et al. (2010).

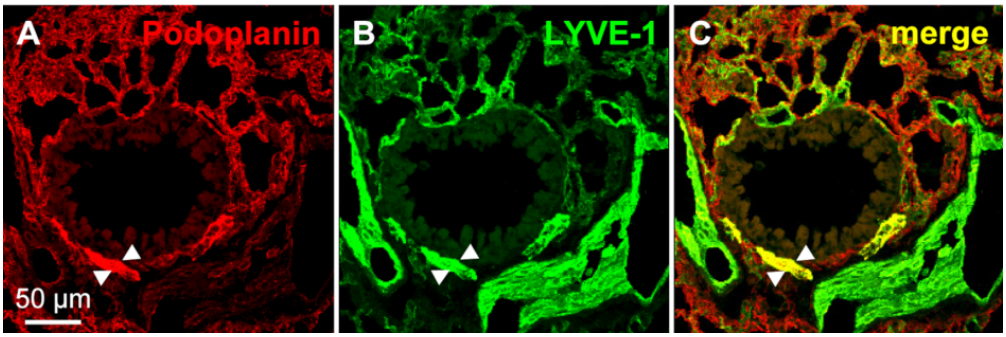

Figure 2: Double labelling of murine lung tissue with both anti-LYVE-1 (Image A) and anti-podoplanin (Image B) marked antibodies. Image C shows the need to use multiple markers to confidently identify LVs (arrowheads). Image modified from Kretschmer S et al. (2013)

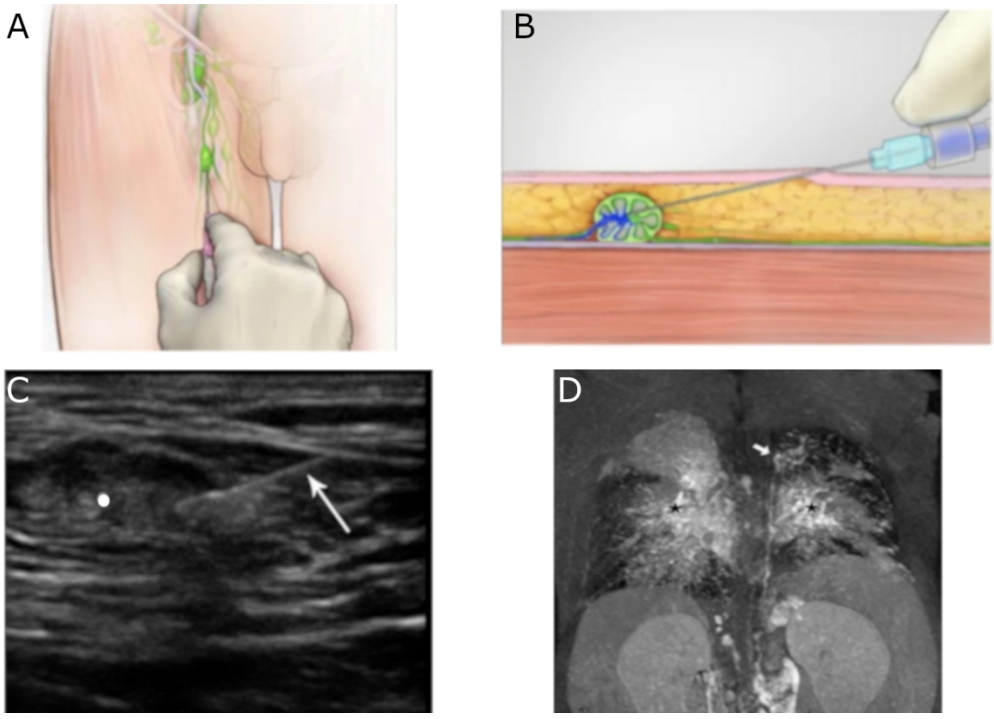

Figure 3: Image A: Illustration of the percutaneous non-guided INL technique. Image B: Illustration of ultrasound-guided delivery of contrast agents to an inguinal lymphatic node. Image C: Ultrasound image of the INL procedure, the lymph node is marked as a white dot, and the arrow indicates the needle. Image D: dynamic contrast lymphatic MRI sequence after INL, showing dye dispersion progression 25 minutes after intranodal administration of a contrast agent. A thoracic duct obstruction (white arrow), as well as alternate pathways (peri-hilar and peri-bronchial lymphatics, black asterisks), can be identified. Image A, B from Itkin & Nadolski (2018). Image C from Nadolski & Itkin (2012). Image D from Pimpalwar et al. (2018).