The Influence of Flow Mechanotransduction on Endothelial Cells in the Lymphatic Valve Sinus

Joshua Daniel Hall
University of Massachusetts Amherst

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The Influence of Flow Mechanotransduction on Endothelial Cells in the Lymphatic Valve Sinus

A Dissertation Presented

by

Joshua Hall

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

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May 2022

MECHANICAL ENGINEERING
The Influence of Flow Mechanotransduction on Endothelial Cells in the Lymphatic Valve Sinus

A Dissertation Presented

by

Joshua Hall

Approved as to style and content by:

Juan Jiménez, Adviser

Shelly Peyton, Member

Yubing Sun, Member

Sundar Krishnamurty, Department Chair

Mechanical and Industrial Engineering
ABSTRACT

The Influence of Flow Mechanotransduction on Endothelial Cells in the Lymphatic Valve Sinus

May 2022

JOSHUA HALL

B.S., WORCESTER POLYTECHNIC INSTITUTE

Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Juan Jiménez

Fluid flow in the cardiovascular and lymphatic systems influences the phenotype of endothelial cells that line the interior to the vessel via mechanotransduction. Geometric features in a vessel such as curvature, bifurcation, and valves promote heterogeneous fluid flow profiles, inducing a heterogeneous endothelial phenotype within a vessel region. Certain flow conditions are associated with vascular dysfunction, and diseases such as atherosclerosis preferentially develop in areas of flow disturbance. Lymphatic vessels are highly analogous to blood vessels, although lymphatic flow characteristics and its effect on lymphatic endothelial cells (LECs) via mechanotransduction have been comparatively less examined. The most significant geometric features that influence fluid flow in lymphatic vessels are bi-leaflet valves present in each lymphangion. In the current study, fluid flow was characterized in murine lymphatic collecting vessels using
fluorescent microparticles to examine the phenotypic difference of LECs exposed to physiological flow conditions in vitro. Fluid flow adjacent to the valve sinus was virtually static, while the wall of the midlymphangion experienced net-antegrade pulsatile flow with a small degree of retrograde flow. LECs were exposed to the midlymphangion flow waveform in a parallel plate flow chamber system and compared to cells cultured in static conditions. LECs exposed to static conditions demonstrated relatively increased expression of cell adhesion molecules via mRNA and protein quantification and leukocyte attracting chemokines via mRNA quantification. The increased expression of cell adhesion molecules and leukocyte attracting chemokines suggests that LECs in the sinus may adopt an inflammatory phenotype. Lymphedema is a notable condition that may result from valvular dysfunction and can significantly alter fluid flow and likely cause phenotypic changes in LECs via mechanotransduction. Characterization of the flow field in a vessel affected by lymphedema in a CLEC2-deficient mouse was found to be quasi-steady with loss of pulsatility. Lymphedema is characterized by changes in pressure, which independently influences endothelial cells (ECs). LECs were exposed in vitro to pulsatile or steady static pressure conditions without flow. Pulsatile and steady pressure chambers were constructed to emulate normal lymphatic pressure and lymphedema-like pressure waveforms, respectively. The effects of exposing LECs to steady versus pulsatile pressure of similar mean values have not previously been examined. This study demonstrated that LECs showed increased intercellular gap formation when exposed to steady pressure and steady flow, but not pulsatile pressure and pulsatile flow of similar mean values. Increased endothelial permeability in a lymphedema afflicted vessel could potentially promote lymph and antigen leakage, exacerbating the already impaired lymph transport, impairing the adaptive immune response, and inducing inflammation in the local interstitial tissue.
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Chapter

1.) Introduction

1.1) Lymphatic System Function

The lymphatic system is part of the circulatory system that performs several essential functions such as removing interstitial fluid from tissues, absorbing and transporting lipids from the digestive system, and trafficking immune cells (Figure 1). Lymphatic vessels are lined with lymphatic endothelial cells (LECs). The lymphatic system is composed of thin-walled lymphatic capillaries that drain extravasated interstitial fluid from most organs to the thoracic duct, which is then returned to the cardiovascular system. Lymph, the fluid that circulates through the lymphatic system, is milky white and protein-rich. It is similar in composition to blood plasma, which it is primarily derived from. Lymph is transported through lymph nodes, where it is cleaned by lymphocytes before returning to the bloodstream via the left subclavian vein. Lymph also transports bacteria and viral particles, which are killed in the lymph nodes. Dietary fats are absorbed via lymphatic capillaries known as lacteals in the villi of the small intestines. During digestion, fats are emulsified by bile and hydrolyzed by
lipase, and converted into a mixture of fatty acids and glycerides. These are then passed from the intestinal lumen into enterocytes. The lipids are then re-esterified to form triglycerides. Chylomicrons are formed by combining the triglyceride with cholesterol ester, phospholipids, and apolipoprotein B48. Chyle is a milky substance that is composed of chylomicrons that have passed into the lacteals. Lacteals then merge to form larger lymphatic vessels that deposit chyle in the thoracic duct where lymph enters the bloodstream at the subclavian vein.

**Figure 1:** Structural and molecular features of lymphatic vessels. Blind-ended lymphatic capillaries remove fluid and immune cells from the interstitial space. Capillary LECs (capLECs) harbour button-like intercellular junctions and discontinuous basement membrane (BM). Pre-collecting and collecting vessels efficiently transport lymph towards the draining lymph node due to zipper-like LEC junctions, continuous basement membrane, and intraluminal valves. Contractile smooth muscle cells surround collecting LVs. The selected molecular markers of capLECs, collecting (collLECs) and valve LECs (vLECs) are shown. From the draining lymph node (LN) the lymph is transported to the next LNs until it reaches the blood circulation. From Ref 1.

### 1.2) Lymphatic System Anatomy
The lymphatic system contains several categories of conduits: capillaries, pre-collecting vessels, collecting vessels, lymph nodes, lymphatic trunks, and ducts. The size of each vessel ranges from 10 µm to 2 mm in diameter. Lymph drains from capillaries to collecting vessels, which typically travel through several clusters of lymph nodes. Collecting vessels lead to trunks, which connect to lymphatic ducts. Lymphatic ducts then return lymph to the bloodstream, finishing the lymphatic circuit.

Lymphatic capillaries are blind-ended vessels that uptake interstitial fluid and particles. They are comprised of a single, typically nonfenestrated endothelial cell layer. Despite this, they generally have a more irregular and wider lumen size than blood capillaries and they possess an extremely attenuated cytoplasm, with the exception of the perinuclear region. Also, unlike blood capillaries, lymphatic capillaries do not have a basal lamina and are not encircled by pericytes. Although tight junctions and adherens junctions are common in blood vessels, they are seen in much lower frequency in lymphatic capillaries. While junctions in blood capillaries primarily serve to maintain firm cell-cell adhesion to connect adjacent endothelial cells (ECs) over entire cell boundaries, in lymphatic capillaries, they function as focal points of adhesion instead. LECs in capillaries are also closely connected to the surrounding tissue by strands of elastic fibers\(^3,4\). These filaments anchor the abluminal surface of the cells to connective tissue, adhering the lymphatic endothelium to the extracellular matrix fibers. LECs in capillaries are also characterized by numerous invaginations and cytoplasmic vesicles that mediate transendothelial transport of molecules into the lumen\(^5-7\).

Lymphatic capillaries have unique primary valves that allow interstitial fluid to flow into them but not out\(^8\). Primary valves are composed of ECs that form the walls of
lymphatic capillaries that open inwards when interstitial fluid pressure is greater than inside the lymphatic capillary (Figure 2). When pressure is greater inside the capillary, the ECs adhere more closely to prevent backflow. Lymph flow in lymphatic capillaries is primarily propelled via extrinsic mechanisms.

After exiting the lymphatic capillaries, lymph drains into pre-collecting lymphatic vessels. Pre-collecting vessels contain bicuspid one-way valves similar to collecting vessels, however, unlike collecting vessels, pre-collecting valves are located at irregular intervals. Collecting lymphatic vessels contain smooth muscle and are not tethered to the extracellular matrix. Collecting vessels contain intraluminal valves that are also essential to regulate net antegrade flow, as lymph propulsion is irregular and is characterized by a low flow rate. Collecting vessels are composed of functional units between valves called lymphangions. Unlike the blood circulatory system, the lymphatic system has no central pumping organ. To regulate flow, mammals rely on both intrinsic and extrinsic forces to generate lymph propulsion. The extrinsic mechanism consists of skeletal muscle contractions that occur during skeletal muscle contractility, arterial pulsations, venous pressure fluctuations, respiration, and peristaltic movement of the intestine. While larger collecting vessels are also affected by extrinsic mechanisms, they are primarily controlled by intrinsic mechanisms regulated by the contraction of smooth muscle cells around the vessel. Smooth muscle contractions involved in lymphatic pumping are activated by high nitric oxide concentrations, which are regulated by wall
LEC expression of eNOS\(^\text{12}\).

All lymphatic collecting vessels pass through a series of lymph nodes where lymph is filtered of dead cells, bacteria, and foreign matter\(^\text{10}\). Antigens are also presented to lymphocytes in lymph nodes to mediate the adaptive immune response. Collecting vessels situated upstream of nodes are afferent vessels, while those downstream are efferent. Purified lymph exits nodes via efferent vessels, where it enters lymphatic trunks. Lymphocytes present in nodes may also exit in efferent lymph during an adaptive immune response.

Lymphatic trunks are much larger than collecting vessels, as large as 2 mm in diameter in humans. The trunks then drain into the final branch of the lymphatic system, the thoracic duct. The thoracic duct passes through the aortic opening of the diaphragm.
and drains fluid back into the bloodstream via the junction of the left jugular and subclavian vein.

1.3) Lymphatic Valve Function

As valve function plays an important role in lymphatic vessel flow, many lymphatic related diseases occur from valve dysfunction. Lymphatic valves are primarily composed of two intraluminal leaflets that are each formed by two layers of LECs separated by an extracellular matrix rich core. Due to the importance of valve function in lymph flow, the physiology of valves and their effect on fluid dynamics is a large area of research interest. Ultrasound technology has elucidated the physics of venous and lymphatic valve operation. Lymphatic valve operation has been suggested to be similar to that of venous valves, which have been much better studied. Four distinct phases occur during the valve cycle: opening, equilibrium, closing, and closed (Figure 3). When distal pressure is greater than proximal pressure, the closed leaflets open towards the vessel wall and allow flow to travel. At peak openness during the equilibrium phase, the cusps form a narrowing of the lumen about 35% smaller than the distal lumen. This increases pressure and causes flow acceleration. After opening, the leaflets remain suspended in the flowing stream and undergo self-excited oscillations that resemble the
flutter of flags in the wind. Flow separation occurs during the equilibrium phase at the leading edge of the cusp with flow reattachment at the wall of the sinus. At the wall of the sinus, the flow bifurcates into two streams at each valve cusp. A portion of the flow is directed into the sinus pocket behind the valve cusp. This forms a vortex along the cusp prior to re-emerging in the main stream of the vessel. The closing phase generally consists of the leaflets moving synchronously toward the center, although in some instances due to lower Reynolds numbers, the lymphatic valves do not fully close\textsuperscript{16–18}. During the closed phase, the valves remain closed and flow is halted.

1.4) The Influence of Mechano-transduction on Vasculature
ECs that compose the innermost lining of vascular walls are constantly exposed to frictional pulsatile wall shear stress (WSS), hydrostatic pressure, and cyclic stretch induced by flowing blood or lymph (Figure 4). A growing body of evidence supports ECs recognize and adapt to different WSS, amplitudes, and patterns via mechanotransduction\textsuperscript{19,20}. Mechanisms located in the cell membrane such as receptor tyrosine kinases, G protein-coupled receptors, integrins, and glycocalyx transduce physical stimuli into intracellular signaling, gene expression, and protein expression. Mechanotransduction allows ECs to adapt to their environment in response to mechanical cues. For instance, lymphangion contraction frequency is positive correlated with transmural pressure, allowing lymphatics to adapt to higher lymph loads\textsuperscript{21,22}. Under pulsatile laminar flow, ECs have been observed to undergo cytoskeletal remodeling and align parallel to the fluid flow\textsuperscript{23}. However, endothelial mechanotransduction can be a double-edged sword, as certain stimuli are believed to cause endothelial dysfunction and

\textbf{Figure 4:} Blood and lymph endothelial cells are constantly exposed to fluid flow, which applies waveforms of wall shear stress, circumferential stress, and hydrostatic pressure. These flow mediated stimuli promote an endothelial biological response via mechanotransduction.
initiate vascular disease\textsuperscript{19}. Although the effects of flow on cardiovascular development and disease are better understood, the lymphatic system is analogous and requires WSS mediated mechanotransduction for normal development. WSS has been established to be necessary for the expression of genes necessary for valve development in LECs (Figure 5). LECs highly expressed the genes necessary for valve development such as Foxc2, Cx37, Itga9, and Gata2 under pulsatile WSS, but insufficiently expressed them in static conditions in vitro \textsuperscript{24,25}. Abolishing lymph flow in mice via Clec2 knockdown similarly impaired valve development.

The WSS magnitude within lymphatics is significantly lower than that in blood vessels\textsuperscript{26}. Additionally, LECs have been reported to respond to lower WSS values than BECs\textsuperscript{27,28}. The mechanisms that allow LECs to respond to lower WSS are not well

\textbf{Figure 5}: Mechanotransduction mediated lymphatic valve morphogenesis. Flow regulated Foxc2 and PROX1 regulate the expression of connexin37, which controls calcineurin/NFAT signaling. Cx37 and calcineurin mediate the development and assembly of lymphatic valves.
understood, although the LEC-specific receptor VEGFR3 has been suggested to form a sensitive mechanosensory complex. Mechanotransduction has also been demonstrated to occur in bone, cartilage, and skeletal muscle, and the cellular mechanisms are largely conserved.

1.5) Heterogeneous Endothelial Phenotype is the Result of Vessel Geometry

Although one would expect ECs in an individual’s vessels to have a generally similar phenotype, vascular diseases have been well documented to selectively develop in specific vessel geometries. For instance, atherosclerosis frequently develops adjacent to vessel bifurcations and regions of small radii of curvature where blood flow separates, but rarely develops in linear vessel regions where blood flow does not separate. ECs in arterial sites where blood flow separates consistently adopt a dysfunctional phenotype and are at a higher risk of developing diseases. In a uniform cylindrical vessel, the WSS, hydrostatic pressure, and circumferential stress experienced by all ECs would be theoretically identical. However, actual vessels contain many irregular geometries, which
cause the endothelium to be exposed to spatially dependent heterogeneous fluid flow, and thus express a heterogenous endothelial phenotype. When considering arterial bifurcations and curved segments, these vascular regions experience flow separation, which can expose the local ECs to a flow waveform characterized by low WSS and a degree of flow reversal. ECs in straighter vessel geometries, such as the common carotid artery, generally do not develop atherosclerosis and are exposed to a fully antegrade flow waveform with higher mean WSS.

Considering lymphatic vessels, the recirculation zone of lymphangion is characterized by flow separation and recirculation, a significantly different flow condition compared to the rest of the vessel. Heterogeneous flow fields caused by vascular geometric features have been well established to induce a differential EC phenotype. Atherosclerosis and chronic inflammation have been well established to occur in ECs adjacent to vessel curvature and bifurcation where flow separation occurs (Figure 6). One study has observed a heterogeneous endothelial phenotype within rat lymphangions \(^{12}\). Fluorescent confocal microscopy was used

**Figure 7:** The low shear stress in the lymphangion sinus induces an altered endothelial cell phenotype compared to endothelial cells in the rest of the vessel. ENOS, a highly flow sensitive endothelial gene, was previously found to be less expressed in the sinus.
to examine LEC expression of eNOS, a gene known to be highly flow-sensitive, within a lymphangion. eNOS expression was found to be low in the sinus, but much higher in the rest of the lymphangion. eNOS is a highly shear sensitive gene, suggesting that other flow sensitive genes share a similar expression profile in the sinus. The flow conditions in the lymphangion sinus may have a significantly altered phenotype compared to LECs in the rest of the vessel (Figure 7).

1.6) Effects of Stimuli Pulsatility on Mechanotransduction in Endothelial Cells

Mechanotransduction mediates mRNA and protein expression of certain genes, thus, certain physical stimuli can promote endothelial dysfunction. For instance, certain flow waveforms may upregulate pro-inflammatory genes (such as TNF-α) or downregulation of genes necessary for normal function\(^{31}\). To elucidate endothelial function, in vitro studies emulate various physiological flow conditions to study endothelial response. However, many in vitro studies investigating mechanotransduction in vasculature prioritize replicating the magnitude of stimuli such as WSS rather than the waveform. A growing body of evidence supports that a change in waveform period or even loss of pulsatility can lead to endothelial dysfunction and clinical complications even when the level of WSS applied is similar. Human umbilical vein endothelial cells (HUVECs) had significantly different expression levels of EDN1, PTGIS, and SOD1 when exposed to steady flow and pulsatile flow at similar mean values\(^{32}\). ECs a dramatic difference in NF-κB expression Oftentimes, deviation from normally experienced physiological physical stimuli in mechanosensitive cells leads to cellular dysfunction via mechanotransduction.
WSS pulsatility also plays a significant role in lymphatic function. The intrinsic contractions of lymphangions are heavily mediated by the mechanical forces imposed on lymphatic vessels. As lymph formation can vary widely depending on external factors such as digestion and respiration, the rate of lymphatic vessel contraction can adapt in response to mechanical cues caused by increased lymph load. Applying steady WSS to a cannulated collecting vessel had a magnitude correlated inhibitory effect on contraction frequency. However, this inhibition was reversed when the applied WSS oscillated, even at similar mean WSS and controlling for hydrostatic pressure. These results demonstrate that not only can lymphatic vessels discriminate between the frequency of WSS, but steady WSS can lead to lymphatic dysfunction in certain cases.

Additionally, endothelial response to stretching is also sensitive to waveform. Differential gene expression was observed in blood endothelial cells exposed to cyclic stretch and constant static stretch at similar mean values. However, no study has directly compared the response of endothelial cells to cyclic and steady hydrostatic pressure of similar mean values.

1.7) Effects of Pulsatile Pressure Vs Steady Pressure on Mechanotransduction in Non-Endothelial Cell Types

Like WSS, hydrostatic pressure experienced by most cells is generally pulsatile in vivo. Accordingly, mechanosensitive cells respond differently to steady pressure than pulsatile pressure. Non-endothelial mechanosensitive cell types have been demonstrated to grow normally under pulsatile pressure but adopt a dysfunctional phenotype under
steady pressure. Considering that mechanosensing is widely conserved across mechanosensitive cell types, ECs possibly respond differently to steady pressure and pulsatile pressure of similar mean values. Osteocytes and chondrocyte cultures, which normally experience pulsatile pressure in vivo, responded differentially to pulsatile pressure and steady pressure of similar mean values\textsuperscript{40,41}. Osteocytes in bone culture exposed to steady pressure initiated catabolic effects on mineral metabolism, while exposing osteocytes to pulsatile pressure promoted anabolic effects. Supporting these findings, a study that exposed chondrocytes to either atmospheric hydrostatic pressure, steady pressure (4MPa), or pulsatile pressure (0.1-0.4MPa) found that steady pressure suppressed cartilage development by substantially reducing chondrocyte expression of collagen II, aggrecan, and sox-9. In all conditions, the column of media contributed a small amount of hydrostatic pressure. Interestingly, not only did the pulsatile pressure waveform promote cartilage development the most despite having the same peak pressure magnitude as the steady pressure condition, chondrocytes exposed to atmospheric pressure showed significantly higher cartilage development than those exposed to steady pressure. These findings demonstrate that not only is steady pressure not a substitute for pulsatile pressure when examining pressure mediated mechanotransduction, but steady pressure can also induce opposite effects as pulsatile pressure even at the same magnitude. As mechanosensing is highly conserved between mechanosensitive cell types, these findings may have implications on ECs. Hydrostatic pressure experienced by endothelial cells is generally physiologically pulsatile, which raises the possibility that ECs may behave abnormally under steady pressure. Steady pressure assays are inherently limited in emulating physiologically relevant hydrostatic pressure. Endothelial cells have
been demonstrated to be sensitive to flow pulsatility, and flow is inherently driven by pressure gradients. Therefore, hydrostatic pressure waveform sensing may contribute to changes in endothelial response to various flow waveforms that are typically attributed to shear sensing. ECs need to be exposed to steady pressure and pulsatile pressure of similar mean values to know if ECs respond to pressure waveforms similarly to chondrocytes and osteocytes.

1.8) Effects of Pulsatile Pressure Vs Steady Pressure on Mechanotransduction in Endothelial Cells

Endothelial response to hydrostatic pressure has been significantly less examined than to WSS. Of the studies that have examined the endothelial response to hydrostatic pressure, most have neglected the influence of pulsatility, and have compared ECs exposed to atmospheric pressure and steady hydrostatic pressure steady pressure. One study found that HUVECs exposed to 200mmHg steady pressure had increased permeability and increased actomyosin formation. Another found that HUVECs exposed to either 50mmHg or 100mmHg steady pressure had increased myosin light chain phosphorylation and cytoskeletal contractility. This contractility response was attenuated by the application of blebbistatin, a myosin II inhibitor, suggesting that actomyosin activation is primarily mediating the contractile response. Fewer studies have compared the effects of exposing ECs to cyclic pulsatile pressure and atmospheric pressure. HUVECs exposed to 60/20mmHg 1Hz sinusoidal pulsatile pressure experienced no change in permeability compared to those exposed to atmospheric
pressure. In the same study, HUVECs exposed to 140/100mmHg pulsatile pressure actually had decreased permeability when exposed to pulsatile pressure. These two independent studies suggest that steady pressure promotes endothelial actomyosin contractility and loss of barrier function, while pulsatile pressure promotes a normal phenotype. However, no study has directly compared the biological effects of exposing ECs to physiologically relevant pulsatile pressure and steady pressure of similar mean values. Additionally, no study has examined the effects of applying hydrostatic pressure to LECs, which may respond to lower pressure magnitudes compared to BECs as they do to WSS.

From a clinical perspective, a change in vascular hydrostatic pressure magnitude resulting from complications like arterial hypertension can promote endothelial dysfunction. However, changes in vessel pressure waveform period or even total loss of pulsatility could potentially lead to a differential endothelial response even when the pressure magnitude is within normal parameters. Therefore, elucidating the effects of hydrostatic pressure pulsatility on ECs has potentially translatable implications. Lymphedema, for instance, is characterized by increased interstitial pressure. Increased hydrostatic pressure inherently always increases lymph load, flow velocity, and hydrostatic pressure. Several studies have measured increased intraluminal lymphatic pressure as a result of lymphedema. Changes in intraluminal pressure and load have implications on lymphangion intrinsic contractility as well, affecting hydrostatic pressure waveform.

Complicating this research, any assay that is used to emulate fluid flow exposes cells to WSS, creating an additional variable. WSS is sensed by different cellular
mechanisms and thus may potentially overshadow or compensate for the endothelial response to altered hydrostatic pressure. For instance, studies have demonstrated that elevated WSS promotes barrier function in ECs, while other studies have shown that elevated steady pressure induces loss of barrier function. When investigating hydrostatic pressure-mediated mechanotransduction, isolating pressure magnitude, waveform, and WSS as separate variables is critical. Elucidating static pressure mechanotransduction could have significant implications in treating pressure-related complications like arterial hypertension and lymphedema.

1.9) Endothelial Barrier Function

Physical barriers are essential to separate different compartments for development and maintenance in a multicellular organism. Barrier function between cells is primarily achieved by junctional complexes between adjacent cells composed of cell-cell junction proteins. Animals have four general types of cell-cell junctions: gap junctions, desmosomes, adherens junctions, and tight junctions. Tight junctions are the most significant contributors of endothelial barrier function. The endothelium forms a barrier between the vessel lumen and tissue that regulates the flow of ions, substances, and fluid into and out of a tissue. Endothelial barrier dysfunction is characterized by increased transendothelial flux, which is a serious problem in most vessels. Barrier dysfunction occurs during many inflammatory conditions such as trauma, sepsis, diabetes, and cardiovascular disease. Endothelial barrier dysfunction is generally characterized as the
result of cell-cell junction disruption in response to inflammatory mediators such as thrombin.

1.10) Lymphatic Collecting Vessel Permeability

Historically, collecting lymphatics were considered to be relatively impermeable to solutes and solely functioned to transport lymph. Findings have demonstrated that collecting vessels are considerably permeable and suggested that regulation of collecting vessel permeability is important to lymphatic function. Collecting vessels in rat mesenteries were found to be similarly permeable to venules in flux of macromolecules like albumin. Additionally, transport of macromolecules across the collecting lymphatic endothelium is coupled to water flux and permeability was a function of hydrostatic pressure. Although physiological role and mechanisms of flux in collecting vessels need to be further elucidated, hyperpermeability is associated with deleterious effects. Lymph transport is impaired during increased collecting vessel permeability, as extravasated lymph becomes interstitial fluid that must reenter lymphatic capillaries. As lymph

Figure 8: Lymphatic endothelial hyperpermeability inhibits lymph transport and causes antigen leakage into surrounding interstitial space.
transport is an essential role of the lymphatic system, several lymphatic functions are impaired when transport is inhibited. Antigens and antigen presenting cell transport to lymph nodes is impaired, weakening the adaptive immune response. Impaired transport results in accumulation of interstitial fluid. As lymphedema is a disease characterized by lymph accumulation, collecting vessel permeability is potentially implicated. Although flux in collecting vessels afflicted by lymphedema has not been measured, increased permeability would likely exacerbate the poor lymph transport that is characteristic of the disease. In rats, increasing permeability in rat collecting vessels reduced capillary interstitial fluid uptake, reducing lymph transport\textsuperscript{50}. Additionally, increased permeability in murine collecting vessels induced local inflammation of surrounding tissue\textsuperscript{51}. Hyperpermeability in LECs could cause antigen leakage that would impair adaptive immunity, reduce lymph transport, and induce inflammation in local interstitial tissue (Figure 8). Regulation of permeability in collecting vessels and its role in lymphatic function requires further study.

1.11) Myosin Light Chain Kinase Dependent Permeability

Apart from downregulation of junction proteins, cytoskeletal tension causes retraction at cell-cell interfaces, which causes endothelial gap formation and disrupts barrier function. An important mechanism of endothelial hyperpermeability is increased myosin light chain kinase (MLCK) activity, which induces cytoskeletal tension via phosphorylation of myosin light chain (MLC)\textsuperscript{52,53}. Actomyosin stress fiber formation mediated cytoskeleton contraction causes cell membrane retraction and intercellular gap
formation, promoting transendothelial flux (Figure 9). Conversely, myosin light chain phosphatase (MLCP) dephosphorylates MLC, which relieves cytoskeletal tension. Either an increase in MLCK or a decrease in MLCP activity can result in increased stress fiber assembly and consequent hyperpermeability. MLCK mediated hyperpermeability occurs in numerous vascular diseases such as sepsis\textsuperscript{54}, trauma\textsuperscript{55}, burns\textsuperscript{56}, and chronic inflammatory conditions. MLCK mediated endothelial hyperpermeability has many consequences: alveolar flooding in lungs\textsuperscript{57}, pancreatitis\textsuperscript{58}, and most significantly, atherosclerosis\textsuperscript{39}. and accordingly, strategies to attenuate MLCK activity have significant clinical potential\textsuperscript{60}. Inhibitors such as ML-7 have been demonstrated to attenuate MLCK mediated hyperpermeability in cell culture and animal models. Atherosclerosis, a disease originating from increased LDL extravasation, can be alleviated in rats by administration of ML-7\textsuperscript{61}. Interestingly, complete deletion of MLCK in mice attenuated endothelial hyperpermeability but caused no significant impairment to vascular function\textsuperscript{62}. Inhibiting endothelial MLCK in human patients to treat vascular diseases is challenging because MLCK is widely expressed and performs many functions in other cell types, leading to significant side effects. Currently, no MLCK inhibitors have been approved for use in humans, although future advances in endothelial-specific targeted drug delivery could potentially allow these inhibitors to treat endothelial hyperpermeability without affecting other cell types\textsuperscript{63}. More studies are needed to identify instances of MLCK mediated endothelial hyperpermeability to elucidate the basic mechanism of vascular dysfunction and develop therapies.
1.12) Lymphedema

Lymphedema is a progressive and debilitating condition that is characterized by interstitial swelling of excess fluid resulting from mechanical insufficiency of the lymphatic system\textsuperscript{[64]}. Lymphedema is classified as primary or secondary. Primary lymphedema occurs as the result of innate dysfunction in lymphatics, such as lymphatic agenesis or hypogenesis. Genetics play a complex role in primary lymphedema, and currently, at least 23 associated genes have been identified\textsuperscript{[65]}. In many cases, defective secondary valves are the underlying cause of primary lymphedema\textsuperscript{[66]}. Secondary lymphedema is significantly more common and results from lymphatic vessels becoming damaged or obstructed. Secondary lymphedema may be the result of inflammatory, surgical, iatrogenic, filarial infection, or traumatic damage. In developed countries, secondary lymphedema is most commonly caused by the removal of or lymph nodes during cancer treatment\textsuperscript{[67]}. Additionally, a rapidly increasing number of obese patients have developed

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure9}
\caption{a) Endothelial cells under normal myosin light chain (MLC) phosphorylation equilibrium between myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP). (b) Increased MLC phosphorylation increases endothelial contractility. The related membrane contraction causes gap formation and decreased barrier function.}
\end{figure}
lymphedema with no other underlying causes\textsuperscript{68,69}. As the obesity epidemic expands, lymphedema cases could similarly increase, emphasizing the importance of studying the physiological consequences of lymphedema. Currently, no studies have accurately quantified the flowrate inside diseased lymphedema vessels. Measuring the flow rate inside of lymphatic vessels is inherently challenging because not only due to the small size scale of lymphatics, but also the diameter and flow velocities vary greatly within an individual. Therefore, the vessel diameter and flow velocity must be simultaneously measured to observe flowrate. Additionally, comparisons between vessels may be limited due to variances. Some studies have induced edema conditions in animals and observed increased lymph flow rates in healthy collecting vessels\textsuperscript{70–72}. However, diseased collecting vessels may behave differently in similar circumstances. The flow conditions within lymphedema vessels need to be elucidated to examine LECs change via mechanotransduction.

1.13) Interstitial and Endolymphatic Pressures in Lymphedema Vessels

In order for lymph to return to the venous system, lymph must overcome the adverse pressure difference. Fluid in interstitial spaces is typically less than atmospheric pressure and is roughly 20 cmH\textsubscript{2}O in humans\textsuperscript{73}. Lymphangions overcome adverse pressure differences via smooth muscle contractions to increase inlet pressure upstream of the valve. Rat mesenteric lymphatic contractions failed to open the valve when transverse pressure gradients exceeded an average of 11 cmH\textsubscript{2}O\textsuperscript{70}. A chain of lymphangions is able to overcome greater pressure gradients, as collecting vessels are able to sense transmural pressure and adjust contractility to compensate for the increased...
lymphatic load. In human legs, lymphangions in series were able to drive flow against approximately 40 cmH₂O pressures. The relevant transmural lymphatic pressure values measured in cannulated murine collecting lymphatics reached a maximum systolic pressure ranging from 5.4 to 8.8 cmH₂O. Lymphatic pressure is highly varied at different anatomical regions due to hydrostatic pressure. The pressure measured in the subcutaneous lymphatics in a human leg while standing were recorded to peak at an average of 61 cmH₂O. Lymphedema can develop when adverse pressure gradients increase to the point when lymphatics become insufficient to drive lymph flow forward. While the maximum pressures that collecting lymphatics can generate has been measured in canulated lymphatic vessels, this does not necessarily mean that adverse pressure gradients greater than this measured will result in complete lymph-stasis. Although accumulation of interstitial fluid will occur, the resulting interstitial pressure increase will contribute to intrinsic lymphatic pressure in vivo, thus overcoming the adverse pressure gradient. Thus, although transmural pressure will be much greater, lymph transport will generally still occur in lymphatic vessels that are not totally occluded. However, interstitial pressure will be the primary driver of flow as lymphangion contractions become less effective at higher pressures.

Lymphedema is a highly varied condition, and pressure values can vary significantly between patients and vessel regions. Pressure is difficult to measure in vivo due to the size scale of lymphatics and the technical limitations of measurement techniques. A common technique used to measure endolymphatic pressure is the application of a pressure cuff. The lymphatics of mouse tails with lymphedema have been measured using this method. Collecting lymphatics in the tails of control mice had
pressures of roughly 11.34 cmH₂O, while mice with lymphedema had as high as 44.95 cmH₂O⁷⁷. However, while applying a pressure cuff is simple and commonly used, it is less accurate than more sophisticated methods.

Pressures in human lymphatic micro-vessels have been measured in patients with varying stages of lymphedema using fluorescent microlymphography⁷⁸. In healthy individuals, interstitial pressure was an average of 0.88cmH₂O, while it rose to 9.78 cmH₂O in individuals with advanced lymphedema. Healthy individuals had an endolymphatic pressure of 5.69 cmH₂O. Micro-vessels with early-stage lymphedema had endolymphatic pressures of roughly 11.14 cmH₂O. Endolymphatic pressures in micro-vessels measured in instances of moderate lymphedema were approximately 13.64 cmH₂O. The highest pressure in patients with severe lymphedema, characterized by incompressible fluid interstitial fluid buildup, had endolymphatic pressures of approximately 14.95 cmH₂O.

In cases of secondary lymphedema, some studies have measured endolymphatic pressure via lymphatic congestion lymphoscintigraphy. As secondary lymphedema is most common in breast cancer patients after lymph node dissection, examining lymphatic pressure in this context is highly relevant to clinical medicine. Women without lymphedema had upper lymph endolymphatic pressures of roughly 31.26 cmH₂O⁷⁹. Women that developed secondary lymphedema had pressures of approximately 54.38 cmH₂O in the same region.

Endolymphatic pressure values measured in human patients are still limited due to the technical challenges of ethically obtaining accurate measurements in lymphatic vessels of living patients. Additionally, the mechanics of the lymphatic system has been
significantly less examined than the cardiovascular system. Furthermore, as most studies measure lymphatic flow in the context of sedated and stationary animals and cannulated vessels, the contributions of extrinsic muscle contractions are not involved. Lymph flow is a complex and dynamic process that is driven by many mechanisms. Thus, obtaining accurate physiologically relevant flow and pressure waveforms is inherently challenging. Nonetheless, as medical imaging and flow quantification techniques improve, the mechanics of fluid flow in lymphatics can be characterized in greater detail to better understand the function of the lymphatic system and study implications of lymphedema in vitro.

1.14) Crosstalk Between Lymphatic Endothelial Cells and Leukocytes or Circulating Tumor Cells

The mechanism of tumor cell extravasation via the endothelium is very similar to the mechanism of leukocyte extravasation. Circulating tumor cells initially bind to cell adhesion molecules on the endothelium such as P-selectin, CD44, or E-selectin with ligands present on the surface of the tumor cell to initiate the rolling phase. Next, the circulating tumor cell is brought to a complete stop at the adhesion step by cell adhesion molecules such as VCAM-1 on the endothelium binding to ligands on circulating tumor cells. However, a major difference between leukocyte diapedesis is that tumor cells damage the endothelium when passing through.
Circulating tumor cells (CTCs) that successfully extravasate and form a metastatic lesion in a distant site are disseminating tumor cells (DTCs), although very few CTCs become DTCs. One study found that a mere 0.02% of injected CTCs became DTCs\textsuperscript{84}. The reason certain CTCs become DTCs remains incompletely understood. Traditionally, lymphatic vessels were believed to play a passive role in cancer metastasis, and that an increased number and size of peritumoral lymphatic vessels might allow increased cancer cell intravasation. However, an increasing body of evidence suggests that crosstalk between both CTCs and LECs can promote a pro-metastatic microenvironment necessary for CTC extravasation, survival, and proliferation (Figure 10). Organ-specific metastasis has been well documented in several cancer types that metastasize via the lymphatic system\textsuperscript{85,86}. Breast cancers and melanomas have been observed to metastasize at distant lymph nodes, bone marrow, lung, and liver sites, despite that these regions are distant from the primary tumor site, no direct vascular routes are present, and metastases in other en route tissues are rare\textsuperscript{87–89}. Targeted metastasis is a complex process that is dependent on both the phenotype of the involved CTCs and LECs. Similar to an inflammation response, the lymphatic endothelium must be primed for diapedesis. CTCs bind to LECs that highly express cell adhesion molecules such as LYVE1, VCAM-1, and certain integrins. CTCs, like leukocytes, are also influenced by endothelial-secreted cytokines that promote adhesion and diapedesis. LECs have been observed to secrete CCL21, CXCL12, and CCL27, which are significant mediators in CTC extravasation\textsuperscript{90,91}. CTCs that express cytokine receptors have increased lymphatic metastatic potential. For instance, in mice, CCR7\textsuperscript{+} breast cancer and melanoma cells metastasize to lymph nodes
at a significantly higher rate than CCR7− CTCs\textsuperscript{86,92}. This suggests that CCR7 specific cytokines natively accumulate in lymph nodes, providing a suitable microenvironment for certain cancer types. Tumor cells may also secrete growth factors received by LECs that cultivate a pro-metastatic microenvironment. For instance, tumor cells expressed VEGF-C, which binds to LEC expressed VEGFR-3 and increases pumping and lymph flow. The increased lymph flowrate increases VEGFR-3 expression, which is known to be flow regulated, causing a positive feedback loop.

The mechanisms that certain lymphatics become pro-metastatic need to be further investigated. Chronic inflammation of tissue is associated with increased metastasis because inflammation induces upregulation of metastatic mediators like chemokines and adhesion molecules in LECs and lymphangiogenesis in draining lymph nodes\textsuperscript{93}. However, certain LECs may have a pro-metastatic phenotype during healthy function, leading to region-specific metastasis. Although the mechanisms that result in pro-

\textbf{Figure 10:} Endothelial cells engage in crosstalk with cancer cells and leukocytes that promotes extravasation. Endothelial crosstalk is complex and implicates many genes and molecules. Chemokines and adhesion molecules are significant mediators of extravasation via inflammation and cancer metastasis.
metastatic niches are likely complex, evidence suggests that mechanotransduction is a contributing factor. The significance of WSS in CTC metastasis is becoming increasingly recognized, although it has been primarily investigated in blood vessels. Some evidence suggests that WSS may be playing an important role in lymphatic metastasis. CCL21, a chemokine that attracts CXCR4\(^+\) and CCR7\(^+\) CTCs, is upregulated by the lymphatic endothelium in response to increased lymphatic flow.  

1.15) In-Transit Metastasis

Metastatic circulating tumor cells (CTCs) in the lymphatic system typically extravasate in the lymph nodes or ultimately the cardiovascular endothelium. Thus, the majority of studies examining lymphatic metastases have focused on lymph node metastases. However, metastatic tumors referred to as in-transit metastases (ITMs) have been recently found to develop in lymphatic vessels between the primary tumor and

**Figure 11:** (a) Lymphatic metastases are conventionally thought to occur when circulating tumor cells from a primary tumor metastasize in the sentinel lymph node and subsequently the secondary lymph nodes. (b) In-transit metastases occur when circulating tumor cells that metastasize in lymphatic vessels between the primary tumor and lymph nodes.
the draining lymph node (Figure 11). ITMs are a serious complication because dormant CTCs can form ITMs after a primary tumor and draining lymph nodes have been removed. Melanoma is the most common originator of ITMs, but carcinomas have also been found to form ITMs in certain cases. These cancer types have all demonstrated a high degree of organ-specific metastatic potential and engage in crosstalk with LECs. The presence of such metastases has suggested that the lymphatic endothelium potentially provides a protective microenvironment for tumor cell survival, particularly CTCs that express a stem cell-like phenotype. One study found that CXCR4+ melanoma cells, that also express the CD133 stem cell marker, were in proximity to CXCL12-producing lymphatic vessels in the metastatic lymph nodes and lungs. This study also found that CXCR4/CD133+ cells had higher metastatic activity than CXCR4+/CD133+ cells. The reasons why certain lymphatic vessels secrete higher concentrations of CTC attracting chemokines is not fully understood. As CTC-LEC crosstalk appears to play a significant role in ITM formation, studies need to elucidate the microenvironment in draining lymphatic vessels. Additionally, the potential role of mechanotransduction in cultivating a pro-metastatic niche in lymph vessels needs to be examined.

1.16) Influence of Lymphedema on Cancer Metastasis

Patients who undergo axillary lymph node dissection for breast cancer have roughly a 14% chance of developing lymphedema. Additionally, lymphedema has been positively associated with the development of certain rare cancer types, such as Stewart-Treves and Kaposi’s syndrome, that do not develop in healthy lymphatics.
Rare cancers like Stewart-Treves are viewed as exotic and generally only examined in the context of novel case studies. Roughly 0.07-0.45% of patients with lymphedema develop Stewart-Treves after five years post lymph node dissection. Thus, the chance of developing a lymphedema-specific rare cancer is generally considered to be trivial and not a significant complication of lymphedema. However, while these types of lymphedema-specific rare cancers have been proven to develop independently of a patient’s primary cancer, lymphedema likely also affects the chance of all cancer metastasis recurrence. As most instances of lymphedema result from cancer intervention, the contribution of lymphedema to cancer recurrence is difficult to determine, and elucidating the relationship between lymphedema and cancer metastasis has received little attention.

The longstanding presumption has been that the immunocompromising effects of lymphedema promote a permissive microenvironment for metastatic tumor development. A 2018 study demonstrated that mice with induced lymphedema developed cancer metastases at a greater rate than control mice. Furthermore, immunodeficient mice showed no difference in metastasis development regardless of the presence of lymphedema, demonstrating that immunocompromise is likely the primary mechanism of lymphedema that promotes cancer metastases. Statistical examination of the rate of cancer metastasis in lymph node dissection patients who develop lymphedema compared to those who do not would provide crucial insight on this phenomenon. However, no longitudinal study to date has examined the rate of cancer metastasis development in patients with lymphedema compared to patients with normally functioning lymphatics.
Although no clinical studies have directly compared cancer recurrence in patients who develop lymphedema and those who do not, some researchers have examined if the manual treatment of lymphedema promotes metastasis. Some concern exists that physical agitation from manual massage of edematous tissue may both increase the dissemination of circulating tumor cells into lymph and the increased lymph transport will deliver metastatic tumor cells to downstream lymph nodes at a higher rate. One study examined the rate of metastasis in patients post lymph node dissection who either developed no lymphedema or who developed lymphedema and were treated by the manual lymphatic drainage technique (MLD) \(^{106}\). This study found no difference between cancer recurrence in either group. Unfortunately, while this study did not include a cohort of patients with untreated lymphedema, the results potentially support the notion that immunocompromise via lymphostasis is the driving mechanism of increased metastasis. MLD may have attenuated adaptive immunity via rescuing lymph transport, preventing a potential increase in metastasis. Hopefully, future clinical cohorts can definitively answer this question. If confirmed, techniques to promote lymphatic drainage may be viewed as essential therapies to reduce cancer recurrence.

**1.17) Potential Mechanosensitive Gene Targets that Promote Lymphatic Cancer Metastasis**

Stewart-Treves syndrome the most notable rare cancer that develops resulting from lymphostasis is characterized as the development of angiosarcomas occurring in chronic lymphedema \(^{107–109}\). While Stewart-Treves was initially understood to occur in
patients having undergone mastectomy as the result of axillary lymph node dissection, the high rate of angiosarcoma occurrence in cases of lymphedema unassociated with breast cancer has demonstrated that lymph stasis is the cause of angiosarcoma development rather than the primary breast cancer. Stewart-Treves may develop in patients with long-standing lymphedema that is either primary or secondary. However, it is most often observed in breast cancer patients with secondary lymphedema after lymph node dissection. Additionally, several malignant tumor types are more likely to develop in edematous limbs, such as Kaposi’s sarcoma, squamous cell carcinoma, malignant lymphoma, and melanoma\textsuperscript{110,111}.

The relationship between lymphedema and lymphatic cancer metastasis is complex. Immune-dysfunction as a consequence of lymphedema is suspected to be a significant contributor to increased lymphedema metastases\textsuperscript{107,112}. Due to impaired lymph transport, immune surveillance of cancer cells is disrupted due to reduced trafficking of immunocompetent cells.

In certain instances, cancer development may directly cause lymphedema via tumor infiltration or compression of lymphatic vessels, referred to as malignant lymphedema\textsuperscript{113}. Malignant lymphedema generally occurs in individuals with advanced or metastatic cancer, and the resulting lymphedema progression is rapid\textsuperscript{114}. In-transit metastasis is also enhanced in lymphedema conditions. Mice with surgically induced lymphedema developed more instances of melanoma in-transit metastases, in addition to distant lymph node and lung metastases\textsuperscript{115}.

As impaired lymphatic transport is the underlying cause of most clinical complications of lymphedema, improving lymphatic transport would certainly be a
promising potential treatment for lymphedema patients. Intuitively, pharmacological stimulation of lymphangiogenesis has been suggested as a potential treatment of lymphedema patients, as the development of new lymphatic vessels and increasing the efficiency of functional lymphatics in a patient could attenuate lymph stasis\textsuperscript{116}. VEGF-C and VEGF-D are two of the most significant regulators of lymphangiogenesis that bind to VEGFR-3 in LECs\textsuperscript{117}. Angiopoietin-1 and fibroblast growth factor 2 additionally are important regulators of lymphatic vessel formation and growth that may be potential therapeutic agents\textsuperscript{118,119}. Human growth factor additionally has considerable lymphangiogenic potency that has been demonstrated to improve lymphatic transport in secondary lymphedema\textsuperscript{120}.

Unfortunately, lymphangiogenic stimulation is strongly associated with increased rates of cancer metastasis in individuals with cancer\textsuperscript{121}. Lymphatic vessel development improves circulation of tumors and metastatic dissemination of primary tumors. While lymphangiogenic stimulation is likely strongly inadvisable in patients with comorbid cancer and lymphedema, it could potentially reduce the risk of cancer development in lymphedema patients without cancer due to improving immune surveillance of cancer cells. Despite the significant negative consequences of lymphangiogenesis in cancer patients, pro-lymphangiogenic therapies are still worth investigating and may provide benefits that outweigh their risks in certain instances.

Addressing cancer metastasis in lymphedema patients requires understanding and elucidation of the mechanisms and pathways of lymphedema progression that ultimately results in lymph stasis. Patients who undergo lymph node dissection require years for lymphedema to develop\textsuperscript{122}. The general hypothesis that surgical trauma from lymph node
removal blocks lymph flow and leads to fluid accumulation is certainly an inadequate oversimplification of the pathophysiology of secondary lymphedema development\textsuperscript{123}. Lymphedema progression requires time and presents itself in several stages characterized by histological changes. Initially, vessels appear identical to healthy vessels histologically, but pathological changes occur in LECs, which is sometimes referred to as occult lymphedema\textsuperscript{116}. Subsequently, the increased endolymphatic pressure leads to flattening of LECs and luminal dilation\textsuperscript{124}. Although advanced lymphedema is characterized by lymph stagnation, vessels with ectasis may actually have increased flowrates to compensate for pressure increases. Advanced lymphedema is characterized by significant hyperplasia of smooth muscle cells and collagen fibers that eventually causes total occlusion of lymphatic vessels.

While very little is known of the mechanism that lymph node dissection leads to smooth muscle cell and collagen hyperplasia, mechanotransduction likely plays a role. If flow sensitive genes involved in lymphatic vessel obstruction can be identified, attenuating therapeutics can be developed to prevent lymphedema progression and resulting in increased metastatic consequences. As the process of intimal thickening in lymphatic collecting vessels appears analogous to neointimal hyperplasia in blood vessels, flow sensitive genes involved in neointimal hyperplasia should be examined in LECs in the context of lymphedema. Neointimal hyperplasia is also known to be a highly flow sensitive process\textsuperscript{125}. For instance, platelet derived growth factor upregulation, MAPK phosphorylation, and PI3K/Akt phosphorylation was observed in venous ECs exposed to flow conditions present in disease prone vessels\textsuperscript{126}. Additionally, the process of neointimal hyperplasia was reduced via broad receptor tyrosine kinase inhibition.
Glutathione S-Transferase a4 is additionally a flow sensitive gene that promotes neointimal hyperplasia\textsuperscript{127}. In addition to LECs, smooth muscle cells and fibroblasts are highly mechanosensitive cell types\textsuperscript{128}. Smooth muscle cells and fibroblasts do not experience luminal wall shear stress but are sensitive to changes in pressure and stretch due to elevated endolymphatic pressure. Fibroblasts mediate extracellular matrix synthesis homeostasis in a mechanotransductive sensitive manner\textsuperscript{129}. For instance, c-Fos is a transcription factor expressed by fibroblasts that is highly upregulated in response to cyclic strain\textsuperscript{130}. Mechanical stretch additionally affects the regulation of CCN2 in fibroblasts\textsuperscript{131}. Future experiments should examine the response of fibroblasts to mechanical conditions in early-stage lymphedema to elucidate if they are playing a role in lymphatic collagen hyperplasia that leads to intimal thickening. While the fibroblast response to mechanical stimuli is most often examined in the context of mechanical stretch, their response to physiological pressure also needs to be further elucidated.

Smooth muscle cell proliferation and hyperplasia is known to be influenced by mechanotransduction and has been studied in the context of arteries\textsuperscript{132}. mTORC1 is a significant mechanosensitive involved in neointimal hyperplasia. As rapamycin is used as an effective pharmaceutical inhibitor for mTORC1 in the context of arterial neointimal hyperplasia, it could potentially be effective if the mTORC1 pathway is significant in lymphedema smooth muscle cell proliferation\textsuperscript{133}. As smooth muscle cells in arteries and lymphatics likely have significant functional overlap, mechanosensitive genes that are known to contribute to neointimal hyperplasia in arteries should be examined in the context of early-stage lymphedema. If the genes and pathways involved in both processes are determined to be primarily conserved, several pharmaceuticals approve to attenuate
arterial neointimal hyperplasia could potentially be used to slow smooth muscle cell proliferation in lymphedema.

2.) Lymphatic Valves Separate Lymph Flow into a Central Stream and a Slow-Moving Peri-Valvular Milieu

2.1) Introduction

The transport of lymph throughout the lymphatic system is an incompletely understood process and can vary significantly from one species to another. Interstitial fluid is taken up by the initial lymphatics, removing cell waste and toxins from the interstitial space. The uptake of fluid, macromolecules, and lymphocytes is aided by flap-like mini-valves formed by overlapping ECs, aquaporin-1 water channels, and highly permeable button-like junctions in the initial lymphatics. The initial lymphatics lack a basement membrane and are composed of a monolayer of lymphatic endothelial cells (LECs) with long cytoplasmic projections anchored to surrounding connective tissue. The initial lymphatics drain into precollector and subsequently collector lymphatic vessels, with an increase in lumen size. The number of initial lymphatics draining into precollector lymphatic vessels varies by organ. The precollector and collector lymphatic vessels are distinguished by the presence of intraluminal valves. However, collectors also exhibit smooth muscle cell layers to aid in the pumping of lymph. Ex vivo and computational experiments have demonstrated that, following smooth muscle cell contraction, lymphatic vessels distend when externally tethered or with positive
transmural pressure. This vessel dilation creates a suction force that propagates to precollector lymphatics enhancing uptake of interstitial fluid from tissues, even at subatmospheric pressures. Overall, lymph is transported via a series of lymphatic vessels that progressively become less permeable, utilizing valves to yield net antegrade flow.

Lymph is propelled through the lymphatic vessel network by transient pressure gradients in conjunction with intraluminal lymphatic valves, lymphatic vessel contraction, and local tissue motion. In the absence of a central pumping organ like the heart, pumping of lymph is highly decentralized with local motion driving the flow, and is subject to intra- and interanimal variation. Although physiologically normal, out-of-sequence contractions of neighboring lymphangions and incomplete closure of intraluminal valves may decrease net antegrade lymph flow and cause local retrograde lymph flow, even in the absence of disease. While it has been observed in unaffected individuals, lymph retrograde flow is more commonly associated with patients suffering from lymphedema. Retrograde lymph flow due to lymphedema is associated with significantly dilated lymphatic vessels, which render valves insufficient. Lymph flow in both directions is commonly observed in dilated lymphatic vessels. The importance of bidirectional lymph flow and its effects on lymphatic homeostasis are largely unknown.

While the directionality of lymph transport may be relevant in cancer metastasis, lymphedema, immunity, and homeostasis, the mechanisms that regulate lymph transport are incompletely understood. One of the mechanisms to propel lymph forward in collector vessels is phasic contractions of lymphangions by the smooth muscle cells in
the tunica media\textsuperscript{142,143}. In the study by Dixon et al., even though the frequency and amplitude of contractions were heterogenous between various mesenteric lymphatic vessels, the relationship between vessel contraction and fluid velocity remained relatively consistent\textsuperscript{144}. Phasic contractility parameters, including amplitude and frequency, have been quantified in vivo using near-infrared tracer imaging and have been suggested to be dependent on WSS\textsuperscript{144,145}. Although difficult to directly measure, the average lymphatic WSS has been estimated to be $0.64 \pm 0.14$ dyn/cm$^2$ in rat mesenteric lymphatic vessels using lymphocyte velocity and vessel diameter measurements\textsuperscript{144}.

Akin to ECs in the blood circulatory system, LECs respond to WSS stimuli by upregulation and downregulation of a variety of genes. WSS has been shown to regulate lymphatic endothelial permeability in an inversely proportional manner where greater WSS magnitudes elicit lower permeability. This WSS effect can be abrogated via inhibition of RAC-1\textsuperscript{146}. In vivo, inducible deletion of the transcription factor Forkhead-box protein C2 (FOXC2) confers abnormal shear stress sensing by LECs resulting in cell–cell junction defects, regression of lymphatic valves, and lymphatic vessel collapse, leading to lymphatic dysfunction and lethality\textsuperscript{147}. C-type lectin-like receptor 2 (CLEC2) deficient mice experience disruption of lymph flow during development leading to lack of WSS stimuli on LECs and failed development of lymphatic valves; this phenotype is also observed with lack of FOXC2\textsuperscript{148}. In contrast, exposing LECs to WSS in vitro upregulated the expression of GATA-binding factor 2 (GATA2) and FOXC2, genes involved in lymphatic valve development, in comparison to LECs in static conditions, which simulates the lack of lymph flow experienced by LECs in CLEC2 deficient mice\textsuperscript{148}. 

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In the circulatory system, WSS stimuli promote either an anti-inflammatory or pro-inflammatory endothelial gene and protein expression environment that regulates transmigration of leukocytes. For example, arterial ECs exposed to atherosusceptible WSS waveforms shift to a pro-inflammatory phenotype characterized by upregulation of inflammatory cytokines, leukocyte attracting chemokines, and surface adhesion molecules that mediate cellular adhesion and extravasation\textsuperscript{149–151}. Various chemokines (IL-8, CCL2, and CXCL2) as well as cellular adhesion molecules (ICAM-1, ICAM-2, JAM-A, and LYVE-1) are known mediators of endothelial transmigration of leukocytes in the blood circulatory system. Akin to the circulatory system, the process of leukocyte migration in the lymphatic system is triggered by an inflammatory response \textsuperscript{152}. The lymphatic system is actively involved in the initiation and resolution of inflammation, with lymphatic vessels serving as a route for lymphocytes and newly activated dendritic cells to lymph nodes, and plays an important role in leukocyte extravasation from tissues during resolution of inflammation \textsuperscript{153}. However, the role of lymphatic flow in the inflammatory response of LECs has not been thoroughly examined.

Several lectins, such as E-selectin and L-selectin, are well established mediators of leukocyte extravasation. \(\beta\)-galactosidase-binding galectins have been increasingly emerging as having an immunoregulative role in ECs. The expression of galectins is significantly altered in diseased EC phenotypes\textsuperscript{154}. Increasing concentrations of LGALS8 and LGALS9 has been shown to enhance EC adhesion of T-cells, B-cells, neutrophils, eosinophils, and monocytes with increasing concentrations\textsuperscript{155}. Whether these galectins are sensitive to flow mediated mechanotransduction has not been determined. CD99L2 is a glycoprotein in the CD99 family that is expressed by both leukocytes and ECs, which
mediates leukocyte-EC adhesion via homophilic bonding\textsuperscript{156}. The phosphatase 2 regulatory subunit A, \(\alpha\)-isoform (PPP2R1A) is expressed on the surface of both LECs and certain CTCs, such as melanoma. PPP2R1A has been demonstrated to mediate EC-CTC binding via homophilic interaction\textsuperscript{157}. PPP2R1A is highly expressed in both collecting vessel LECs and tumor cells of melanoma in-transit metastases and has been suggested to potentially be a mediator in lymphatic in-transit metastasis. Whether PPP2R1A is mechanosensitive has not been investigated. Mannose receptor 1 (MRC1) is one of few proteins on collecting lymphatic ECs that has been shown to mediate leukocyte trafficking. Lymphocytes bind to LEC expressed MRC1 via CD44.

In order to elucidate the effects of WSS on lymphatic mechanobiology, it is crucial to characterize the fluid flow environment that dictates fluid forces. To address this need, to the best of our knowledge, the work presented herein is the first time that the lymph flow field distal from the inguinal lymph node has been characterized in vivo. In addition, the lymph flow field was recreated in vitro to demonstrate a WSS-dependent pro-inflammatory LEC phenotypic change. In vivo characterization of lymphatic flow is important to understanding a variety of diseases with affected lymph flow, and normal drug transport in the lymphatic system. Herein, we experimentally examined how lymph transport is affected by intraluminal bileaflet valves in vivo. Results from particle tracking and wall contractility measurements in lymphatic vessels of mice are presented along with in vitro LEC gene and protein WSS-dependent expression.

2.2) Methods
2.2.1) Cell Culture

Human dermal lymphatic endothelial cells (PromoCell; Heidelberg, Germany) in passages 2–9 were cultured in EGM-2 MV cell medium (Lonza; Basel, Switzerland). Cells were grown to confluence on 75 × 38 mm² borosilicate glass slides (Fisher Scientific; Waltham, MA) coated with gelatin (Eastman; Rochester, NY) to promote adhesion. Experiments were conducted at least 2 days after cells reaching 100% confluence for 48 hours in a parallel plate flow chamber system (Figure 12).

2.2.2) Quantitative Reverse Transcription Polymerase Chain Reaction

Reverse transcription and quantitative real-time polymerase chain reaction was conducted using the CFX Connect Real-Time Polymerase Chain Reaction (PCR) Detection System (Bio-Rad Laboratories; CA). Immediately upon termination of the in vitro flow experiments, the in vitro experimental flow set-up. A computer-operated script relays signals to a data acquisition card modulating the peristaltic pump with flow rate data from a flowmeter. A repeatable waveform is applied to the PPFC and endothelial cells inside. Endothelial cells cultured on a glass substrate are exposed to a flow waveform for 48 hours.

Figure 12: In-vitro experimental flow set-up. A computer-operated script relays signals to a data acquisition card modulating the peristaltic pump with flow rate data from a flowmeter. A repeatable waveform is applied to the PPFC and endothelial cells inside. Endothelial cells cultured on a glass substrate are exposed to a flow waveform for 48 hours.
total RNA was extracted from cells in static culture and from cells exposed to flow using the RNEasy Plus Mini Kit isolation reagents (QIAGEN; Hilden, Germany). Extracted RNA was reverse transcribed to complementary DNA using iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad Laboratories; CA) and amplified with the iQ SYBR Green Supermix (Bio-Rad Laboratories; CA) to identify relative expression of IL-8, CCL2, CXCL2, ICAM-1, ICAM-2, JAM-A, and LYVE-1 mRNA. Results from the in vitro cultured LECs were normalized to those of human GAPDH. The sequences of the primers used are shown in Table 1.

2.2.3) Western Blotting

After the 48 hour experiment of either static or lymphatic-like waveform, protein was extracted by lysing the cells for 1 h in 10% RIPA (Cell Signaling Technology; Danvers, MA) containing MG132 protease inhibitor (Tocris Bioscience; Bristol, UK), Halt protease inhibitor (Life Technologies; Carlsbad, CA), and Halt phosphatase inhibitor (Life Technologies; Carlsbad, CA) at 4 °C, centrifuging at 13,000 × g, and discarding the cell pellet. The Western blot was conducted by separating 20 µg of protein by 10% SDS-PAGE gel (Bio-Rad Laboratories; CA) and transferring to a PVDF membrane (Invitrogen; Carlsbad, CA). After transferring, the PVDF membrane was incubated in a blocking buffer of PBS with 0.1% Tween-20 and 5% milk for 1 h at room temperature and probed overnight at 4 °C with the primary and secondary antibodies listed in Table 2. The membrane was rinsed and incubated with HRP-conjugated antibody for 1 h at room temperature. Femto-Chemiluminescent substrate (Thermo Fisher Scientific; Waltham, MA) was used. Digital images were acquired with the ChemiDoc Touch imager (Bio-Rad Laboratories; CA). Densitometric band analysis was performed with Image Multi-Gauge.
Software (Fujifilm; Tokyo, Japan) and normalized to the levels of β-tubulin or β-actin. The antibodies used are listed in Table 2.

Table 1: Primers used for qPCR.

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<th>Reverse primer (5′–3′)</th>
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Table 2: Antibodies used for western blotting.
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2.3) Results

The flow waveforms in the valvular sinus region and mid-lymphangion region were characterized for use in in vitro experiments (Figure 13). Flow in the sinus was found to be virtually static, while flow in the midlymphangion region was pulsatile net-antegrade with a small amount retrograde flow (Figure 14). Because of the importance of the lymphatic system in regulating an inflammatory response, the gene and protein expressions of various chemokines (IL-8, CCL2, and CXCL2) and cellular adhesion molecules (ICAM-1, ICAM-2, JAM-A, and LYVE-1) known to mediate endothelial transmigration of leukocytes in the blood circulatory system were probed. Of the various genes probed, only LYVE-1 expression has been shown in LECs to be sensitive to lymphatic flow in vitro\textsuperscript{148}, ICAM-1, IL-8, CCL2, and CXCL2 expression have been shown to be shear sensitive in circulatory ECs, but it is unknown if LECs will respond similarly, especially in the context of lymphatic relevant flow conditions\textsuperscript{158–162}. To our
knowledge, expression of ICAM-2 and JAM-A have not been previously demonstrated to be regulated by fluid shear stress in ECs originating from lymphatic or blood vessels.

The LECs subjected to the static VS condition for 48 h uniformly expressed higher mRNA expression of cellular adhesion molecules ICAM-1, ICAM-2, JAM-A, and LYVE-1 by 3.9-, 1.8-, 1.4-, and 13.5-fold, respectively, in comparison to the pulsatile ML waveform (Figures 15 and 16). Similarly, the LEC mRNA expression of transmigration relevant chemokines IL-8, CCL2, and CXCL2 was upregulated 8.2-, 5.8-, and 4.3-fold, respectively, when exposed to the static VS condition. LEC protein expression was also uniformly upregulated by 1.74-, 1.46-, 1.37-, and 1.48-fold for cellular adhesion molecules ICAM-1, ICAM-2, JAM-A, and LYVE-1, respectively, under static VS conditions. Protein measurements for secreted transmigration relevant chemokines IL-8, CCL2, and CXCL2 was not possible given the low protein concentration in our high cell medium volume system.

Figure 14: Pulsatile ML (+ + +) or static lymphatic sinus (○ ○ ○) representative flow conditions.
LGALS8 and LGALS9 mRNA expression increased 1.4-fold and 3.7-fold respectively under static conditions VS flow conditions. CD99L2, PPP2R1A, and MRC1 mRNA expression was not significantly altered between static conditions vs flow conditions (Figure 17).

2.4) Discussion

To the best of our knowledge, this is the first study to characterize in vivo the local flow field in the inguinal lymph node efferent lymphatic vessels of adult mice and to connect different flow environments present in the lymphangion to a LEC pro-inflammatory phenotype. This study demonstrates the heterogeneous nature of lymphatic flow and shows that valve morphology affects the directionality of lymph flow. It is the first demonstration that the unique anatomy of lymphatic valves promotes higher flow velocity in the center of the vessels accompanied by near zero velocity extending beyond the near-wall region, significantly veering from the Poiseuille flow. The lymphatic valve to lymph flow relationship also greatly influences local shear forces and transport of
molecules with unknown consequences. Exposing LECs in vitro to the near stagnant lymph flow conditions present in the valve sinus elicited a pro-inflammatory phenotype in comparison to midlymphangion pulsatile flow conditions.

Lymph propulsion is regulated by local and distant elements, such as lymph vessel contractions, skeletal muscle movement, and changes in interstitial pressure. As the lymph intraluminal pressure increases, the circumferential stress that smooth muscle cells experience increases, activating contractility pathways. These contractility pathways, regulated by ion channels, are critical for the propulsion of lymph.\textsuperscript{163–165}

Although maximum lymph propulsion may be expected when neighboring lymphangions contract sequentially, our studies demonstrate that maximum propulsion is less predictable, emphasizing the interdependence of lymph propulsion with local and distant elements.
Fluid shear stress is known to regulate cardiovascular EC expression of cellular adhesion molecules that facilitate leukocyte binding, such as VCAM-1 and ICAM-1. ICAM-1 functions as a primary receptor for LFA-1, which is present on T cells, monocytes, neutrophils, dendritic cells, and macrophages. Dendritic cell expressed Mac-1 binds to ICAM-1 to mediate adhesion and transmigration, while blocking of ICAM-1 in LECs affects T cell adhesion, crawling, and transmigration. Although ICAM-1 is generally considered to be the primary LFA-1 receptor in ECs, ICAM-2 also functions as a redundant receptor in lymphocyte trafficking and plays a role in neutrophil transmigration in blood ECs. Neutrophils express both LFA-1 and Mac-1, which facilitate adhesion to the endothelium. LFA-1 and Mac-1 are each able to adhere to either to endothelial ICAM-1 or ICAM-2. The VS static conditions upregulated LEC ICAM-1 and ICAM-2, suggesting that LECs in the VS region will be primed toward a pro-inflammatory state. In circulatory system ECs, the tight junction protein JAM-A has been shown to mediate extravasation via interaction with LFA-1 on leukocytes during diapedesis, while in LECs one study suggested that
JAM-A plays a positive role in neutrophil transmigration\textsuperscript{176–178}. Although the role of JAM-A in lymphatic endothelial transmigration has not been thoroughly examined, we observed that the VS static conditions upregulated LEC JAM-A expression, suggesting that transendothelial migration may be more favorable in this region. Similar results were observed for the major lymphatic hyaluronic acid (HA) receptor LYVE-1 that mediates leukocyte docking via interaction with HA as a surface glycocalyx. LYVE-1 HA interaction has been demonstrated to mediate lymphatic transendothelial migration of both dendritic cells and macrophages\textsuperscript{179,180}. Although a LYVE-1 mediated mechanism has yet to be reported in the lymphatic transendothelial migration of lymphocytes, HA is also synthesized as a glycocalyx in T cells suggesting that T cells potentially engage in a similar transmigratory mechanism\textsuperscript{181–183}.

\textbf{Figure 18}: Proposed lymphangion fluid flow model where the lymphatic valves introduce spatial fluid flow heterogeneity. Lymph accelerates as it approaches the lymphatic valve reaching a local maximum speed near the tip of the valve leaflets where the effective area is smallest. Lymph flow separates from the valve tips creating recirculation zones with long particle residence times. Lymph flow reattaches in the ML region and travels parallel to the wall. Near stagnant flow conditions persist in the VS region, especially in the vicinity of the lymphatic valve origin. The low WSS conditions in the VS and recirculation zone serve to prime the LECs toward a pro-inflammatory phenotype by upregulating the expression of cellular adhesion molecules ICAM-1, ICAM-2, JAM-A, and LYVE-1 and secreted transmigration relevant chemokines IL-8, CCL2, and CXCL2. The long particle residence time in the VS region allows leukocytes to interact with a potentially primed pro-inflammatory lymphatic endothelium.
A potentially pro-inflammatory milieu generated by the flow field in the vicinity of lymphatic valves was also assessed by measuring the gene expression of transmigration relevant chemokines IL-8, CCL2, and CXCL2. IL-8 is a neutrophil attracting chemokine that regulates transendothelial migration and has been shown to be shear sensitive in HUVECs\textsuperscript{184–186}. The chemokine CXCL2 is another potent regulator of neutrophil transendothelial migration and its expression in HUVECs has also been shown to be shear sensitive\textsuperscript{159,187}. Similarly, the expression of CCL2, a chemokine that recruits monocytes, T cells, and dendritic cells has been shown to be shear sensitive in HUVECS\textsuperscript{188–191}. The static VS flow conditions upregulated the LEC mRNA expression of IL-8, CCL2, and CXCL2. Interestingly, both CXCL2 and IL-8 are preferentially expressed on the luminal rather than abluminal membrane facilitating luminal recruitment of leukocytes within the vessel\textsuperscript{192}. A recent study has shown T cell extravasation into the surrounding tissue from murine lymphatic vessels\textsuperscript{193}. Although this study observed leukocyte extravasation from initial lymphatics into the surrounding tissue, we propose that entrapment of leukocytes in the low shear stress near-stagnant environment of the valvular sinus along with potentially higher concentrations of secreted chemokines IL-8, CCL2, and CXCL2 and primed LECs with upregulated expression of cellular adhesion molecules ICAM-1, ICAM-2, JAM-A, and LYVE-1 creates conditions favorable for transendothelial migration (Figure 18).

An increasing body of research has suggested that galectins play a role in inflammation and leukocyte-EC adhesion\textsuperscript{154}. This is the first study to our knowledge that has reported differential galectin expression in response to fluid flow. The increased expression of LGALS8 and LGALS9 in static conditions suggests that flow conditions in
the lymphangion sinus may cultivate a phenotypic niche that promotes leukocyte trafficking and inflammation in local LECs.

In the cardiovascular system, leukocytes are affected by shear stresses as low as 1–10 dyn/cm\(^2\). Reduced shear stress induces pseudo-pod projection in leukocytes, increasing spreading of leukocytes over ECs, and thus potential transendothelial migration\(^{195}\). Therefore, leukocytes may undergo diapedesis at a higher rate in ECs adjacent to recirculation zones and near the valve origins in the valvular sinus, where fluid stress is lower than other regions of the vessel. Given that LECs are also sensitive to fluid flow\(^{148}\), it is possible that recirculation zones and the valvular sinus regions in the lymphatic system are characterized by a pro-inflammatory endothelium interacting with lymphocytes. The long particle residence time and pro-inflammatory endothelial milieu allows for lymphocytes to interact for extended periods and may facilitate transmigration. However, this process is not expected to be exclusive for lymphocytes, but may be relevant to any other cell circulating in the lymphatic system, for example, circulating tumor cells, and have potential clinical effects on in-transit metastasis\(^{196,197}\). The model proposed in Figure 18 is based on the in vitro results, which suggest the presence of an underlying pro-inflammatory lymphatic endothelial phenotype in the valvular sinus. Future studies are needed to determine if recirculation zone flow indeed mediates diapedesis of leukocytes or other cell types in the lymphatic system.

Stagnation points have been previously shown to affect the morphology, migration, and density of ECs\(^{198}\). This may be because stagnation points create gradients of WSS and increase the total pressures experienced by ECs\(^{198,199}\). Vascular sites that coincide with stagnation points have also been shown to affect the adhesion of
monocytes, as well as the functionality of endothelial gap junction proteins\textsuperscript{199,200}. These combined effects may shift the lymphatic endothelium phenotype\textsuperscript{198–202}.

To the best of our knowledge, this is the first time that the local fluid flow properties of the lymphatic vessels distal from the inguinal lymph node have been characterized in vivo and connected to an LEC pro-inflammatory phenotype in vitro via upregulation of cellular adhesion molecules and secreted transmigration relevant chemokines. We quantified lymph flow using particle tracking of fluorescent microparticles in the lymphatic vessels of anesthetized mice. We compared the fluid flow properties between a vessel with functioning bileaflet valves. We observed that normal bileaflet valves significantly affect the flow field by generating a relatively higher-momentum lymph stream at the core of the vessel compared to slow-moving recirculation zones in the valvular sinus region. These studies have provided unique and detailed insight into lymphatic flow, with potential applications to a variety of diseases that affect lymph transport and drug delivery.

3.) Lymphedema-like Conditions Increase Endothelia Permeability In Vitro

3.1) Introduction

The lymphatic system is a subsystem of the circulatory system that regulates fluid homeostasis, immune cell trafficking, and the absorption of dietary fats\textsuperscript{203}. The initial uptake of interstitial fluid, antigens, proteins, lipids, and inflammatory cells into
lymphatic capillaries, also called initial lymphatics, is regulated by a pressure differential between the interstitium and the lymphatic capillaries. This transient pressure differential further drives lymph to the precollector lymphatic vessels. Transport of lymph through the precollectors and larger collecting lymphatic vessels is primarily propelled by the local smooth muscle cells (SMCs) at relatively low flow rates and secondarily by skeletal muscle contractions, respiration, and external compressions, but not by a central pump\textsuperscript{204}. Lymph flow direction in precollectors and collecting vessels is aided by sequential contraction of SMCs and one-way bileaflet valves to maintain net antegrade flow\textsuperscript{11}. Consequently, lack of SMC contraction and valvular dysfunction can affect lymph transport and may cause lymphedema.

Lymphedema is the accumulation of fluid in the interstitium and is generally caused by lymph transport insufficiency as a consequence of impaired lymphatic function\textsuperscript{205}. Lymphedema is classified into primary lymphedema, which is an inherited or congenital condition resulting from malformation of the lymphatic system, while secondary lymphedema is more common and results from injury or damage to the lymphatic system. The initial stages of lymphedema are not completely understood, although ultrasonography, histology and microscopy studies have elucidated the physiological and structural changes of collecting vessels during the onset of the disease\textsuperscript{124,206}. Initially, the collecting lymphatic vessels transition from the normal to the ectasis type where the vessel becomes dilated and the lumen greatly enlarged. The vessel transitions from ectasis to the contraction type where the lymphatic vessel lumen is partially reduced due to thickening of the smooth muscle layer and then progresses to the
sclerosis type where the vessel lumen is narrowed accompanied by loss of elasticity and lymphatic contractility.

The ECs that line the inner layer of blood and lymphatic vessels are exposed to mechanical stresses, such as wall shear stress (WSS), pressure, and hoop stress, induced by the fluid flow. In the blood circulatory system, it has been demonstrated that ECs exposed to disturbed fluid flow experience a constantly changing WSS direction and are more susceptible to atherosclerosis\textsuperscript{207,208}. In hypertensive patients, the increase in mean blood arterial pressure yields an increase in hoop stress and causes an increase in endothelial reactive oxygen species, inflammation, and apoptosis\textsuperscript{209}. In vitro and in vivo, blood ECs respond differently to pulsatile and steady blood flow, with pulsatile flow conferring a protective phenotype clinically\textsuperscript{210–213}. Patients that underwent cardioplegic arrest for cardiac surgery and were provided pulsatile cardiopulmonary bypass blood (CPB) flow instead of steady CPB flow experienced reduction in endothelial dysfunction and renal replacement therapy\textsuperscript{214}. Pulsatile flow increases basal release of endothelium-derived nitric oxide and decreases vascular resistance in comparison to steady blood flow\textsuperscript{215}. During the different degenerative stages of collecting lymphatic vessels in regions of lymphedema, lymph flow rates and flow characteristics change and consequently the mechanical stresses experienced by the LECs change. In lymphedema patients, increased lymphatic flow rates have been observed\textsuperscript{216,217}. In addition to flow rates, pulsatility can be affected by lymphedema conditions. Flow in normal lymphatic vessels is pulsatile, while predominantly steady under lymphedema conditions\textsuperscript{17,76,218–220}.

Although normal pulsatile lymph flow regulates LEC barrier function, it is unclear how the loss of pulsatility that may occur during lymphedema can affect LECs
and further exacerbate lymphedema. A possible consequence of steady lymph flow is impairment of the lymphatic barrier function, which has been observed to increase lymphatic permeability and may further contribute to lymphedema\textsuperscript{221}. In blood ECs barrier function is regulated by WSS with pulsatile flow downregulating permeability in comparison to steady flow\textsuperscript{222}. The disruption of blood EC barrier function and consequent hyperpermeability is influenced by several mechanisms, including intercellular gap formation caused by contractility of actomyosin stress fibers\textsuperscript{52}. Disturbed flow characterized in atherosclerosis prone arteries has been shown to induce EC hyperpermeability via cytoskeletal tension\textsuperscript{223,224}. Actomyosin-driven cytoskeletal tension is driven by F-actin stress fiber formation and phosphorylation of myosin light chain (MLC). MLC may be single or double phosphorylated (pMLC and ppMLC) at Ser19 or Thr18/Ser19 sites by several mediators of MLC phosphorylation, especially myosin light chain kinase (MLCK). pMLC and ppMLC play distinct roles in blood endothelial barrier dysfunction\textsuperscript{225}. pMLC is constitutively present in the endothelial cytosol during normal conditions but becomes localized with actin stress fibers during inflammation. ppMLC is scarcely detected in blood ECs under normal conditions however is observed in greater quantities during inflammation where it predominantly localizes in peripheral stress fibers and contributes to cellular tension.

Apart from WSS, it is unclear how changes in pressure magnitude and pulsatility could further exacerbate lymphedema. As lymphedema is often associated with changes in transmural pressure and pulsatility, lymphedematous conditions may influence LEC phenotype via pressure mechanotransduction\textsuperscript{219,226}. In the absence of flow, steady
pressure increases cause blood EC barrier function disruption due to MLC phosphorylation\textsuperscript{42,227}. In a separate study, blood ECs exposed to pulsatile pressure showed decreased or no change in permeability\textsuperscript{44}. Given that EC barrier function is regulated by pressure and WSS independently, it is possible that fluid mechanics stresses cause pMLC and ppMLC to localize to stress fibers and play a role in lymphatic barrier function.

In the present study, we have used a CLEC2-deficient mouse, which experiences an adverse pressure gradient that significantly affects lymph flow and drainage due to a hemostatic defect where the thoracic duct drains into the venous system\textsuperscript{228}. In the early stages of development, the adverse pressure gradient obstructs lymph flow and affects functional valve development, a flow dependent process\textsuperscript{148}. The reduction of lymph flow due to the adverse pressure gradient causes lymphedema. The lymphatic flow field downstream of the inguinal lymph node of a normal and a CLEC2-deficient mouse were characterized \textit{in vivo}. The \textit{in vivo} lymph flow characteristics were recreated \textit{in vitro} to address the role of normal pulsatile lymphatic flow and quasi-steady lymphedema flow conditions on LEC barrier function. Furthermore, the effect of steady and pulsatile static pressure was decoupled from the fluid flow to elucidate the role of static pressure on LEC barrier function. Here, we report the differential response in phenotype and morphology of LECs exposed to physiologically relevant steady flow, pulsatile flow, atmospheric pressure, pulsatile pressure, and steady pressure.

3.2) Methods
3.2.1) Cell Culture

Human dermal lymphatic endothelial cells (LECs; passage 2 – 10; PromoCell; Heidelberg, Germany) were cultured in EGM-2 MV cell medium (Lonza; Basel, Switzerland) at 37 °C in 5% CO₂. For parallel plate flow chamber (PPFC) experiments, cells were grown to confluence on 75 × 38 mm² glass slides (Fisher Scientific; Waltham, MA) coated with 0.2% gelatin (Eastman; Rochester, NY) to enable cell adhesion. For pressure chamber experiments cells were cultured either on 6 well cell culture plates (Trueline; USA) for mRNA and protein isolation or on #1 glass cover slips (Chemglass Life Sciences; Vineland, NJ) coated with 0.2% gelatin for immunofluorescence. All experiments were started at least 48 hours after cells reached 100% confluence.

3.2.2) Mouse Experiments

All animal experiments were approved by the University of Pennsylvania Institutional Animal Care

![Figure 19](a) A schematic of the mouse model highlights the ROI distal from the inguinal lymph node, (b) which was imaged using a dissecting epifluorescence microscope.

![Figure 20](Lymphedema Collecting Vessel Normal Collecting Vessel) Fluorescent microparticle tracking in (a) CLEC-2 deficient mouse with undeveloped valves and (b) wild type mouse.
and Use Committee. The Clec2^{−/−} and Prox1-GFP BAC transgenic mice were obtained from the Mutant Mouse Regional Resource Centers (MMRRC)\textsuperscript{229}. CLEC2-deficient mice have been previously reported to develop lymphedema and were used in the present study\textsuperscript{228,230,231}. The Prox1-GFP (normal) mouse weighed approximately 14.5 grams, while the Clec2^{−/−} (lymphedema) mouse weighed about 25 grams due to interstitial fluid accumulation as a consequence of lymphedema. Lymph flow was observed and characterized in the collecting vessel downstream of the inguinal lymph node. Fluorescent microparticles were injected into the inguinal lymph node, transported by the lymph flow, imaged with epifluorescence microscopy, and tracked using ImageJ software as previously described\textsuperscript{17}. Flow was observed and characterized in a normal lymphatic vessel of a wild-type (WT) mouse and a collecting vessel with lymphedema-like conditions in a CLEC2-deficient mouse (Figures 19 and 20).

3.2.3) Steady Pressure System
A steady pressure system was designed to emulate steady pressure in the absence of flow (Figure 21). The pressure chamber was assembled using a commercially available 4 quart pressure cooker with three 1/16” barbed connections fitted. A PhD Ultra syringe pump (Harvard Apparatus; Holliston, MA) with dual 60mL syringes was used to precisely infuse or withdraw gas from the chamber to maintain constant hydrostatic pressure with high accuracy via LabVIEW software. All gas connections were joined using 3/16” OD 73A Food Grade Santoprene tubing. To allow the pump to infuse or withdraw from atmospheric air when full or depleted, two ET-2-12 solenoid valves (Clippard; Cincinnati, OH) used. During standard operating conditions, solenoid 1 was

![Figure 21: Schematic diagram of the computer-operated steady pressure system. The gauge pressure within the pressure chamber was monitored via a pressure transducer. A humidifier was inserted inside the pressure chamber. A computer-operated dual syringe pump could infuse or withdraw to compensate for respective gas pressure decreases or increases. The solenoid valve adjacent to the pressure chamber could close while a second solenoid valve opened to atmosphere to allow the syringe pump plungers to reset at the median while maintaining the gauge pressure in the chamber. The syringe pump and solenoid valves were controlled using Labview software.](image-url)
open while solenoid 2 was closed. When the syringes were full or depleted, solenoid 1 close and solenoid 2 would open to allow for the syringes to reset to the median without altering the pressure in the chamber.

The solenoid valves were powered by a 12V power supply and toggled independently by ZGT-25DD solid state relays (Xiqi Electrical; Wenzhou, China) (Figure 22). The pressure transducer complex was assembled by mounting a PX26-005GV pressure transducer (Honeywell; Charlotte, NC) to a SEK002 (Honeywell; Charlotte, NC), which was mounted to an Arduino Uno (Arduino; Somerville, MA) (Figure 23). The pressure transducer was connected to the pressure chamber to monitor the pressure waveform using LabVIEW software via USB connection to a personal computer. The LINX software modification package was needed to interface between the Arduino Uno and LabVIEW using
Makerhub. LabVIEW toggled the solid state relay to regulate the opening of the solenoid valve via DAQ I/O device when the corresponding state was reached in response to pressure sensing. The truth table and state table for the steady pressure system is shown in Tables 3 and 4 respectively. The logic flowchart is shown in Figure 24. During experiments with cells, for temperature control, the humidifier and pressure chamber was placed in an incubator to maintain the system at 37°C.

Table 3: Steady pressure system truth table.

<table>
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<tr>
<th>State</th>
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<th>Binary</th>
</tr>
</thead>
<tbody>
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</table>
Table 4: Steady pressure system states.

<table>
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<th>State</th>
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<th>Solenoid 2</th>
<th>Syringe Pump</th>
<th>Count</th>
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</thead>
<tbody>
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<td>-1</td>
</tr>
<tr>
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<td></td>
<td>Closed</td>
<td>Opened</td>
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<tr>
<td>4</td>
<td></td>
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<td>Opened</td>
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<td></td>
<td>Opened</td>
<td>Closed</td>
<td>Hold</td>
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</tr>
</tbody>
</table>

3.2.4) Pulsatile Pressure System
The previously described pressure system was modified to produce pulsatile pressure waveforms by replacing the syringe pump with a gas mixer controlled by a solenoid valve (Figure 25). A Pegas 4000 gas mixer (Columbus Instruments; Columbus, OH) supplied 5%CO2/95% air at a flowrate 2,800 cm³/min of through an ET-2-12 solenoid valve (Clippard; Cincinnati, OH) and into a humidifier reservoir that was joined to the chamber. The solenoid valve was powered by 12V power supply and toggled by a ZGT-25DD solid state relay (Xiqi Electrical; Wenzhou, China). The pressure transducer complex was assembled by mounting a PX26-005GV pressure transducer (Honeywell; Charlotte, NC) to a SEK002 (Honeywell; Charlotte, NC), which was mounted to an Arduino Uno (Arduino; Somerville, MA). The pressure transducer was connected to the pressure chamber to monitor the pressure waveform using LabVIEW software via USB connection to a personal computer. The LINX software modification package was needed to interface between the Arduino Uno and LabVIEW using Makerhub. LabVIEW toggled Figure 25: Schematic diagram of the computer-operated pulsatile pressure system. The gauge pressure within the pressure chamber was monitored via a pressure transducer. A constant stream of pressurized 5%CO2/95% air was emitted from a gas mixer and passed through a humidifier. The pressure waveform was controlled by operating an inlet solenoid valve using Labview software.
the solid state relay to regulate the opening of the solenoid valve via DAQ I/O device when the corresponding state was reached in response to reading a pressure value above or below a maximum or minimum value, respectively. A maximum pressure value of 11.601 cmH₂O and a minimum of 9.491 cmH₂O was used. The truth table for the pulsatile pressure system is shown in Table 5. The logic flowchart is shown in Figure 26. The humidifier reservoir was composed of a 1000mL narrow mouth Erlenmeyer flask (Kimble Chase; Vineland, NJ) filled with 850mL DI water and sealed with a rubber stopper housing two 1/16” barbed fittings. 60mL DI water was replenished to the incubator every 12 hours to compensate for evaporation. During experiments with cells, for temperature control, the humidifier and pressure chamber was placed in an incubator to maintain the system at 37°C.

Table 5: Pulsatile pressure system truth table and state outputs.

<table>
<thead>
<tr>
<th>State</th>
<th>Output</th>
<th>Decimal</th>
<th>Binary</th>
</tr>
</thead>
<tbody>
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<td>B₁</td>
</tr>
<tr>
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<td>Opened</td>
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<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Closed</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

**Figure 26:** Logic flowchart of the pulsatile pressure system. State 1 is reached when the pressure in the chamber is below a certain minimum value to allow pressure to increase. State 2 is reached when the pressure rises above a certain maximum value to allow pressure to decrease. The feedback signal retains the most recent state while pressure is between the minimum and maximum values.
3.2.5) Immunofluorescence

Fixed cells were permeabilized with 0.1% Triton X in TBS for 15 minutes. Cells were blocked in blocking buffer prepared from 10% w/v bovine serum albumin in TBS. The primary antibodies listed in Table 6 were diluted in blocking buffer and incubated overnight at 4°C. Cells were washed with TBS three times for 5 minutes on an orbital shaker. Secondary antibody was diluted in blocking buffer and incubated for one hour at room temperature in light blocking conditions. Texas-Red phalloidin (Invitrogen; Waltham, MA) Cells was diluted 1:40 with the secondary antibodies if applicable. Cells were washed again with TBS three times for 5 minutes on an orbital shaker. DAPI (Sigma-Aldrich; St. Louis, MO) was diluted 1:10,000 in blocking buffer and incubated for 10 seconds. Cells were washed again with TBS three times for 5 minutes on an orbital shaker. A small drop of Fluoroshield (Sigma-Aldrich; St. Louis, MO) was applied to each sample, which was placed face down on a glass slide and permanently sealed via application of nail polish around the perimeter. Exposure and contrast settings were adjusted to emphasize cellular morphology and protein localization, as immunofluorescence is not reliable for protein quantification. Western blotting was relied upon for protein quantification. Images were taken with Metamorph software, and Pearson correlation coefficient was determined with the ImageJ using the JACoP plugin.
3.2.6) Endothelial Permeability Under Pressure

LECs were seeded on cell culture inserts (Corning Incorporated; Corning, NY) with 1.0 μm sized pores that were placed in 24 well cell culture plates (Trueline; USA). Cells were grown to confluence and experiments were not run for at least 48 hours after achieving 100% confluence. Cells were placed inside of pressure chamber and exposed to a pressure condition for 48 hours. Each condition was replicated including 10μM of the MLCK inhibitor ML-7 (Abcam; Waltham, MA) in the medium. After 48 hours, the medium of the upper chamber was replaced with 150 μL of medium with 10kDa FITC-Dextran (TCI; Portland, OR) at 1:40 dilution. The cell culture plates were placed back in the incubator for 1 hour to allow transendothelial flux of FITC-Dextran. After incubation, cell culture inserts were discarded and 100μL of medium in the bottom well for each condition was transferred to a 96 well plate in triplicate. Fluorescence measurements were taken using a plate reader (BioTek; Winooski, VT) using excitation/emission wavelengths of 485/530nm.

3.2.7) Western Blotting

After exposing cells to an experimental condition for 48 h experiment of either static, normal lymphatic flow, lymphedema flow, steady pressure, or pulsatile pressure, protein was extracted by lysing the cells for 1 h in 10% RIPA (Cell Signaling Technology; Danvers, MA) containing MG132 protease inhibitor (Tocris Bioscience;
Bristol, UK), Halt protease inhibitor (Life Technologies; Carlsbad, CA), and Halt phosphatase inhibitor (Life Technologies; Carlsbad, CA) at 4 °C, centrifuging at 15,000 × g, and discarding the cell pellet. The Western blot was conducted by separating 20 μg of protein by 10% SDS-PAGE gel (Bio-Rad Laboratories; CA) and transferring to a PVDF membrane (Invitrogen; Carlsbad, CA). After transferring, the PVDF membrane was incubated in a blocking buffer of PBS with 0.1% Tween-20 and 5% milk for 1 h at room temperature and probed overnight at 4 °C with the primary and secondary antibodies listed in Table 7. The membrane was rinsed and incubated with HRP-conjugated antibody for 1 hour at room temperature. Femto-Chemiluminescent substrate (Thermo Fisher Scientific; Waltham, MA) was used. Digital images were acquired with the ChemiDoc Touch imager (Bio-Rad Laboratories; CA). Densitometric band analysis was performed with Image Multi-Gauge Software (Fujifilm; Tokyo, Japan) and normalized to the levels of β-actin or β-Tubulin.

3.2.8) Quantitative Reverse Transcription Polymerase Chain Reaction

Reverse transcription and quantitative real-time polymerase chain reaction was conducted using the CFX Connect Real-Time Polymerase Chain Reaction (PCR) Detection System (Bio-Rad Laboratories; CA). Immediately upon termination of the in vitro flow experiments, total RNA was extracted from cells in static culture and from cells exposed to flow using the RNEasy Plus Mini Kit isolation reagents (QIAGEN; Hilden, Germany). Extracted RNA was reverse transcribed to complementary DNA using iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad Laboratories; CA) and amplified with the iQ SYBR
Green Supermix (Bio-Rad Laboratories; CA) to identify relative expression of MYH9, MYH10, FLNB, MYL9, MYL12a, and MYL12b mRNA. Results from the target sequences quantified from in vitro cultured LECs were normalized to those of human GAPDH. The sequences of the primers used are shown in Table 8.

3.3) Results

3.3.1) Flow and Pressure Waveform Generation

The pulsatile flow waveform was previously characterized in a healthy mouse collecting vessel by our group\textsuperscript{17}. The steady flow waveform was characterized in the CLEC-2 deficient mouse used in this study. Each flow waveform was conducted in the PPFC, and the pressure values were measured using a pressure transducer (Figure 27). The pulsatile flow waveform exposed LECs to a 1-Hz waveform of \(-0.15-0.45\) dyn/cm\(^2\) WSS and \(8.64-12.30\) cmH\(_2\)O pressure with a mean pressure of \(10.53\) cmH\(_2\)O. The steady flow waveform exposed LECs to a constant WSS of [Need WSS Value for Steady Flow] and a mean pressure of \(10.68\) cmH\(_2\)O. Each pressure waveform was applied to LECs in the absence of flow using the custom designed pulsatile pressure and a steady pressure systems. The pulsatile pressure waveform generated a 1-Hz pressure waveform of approximately \(8.51-12.35\) cmH\(_2\)O with a mean value of \(10.57\) cmH\(_2\)O. The steady pressure waveform maintained a mean pressure of approximately \(10.65\) cmH\(_2\)O. These pressure values were in line with literature values for functionally relevant transmural pressure measured in cannulated collecting lymphatics, which generally reach a maximum systolic pressure range from \(5.4\) to \(8.8\) cmH\(_2\)O\textsuperscript{75}. 
Elevated Steady Pressure and Steady Flow Upregulated mRNA Expression of Myosin Regulatory Light Chain Subunits MYL9 and MYL12B

LECS were exposed for 48 hours to atmospheric, elevated, and pulsatile pressure conditions without flow, and steady and pulsatile flow conditions to determine the role of transient mechanical stresses on MLC mRNA expression. As MLC is encoded by several isoforms, the expression of MYL9, MYL12A, and MYL12B mRNA were quantified. The mRNA expression of MYL9 and MYL12B mRNA were significantly upregulated in response to steady pressure and steady flow compared to atmospheric pressure, pulsatile flow, and pulsatile pressure (Figure 28). MYL12A was upregulated exclusively by steady pressure compared to all other conditions.
The mRNA expression of myosin heavy chains MYH9 and MYH10 was also quantified, but no significant expression change was observed in response to any condition. As actin crosslinkers contribute to actomyosin mediated cellular stiffening, mRNA expression of the actin crosslinker FLNB was examined. FLNB is an isoform of FLNA, which was previously shown to be upregulated in response to pressure in ECs\textsuperscript{42}. However, no FLNB mRNA expression change was measured in response to any experimental condition. Taken together, these results suggest that MYL12B and MYL9 are sensitive to steady pressure and flow but are not influenced by the lower atmospheric steady pressure, pulsatile flow, or pulsatile pressure.

\textbf{Figure 28}: In vitro LEC mRNA expression of FLNB, MYH10, MYH9, MYL9, MYL12A, and MYL12B after exposure to each experimental condition normalized by atmospheric pressure.
3.3.3) Steady Pressure and Steady Flow Promote MLC Phosphorylation

The effect of atmospheric, elevated, and pulsatile pressure conditions without flow, and steady and pulsatile flow conditions on MLC protein expression was also tested. The total amount of MLC expression did not significantly change after exposure to any condition (Figure 29). As increased MLC phosphorylation state is a hallmark of endothelial hyperpermeability and inflammation, the single and double phosphorylation states of MLC was assessed. The quantity of pMLC in LECs exposed to steady pressure was significantly greater than in LECs exposed to atmospheric pressure, pulsatile pressure, and pulsatile flow. Steady flow induced an increase in pMLC compared to pulsatile pressure and atmospheric pressure. Following a similar trend, both steady pressure and steady flow caused a significant increase in the quantity of LEC ppMLC compared to atmospheric pressure, pulsatile pressure, and pulsatile flow. These data show that steady elevated pressure and steady flow increase pMLC and ppMLC, suggesting a contractile phenotype with increased permeability.
3.3.4) Elevated Steady Pressure and Steady Flow Promote Phosphorylated MLC to Colocalize with Peripheral Actin Network and Disrupt Barrier Function

Studies have reported that colocalization of phosphorylated MLC with the peripheral actin network disrupts barrier function\textsuperscript{232}. To determine whether mechanical stresses modulate MLC phosphorylation and actin network formation in the cell periphery, LECs were exposed to the five different experimental conditions (Figures 30 -

**Figure 29:** In Vitro LEC Protein quantification of MLC, pMLC, and ppMLC after exposure to each experimental condition normalized by atmospheric pressure.
Under exposure to atmospheric pressure, pMLC was observed in the perinuclear cytoplasm consistent with reported findings (Figures 30 - 31). These cells were also characterized by continuous VE-cadherin expression at the junctions. Consistent with previous studies, the actin stain revealed prominent circumferential actin filaments, and negligible stress fiber formation (Figure 31). ppMLC was sparsely distributed and localized in the periphery (Figures 32 and 33). LECs exposed to pulsatile pressure developed a small degree of central stress fiber formation (Figures 31 and 33). pMLC colocalized in the cytoplasm with the central stress fibers, while ppMLC was sparsely present in the periphery, similar to LECs exposed to atmospheric pressure. Colocalization of pMLC and ppMLC with actin or VE-Cadherin was quantified using a Pearson correlation coefficient and values are shown in Table 9.

LECs exposed to both steady elevated pressure and steady flow showed similar morphological responses. Actin assembly was observed primarily in the peripheral cortex.

Figure 30: Immunofluorescence analysis of pMLC and actin in LECs exposed to different pressure and fluid flow profiles. Contrast was adjusted to emphasize localization and morphology.
with minimal central stress fiber assembly. pMLC and ppMLC both colocalized with the peripheral actin. LECs demonstrated discontinuous VE-cadherin junction expression and exhibited intercellular gaps. The peripheral pMLC and ppMLC localization and gap formation suggest a significant degree of cellular tension.

Similar to pulsatile pressure, pulsatile flow caused pMLC localization with central actin stress fibers. ppMLC was observed in central stress fibers as well as peripheral stress fibers. The continuous VE-cadherin junction expression did not suggest cellular tension or gap formation characteristic of a cytoskeletal tension driven permeability increase. Therefore, steady elevated pressure and steady flow induced intercellular gap formation, while pulsatile pressure and pulsatile flow did not exhibit intercellular gap formation, suggesting that pulsatility protects LEC barrier function.

**Figure 31:** Immunofluorescence analysis of pMLC and VE-Cadherin in LECs exposed to different pressure and fluid flow profiles. Arrows indicate endothelial gap formation. Contrast was adjusted to emphasize localization and morphology.
3.3.5) Exposure of LECs to Steady Pressure, but not Pulsatile Pressure, Causes Barrier Function Loss that is Rescued by MLCK Inhibitor ML-7

An *in vitro* transwell assay was used to determine the functional change of permeability in LEC monolayers after exposure to 48 hours of atmospheric, elevated, and pulsatile pressure conditions without flow (Figure 34). Steady elevated pressure induced a significant increase in LEC permeability compared to both atmospheric pressure and pulsatile pressure. LECs exposed to pulsatile pressure showed no change in permeability compared to those exposed to atmospheric pressure. As increased EC permeability is often the result of MLC phosphorylation via MLCK, each experimental condition was simultaneously performed using the MLCK inhibitor ML-7. LECs treated with ML-7 exposed to atmospheric pressure, steady pressure, and pulsatile pressure showed no

![Immunofluorescence analysis of ppMLC and VE-Cadherin in LECs exposed to different pressure and fluid flow profiles. Arrows indicate endothelial gap formation. Contrast was adjusted to emphasize localization and morphology.](image-url)
significant change in permeability. These data suggest that steady elevated pressure, but not pulsatile pressure, caused increased permeability that is rescued by MLCK inhibition.

3.4) Discussion

3.4.1) Endothelial Cells can Discriminate Between Steady and Pulsatile Pressure

While the literature has well established that ECs respond to pressure, little is known about the biological response of ECs to pressure exposure. As flow is inherently driven by pressure gradients, cellular responses assumed to be WSS driven may be partially or entirely regulated by pressure. In this study, we designed custom pressure systems to examine the response of LECs to physiologically relevant pressure waveforms in the absence of flow. To the best of our knowledge, this is the first study to directly compare the effects of exposing ECs in vitro to physiologically relevant pulsatile pressure...
and steady pressure of similar mean values. Our results suggest that not only do LECs respond differently to pulsatile and steady pressure but that they respond differently even at similar mean pressure values. Our findings are consistent with previous reports that show increased EC increased permeability via MLC phosphorylation in response to sustained pressure\textsuperscript{42,227}. This permeability increase was rescued via MLCK inhibition, suggesting that this pressure sensitive response was mediated in a MLCK dependent manner. Interestingly, LECs exposed to pulsatile pressure of similar mean values exhibited neither the permeability increase, increased MLC phosphorylation state, nor exhibited intercellular gap formation observed in LECs exposed to steady pressure. These

![Lymphatic Endothelial Permeability](image)

**Figure 34:** Transwell permeability assay. LEC monolayers were exposed to a pressure condition and permeability was measured via flux of FITC-labeled dextran and normalized by atmospheric pressure. Each experimental condition was additionally performed using the MLCK inhibitor ML-7.
results suggest that steady pressure at physiologically relevant values promotes EC barrier function disruption, while pulsatile pressure does not.

Exposure of LECs to either the steady pressure or steady flow condition caused upregulation of MYL12B and MYL9 mRNA, suggesting that pressure, not WSS, primarily influenced the mRNA upregulation. Additionally, the lack of mRNA upregulation in response to pulsatile pressure suggests that the transcriptional regulatory mechanisms can discriminate between steady and pulsatile pressure. MYL12A mRNA was upregulated by steady pressure but not steady flow, suggesting that WSS and

Table 6: Antibodies used in immunofluorescence.

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pressure both influence expression. While the total amount of MLC protein did not significantly change after exposure to any condition, mRNA expression does not necessarily correlate to protein expression as translational regulatory mechanisms can be complex.

The MLC phosphorylation state of LECs exposed to the different pressure and flow conditions followed a similar trend. pMLC and ppMLC quantity were significantly increased after exposure to steady pressure and steady flow, which often occurs in ECs in response to inflammatory stimuli. This is in line with previous reports of increased MLC phosphorylation in ECs exposed to steady pressure\textsuperscript{42,227}. Pulsatile pressure and pulsatile flow did not induce significant MLC phosphorylation, suggesting these conditions do not promote a hyperpermeable EC phenotype.

3.4.2) WSS and Pressure Waveforms Induce Different LEC Phenotypes and Morphology

Another goal of this study was to examine the effect pressure and flow have on LEC actomyosin dynamics and morphology via immunofluorescence. While pMLC and ppMLC function and dynamics have been explored in ECs exposed to inflammatory mediators such as thrombin, they have not been thoroughly examined in

Table 7: Antibodies used in western blotting.

<table>
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<th>Antibody Target</th>
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<th>Host Species</th>
<th>Vendor</th>
<th>Catalogue #</th>
<th>Working Dilution</th>
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<td>Mouse</td>
<td>Life Technologies</td>
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<td>1:20,000</td>
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</table>
Phospho-Myosin Light Chain 2 (Ser19) | Primary | Rabbit | Cell Signaling Technology | 3675S | 1:200

Phospho-Myosin Light Chain 2 (Thr18/Ser19) | Primary | Rabbit | Cell Signaling Technology | 3674S | 1:200

Anti-MYL9/MYL12A/B | Primary | Mouse | Santa Cruz Biotechnology | SC-48414 | 1:1000

Anti-Mouse HRP | Secondary | Goat | Thermo Fisher Scientific | 31430 | 1:5000

Anti-Rabbit HRP | Secondary | Goat | Thermo Fisher Scientific | 31460 | 1:5000

response to mechanical stimuli. MLC phosphorylation, localization, and actomyosin stress fiber assembly play a complex role in mediating EC function. Exposure of ECs to physiological flow generally induces stress fiber assembly, which may have a beneficial effect on barrier function. However, excessive cytoskeletal tension via MLC phosphorylation in ECs induces hyperpermeability.

Our report shows that pulsatility pressure and pulsatile flow did not induce a morphology associated with barrier function disruption, while both steady pressure and steady flow showed VE-Cadherin disruption and gap formation. Pulsatile pressure appeared to have a small effect on central stress fiber assembly, but the overall morphology appeared comparable to LECs exposed to atmospheric pressure. Significant
central stress fiber assembly was observed in LECs exposed to pulsatile flow, but the continuous VE-cadherin lining suggests that this did not promote permeability.

Although our PPFC does not allow direct EC permeability measurements, the disrupted VE-cadherin lining and intercellular gap formation in LECs exposed to 48

Table 8: Primers used in qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus ID</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (3’-5’)</th>
<th>Fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAP DH</td>
<td>NM_00204 6.1</td>
<td>TGTAGTTGGAGGTCAA TGAAGGG</td>
<td>ACATCGCTCAGACAC CATG</td>
<td>143</td>
</tr>
<tr>
<td>MYL 9</td>
<td>NM_18152 6.1</td>
<td>TGATGGCTTCATTGA CAAGGAG</td>
<td>TCGTCCACTTCTCTCA TCTGT</td>
<td>126 or 288</td>
</tr>
<tr>
<td>MYH 9</td>
<td>NM_00247 3.1</td>
<td>CAAGACGAGAAGAT CAATCCA</td>
<td>CCACGTACGCCAGAT ACTG</td>
<td>97</td>
</tr>
<tr>
<td>MYH 10</td>
<td>NM_00125 6012.3</td>
<td>CAGGGATGAGCAGA ATGAAGA</td>
<td>CTTTGAAGCTACAGC AAGCG</td>
<td>112</td>
</tr>
<tr>
<td>MYL 12B</td>
<td>NM_03354 6.1</td>
<td>CCTAACGCTCCTCAGC TGTC</td>
<td>TTGGATGTTCACGC TGA</td>
<td>144</td>
</tr>
<tr>
<td>MYL 12A</td>
<td>NM_00647 1.1</td>
<td>ATITCCACCATGTTCT CCTCCTT</td>
<td>GCTCTCTCAAGTAAT CTTG</td>
<td>132</td>
</tr>
<tr>
<td>FLNB</td>
<td>NM_00116 4317.4</td>
<td>ACTTCGTGGTAGAAT CCATTGG</td>
<td>GTCGTTGTAACGAT CTTG</td>
<td>89</td>
</tr>
</tbody>
</table>
hours of steady flow suggests barrier function compromise. The increase in pMLC and ppMLC after exposure to this condition supports this conclusion. Only one study to our knowledge has subjected LECs to WSS and examined its effect on permeability, and found that barrier function increased after 30 minutes of exposure. However, this does not necessarily contradict our findings. Considering the short time scale of the experiment, those observations do not necessarily translate to the response on LECs following 48 hours of exposure to flow or pressure. Mechanical stimuli have been shown to induce initial permeability changes that can return to baseline or even reverse following long-term exposure. For instance, bovine aortic ECs show increased permeability in response to steady flow after 1 hour in a cytoskeletal stiffness dependent manner but return to baseline after 4 hours. Another study found that gap formation occurred in ECs after 8.5 hours of shear exposure. Actomyosin mediated cellular stiffening in ECs is a complex and time dependent mechanosensitive response that requires.

3.4.3) Cellular Mechanisms of Pressure Sensing

Although the pressure values involved in this study are lower than the pressure values in studies examining the response of blood ECs to pressure, LECs have been well established to respond to mechanical stimuli an order of magnitude lower than blood ECs. Mesenteric collecting vessels have been shown to discriminate between pressure changes between 1-9 cmH\textsubscript{2}O.
Demonstrating that LECs can discriminate between pulsatile and steady pressure is not surprising as LECs respond differently to pulsatility in other mechanical stimuli. For instance, lymphatic collecting vessel contraction occurs when LECs sense pulsatile flow, but contractions are inhibited by steady flow even at similar mean WSS values. 

Table 9: Pearson Correlation Coefficient for Immunofluorescence Co-staining of MLC with Actin or VE-Cadherin.

<table>
<thead>
<tr>
<th>MLC Phosphorylation State</th>
<th>Co-localization Target</th>
<th>Atmospheric Pressure</th>
<th>Pulsatile Pressure</th>
<th>Steady Pressure</th>
<th>Steady Flow</th>
<th>Pulsatile Flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMLC</td>
<td>VE-Cadherin</td>
<td>-0.34</td>
<td>-0.124</td>
<td>0.034</td>
<td>0.138</td>
<td>-0.131</td>
</tr>
<tr>
<td>ppMLC</td>
<td>VE-Cadherin</td>
<td>0.184</td>
<td>0.078</td>
<td>0.047</td>
<td>0.119</td>
<td>0.078</td>
</tr>
<tr>
<td>pMLC</td>
<td>Actin</td>
<td>0.35</td>
<td>0.675</td>
<td>0.867</td>
<td>0.828</td>
<td>0.867</td>
</tr>
<tr>
<td>ppMLC</td>
<td>Actin</td>
<td>0.389</td>
<td>0.587</td>
<td>0.821</td>
<td>0.791</td>
<td>0.349</td>
</tr>
</tbody>
</table>

This response is impaired when LECs are denuded, demonstrating that LECs are the primary mechanosensors. Additionally, pulsatile stretch influences lymph vessel orientation via lymphangiogenesis in LECs.

Although previous studies have not directly compared the response of ECs to pulsatile and steady pressure, other mechanosensitive cell types have demonstrated the ability to discriminate between steady and pulsatile pressure. Osteocytes and chondrocytes, which are mechanosensitive cell types with conserved mechanosensing
mechanisms, have been thoroughly demonstrated to adopt phenotypic differences in response to growing under pulsatile and steady pressure at similar mean values\textsuperscript{40,41}. Thus, it is generally accepted that culturing chondrocytes under pulsatile pressure is favorable to culturing them under steady or atmospheric pressure, possibly because it is more similar to physiological joint load\textsuperscript{244}. Similarly, steady pressure and steady flow are generally not present in healthy lymphatic vessels and induced a dysfunctional LEC phenotype.

The implications of the ability of ECs to discriminate between pulsatile and steady pressure are not limited to lymphatics. The findings of this study emphasize a need for more studies to elucidate the effect of subjecting other EC types to various pressure waveforms. As the cardiovascular system experiences significant fluctuations in pressure magnitude and waveform during physiological states like rest and exercise, independently examining the effect of pressure could provide novel insight in EC function. Notably, regarding pulmonary ECs, pulmonary hypertension has been reported to be associated with pulmonary EC hyperpermeability\textsuperscript{245–247}.

The mechanism by which ECs sense pressure stimuli remains incompletely understood and requires further elucidation. Several studies have suggested that ion channels such as ENaC may sense changes in pressure via ion flux\textsuperscript{42,227}. Water efflux through aquaporin 1 has also been demonstrated to be a pressure sensing mechanism in ECs\textsuperscript{248}. Considering the large number of putative mechanosensors involved in EC sensing WSS, the cellular mechanism of pressure sensing may be similarly complex. Future work is needed to elucidate how ECs can discriminate between pulsatile and steady pressure.
3.4.4) Flow Characterization

This is the first study to our knowledge that has characterized lymphedema-like flow in the collecting vessel of an animal model with dysfunctional valves. The vessel with lymphedema-like flow demonstrated a high flowrate and the complete absence of intrinsic lymphatic contractions. The intrinsic contractions of lymphatic collecting vessels are inhibited in response to imposed flow, and that contractile frequency decrease linearly as flow increases. Contractions will cease entirely if the flowrate is high enough, presumably because they are unnecessary when lymph transport is already sufficient\(^\text{249}\). However, without observing the vessel prior to contraction cessation, the cause of contractile dysfunction cannot be definitively established. Additionally, intrinsic contractile dysfunction is commonly reported in clinical lymphedema and remains poorly understood\(^\text{250}\). Whether lymphatic pump dysfunction occurs before the development of lymphedema or edematous conditions result in pump failure remains to be determined.

3.4.5) Clinical Implications

While the murine lymphedema model used in the present study retained a considerable lymph flowrate, lymphedema is a highly varied and irregular condition. Increased lymph flowrates have been reported in some cases of lymphedema, particularly in the lower extremeties\(^\text{251}\). In many cases, lymph flow is slow, stagnant, or retrograde\(^\text{252,253}\). The phenotype and morphology of LECs exposed to the steady pressure condition may provide a relevant comparison to LECs in lymphedema vessels when lymph flow is chronically stagnant. Considering the large degree of irregularity in terms
of vessel geometry and flowrates in collecting vessels of a lymphedema patient, one potentially has a spectrum of LEC phenotypes and morphologies. The findings of this study emphasize the sensitivity of LEC morphology to changes in flow and pressure and highlight the need for further examination of the mechanotransductive implications of lymphedema.

Increased LEC hyperpermeability could potentially explain symptoms and clinical complications in lymphedema patients. One obvious consequence of LEC hyperpermeability is reduced lymph transport, as collecting permeability reduces the lymph pressure gradient. In a state of increased LEC permeability, lymph exits the collecting lymphatic lumen to interstitial space and must reenter initial lymphatics. As lymph transport is an essential function of the lymphatic system, several lymphatic functions are impaired when transport is impaired. Although lymphatic insufficiency is already present in lymphedema cases, increased LEC permeability could exacerbate the reduced lymph transport. The transport of antigens and antigen presenting cells to lymph nodes is essential for the adaptive immune response to function. As lymphedema is clinically associated with an impaired adaptive immune response, endothelial hyperpermeability could potentially contribute to this symptom. When lymph transport is impaired, antigens and leukocytes are inefficiently delivered to lymph nodes, which weakens the adaptive immune response\textsuperscript{254}. Additionally, lymphedema is a condition characterized by chronic interstitial inflammation. A mouse demonstrated that induced LEC hyperpermeability in mice causes inflammation in local tissue due to antigen leakage\textsuperscript{51}. Thus, increased LEC permeability could play a role in chronic inflammation.
that is described in clinical lymphedema patients. Regulation of permeability in collecting vessels and its role in lymphatic function requires further study.

While the findings of this study need to be corroborated in vivo, these observations suggest that MLCK may be a potential therapeutic target for patients with lymphedema. Inhibition of MLCK has attracted interest in treating several vascular conditions\textsuperscript{60}. Interestingly, total deletion of MLCK in ECs of rats caused no observed deleterious effects\textsuperscript{62}. Despite this, due to the importance MLCK in numerous other cell types, inhibition of MLCK is associated with significant side effects, so no MLCK inhibitor has been approved for use in human patients. With future advances in targeted drug delivery, specific delivery of an MLCK inhibitor to LECs may be a potential therapy in lymphedema patients.

4.) Conclusion and Future Work

4.1) Summary and Conclusion

While mechanotransduction in blood vessels has been thoroughly examined, Fluid flow in a murine collecting lymphatic vessel was characterized via fluorescent microparticles injection into the inguinal lymph node. Flow waveforms were characterized in the midlymphangion region of the lymphangion and the valvular sinus. The flow waveform in the midlymphangion region was found to be pulsatile net-antegrade flow with a small amount of retrograde flow, while flow in the valvular sinus was virtually static. The resulting flow waveforms were applied to LECs in vitro to elucidate effects on LECs via mechanotransduction. The resulting mRNA and protein
expression changes were quantified via RT-qPCR and western blotting, respectively. Cell adhesion molecules ICAM-1, ICAM-2, JAMA, and LYVE-1, which play a role in leukocyte and CTC extravasation, were quantified. mRNA and protein of all cell adhesion molecules were upregulated in all genes in LECs exposed to static conditions compared to the midlymphangion waveform. Additionally, the mRNA expression of the leukocyte and CTC attracting chemokines CCL2, CXCL2, and IL-8 were also quantified and found to be expressed more in LECs exposed to static conditions compared to the midlymphangion waveform. These in vitro results suggest that these cell adhesion molecules and chemokines may be upregulated in the lymphangion sinus compared to the rest of the vessel, promoting a pro-inflammatory niche. Furthermore, the static flow conditions would increase the residence time of secreted molecules, promoting chemokine accumulation. These factors would make leukocyte transmigration more favorable in the sinus, suggesting that this region may play an important role in leukocyte trafficking and transport. Additionally, few studies have examined the mechanism of in-transit metastasis, which results from CTC extravasation in lymphatic collecting vessels. The sinus may provide a preferable microenvironment for cancers, such as melanoma, to extravasate and form secondary tumors.

Additionally, as lymphedema greatly alters lymph flow, the flow profile in a lymphedema-like vessel may be significantly influencing LEC phenotype via mechanotransduction. As lymphedema generally results from valve incompetence, flow was measured in a CLEC-2 deficient mouse, which does not develop lymphatic valves. Flow was observed to have a steady unidirectional antegrade profile. Additionally, pressure is known to influence mechanotransduction but has been relatively
underexamined compared to WSS. As fluid flow is inherently driven by pressure gradients, mechanosensitive responses attributed to WSS may be partially or entirely the result of pressure. To isolate pressure as a variable, steady and pulsatile pressure systems were developed to apply the lymphedema-like and midlymangion pressure waveforms to LECs in the absence of flow. As steady pressure has been previously shown to influence actomyosin dynamics in ECs, expression of myosin genes and myosin phosphorylation state were quantified. mRNA expression of MYL9 and MYL12B were found to be upregulated both by steady pressure and steady flow, but not pulsatile pressure and pulsatile flow of similar mean pressure values. Protein analysis showed that the protein expression of myosin light chain did not increase under exposure to any condition. The upregulation of mRNAs suggests transcriptional regulatory mechanisms that are sensitive to pressure and pulsatility. Additionally, quantities of pMLC and ppMLC were examined as each are involved in actomyosin contractility and associated with endothelial inflammation and hyperpermeability. Both pMLC and ppMLC were present in higher quantities in LECs exposed to steady pressure and steady flow compared to pulsatile pressure and pulsatile flow. Immunofluorescence analysis revealed intercellular gap formation in LEC monolayers exposed to steady pressure and steady flow but not when exposed to other conditions. Additionally, permeability was significantly increased in LEC monolayers exposed to steady pressure compared to pulsatile flow, but that this permeability increase was rescued via MLCK inhibitor ML-7. These results suggest that steady pressure and steady flow induce LEC hyperpermeability. Increased permeability in collecting vessel LEC promotes lymph and antigen leakage that has several deleterious effects, such as reducing lymph transport,
impairing the adaptive immune response, and causing inflammation of local tissue. To our knowledge, this study is the first that has compared the effects of exposing ECs to pulsatile and steady pressure of similar mean values.

4.2) Future Work

While the lymphatic system has been historically viewed as a passive system, contemporary perspectives are increasingly recognizing that the lymphatic system plays an active and adaptive role in leukocyte trafficking and antigen transport. The response of LECs to different physiologically relevant flow waveforms certainly requires further examination. Apart from molecules that enable leukocyte transmigration, other important genes and pathways are likely activated under certain flow conditions. Additionally, the in vitro findings of this study need to be validated in vivo. Furthermore, lymphatic vessels have highly variable geometry and flowrates, even within the same animal. Lymph flow profiles in more animal models need to be characterized. While collecting vessels are of similar size between mammalian species, characterizing flow in human collecting vessels would be ideal. However, due to the inherent difficulties in examining lymphatic vessels, no ethical way exists to characterize flow waveforms in a human lymphatic vessel to the degree of accuracy presented in this study.

LECs adopting a hyperpermeable state via actomyosin contractility in response to sustained pressure is a discovery that could potentially lead to improved treatment of clinical lymphedema. Increased LEC permeability in collecting vessels could potentially exacerbate symptoms associated with lymphedema such as impaired lymph transport,
adaptive immunity, and chronic inflammation. Examining the effects of inhibiting actomyosin contractility in a lymphedema model would be an interesting experiment.

Discovering that ECs can discriminate between steady and pulsatile pressure of similar mean values likely has important implications in blood and pulmonary ECs. As lymphedema is analogous to arterial hypertension, activation of certain genes in ECs may be conserved between the two conditions. Similar experiments should be conducted using blood ECs at physiologically relevant blood pressure values to further elucidate the role of pressure in blood EC mechanosensing. Such experiments could increase understanding of the basic mechanisms of EC mechanosensing provide insight that may improve clinical care.
Appendix

Appendix Figure 1: Labview block diagram of pulsatile pressure system.

Appendix Figure 2: Labview block diagram of steady pressure system.
Bibliography


