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INSIGHTS

An in-depth look at lung lymphatics

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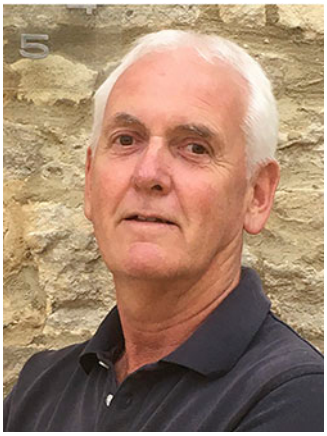
In this issue of *JEM*, Cleary et al. (https://doi.org/10.1084/jem.20241359) present a new intravital imaging technique using a 3D-printed window device that enables lung lymphatics and their participation in immune cell trafficking events to be visualized in action for the first time in mechanically ventilated mice.

The lungs play an essential role in the maintenance of life through the cyclic inhalation of oxygen and release of carbon dioxide. Exposed to the external environment, the airways are constantly open to invasion by pathogens and must maintain continuous immune surveillance while avoiding the development of inflammation and edema that would be detrimental to respiration. Here, the lung lymphatics play a dual role by enabling the trafficking of antigen-presenting cells, neutrophils, monocytes, and lymphocytes for immune protection as well as fluid drainage (Maisel and Outtz Reed, 2025). Indeed, defects in lymphatic function are implicated in lung disorders ranging from asthma, chronic obstructive pulmonary disease (COPD), and acute respiratory/fibrotic syndromes, such as SARS COVID-19 (MacDonald et al., 2022; Maisel and Outtz Reed, 2025). Nevertheless, the lymphatics of the lung have been largely overlooked, and basic aspects of their structure and function remain obscure. In other tissues, these begin as initial capillaries with discontinuous buttoned endothelial junctions specialized for fluid and chemokine (CCL21)-directed cell uptake (Baluk and McDonald, 2022; Pflicke and Sixt, 2009) and continue into contractile collectors sealed by tight zippered endothelial junctions (see figure) that propel downstream lymph flow (Baluk and McDonald, 2022). Whether such architecture is conserved in lymphatics within the more compliant environment of lung, and particularly those in bronchovascular cuff spaces—perivenous hubs specialized for leukocyte and interstitial fluid uptake—has yet to be demonstrated (Dahlgren and Molofsky, 2019). Although RNA sequencing and spatial transcriptomics are shedding new light on the composition of human and mouse lung microvascular and immune cell populations (Jiang et al., 2022), a proper understanding of their dynamics requires visualization in the intact organ by intravital microscopy. Major obstacles are the inaccessibility of the lungs in living animals and their constant motion from the breathing actions of intercostal muscles. Previous attempts to surmount these problems in mice employed surgical implantation of thoracic windows or direct placement of glass coverslips to the exposed lungs, either in situ or isolated within a synthetic ribcage (Looney et al., 2011; Kreisel et al., 2010; Banerji et al., 2023). However, even these innovations still restricted imaging to the first 100 µm beneath the pleural lung surface, precluding observation of the deeper vasculature.

In this issue, Cleary et al. (2025) have overcome such constraints using a new 3D-printed window device attached to the pleural surface of the stabilized lung in mechanically ventilated mice. Using the lineage-specific Prox1 eGFP and ubiquitous Rosa 26^{mTmG} fluorescent reporters, they could image the deep bronchovascular collectors for the first time and view dynamic events such as the opening and closing of valves and the flow of lymph and cells within them. The first key issue addressed was the mechanism of lymph propulsion. The prevailing view, supported by classical



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studies in dogs (Warren and Drinker, 1942), was that the pulmonary lymphatics rely on the extrinsic muscle contractions of normal breathing, because they lack the smooth muscle investment of conventional collectors. By controlling mechanical ventilation (see figure), the authors could show that lymph flow and valve operation worked in synchrony with the ventilation rate, pausing and restarting when this was halted and reinitiated, thus confirming that lymph propulsion was indeed controlled extrinsically. Surprisingly, this was not the case upon induction of acute lung inflammation by intratracheal LPS, whereupon lymph flow continued autonomously after ventilation was paused. Yet, when the amplitude of ventilation was raised in the same inflamed lungs to model increased tidal volume, it exerted an increase in lymph flow. The authors speculate the autonomous component

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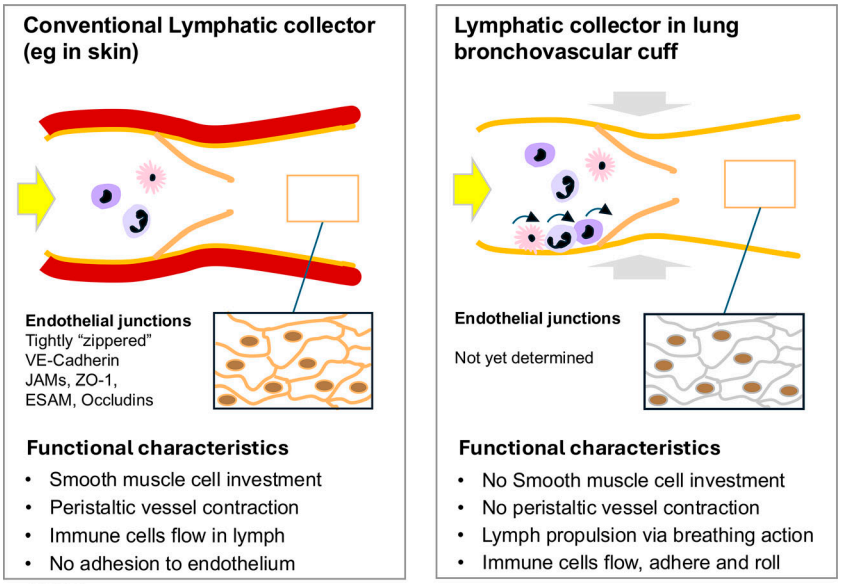

Lymphatic collectors in the mouse bronchovascular cuffs have a different architecture to conventional collectors. The constituent lymphatic endothelial cells (orange) are more compliant, lack the contractile smooth muscle cell coating (brown) of conventional collectors, and lymph pumping is exerted instead by the rhythmic breathing action of intercostal muscles (grey arrows). It is not yet known whether bronchovascular cuff collectors have tight zippered endothelial junctions containing the adhesion molecules shown.

of lymph propulsion resulted from an inflammation-induced increase in interstitial fluid pressure, but it could also be indicative of some auxiliary downstream pumping activity during inflammation. Clearly, more remains to be learned about how pulmonary lymph flow is coupled to breathing. Further research using the systems described in this paper may even help optimize parameters for mechanical ventilation of human patients with inflammatory lung diseases such as acute respiratory distress syndrome (ARDS), where the current procedure has significant mortality.

Cleary et al. (2025) next focused on the trafficking of immune cells via the pulmonary lymphatics and its induction by inflammation. Again, by tracking the fluorescently labeled cells, they could measure a significant increase in the numbers of leucocytes flowing within the bronchopulmonary collectors as well as an uptake of extravasated proteins 24 h after LPS administration. Using appropriate (CD11c, CSF-1R, MRP8, and platelet factor 4) reporter lines, these were identified as mainly type 1 dendritic cells (DCs) along with significant numbers of neutrophils and a very small numbers of platelets. Here, the importance of neutrophils is that they are early responders to microbial infections in lung, where they enter pulmonary lymphatics, traffic to draining LNs, and can form fibrin clots through the release of neutrophil extracellular traps (MacDonald et al., 2022). In contrast, no resident alveolar macrophages were detected in the bronchovascular collectors after in situ PKH26 dye labeling and LPS administration, despite an earlier report that these lung-resident phagocytes drain to lung LNs during the resolution of lung injury (Kirby et al., 2009), although it is possible they were missed due to the likely small numbers involved. Intriguingly, the authors also observed that significant numbers of immune cells (monocyte/macrophages and neutrophils) adhered and rolled on the luminal surface of the collectors in the direction of flow (see figure). This is a novel finding, as thus far such adhesive interactions have only been seen in initial lymphatic capillaries during intraluminal crawling of DCs (Collado-Diaz et al., 2022) and never within lymphatic collectors. Disappointingly, the authors could not determine how many immune cells had entered the collectors directly, rather than via upstream initial capillaries, as the high frame rates used for image acquisition precluded the capture of slower transendothelial migration events. It was also unclear whether the endothelial junctions in the bronchovascular collectors were conventionally zippered or more freely permeable, as diagnostic imaging with appropriate VE-cadherin or LYVE-1 reporters was not included.

Confirming the likelihood that interstitial migration of immune cells and entry to the pulmonary lymphatics is directed primarily by the chemokine CCL21 and its Gαi protein–linked receptor CCR7 (as, e.g., in skin), the authors showed that genetic deletion of the CCR7 gene or inhibition of signaling via pertussis toxin caused a marked accumulation of MHC II–positive immune cells within the bronchovascular cuff spaces and a massive reduction in their numbers within the collectors. Similar effects were seen with the small molecule inhibitor navarixin, although oddly, lung administration of CCR7 function-blocking mAbs did not prevent immune cell entry, but instead led to Fc receptor–mediated aggregation, an unexpected effect that was attributed to poor penetration of the lung interstitium by the inhaled antibody.

Complementing these studies of normal immune cell trafficking, the authors also used their imaging window to follow dissemination of intravenously injected fluorescent B16.F10 melanomas to the lung and visualized tumor cells, both within the bronchopulmonary lymph and, in some cases, directly adjacent to a collector, potentially in the act of lymphatic invasion. Moreover, they could see evidence of the host immune response in the form of phagocytes, many carrying tumor fragments, in the bronchovascular cuff spaces and adjacent lymphatics. Although they did not perform similar intravital imaging of orthotopically implanted lung tumors, one can anticipate future studies will enable the imaging of early events in lymphatic invasion/metastasis and analysis of the molecular mechanisms involved. Finally, to showcase the wider applicability of the stabilization window for intravital imaging of the vasculature in other less accessible internal organs, they demonstrated its use for visualizing lymphatics in the hilum of the liver and spleen and in the ventricular wall of the beating heart.

In summary, the intravital imaging technique developed by Cleary et al. (2025) is a significant advance that should greatly

facilitate further research into the roles of pulmonary lymphatics in normal and pathological lung function. In the first instance, the current findings already raise some basic questions. For example, do the bronchovascular collectors have a different junctional architecture to conventional tightly zippered collectors, and if so, how is this tailored to their functions within the lung? What is the significance of immune cell adherence and rolling within the pulmonary collectors? Might the endothelial cells participate in antigen presentation and immune tolerance via class I MHC and PDL1 expression in a similar manner to LN lymphatic endothelial cells (Rouhani et al., 2015)? In the context of lung pathology, what are the patterns of lymphatic trafficking for individual immune cell populations, including DCs, T effector cells, T regulatory cells, neutrophils, and monocyte/macrophages during the course of lung infection, inflammation, and injury in mice, and are these informative for human lung diseases? What are the dynamics within ectopic lung lymphoid structures such as the inducible bronchus-associated lymphoid tissues (iBALT) that can develop during inflammatory lung diseases, and what roles do they play in disease pathology? Can the new intravital imaging window be used to assess the potential therapeutic effects of blocking adhesion receptor, inflammatory cytokine, or chemokine function on lung pathology in models of asthma, COPD, or COVID-19, or models of primary lung tumor metastasis? One can anticipate that these and other important questions will soon be answered.

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