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Mechanical Forces and Lymphatic Transport

Jerome W. Breslin

Department of Molecular Pharmacology and Physiology, Morsani College of Medicine, University of South Florida, Tampa, FL

Abstract

This review examines current understanding of how the lymphatic vessel network can optimize lymph flow in response to various mechanical forces. Lymphatics are organized as a vascular tree, with blind-ended initial lymphatics, precollectors, prenodal collecting lymphatics, lymph nodes, postnodal collecting lymphatics and the larger trunks (thoracic duct and right lymph duct) that connect to the subclavian veins. The formation of lymph from interstitial fluid depends heavily on oscillating pressure gradients to drive fluid into initial lymphatics. Collecting lymphatics are segmented vessels with unidirectional valves, with each segment, called a lymphangion, possessing an intrinsic pumping mechanism. The lymphangions propel lymph forward against a hydrostatic pressure gradient. Fluid is returned to the central circulation both at lymph nodes and via the larger lymphatic trunks. Several recent developments are discussed, including: evidence for the active role of endothelial cells in lymph formation; recent developments on how inflow pressure, outflow pressure, and shear stress affect pump function of the lymphangion; lymphatic valve gating mechanisms; collecting lymphatic permeability; and current interpretations of the molecular mechanisms within lymphatic endothelial cells and smooth muscle. Improved understanding of the physiological mechanisms by which lymphatic vessels sense mechanical stimuli, integrate the information, and generate the appropriate response is key for determining the pathogenesis of lymphatic insufficiency and developing treatments for lymphedema.

Keywords

Lymphatic endothelium; lymphatic muscle; lymphatic myogenic response; lymphatic contractile cycle; lymphedema

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Address Correspondence to: Jerome W. Breslin, PhD, Associate Professor, Molecular Pharmacology and Physiology, University of South Florida, 12901 Bruce B Downs Blvd MDC8, Tampa, FL 33612, +1 (813) 974-7631, jbreslin@health.usf.edu.

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Introduction

The mammalian lymphatic system has an important role in overall health, collectively through its contributions to extracellular fluid and protein homeostasis, lipid transport, and immunity. Lymphatic insufficiency causes lymphedema, which in its worst form is a deforming and debilitating disease with severe swelling throughout the body. Mild forms of lymphedema are more prevalent, and certain populations, like cancer survivors, are at-risk for development of lymphedema (Beesley et al., 2007; Petrek et al., 2001). As with all living tissues, organs, and organ systems, a key characteristic of the lymphatic system is its ability to detect and respond to a variety of physical and chemical cues, in order to optimize function under varying conditions. Lymph flow can vary widely, with 10–15 fold changes recorded, when standing up from a supine position, and as high as 63-fold by foot warming in standing individuals (Olszewski et al., 1977). Others have reviewed the impact of mechanical forces on lymphatic development and lymphangiogenesis in detail (Planas-Paz and Lammert, 2013; Wiig and Swartz, 2012). This review will highlight recent advances to our understanding of how lymphatic vessels optimize lymph flow in response to mechanical forces.

Organization of the lymphatic system

Lymphatics are organized as a vessel tree (Fig. 1), with the most distal, blind-ended vessels serving as the site where interstitial fluid enters the system to form lymph. Terms to describe these blind-ended vessels include lymphatic capillaries (based on fluid exchange function), terminal or peripheral lymphatics (both based on location at the end of the vascular tree), and initial lymphatics (based on being the site of lymph formation). The initial lymphatics are thin-walled vessels composed of a single layer of endothelial cells, with an incomplete basement membrane. They typically form interconnected networks with features that are tissue specific, like the blind-ended lacteals inside intestinal villi (a comprehensive review of tissue-specific initial lymphatic networks is featured in (Schmid-Schonbein, 1990)). The diameters of initial lymphatics vary widely depending on tissue and species. The smallest initial lymphatics reported are the lacteals within rat intestinal villi, as small as 5–15 μm in specimens fixed and viewed by electron microscopy, or 15–30 μm when filled with fluid and viewed by intravital microscopy (Lee, 1979; Ushiki, 1990). Much larger initial lymphatic diameters, as high as 450 μm , have been observed in the bat wing (which are also exceptional in that they possess a smooth muscle layer) (Webb and Nicoll, 1944). Rat mesenteric and human skin initial lymphatics typically fall within ranges of 25–50 and 35–70 μm , respectively (Fischer et al., 1996; Zweifach and Prather, 1975).

The endothelial cells of initial lymphatics feature specialized intercellular junctions, described as “buttons” that give each cell an “oak leaf” shape (Baluk et al., 2007; Murfee et al., 2007). These button junctions display an alternating pattern of the adhesion proteins VE-cadherin and PECAM-1, forming overlapping leaflets of cell membrane between adjacent cells (Baluk et al., 2007), and are thought to act as microscopic one-way valves, termed primary valves, that favor entry of fluids, macromolecules, and lymphocytes into the lymphatic lumen (Murfee et al., 2007; Schmid-Schonbein, 1990; Trzewik et al., 2001). In addition to this paracellular route of fluid entry, the initial lymphatic endothelial cells have

significant expression of the water channel aquaporin-1, suggesting transcellular water transport across the initial lymphatic may contribute to lymph formation (Gannon and Carati, 2003).

The initial lymphatic vessel segments coalesce into larger, collecting lymphatics. The collecting lymphatics are distinguished by having both endothelial and smooth muscle layers, and an adventitia that can contain fibroblasts, connective tissue, MHC-II+ antigen presenting cells, nerves that innervate the vessel, and vasa vasorum (Bridenbaugh et al., 2013; Schmid-Schonbein, 1990; Zawieja et al., 2008). While the lymphatic smooth muscle cell organization varies between species, in general the amount of smooth muscle increases when moving centrally along the lymphatic tree. The functional importance of the smooth muscle layer is that it establishes both vessel tone and a phasic contractile cycle that drives lymph flow (von der Weid, 2001; Zawieja et al., 2008). Another important feature of collecting lymphatics is their organization into segments, termed lymphangions, which have unidirectional valves at each end. These valves, which are bicuspid flaps composed of endothelial cells and connective tissue, prevent backflow of lymph. This is of particular importance in humans standing upright, in which lymph must flow against a significant hydrostatic gradient. These luminal unidirectional valves are referred to as “secondary valves” to distinguish them from the microscopic leaflets between endothelial cells of initial lymphatics (Schmid-Schonbein, 1990; Zawieja et al., 2008). The transition from initial lymphatic to collecting lymphatic is easily distinguishable in some tissues, like mesentery. However in the skin, an intermediate network of lymphatics known as precollectors lies between the initial and collecting lymphatics. Notable features of precollectors is the appearance of secondary valves, yet there is no smooth muscle layer or phasic contractions (Schmid-Schonbein, 1990). Also, the precollector endothelial cells express lower levels of the membrane protein podoplanin than the endothelium of initial lymphatics, suggesting functional differences (Wick et al., 2008).

The anatomical pattern of collecting lymphatics, organized as a chain of lymphangions in series, combined with smooth muscle-generated contractions, enables collecting lymphatics to work as pumps. Each lymphangion is individually capable of propelling lymph forward to the next in series, in a rhythmic manner analogous to the pumping of the heart chambers, with systolic and diastolic phases (Benoit et al., 1989; Davis et al., 2012; Scallan et al., 2012). This active, intrinsic lymphatic pump plays a critical role in normal lymph flow from the extremities in humans (Olszewski and Engeset, 1980), and structural defects in the secondary valves that impair the ability of the pump to propel lymph forward results in lymphedema (Kriederman et al., 2003).

The collecting lymphatics deliver the lymph to one or more lymph nodes. These organs are meeting centers for lymphocytes and antigen-bearing cells, where the profile of antigens arriving from the feeding lymphatics can be sampled (Kogan and von Andrian, 2008). As a result, the cellular content of lymph changes dramatically at the lymph node, with the significant numbers of monocytes, macrophages, and dendritic cells found in prenodal (afferent) lymph essentially absent in postnodal (efferent) lymph (Hay and Andrade, 1998). In addition, lymphocytes exit the lymph nodes primarily through postnodal lymphatics, causing lymphocyte counts to be higher in postnodal lymph than in prenodal lymph (Kogan

and von Andrian, 2008). The lymph nodes also alter the protein concentration of lymph. Protein-free fluid can cross the blood-lymph barrier when there is disequilibrium in Starling forces (hydrostatic and colloid oncotic pressures). Prenodal lymph with a relatively low colloid osmotic pressure becomes more concentrated when passing through the lymph node, due to loss of protein-free fluid into the blood. (Adair and Guyton, 1983; Adair et al., 1982). The combined modification of protein concentration and cellular composition thus make lymph in postnodal collecting lymphatics different from that seen in prenodal collecting lymphatics.

The postnodal collecting lymphatics coalesce into larger lymphatic trunks. The right lymphatic duct drains the right arm, right side of the head, and right thoracic cavity, and empties into the right subclavian vein. The thoracic duct drains lymph from all other parts of the body, emptying into the left subclavian vein (Schmid-Schonbein, 1990). Like the smaller collecting lymphatics, these vessels possess a smooth muscle layer with phasic and tonic contractile activity (Gashev et al., 2004).

Influence of mechanical forces on lymph formation

Tissue fluid volume is determined upon fluid delivery minus removal. While arteries are responsible for fluid delivery, removal by veins is incomplete due to the filtration of plasma in the capillaries and postcapillary venules. Lymphatics remove the remainder, with interstitial fluid accumulation serving as a driving force for lymph formation. When the rate of fluid entering the interstitium increases, the limited space available facilitates the hydraulic conductivity of additional fluid through the interstitium toward the lymphatics (Bert and Reed, 1995; Guyton et al., 1966). This is readily apparent in the demonstration that continuous intravenous infusion of saline, which expands plasma volume, produces a significant increase in lymph flow (Benoit et al., 1989; Rahbar et al., 2014). Interstitial fluid flow and lymph formation serve as compensatory mechanisms that establish a “margin of safety” against edema formation (Granger, 1979). Without these mechanisms, uncompensated expansion of interstitial fluid volume will lead to tissue edema. Recent advances and current understanding of the lymph formation mechanisms will be outlined here. For a more in-depth analysis, a recent review by Wiig and Swartz is an excellent resource (Wiig and Swartz, 2012).

Initial lymphatics typically lie a short distance (hundreds of μm) from the capillaries and postcapillary venules (Schmid-Schonbein, 1990). In order to produce lymph, a gradient must be established for fluid movement from these sites of blood-tissue exchange, across the interstitium, and into the lymphatic lumen. The movement of fluid and various solutes across the microvascular barrier is determined by the Starling forces (hydrostatic and osmotic pressure gradients) and the permeability of the endothelium, favoring net filtration into the interstitium (Durán et al., 2008). Pressure gradients favoring movement of interstitial fluid toward lymphatics have also been reported (Hogan, 1981b; Zhang et al., 2000). However, the mechanism for passage of fluid across the interstitium and into the lymphatics has been less straightforward.

It is generally accepted that average interstitial fluid pressure is slightly lower than atmospheric pressure, as originally observed by Guyton using subcutaneously implanted perforated capsules (Guyton, 1963). Early measurements of intraluminal pressures of mesenteric initial lymphatics were performed by Zweifach and colleagues and were on average slightly higher than atmospheric pressure (Hargens and Zweifach, 1977; Zweifach, 1973; Zweifach and Prather, 1975). Later measurements in human skin lymphatics produced comparable findings, with a range including both subatmospheric (negative) and positive values (Spiegel et al., 1992). These measurements presented the problem of an apparent uphill pressure gradient for lymph formation. An osmotic pressure gradient hypothesis was proposed, however accumulated experimental evidence has not supported this (Casley-Smith, 1975; Schmid-Schonbein, 1990; Wiig and Swartz, 2012; Zawieja et al., 2008).

An alternative explanation takes into consideration that the interstitial and lymphatic fluid pressures are not static, but fluctuate with tissue movements. The hydrostatic hypothesis postulates that oscillating movements like contraction of the heart or tidal movements of the lungs transmit force to the tissues, which ripples through the interstitium toward lymphatics. These oscillating forces transiently change the interstitial-intraluminal fluid pressure gradient, producing periodic movement into initial lymphatics. This hypothesis originated from the demonstration that arterial pulsations were required for local lymphatic removal of subcutaneously injected tracer molecules in a rabbit ear model; steady perfusion of the artery at the same mean arterial pressure stopped lymph flow (Cressman and Blalock, 1939). Transient changes in pressure gradients favoring lymph formation were subsequently shown in the bat wing, which has atypical contractile initial lymphatics (Hogan, 1981a; Hogan, 1981b). More recent evidence has come from study of rodent diaphragmatic and intercostal lymphatics, which receive cyclic pressure waves from cardiac contractions and the pulmonary tidal movements (Grimaldi et al., 2006; Moriondo et al., 2005; Negrini and Del Fabbro, 1999; Negrini and Moriondo, 2013; Negrini et al., 2004). In some of these studies, simultaneous measurement of interstitial and intralymphatic pressure revealed an apparent pressure gradient favoring lymph formation (Moriondo et al., 2005; Negrini et al., 2004). Intralymphatic pressure decreased and increased significantly during spontaneous inspiration and expiration, respectively, but not during paralysis and mechanical ventilation of the lung (Moriondo et al., 2005). Tissue deformation in other organs, due to activities such as walking, passive limb movements, or gentle skin massage also promotes lymph formation (Jacobsson and Kjellmer, 1964; McGeown et al., 1988b; Olszewski et al., 1977; Olszewski and Engeset, 1980). In heart, skeletal muscle, lung, and intestine, non-contracting initial lymphatic networks predominate, with collecting lymphatics appearing near the point where lymphatic vessels exit from the tissues (Schmid-Schonbein, 1990). Thus it is conceivable, but remains to be proven, that the lymph formation mechanism involving oscillating pressures and gradients is universal. At least one study has suggested that in the sheep hindlimb, the arterial pulsation-driven pressure gradient mechanism does not drive lymph formation (McGeown et al., 1988a), suggesting other tissue-specific mechanisms.

A key aspect of initial lymphatic structure that can enable lymph formation is the presence of the microscopic primary valves (Fig. 2) between endothelial cells (Lynch et al., 2007; Mendoza and Schmid-Schonbein, 2003; Schmid-Schonbein, 2003; Trzewik et al., 2001). The alternating pattern of VE-cadherin and PECAM-1 labeling at the junctions of the oak

leaf-shaped initial lymphatic endothelial cells defines the locations of the primary valves (Baluk et al., 2007; Murfee et al., 2007). In the case of hydrostatic oscillations in interstitial fluid pressure, these valves would prevent back-and-forth flow of fluid between the interstitium and lymphatic lumen. While anchoring filaments help to maintain the patency of the initial lymphatic lumen, these one-way leaflet structures are thought to allow fluid to enter lymphatics when fluid pressure gradients favor lymph formation, and close when intralymphatic fluid pressure exceeds the interstitial fluid pressure (Lynch et al., 2007; Trzewik et al., 2001).

Recent evidence also suggests active involvement of lymphatic endothelial cells in lymph formation. On one hand, transport vesicles have long been postulated to serve as a transendothelial transport mechanism (Dobbins, 1971; Dobbins and Rollins, 1970; Leak, 1971). A recent study using a bioengineered intestinal lymph lacteal model, in which an abundance of lipid-containing vesicles appeared to shuttle across endothelial cells, and basal-to-apical transport of chylomicrons was favored, strongly suggests such mechanisms contribute to lymph formation (Dixon et al., 2009). On the other hand, various chemical and physical stimuli cause lymphatic endothelial cells to alter their shape and barrier function (Breslin, 2011; Breslin and Kurtz, 2009; Breslin et al., 2007b; Cromer et al., 2013; Miteva et al., 2010). One of these studies showed that increased transmural flow across lymphatic endothelium upregulates aquaporin-2, an adaptation which may increase permeability in order to optimize drainage (Miteva et al., 2010). Future studies aimed at elucidating how lymphatic endothelial cells adapt to local mechanical forces and chemical cues will hopefully illuminate how these processes may optimize lymph flow, immune cell entry, and lipid transport.

Lymph flow in initial lymphatic networks

Initial lymphatics may connect directly to a collecting lymphatic or form hexagonal networks in which the components of lymph can move freely. The direction of net lymph flow will be determined again by fluid pressure gradients. In studies where a fluorescent tracer is subcutaneously injected in the distal tail of the mouse, a gradual uptake of this tracer into the cutaneous lymphatic network was observed, followed by central movement along the network toward the body. This gradual movement of tracer is thought to reflect the lymph flow occurring in deeper collecting lymphatics, due to the frequent connections with the initial lymphatic network (Hagendoorn et al., 2004; Swartz et al., 1996).

Impact of mechanical forces on collecting lymphatic contractions

Once lymph enters the collecting lymphatics, it must be propelled against a hydrostatic gradient. While passive compression of lymphatics due to extrinsic events like skeletal muscle movements can aid in this process, the primary mechanism for lymph flow is the intrinsic pumping of lymphangions (Olszewski and Engeset, 1980; von der Weid and Zawieja, 2004; Zawieja et al., 2008). The collecting lymphatic smooth muscle layer provides the driving force to move lymph, through its generation of a phasic contractile cycle (Fig. 3). These phasic contractions compress the lumen, producing fluid movement, and the unidirectional, secondary valve system forces the fluid movement downstream

towards the central circulation (Schmid-Schonbein, 1990). The uphill gradient to be overcome may be substantial, as in the case of the human legs while standing. However, the organization of lymphatic vessels as a series of lymphangions, separated by secondary valves, prevents the large buildup of pressure due to gravity (Olszewski et al., 1977; Olszewski and Engeset, 1980). With this pattern, each lymphangion only needs to overcome the outflow pressure imposed by the subsequent, downstream lymphangion to open the downstream valve and propel its contents forward (Hargens and Zweifach, 1977). Lymphangions in series may phasically contract in a peristaltic or segmented manner; both can achieve forward of lymph (Armenio et al., 1981; Zawieja et al., 2008). Zweifach and colleagues measured pressures of mesenteric lymphatic networks and found that gradually increasing pressures in each downstream segment (Hargens and Zweifach, 1976; Hargens and Zweifach, 1977; Zweifach and Prather, 1975). In addition, they also observed that elevated luminal pressure caused an increase in phasic contraction frequency (Hargens and Zweifach, 1977). A later study by Benoit and colleagues showed that edemagenic stress caused by intravenous infusion of excess fluids also increased contraction frequency of mesenteric lymphatics (Benoit et al., 1989). As lymph formation can rise and fall, the ability of lymphangions to respond appropriately to optimize lymph flow represents an important mechanism for energy efficiency of the system (Gasheva et al., 2006).

The collecting lymphatic contractile cycle can be described as having periodic, phasic contractions superimposed over vessel tone, as the vessel does not relax completely between phasic contractions. As in arterioles, changes in tone can be elicited by vasodilators or vasoconstrictors, increased luminal pressure produces a myogenic constriction, and elevations in flow elicit an endothelium-dependent relaxation (Zawieja, 2009). The mechanism governing tone has some similarities with that seen in other types of smooth muscle, in that extracellular Ca^{2+} is a requirement and Ca^{2+} -dependent activation of myosin light chain (MLC) kinase (MLCK) drives the development of actin-myosin mediated tonic contraction (Wang et al., 2009). Rho Kinase (ROCK), which phosphorylates the MLC phosphatase (MLCP) targeting subunit MYPT-1, leading to inactivation of MLCP, also promotes tonic constriction of lymphatics (Hosaka et al., 2003; Kurtz et al., 2014b; Souza-Smith et al., 2013). There is also evidence that PKC increases Ca^{2+} -sensitivity by activating CPI-17-mediated inhibition of MLCP to increase tone (Dougherty et al., 2008; Dougherty et al., 2014). New evidence also suggests that lymphatic tone can be sustained by slow-cycling latch bridges (Dougherty et al., 2014), as seen in vascular smooth muscle (Dillon et al., 1981).

The mechanisms controlling phasic contractions are less clear. Action potentials and transient increases in intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) have been shown to precede phasic contractions of lymphatic vessels (Imtiaz et al., 2007; Shirasawa and Benoit, 2003; Souza-Smith et al., 2011; von der Weid et al., 2008). The pacemaker may involve Ca^{2+} -activated Cl^- currents or hyperpolarization-activated inward current (McCloskey et al., 1999; Toland et al., 2000; von der Weid et al., 2008). In addition, recent work shows that inhibition of ROCK can prevent phasic contractions while allowing the Ca^{2+} transients to persist (Kurtz et al., 2014b). The shortening velocity of lymphatic smooth muscle is much greater than observed in other smooth muscle types and much closer to that of striated muscle (Benoit et al., 1989; Zhang et al., 2013). This appears to be due to the presence of cardiac muscle

contractile proteins in addition to those typically found in smooth muscle. For example, fast isoforms of myosin, such as the smooth muscle B myosin heavy chain (MHC) and the fetal cardiac/skeletal slow-twitch β -MHC, have been identified within lymphatic smooth muscle (Muthuchamy et al., 2003). Cardiac Troponin-C (cTN-C) and cTN-T may also play a role in the phasic contractile nature of lymphatic vessels (Muthuchamy and Zawieja, 2008). The brisk contraction of lymphatic smooth muscle fibers, oriented circumferentially on the vessel, reduces luminal diameter, but can also cause some axial shortening of the lymphangion. Phasic contractions may be propagated to an adjacent lymphangion (upstream or downstream) through gap junctions (Zawieja et al., 1993). Recent work also shows that basal endothelial-derived NO, under conditions in which lymphatics are not stimulated by various mechanical forces or with agents like acetylcholine, reduces phasic contraction amplitude (Scallan and Davis, 2013).

Luminal Pressure and Collecting Lymphatic Contractions

Studies of the lymphatic pressure-diameter relationship show that like in blood vessels, at any given diameter the total pressure is the sum of the passive pressure due to the composition of the lymphatic wall and active pressure generated by the smooth muscle layer (Ohhashi et al., 1980; Zhang et al., 2006). Passive tension is dependent upon the connective tissue in the lymphatic wall, which contains an abundance of collagen and elastin fibers (Rahbar et al., 2012). Recent studies of the passive properties of rat mesenteric lymphatics showed that these vessels are compliant at the low intraluminal at which they typically operate (1–5 cm H₂O). However, a sharp stiffness transition occurs in lymphatics at pressures above 5 cm H₂O (Rahbar et al., 2012). The lymphatic wall is impacted by three main stresses. First, there is the wall tension, which is the circumferential stress (hoop stress) across the entire lymphatic wall. Second, stretching along the length of the lymphangion causes axial stress. Both of these are strongly influenced by the transmural pressure (luminal pressure minus outside pressure). The third stress on the wall is the shear stress caused by the flow of lymph parallel to and in contact with the wall (Dixon, 2010). Under normal conditions, lymphangions sense when luminal pressure and flow change, integrate the information, and readjust their pump function to handle the fluid load. In the *in vivo* setting, these parameters are influenced by the rate of delivery of new lymph into the lymphangion, and the load against which the lymphangion must work to propel lymph forward.

Studies utilizing isolated, cannulated collecting lymphatics have significantly advanced our understanding of how lymphangions respond to changes in lymph pressure and flow. These procedures allow for the tight control and independent manipulation of the imposed pressure and flow, chemical composition of the bathing solution, and temperature. The studies have revealed separate mechanisms for how lymphangions react to changes in 1) luminal pressure (and by extension, transmural pressure when the outside pressure is held constant), and 2) shear stress.

The response of most collecting lymphatics to changes in transmural pressure involves intrinsic readjustment of both tonic and phasic contractions. Generally, an elevation in transmural pressure increases both the frequency and force of phasic contractions, until a maximum is reached at which phasic contractions become weaker against the increasing

load (Davis et al., 2009b; Eisenhoffer et al., 1994; McHale and Roddie, 1976; Ohhashi et al., 1980; Reddy and Staub, 1981; Zhang et al., 2007). The development of more sophisticated protocols to impose step increases in luminal pressure revealed a myogenic constriction characterized by a reduction of the end-diastolic diameter and indicative of a stretch-induced increase in lymphatic tone (Davis et al., 2009a; Souza-Smith et al., 2010). A recent series of elegant experiments by Davis and colleagues has examined the contributions of both inflow pressure (preload) and outflow pressure (afterload) on isolated collecting lymphatics with two secondary valves. The protocols utilized step or ramp changes in either of these pressures, while also monitoring the luminal pressure of the lymphangion between the two valves with a smaller servo-null pipette. The pressure data were recorded together with video that was used to obtain the intraluminal diameters in multiple segments, and open/closed state of the valves (Davis et al., 2012; Scallan et al., 2013b; Scallan et al., 2012).

First considering afterload, some isolated rat mesenteric lymphangions possess the ability to propel lymph forward when outflow pressures are as high as 12 cm H₂O when an inflow pressure of 1 cm H₂O is imposed. In these lymphangions, when the inflow pressure was clamped at 1 cm H₂O while ramping the outflow pressure from 1 to 12 cm H₂O, the output segment distended while the preceding lymphangion showed a slight myogenic constriction. Both segments displayed increased contraction frequency and decreased phasic contraction amplitude (due to a progressive rise in end systolic diameter) (Davis et al., 2012; Scallan et al., 2013b). The decrease in amplitude is due to the greater amount of stroke work needed to compress the lumen against higher pressures (Zhang et al., 2007). As the luminal pressure increased, with each phasic contraction, the central lymphangion was able to produce a slightly higher pressure than the outflow pressure, opening its outflow valve. (Davis et al., 2012). The pressure-volume loops constructed from the data in this study resembled those typically observed for the heart. Quantification of end-systolic pressure-volume relationship (ESPVR) showed that step increases in outflow pressure caused a leftward shift, while the rate of rise in luminal pressure during systole was also increased, suggesting that lymphatic smooth muscle contractility increases in response to elevated afterload, or homeometric autoregulation (Davis et al., 2012).

Considering preload, several studies have shown that increased inflow pressure enhances pump function of chains of lymphangions by the Frank-Starling mechanism (heterometric autoregulation) (Eisenhoffer et al., 1995; Elias et al., 1990; Li et al., 1998; McHale and Roddie, 1976). Recent studies by Scallan and colleagues tested how single lymphangions respond to increases in preload (Scallan et al., 2012). Step increases in inflow pressure increased the luminal pressure, phasic contraction frequency, and end diastolic diameter, and initially decreased phasic contraction amplitude and stroke volume, which both recovered after a few minutes. The pressure-volume loop analysis revealed a leftward shift in ESPVR, suggesting an increase in lymphatic contractility (Scallan et al., 2012). In addition, in some lymphatics in which afterload was progressively increased by a ramp protocol, a critical pressure could be reached at which they failed to eject their contents, resulting in pump failure. The pressure-volume loops for these lymphangions changed drastically as they reached a limiting outflow that resulted in negative stroke work, i.e. retrograde filling (Davis et al., 2012). This limiting afterload for successful ejection is dependent upon preload. In addition, when preload is very small, an afterload-induced increase in contractility may be

insufficient to cause ejection due to low luminal pressure. In this case, a lymphangion could accommodate, by regulating diastolic filling, to increase contraction strength to overcome the afterload (Scallan et al., 2012). These studies show that the response of single lymphangions to preload conditions is complex and involves multiple mechanisms, including both the Frank-Starling mechanism and true changes in contractility.

The cellular mechanism for the myogenic response of collecting lymphatics must involve the ability of the smooth muscle cells to sense stretch, yet a specific mechanism for this in lymphatics has not yet been identified. However, downstream signals have been identified, such as changes in $[Ca^{2+}]_i$ oscillations after step increases in luminal pressure (Shirasawa and Benoit, 2003). Recent studies show an increased frequency of calcium transients coupled to the elevated contraction frequency, and a gradual increase in the diastolic $[Ca^{2+}]_i$ (between transients) that correlated with the myogenic constriction (Souza-Smith et al., 2011; Souza-Smith et al., 2013). Downstream, both MLCK and RhoA/ROCK have been implicated by studies utilizing pharmacologic agents (Hosaka et al., 2003; Souza-Smith et al., 2013; Wang et al., 2009). An additional recent observation of particular interest is that pressure increases in a downstream lymphangion can cause myogenic constriction and increased phasic contraction frequency and force in the preceding lymphangion (Scallan et al., 2013b). This response can occur even if the valve separating the two lymphangions is closed, indicating the signal must be transmitted via the lymphatic wall. Normal function of the downstream lymphangion's smooth muscle layer was critical, because if it is selectively impaired by a kinase inhibitor or by pharmacologic hyperpolarization, the upstream response was also inhibited (Scallan et al., 2013b). The cellular and molecular mechanisms that mediate lymphatic myogenic constriction and relaxation represent an important future area of focus for the field.

Fluid Shear Stress from Lymph Flow and Collecting Lymphatic Contractions

Like small arteries and arterioles, collecting lymphatics possess a flow-mediated vasodilator response. Studies using lymphatics from different tissues of the rat, in which flow changes were imposed, while luminal pressure was held constant, revealed a flow-induced inhibition of the lymphatic pump (Gashev et al., 2004; Gashev et al., 2002; Gasheva et al., 2006). This response has been considered as an energy-conserving mechanism for periods when lymph formation is high and pressure gradients favor forward flow. If the lymphatics were to continue to pump phasically under such conditions, this activity would be counterproductive, as the periodic constrictions in diameter would actually increase resistance to forward flow (Gashev et al., 2004; Gashev et al., 2002). Several studies have identified that the mechanism for flow-mediated modulation of the lymphatic contractile cycle requires endothelial production of NO (Gashev et al., 2002; Mizuno et al., 1998; Shirasawa et al., 2000; von der Weid et al., 1996; von der Weid et al., 2001). Presumably the action of NO is activation of soluble guanylate cyclase, accelerated production of cGMP, and activation protein kinase G (Gasheva et al., 2013; Kurtz et al., 2014a). Interestingly, in aged rats (22 months old), shear stress-induced relaxation of lymphatic smooth muscle is only partially inhibited by blockade of NO synthase, unlike the 9-month old controls (Nagai

et al., 2011). In the aged rats, histamine appears to also serve as an endothelium-derived lymphatic relaxing factor (Nizamutdinova et al., 2014).

The flow of lymph through collecting lymphatics is influenced by both extrinsic and intrinsic forces. The intrinsic forces generated by phasic contractile activity of lymphangions is characterized by brief periods of rapid forward movement of fluid and an interphase of either no flow or very slow flow in the forward or reverse direction (Dixon, 2010; Dixon et al., 2007; Dixon et al., 2006; Dixon et al., 2005). Experiments in which an electrode sensitive for NO was placed adjacent to a pumping lymphatic in the rat mesentery *in vivo* showed that with each phasic contraction, the brief increase in luminal fluid shear stress caused a local increase in NO synthesis (Bohlen et al., 2009). This mechanism may assist in timing the local relaxation of lymphatic smooth muscle during the contractile cycle. Extrinsic forces, such as very high rates of lymph formation, may cause periods of forward flow (observed by movement of leukocytes contained within the lymph) in the absence of but lymphangion pumping. Inhibition of NO, or deletion of eNOS, has been shown to attenuate the forward movement of lymph in such cases (Hagendoorn et al., 2004). A recent study of collecting lymphatic vessels isolated from eNOS^{-/-} mice suggests that basal NO (unstimulated conditions) only impacts phasic contraction amplitude, while elevated NO (acetylcholine stimulation) affects all other aspects of lymphatic contractility (Scallan and Davis, 2013), suggesting that the action of NO is context-specific.

Collecting Lymphatic Valve Gating

The requirement of the secondary valve system for forward flow of lymph is well established, but only recently have secondary valves become an object of intense study. This interest has been driven in part by the discovery that certain genetic mutations, such as those of the FOXC2 gene that cause lymphedema distichiasis, produce malformation of the secondary valves (Brice et al., 2005; Mellor et al., 2011; Petrova et al., 2004). FoxC2^{+/-} mice recapitulate the symptoms of the human disease (Kriederman et al., 2003).

The secondary valves are thought to act as passive gates to retrograde fluid flow largely through passive mechanisms determined by their structure, low Reynolds number and highly viscous flow, and the trans-valve pressure gradient (Schmid-Schonbein, 1990). The valve leaflets are composed to a layer of endothelial cells, folded so that the basal sides of the cells face each other, with a matrix composed in large part of elastin fibers in between. At the base of each valve, collagen fibers support the interface between the leaflets and lymphatic wall (Mazzoni et al., 1987; Rahbar et al., 2012). Recent studies have also provided new insights about the valve gating mechanisms. When no trans-valve pressure gradient is present, both the inflow and outflow valves of a lymphangion typically remain open for much of diastole. In contrast, when the outflow pressure is higher than the inflow, the gating pattern of the valves is more like the ventricular valves in the cardiac cycle (Davis et al., 2011). In the same study, it was also observed that a certain minimal pressure gradient (inflow pressure < outflow pressure) is required for valve closure, and that this gradient is dependent upon the degree to which the vessel is distended. In addition, the valves could be opened in the presence of a negative trans-valve pressure gradient (inflow pressure < outflow pressure) that was smaller than the pressure gradient for closing but was also

dependent to a lesser degree on vessel distension (Davis et al., 2011). Combined, these data indicate that the valves display hysteresis, have a bias to remain open, and the trans-valve pressure gradients for valve closure are dependent upon transmural pressure. Additional data also indicates that lymphatic vessel tone can impact valve gating (Scallan et al., 2013a). These characteristics imply a complex gating pattern that is susceptible to failure at high outflow pressures (Bertram et al., 2014; Bertram et al., 2011; Bertram et al., 2013). Indeed, a “valve lock” mechanism can occur, in which an output valve remains open when phasic contractions at high outflow pressures become insufficient to drive lymph forward (Scallan et al., 2013b). With the valve locked open, the outflow pressure would then be transmitted through the lymphangion to the inflow valve. If this series of events were to repeat multiple times in upstream lymphangions, the chain of lymphangions that normally prevents against large pressure gradient formation would be lost, causing catastrophic failure of the pumping mechanism (Scallan et al., 2013b).

Permeability of Collecting Lymphatics

In recent years the rat mesenteric collecting lymphatic wall was also shown to also be permeable to macromolecules (Scallan and Huxley, 2010). Of particular interest, the collecting lymphatic permeability could be altered by atrial and brain natriuretic peptides, indicating a potential physiological function for this lymphatic-tissue exchange (Scallan et al., 2013a). Likewise, the impacts of inflammatory agonists or pharmacological agents on the permeability/barrier function of cultured lymphatic endothelial cells, grown as monolayers or in tubes, have also been reported (Breslin, 2011; Breslin et al., 2007b; Cromer et al., 2013; Price et al., 2008). The permeability coefficients for albumin determined from single-perfused rat mesenteric lymphatic vessels lie within the same range as those for postcapillary venules (Scallan and Huxley, 2010). Like postcapillary venules, collecting lymphatics have continuous VE-cadherin labeling at their endothelial intercellular junctions (Baluk et al., 2007; Kurtz et al., 2014a; Wong et al., 1999). This pattern is also seen in cultured lymphatic endothelial cells, grown either as monolayers or in tubes (Breslin et al., 2007a; Price et al., 2008). Enhancing the continuous pattern of VE-cadherin at junctions by experimentally elevating cAMP caused a concomitant decrease in permeability (Price et al., 2008). Thus, VE-cadherin crucial role in limiting paracellular transport across the lymphatic vessel wall as it does in the central circulation (Corada et al., 2001).

The determination of permeability to albumin in single-perfused rat mesenteric lymphatics was performed over a range of luminal hydrostatic pressures, corresponding to the native pressure measured prior to each experiment (Scallan and Huxley, 2010). Plotting the permeability data against luminal hydrostatic pressure revealed a positive correlation that would be expected if fluid and solute move together through common pathways (Huxley and Scallan, 2011; Scallan and Huxley, 2010). In addition, the repeated increases and decreases in luminal hydrostatic pressure, when balanced with the other Starling forces, suggest that the direction of albumin flux across the lymphatic wall could go in either direction. The existing data suggest that albumin flux is outwards except when the luminal pressure falls below ~2 cm H₂O (Huxley and Scallan, 2011; Scallan and Huxley, 2010).

Another mechanical force that may influence permeability of collecting lymphatic vessels is shear stress on the endothelium. While studies utilizing intact lymphatic vessels remain to be performed, a study of cultured lymphatic endothelial cells grown in flow chambers suggests that shear stress influences barrier function (Breslin and Kurtz, 2009). In that study, step increases in shear stress enhanced barrier function, while stepping shear stress back down to baseline caused a reduction in barrier integrity. While the mechanism is largely unknown, inhibition of the small GTPase Rac1, which directs cytoskeletal reorganization at intercellular junctions, attenuated the shear-induced barrier enhancement (Breslin and Kurtz, 2009). While adaptations of the initial lymphatic endothelial barrier can optimize lymph formation, it is less clear at this point what physiological role permeability of the collecting lymphatic wall may have in signaling to the surrounding tissues. In both cases, much remains to be discovered about what cellular and molecular mechanisms control lymphatic permeability.

Summary and Perspective

Significant advances have been made in the last decade to our understanding of lymphatic vessel function. Most of these advances have uncovered previously unseen phenomena by utilizing innovative techniques to visualize lymphatic function *in vivo* or perform technically challenging measurements on isolated lymphatic vessels. Recent innovations to study pump function of lymphatic vessels isolated from mice opens the door to a wide variety of transgenic and knockout models to expand our knowledge (Liao et al., 2011; Scallan and Davis, 2013). New techniques to non-invasively study lymphatic function in both animals and humans are also emerging and will aid with understanding human lymphedema (Aldrich et al., 2012; Maus et al., 2012; Nelson et al., 2014; Rasmussen et al., 2010; Tan et al., 2011; Weiler and Dixon, 2013; Weiler et al., 2012). These new approaches will help with the continued need to investigate the cellular and molecular mechanisms by which lymphatic vessels respond to mechanical cues to optimize lymph flow.

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Highlights

- The impact of mechanical forces within the body on lymph flow is discussed.
- Lymph formation involves pressure gradients and endothelial cell activities.
- Preload, afterload, and shear stress affect contractility in collecting lymphatics.
- Lymphatic tone and trans-valve pressures affect valve gating.
- Peptide hormones can change collecting lymphatic permeability to macromolecules.

Fig. 1.

Schematic view of lymph formation and transport. The interstitial fluid formed by capillary filtration moves toward initial lymphatics (large blue arrows). This fluid then moves along the initial lymphatic network into contractile prenodal collecting lymphatics (small blue arrows). The collecting lymphatics frequently are surrounded by adipocytes, and bring lymph to one or more lymph nodes, where some of the lymph fluid is lost to the blood circulation. A single postnodal collecting lymphatic exits a lymph node, eventually coalescing with other collecting lymphatics into a larger lymph trunk. The two largest, main trunks of this vascular tree (thoracic duct, right lymph duct) empty into the left and right subclavian veins, respectively.

Fig. 2.

Initial lymphatics and lymph formation. The initial lymphatics are composed of a single layer of oak-leaf shaped endothelial cells with discontinuous basement membrane and anchoring filaments that project into the interstitium, and are the site of interstitial fluid entry (blue arrows). Fluid can pass through leaflets of cell membrane at adjacent endothelial cells when the interstitial pressure (P_i) exceeds luminal pressure (P_L). The leaflets are closed when $P_L > P_i$ (bottom left; light blue arrows represent net pressure gradient), and open when $P_L < P_i$. The sites where these leaflets are thought to open are along the far edges of the “oak leaf” junction lobes (bottom right). The yellow arrows show the direction of the leaflet movement when $P_L < P_i$ and the small blue arrows show where fluid is thought to pass across these specialized lymphatic endothelial junctions.

Fig. 3.

Collecting lymphatic phasic contractions. Like the heart, collecting lymphatics have a period of brisk contraction (systole) and a period of relaxation (diastole) between each phasic contraction. Each lymphangion, defined as the segment between two unidirectional valves, can typically exhibit systole and diastole. When a lymphangion is relaxed, the inflow (or upstream) valve will open (given sufficient inflow pressure) for filling of the lymphangion to occur. During systole, the phasic contraction pushes the lymph both upstream and downstream, but the inflow valve closes, so that lymph is forced forward through the outflow valve. The exact valve gating and phasic contraction frequency and force depend heavily on the outflow pressure (afterload), inflow pressure (preload), and the flow of lymph (shear stress), as discussed in the text.