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TOPICAL REVIEW

Lymphatic pumping: mechanics, mechanisms and malfunction

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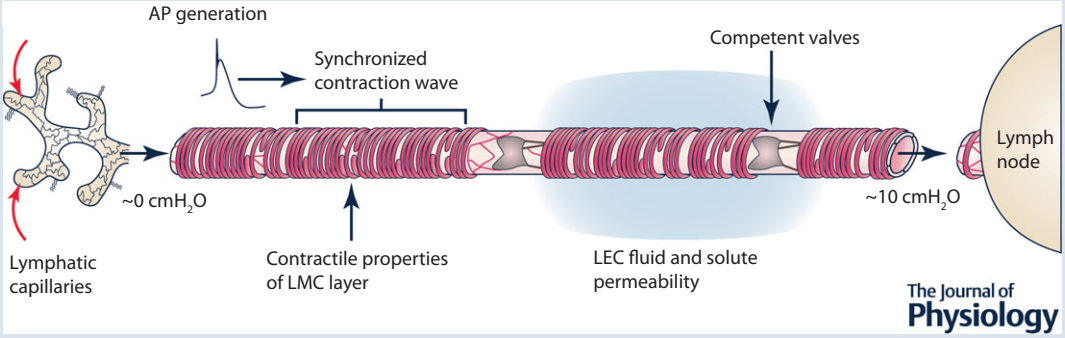

Abstract A combination of extrinsic (passive) and intrinsic (active) forces move lymph against a hydrostatic pressure gradient in most regions of the body. The effectiveness of the lymph pump system impacts not only interstitial fluid balance but other aspects of overall homeostasis. This review focuses on the mechanisms that regulate the intrinsic, active contractions of collecting lymphatic vessels in relation to their ability to actively transport lymph. Lymph propulsion requires not only robust contractions of lymphatic muscle cells, but contraction waves that are synchronized over the length of a lymphangion as well as properly functioning intraluminal valves. Normal lymphatic pump function is determined by the intrinsic properties of lymphatic muscle and the regulation of pumping by lymphatic preload, afterload, spontaneous contraction rate, contractility and neural influences. Lymphatic contractile dysfunction, barrier dysfunction and valve defects are common themes among pathologies that directly involve the lymphatic system, such as inherited and acquired forms of lymphoedema, and pathologies that indirectly involve the lymphatic system, such as inflammation, obesity and metabolic syndrome, and inflammatory bowel disease.

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Abstract figure legend Diagram depicting the major factors regulating the effective pumping ability of a prenodal collecting lymphatic vessel as it transports lymph formed in lymphatic capillaries to the subcapsular sinus of a lymph node. Pressures indicate approximate hydrostatic pressures measured in the interstitium and at the entrance to the lymph node, respectively, that have been recorded in many regions of the lymphatic system. Cut-away sections show the locations of two valves. The blue shaded region depicts the relatively modest net filtration of fluid and solute that occurs under normal conditions all along the length of the collecting vessel. Each of these factors can also become a target of lymphatic dysfunction. AP, action potential; LEC, lymphatic endothelial cell; LMC, lymphatic muscle cell.

Abbreviations AMP, contraction amplitude; AP, action potential; *ApoE*$^{-/-}$, apolipoprotein E knockout; Cx, connexin; *db*/*db*, leptin receptor knockout; EC, endothelial cell; EDD, end-diastolic diameter; EF, ejection fraction; eNOS, endothelial nitric oxide synthase; ESD, end-systolic diameter; ESV, end-systolic volume; FPF, fractional pump flow; FREQ, contraction frequency; GFP, green fluorescent protein; iNOS, inducible nitric oxide synthase; K_{ATP}, ATP-sensitive potassium channel; LEC, lymphatic endothelial cell; LMC, lymphatic muscle cell; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; NO, nitric oxide; NIRF, near infrared fluorescence; ΔP, hydrostatic pressure gradient; P_{in}, inflow pipette pressure; P_L, luminal hydrostatic pressure; P_{out}, outflow pipette pressure; ROS, reactive oxygen species; VIP, vasoactive intestinal peptide; VSM, vascular smooth muscle; WT, wild-type.

Introduction

An extensive network of lymphatic vessels runs in parallel to the blood vascular system, composed of initial lymphatic capillaries that serve an absorptive role, collecting vessels that transport lymph, and lymph nodes/lymphoid organs that facilitate immune responses. Lymphatic vessels or lymphatic-like structures with fluid and/or immune cell transport function(s) have been identified in almost every organ, including the brain and eye (Schroedl *et al.* 2014; Aspelund *et al.* 2015; Louveau *et al.* 2015). Ultimately, lymphatic collecting vessels coalesce into central lymphatic ducts that return lymph to the venous circulation at the confluence of the great veins in the neck. In humans, 8–12 litres of fluid and protein per day that otherwise would accumulate in extravascular compartments are returned to the blood through the lymphatic system (Renkin, 1986; Wiig & Swartz, 2012).

A combination of extrinsic and intrinsic forces move lymph against a hydrostatic pressure gradient in most regions of the body. At rest, approximately 1/3 of lymph transport in the human lower extremities results from compression by skeletal muscle contractions (extrinsic pump) and 2/3 to active pumping (intrinsic pump) of the collecting vessel network (Engeset *et al.* 1977). The robust contractions of lymphatic muscle cells are the driving force for active lymph propulsion against adverse pressure gradients (Zweifach & Prather, 1975), which can be particularly large in dependent extremities (Olszewski & Engeset, 1980). Backflow within the lymphatic network is minimized by a system of one-way valves (Davis *et al.* 2011).

Overload of the intrinsic lymphatic pump or failure of lymphatic valves leads to, and/or results from, chronic lymphoedema (Olszewski, 2002). Observations in the limbs of patients with lymphoedema suggest failure or weakening of the active lymphatic contractions, chronic distension of the collecting vessels and incompetence of the valves (Olszewski *et al.* 1968; Olszewski, 2002). Current therapies for lymphoedema are palliative in nature, promoting passive lymph transport through rigorous and daily deep tissue massage. The limitations in lymphoedema treatments are, in large measure, due to our lack of understanding of the molecular and mechanical properties of lymphatic muscle cells (LMCs).

The focus of this review is the intrinsic, contractile properties of collecting lymphatic vessels in relation to their ability to actively transport lymph. The effectiveness of the lymph pump system impacts not only interstitial fluid balance but other aspects of overall homeostasis such as fat absorption (Dixon, 2010), reverse cholesterol transport (Martel *et al.* 2013) and immune cell trafficking (Angeli *et al.* 2004; Lim *et al.* 2009; Cromer *et al.* 2015; Chakraborty *et al.* 2015*b*).

Normal lymphatic pumping

Active lymph transport by collecting lymphatic vessels depends critically on a combination of factors that combine to produce propulsive and centripetal movement of lymph. Lymph propulsion requires not only robust, spontaneous contractions of LMCs, but contraction waves that are coordinated over the length of a lymphangion, which is the segment of a collecting lymphatic vessel containing two intraluminal valves comprising the

elementary pumping unit. In conjunction, unidirectional valves in the vessel lumen must operate normally to minimize backflow. For the purpose of this discussion, lymphatic pumping is defined as the net outflow of a collecting lymphatic segment due to active contraction of the LMC layer(s). Net outflow is equal to forward (centripetal) flow due to a propulsive contraction minus any reflux through the valves during the contraction cycle.

Pumping behaviour can be visualized in video movies of isolated lymphangions held at constant pressure. The images in Fig. 1 and linked movies (Movies S1 and S2 in the online Supporting information) show contractions of a popliteal afferent lymphatic from a mouse expressing green fluorescent protein (GFP) in lymphatic endothelial cells (LECs) under the control of the LEC-specific transcription factor, *Prox1*. The outer edges of the fluorescent border demarcate the inner diameter of the vessel, which becomes clear when the fluorescence (Fig. 1*B*) and brightfield (Fig. 1*A*) images are compared. Both valve leaflets are visible as they open and close during the contraction cycle. Robust and nearly synchronous contractions of a single layer of LMCs are evident.

Lymphatic muscle is non-striated and usually classified as vascular smooth muscle, but it shares biochemical and functional characteristics with both vascular and cardiac muscle (von der Weid & Zawieja, 2004). Like vascular smooth muscle, lymphatic muscle contraction is regulated primarily by the balance of myosin light chain kinase (MLCK)/myosin light chain phosphatase (MLCP) activity controlling myosin light chain phosphorylation (reviewed in Chakraborty *et al.* 2015*a*). Lymphatic vessels resemble arterioles in that they develop basal tone and exhibit myogenic constriction to pressure elevation (Davis *et al.* 2009). Although the physiological role of the myogenic response in lymphatic vessels is not known, myogenic tone/constriction may serve primarily to preserve valve function (Scallan *et al.* 2012*a*), as discussed below. Like blood vessels, lymphatic behaviour is regulated by nitric oxide (NO) and other endothelium-derived factors such as prostaglandins and histamine (Gasheva *et al.* 2013; Nizamutdinova *et al.* 2014). Both lymphatic tone and spontaneous contractions are inhibited by NO produced as a result of shear stress on the endothelium (in response to either forward or reverse lymph flow) (Gashev *et al.* 2002). Like cardiac muscle, LMCs express troponin C and I as well as cardiac isoforms of tropomyosin (Muthuchamy *et al.* 2003); however, the functional roles of these contractile proteins remain unclear, as are the ways in which they might complement or interact with MLCK/MLCP to control the contraction cycle.

Lymphatic muscle shares several electrophysiological properties with both vascular smooth muscle (VSM) and cardiac muscle. LMC contractions depend predominantly on Ca^{2+} influx through L-type, voltage-gated Ca^{2+} channels (Telinius *et al.* 2014*c*), while the resting membrane potential is influenced substantially by Cl^- (van Helden, 1993; von der Weid *et al.* 2008*b*) and voltage-gated K^+ channels (Allen & McHale, 1988; Telinius *et al.* 2014*b*). Further, the activation of ATP-sensitive potassium (K_{ATP}) channels (Mizuno *et al.* 1999; Mathias & von der Weid, 2013) and Ca^{2+}-activated K^+ channels (Cotton *et al.* 1997) in lymphatic muscle can dramatically modulate spontaneous contractile activity. Like cardiac muscle, the spontaneous contractions of LMCs are initiated by action potentials (APs) that probably originate in LMCs, but also might be generated by a network of interstitial cells (McCloskey *et al.* 2002; Sanders & Ward, 2008; Briggs Boedtkjer *et al.* 2013). LMCs also express several types of ion channels that are similar to those that control pacemaking in the sino-atrial node, e.g. fast Na^+ channels (Hollywood *et al.* 1997*b*; Telinius *et al.* 2015), T-type Ca^{2+} channels (Hollywood *et al.* 1997*a*; Lee *et al.* 2014), hyperpolarization-activated cyclic nucleotide-gated (HCN) channels (McCloskey *et al.* 1999) and *ether-à-go-go* related gene (ERG) channels (Gui *et al.* 2014). The roles of these channels in lymphatic pacemaking are not yet fully understood.

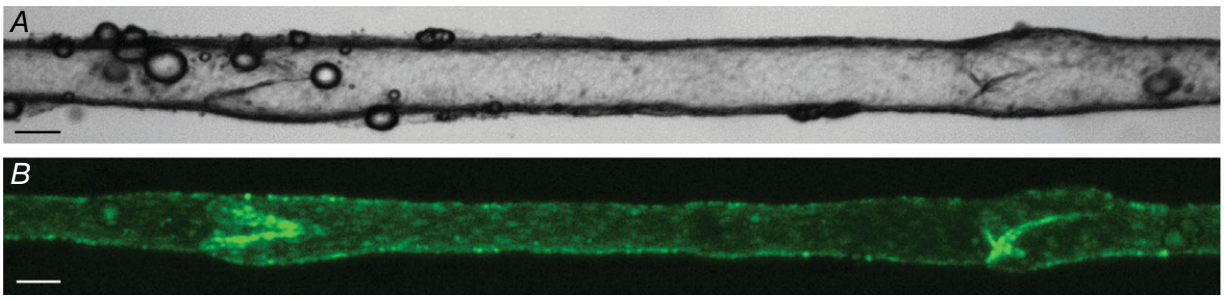

Figure 1. Brightfield (*A*) and fluorescence (*B*) images of a popliteal afferent lymphangion from a Prox1GFP mouse after dissection, cannulation and partial cleaning
The vessel is pressurized to 3 cmH_2O from cannulation pipettes on either end (out of field of view). Calibration bars = 50 μm. Movies S1 and S2 in the online Supporting information show contraction sequences in each imaging mode.

Use of cardiac analyses to evaluate lymphatic contractile function

Functionally, many aspects of the lymphatic pump resemble those of the cardiac ventricular pump. At the beginning of a lymphatic pump cycle both valves are closed so that contraction of the LMC layer (i.e. systole) results in a rapid rise in intraluminal pressure; once pressure exceeds outflow pressure the outflow valve opens, ejecting lymph. When the LMC layer relaxes (i.e. diastole), intraluminal pressure falls, the outflow valve closes, and the inflow valve then opens to allow filling of the lymphangion. This sequence of events is illustrated by the experimental recording in Fig. 2 of two contraction cycles. Internal diameter is measured from high-resolution video images of the vessel using edge detection while valve positions (inflow valve, blue arrow; outflow valve, red arrow) are measured using densitometry (Davis *et al.* 2012). Pressures at the inflow and outflow ends are controlled by a servo system and intraluminal pressure (P_L) is measured through a 3 μm servo-nulling pipette advanced through the wall. To evaluate pump function inflow and outflow pressures are manipulated independently or in unison. In this case, a slow rise in outflow pressure (P_{out}) is imposed while holding inflow pressure (P_{in}) constant. Both pressures are referenced to external pressure (atmospheric). The contractions of the entire lymphangion are essentially synchronized, with a delay of only a few milliseconds for spread of the contraction wave. The contraction amplitude (AMP) for this vessel, as determined from end-diastolic diameter (EDD) minus end-systolic diameter (ESD), is larger on the outflow than inflow side of the outflow valve; note that EDD slowly rises as P_{out} is raised, but remains approximately constant on the inflow side because P_{in} is held low and the central segment is protected from reverse flow in diastole by the outflow valve, which is closed. The ejection fraction (EF) is calculated as $(EDD^2 - ESD^2)/EDD^2$, thereby converting the diameter change to a volume change over a constant length. EF can be as high as 80% for isolated lymphangions from rat and mouse (Scallan *et al.* 2012*b*; Scallan & Davis, 2013). Although pump flow in mouse and rat vessels is not able to be measured directly with current methods (as it is in lymphatics from larger animals; McHale & Roddie, 1976; McHale & Meharg, 1992), pump output (FPF, fractional pump flow) can be estimated from the product of EF and contraction frequency (FREQ).

Modulators of the lymph pump

Like the heart (West, 1991), lymphatic pumping is regulated by four major factors: preload, afterload, spontaneous contraction frequency and contractility. The influence of each factor is addressed briefly in the next section.

Preload. Preload, which is set by end-diastolic pressure (or volume), is a significant determinant of lymphatic pump function. Increasing the filling pressure over a certain range enhances pump output, analogous to the Frank–Starling relationship for the heart (Smith, 1949; Mislin & Schipp, 1966; McHale & Meharg, 1992). In rat

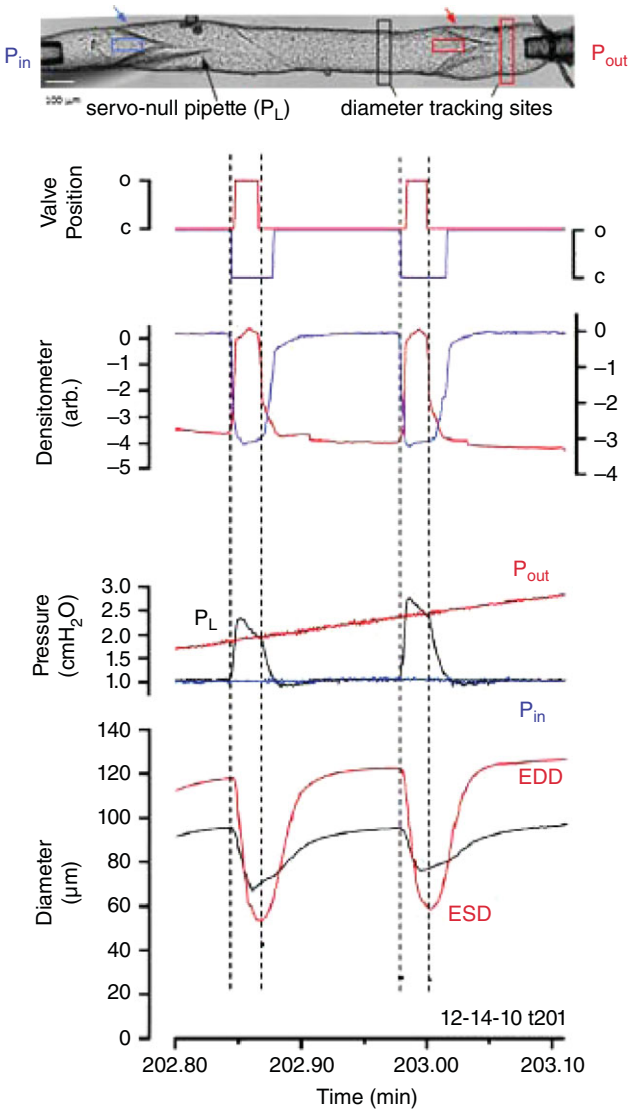

Figure 2. Pump cycle of an isolated, cannulated (2-valve) lymphangion from rat mesentery when P_{out} is elevated ramp-wise while P_{in} is held constant
Normal direction of flow is left to right. Red and black diameter traces correspond to red and black tracking windows on each side of the output valve in the video image at the top. Blue and red densitometer traces correspond to blue and red densitometer windows positioned just upstream of the input and output valves, respectively. Valve position traces represent the binary state of each valve based on thresholding of the respective densitometer traces. Black pressure trace is the intraluminal pressure (between the valves) measured by a sharp servo-nulling pipette advanced through (and sealed into) the wall. Modified from Davis *et al.* (2011).

and mouse lymphangions FREQ increases with pressure over the range 0–5 cmH_2O, reaching a plateau at higher pressures. AMP increases over 0–3 cmH_2O and then declines at higher pressures (Gashev *et al.* 2004; Scallan *et al.* 2012*b*). Examples are shown in Fig. 3*A*. Like the cardiac ventricles, lymphatic EDD increases in a curvilinear fashion with pressure, reflecting the underlying passive pressure–diameter relationship, in contrast to ESD, which increases linearly with pressure (Fig. 3*B*). FPF peaks at around 5 cmH_2O, which is consistent with results from isolated chains of lymphatic segments from larger species (McHale & Roddie, 1976; Elias *et al.* 1990; Eisenhoffer *et al.* 1995; Li *et al.* 1998). The FREQ response to a change in preload is also rate sensitive, as shown in Fig. 3*C*, where a rapid pressure step evokes a burst of contractions followed by a subsequent decline in FREQ; increasing preload at a slower rate will eliminate the bursting (Davis *et al.* 2008*a*).

Afterload. The lymphatic pump must adapt to elevated outflow pressures resulting from partial outflow obstruction, increased central venous pressure and/or gravitational shifts. Lymphangions in series can propel lymph against higher pressures than individual lymphangions (Jamalian *et al.* 2016), which is required in

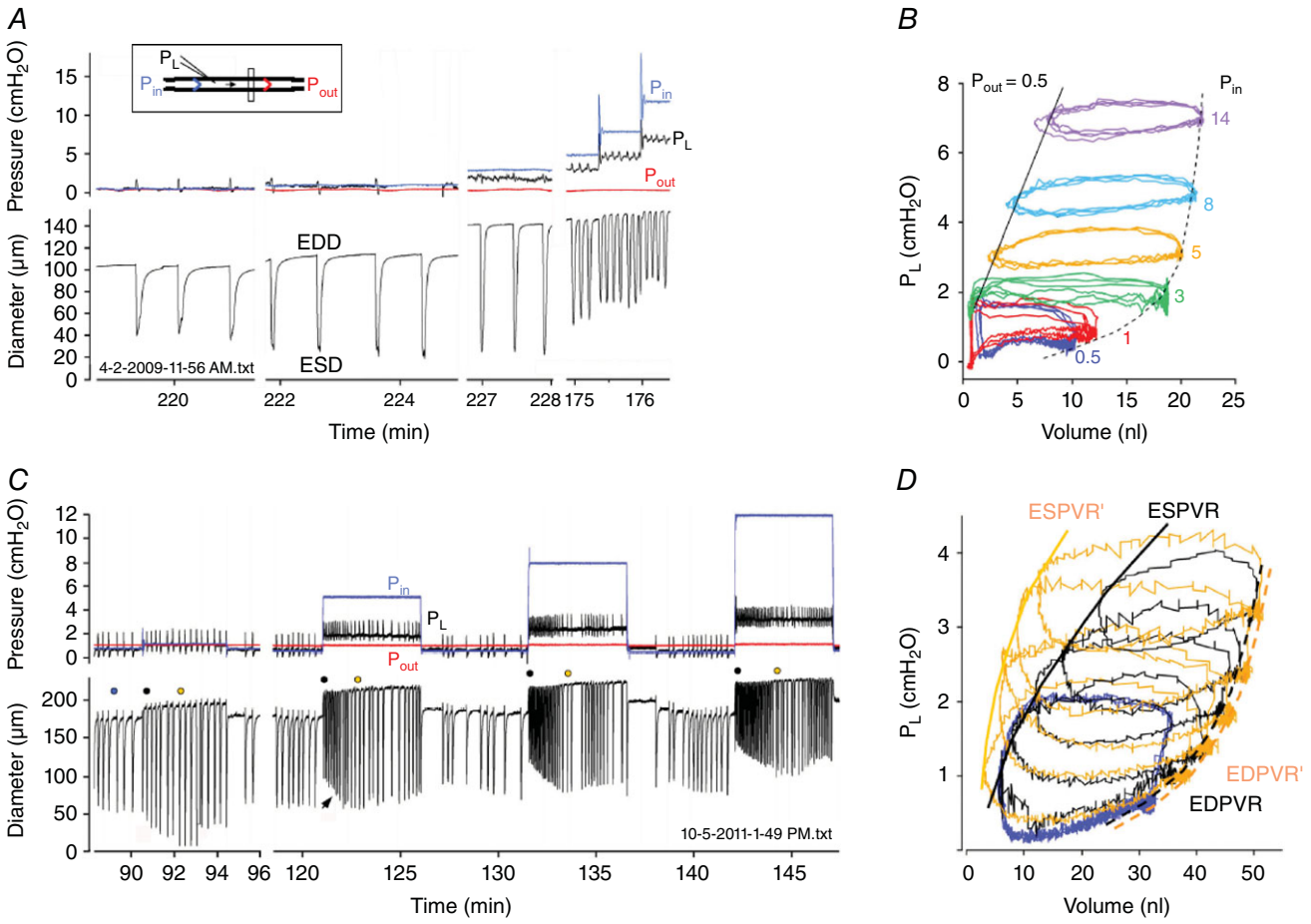

Figure 3. Effect of elevating preload independently of afterload on the contractile function of an *ex vivo* mesenteric lymphangion from rat
A, P_{in} was elevated to various levels while P_{out} was held constant. Pipette resistances were purposely kept to relatively high values to limit the inhibitory effect of forward flow produced by $P_{in} > P_{out}$ gradient. Inset shows diagram of pressure and diameter measurement sites. *B*, pressure–volume loop constructed from a portion of the data in *A*, showing the curvilinear *P–V* relationship for EDD (dashed line) and linear *P–V* relationship for ESD. *C*, time course of spontaneous contraction AMP and FREQ changes after a series of step elevations in P_{in} (P_{out} held constant). After each step, AMP falls but then recovers (or gets even larger) over the course of ~1 min (arrow). Also, a burst of high FREQ contractions occurs, with FREQ subsequently slowing slightly. *D*, *P–V* plot of some of the data in *C*; blue traces represent data from 3 contraction cycles prior to the P_{in} steps (corresponding to time indicated by blue dot in *C*), black and gold traces represent single contraction cycles corresponding to the black and gold dots in *C*, immediately after the pressure step (black dots in *C*) or ~1 min later (gold dots in *C*). The shift in the end-systolic *P–V* relationship (ESPVR) with time after P_{in} elevation, with unchanged end-diastolic (ED)PVR reflects an increase in contractility. Modified from Scallan *et al.* (2012*b*).

dependent extremities where outflow pressures can reach relatively high levels (Olszewski, 2002). An elevation in lymphatic outflow pressure is analogous to an elevation in aortic pressure for the ventricular pump, as it increases the load against which the pump must eject. For rat mesenteric vessels studied ex vivo, the limit against which a single lymphangion pumped was determined by slowly raising P_{out} in ramp-wise fashion while monitoring the opening of the output valve in systole (Davis *et al.* 2012). On average the P_{out} level before reaching pump failure, denoted by cessation of valve opening with each contraction cycle, was ~11 cmH_2O higher than P_{in}, with considerable variation between lymphangions (range 2–18). Given that the inter-valve distance is only ~1 mm and the difference in pressures between lymphangions is only 1–2 cmH_2O *in vivo* (Zweifach & Prather, 1975), there is seemingly a large margin of safety built into the system.

Contraction frequency. In the heart, cardiac output = stroke volume × heart rate. The analogous expression for the lymphatic pump is FPF = EF × FREQ. The contraction FREQ of collecting lymphatics is exquisitely sensitive to pressure, and changes as small as 0.5 cmH_2O can double FREQ (Scallan *et al.* 2012*b*). In some cases FREQ increases 10-fold over the pressure range 0–5 cmH_2O. Striking examples of this response can be observed in Fig. 3*C* at time = 132 and 142 min. As in the heart, high FREQ can limit filling (West, 1991) and thus have a negative effect on AMP. This effect is illustrated in Fig. 4*B* at time = 391 min, where an extended pause between contractions allows EDD to increase to a higher value.

Contractility. 'Contractility' is often used in a broad sense in the lymphatic literature to describe the enhancement of AMP or FREQ in response to a pressure increase or agonist activation (McHale *et al.* 1980; Benoit *et al.* 1989; Gashev *et al.* 2002; Muthuchamy & Zawieja, 2008; von der Weid *et al.* 2008*a*). In contrast, the term 'contractility', and the related term, 'positive inotropy', have very specific definitions in the cardiac literature. Positive inotropic agents produce an increase in cardiac muscle contractility: an increase in the strength and velocity of force development at constant preload. Likewise, an increase in aortic pressure (afterload) leads to an intrinsic increase in contractility, which is also called the ANREP effect (Sarnoff *et al.* 1960; West, 1991). A similar phenomenon can be observed in isolated lymphangions in response to elevated outflow pressure. Ramp-wise elevation in P_{out} is associated with a constriction on the upstream side of the valve and a gradual decline in AMP; however, when P_{out} is returned to control, unusually large amplitude contractions are observed for a limited period of time (see Fig. 8 in Davis *et al.* (2012). Likewise, a step-wise increase in P_{out} produces an initial reduction in ESD that gradually recovers over the course of a few minutes (Fig. 4*B*) and, if the pressure–diameter relationship is plotted (with diameter converted to volume), the line describing the end-systolic volume (ESV) *vs.* pressure relationship shifts gradually, increasing its slope over time; the slope increase is indicative of an increase in contractility (Fig. 4*C*). Interestingly, unlike in the heart, step increases in preload can also transiently increase contractility in *ex vivo* lymphangions (Fig. 3*D*). The mechanistic bases of

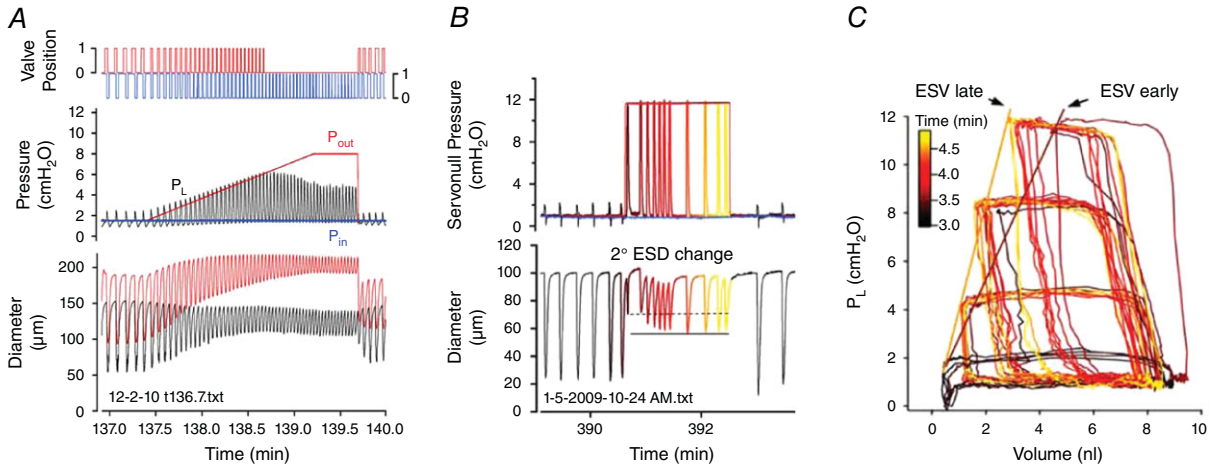

Figure 4. Effect of elevating afterload in an *ex vivo* mesenteric lymphangion from rat
A, ramp-wise elevation in P_{out} (with P_{in} held constant) leads to a progressive increase in the peak systolic pressure developed in the lumen (P_L, black trace). Note also a modest, progressive constriction on the input side of the valve during the pressure ramp. Opening of the output valve (top red trace) is indicative of ejection during systole, until P_{out} reaches ~6.2 cmH_2O, at which point the pump limits (fails). *B*, response to a step increase in P_{out}. Contraction AMP declines initially but then partially recovers over the next ~1 min. *C*, *P–V* plot of the data in *B* showing time-dependent leftward shift in the curves after a P_{out} step (data in *B* represent the top set of curves). See diagram in Fig. 2 for explanation of pressure, diameter and valve position measurements. ESV, end-systolic volume. Modified from Davis *et al.* (2012).

these responses are not known but may involve changes in calcium sensitivity, as shown for cardiac muscle (Solaro, 2011).

At least four additional factors have a significant impact on lymphatic pumping. Each of these is addressed in the following sections.

Neural modulation. While not required for lymphatic contractions *per se*, the effects of neural signalling molecules appear to be keenly involved in regulating lymphatic contractions. However, direct observations of lymphatic innervation and the mechanisms regulating this process are under-studied, with much of our current understanding stemming largely from experiments performed in the 1980s and 90s in vessels from different regions and species. A comprehensive discussion of this topic merits its own review (Zawieja *et al.* 2011).

Sympathetic adrenergic nerve fibres appear to be the dominant neural innervation of the lymphatic vasculature (Todd & Bernard, 1973; Alessandrini *et al.* 1981; McHale, 1990; Hollywood & McHale, 1994). α-Adrenergic stimulation of contractile lymphatic vessels consistently increases tone, AMP and FREQ (McHale, 1990), and these effects are countered by β-adrenergic receptor activation (von der Weid, 1998). Muscarinic receptors on LMCs also promote an increase in FREQ; however, this action of a muscarinic receptor agonist is usually masked by a dominant, inhibitory effect of NO as a consequence of endothelial nitric oxide synthase (eNOS) activation in lymphatic endothelial cells (Ohhashi & Takahashi, 1991; Scallan & Davis, 2013). Interestingly, serotonin (5-HT) can either inhibit or increase spontaneous lymphatic contractions depending on the species and the specific pattern of serotonin receptor expression (Miyahara *et al.* 1994; McHale *et al.* 2000; Chan & von der Weid, 2003). The inhibitory effects on contraction by both serotonin and vasoactive intestinal peptide (VIP) appear to be mediated through cAMP/cGMP and downstream activation of K_{ATP} channels (Ohhashi *et al.* 1983; Chan & von der Weid, 2003; von der Weid *et al.* 2012). Calcitonin gene related peptide also produces inhibitory effects on lymphatic contractions through signalling mechanisms in both LEC and LMC layers that appear to be mediated by the cAMP-K_{ATP} axis (Hosaka *et al.* 2006). The neurotransmitter substance P (SP), commonly associated with afferent nerve endings, also has profound effects on lymphatic function, promoting extensive tone generation and increased FREQ (Amerini *et al.* 2004; Davis *et al.* 2008*b*), although this enhancement comes at the expense of reduced AMP. Direct evidence for SP+ and VIP+ peptidergic innervation has been demonstrated in guinea pig (Guarna *et al.* 1991) and bovine (Ohhashi *et al.* 1983) mesenteric collecting lymphatics, but further work is required to determine if those findings are representative of other species and tissue beds. The human thoracic duct has both sympathetic and parasympathetic innervation (Mignini *et al.* 2012) that appears to decrease with ageing. This innervation is functional because the fibres can be activated via electrical field stimulation and affect contractions in isolated human thoracic ducts (Telinius *et al.* 2014*a*), similar to previous results in sheep lymphatics (Hollywood & McHale, 1994).

Contraction synchrony. The LMC layer of a lymphangion must contract in a coordinated, nearly synchronized manner to generate a systolic pressure pulse that can open the outflow valve and eject lymph; synchronization may be even more critical as outflow pressure is elevated. After lymphatic contractions are triggered by an AP in one of the LMCs, the AP propagates rapidly from cell to cell (at $\geq$8 mm s^{-1}), and in either direction, over the length of the lymphangion (McHale & Meharg, 1992; Zawieja *et al.* 1993; Venugopal *et al.* 2007). Synchronized contractions require electrical coupling between the LMCs, presumably through connexins that form intercellular gap junctions. Although electrical coupling between LM cells has been documented (von der Weid *et al.* 1996; Crowe *et al.* 1997), the connexin isoform(s) in the LMC layer have yet to be identified. In mesenteric lymphatic vessel segments studied either *ex vivo* or *in vivo*, the application of gap-junction blockers (e.g. n-heptanol, oleic acid) leads to uncoordinated contractions of different parts of a lymphatic chain (McHale & Meharg, 1992; Zawieja *et al.* 1993). However, the particular gap junction blockers used in early studies were notoriously non-specific; in the future, targeted deletion of specific connexin isoforms using gene knockout models will probably clarify the specific gap junctions involved in coordinating contractions of the lymphatic muscle layer.

In blood vessels, signals for vasodilatation are conducted primarily along the endothelium because endothelial cells (ECs) are well-coupled electrically, particularly by connexin (Cx) 40 (Cx40) (Simon & McWhorter, 2002; de Wit *et al.* 2003; Wagner *et al.* 2007). A focal hyperpolarization induces a hyperpolarization wave that spreads upstream rapidly along the EC and across the internal elastic lamina to the VSM cell layer, which is coupled through connexins in EC projections (myoendothelial gap junctions, MEGJs) to VSM cells (Emerson & Segal, 2000; Sandow *et al.* 2012). In contrast, LECs express Cx37, Cx47 and Cx43 but not Cx40 (Simon & McWhorter, 2002; Kanady *et al.* 2011). Presumably, hyperpolarizing current can spread along the LEC layer in the same manner as in blood vessel endothelium, but that has not been tested; neither is the functional benefit of conducted hyperpolarization in a collecting lymphatic vessel obvious. Interestingly, Cx43 and Cx47 mutations are associated with the development of human lymphoedema for reasons that could be related to contraction wave dyssynchrony, valve

development and/or valve maturation (Ferrell *et al.* 2010; Finegold *et al.* 2012).

An important difference between arterioles and lymphatics is the very limited degree of electrical coupling between LECs and LMCs (von der Weid *et al.* 1996; Crowe *et al.* 1997). We have confirmed that LMCs of pressurized rat lymphatic vessels have a resting membrane potential around −40 mV and fire spontaneous APs, while LECs have a stable resting potential around −70 mV (von der Weid & Van Helden, 1997) that does not oscillate during contractions of the overlying muscle layer (J. P. Scallan, M. J. Davis & S. D. Zawieja, unpublished observations). A possible advantage of this arrangement is that weak electrical coupling between LECs and LMCs may promote more efficient spread of the AP along the LMC layer if less electrical signal is shunted to LECs, which, collectively, would act as an electrical sink.

Valve function. Collecting lymphatics contain bicuspid valves (Figs 1 and 2) whose leaflets extend from a ring-shaped base and insert as two buttresses into the vessel wall (Schmid-Schönbein, 1990; Bazigou *et al.* 2014). The valve opening is a tapered funnel (Fig. 4 in Davis *et al.* 2011 and Movie S3 in the online Supporting information). Downstream from the valve is an enlarged sinus that facilitates valve opening and partially balances the high resistance of the narrow orifice created by the valve leaflets (Bazigou & Makinen, 2013; Wilson *et al.* 2015). Valves are spaced at semi-regular intervals and the factors that control their spacing are not known (Kanady *et al.* 2011), but may involve Notch1 (Murtomaki *et al.* 2014) and semiphorin3A/neuropilin-1/plexinA1 (Bouvree *et al.* 2012; Jurisic *et al.* 2012). Valves begin developing at embryonic day (E)15–E16 in the mouse and mature postnatally under a genetic programme that includes the transcription factors *GATA2*, *PROX1* and *FOXC2* (Petrova *et al.* 2004; Bazigou & Makinen, 2013; Bazigou *et al.* 2014; Sabine & Petrova, 2014; Kazenwadel *et al.* 2015; Sweet *et al.* 2015). Fluid shear stress is a key regulator of valve development (Sabine *et al.* 2012, 2015; Sweet *et al.* 2015) and mature valves exhibit a differential distribution of connexins, eNOS and other proteins across the valve (Petrova *et al.* 2004; Bohlen *et al.* 2011; Sabine & Petrova, 2014; Sabine *et al.* 2015). Many of the same factors regulate the development of the lymphovenous valves (Srinivasan & Oliver, 2011; Geng *et al.* 2016), which form earlier (E12) and whose integrity is critical for preventing backflow of blood into the central lymphatic ducts and thus maintaining separation between the lymphatic and blood circulations (Hess *et al.* 2014; Sweet *et al.* 2015).

The ultrastructure of lymphatic valves was described extensively in the 1970s–1980s (Lauweryns, 1971; Vajda & Tomcsik, 1971; Gnepp & Green, 1980; Albertine *et al.* 1982), yet no functional studies were published until recently. We developed tests of isolated valves for rat (Davis *et al.* 2011) and mouse vessels (Sabine *et al.* 2015) in which *ex vivo* segments contain a single valve to enable pressure control through cannulation pipettes on either side. A servo-nulling pipette, inserted through the wall on the input side and positioned upstream from the valve leaflets, allows measurement of small, local pressure changes upstream from the valve (Fig. 5). With this protocol the adverse pressure gradient (ΔP) required to close the valve can be determined with high precision. The first surprising observation is that the valves have an open bias, meaning they are predisposed to be open when the trans-valve pressure gradient is zero. Although this property may seem inefficient from a conceptual viewpoint (permitting reflux at certain times in the contraction cycle), it results in lower resistance to forward flow under conditions of little or no adverse pressure gradient. A second surprising observation is that the ΔP for valve closure is substantially dependent on the vessel diameter. At low diameters (associated with systole or a high level of basal tone), adverse pressures as small as 0.1–0.3 cmH_2O are sufficient to close the valve; however, as vessel diameter approaches its maximum (as it does when the vessel is distended in chronic lymphoedema; Olszewski, 2002), pressures of several cmH_2O are required. One caveat of the valve closure measurement is that the resistances of the small micropipettes used to cannulate rodent vessels can lead to an overestimation of the ΔP for valve closure when substantial flow occurs. During the normal lymphangion pump cycle, the diameter at the end of systole/early diastole reduces to a value that will facilitate valve closure if there is even a slight (<0.3 cmH_2O) adverse pressure gradient. This implies that the valve may not close properly in lymphangions that are dilated and/or unable to generate a systolic pressure greater than that in the adjacent lymphangion downstream. The simple observation of reflux occurring in a collecting lymphatic network *in vivo* (Brice *et al.* 2002; Kriederman *et al.* 2003; Normen *et al.* 2009), when pressures are unknown, does not provide incontrovertible evidence of an abnormal valve. Even modest valve defects can have enormous effects on the relationship between ΔP for closure and vessel diameter (Sabine *et al.* 2015), as discussed below.

Barrier function. It is now appreciated that filtration disequilibrium exists across blood capillaries in skin, muscle and other tissues, where the combined hydrostatic and oncotic pressure gradient favours net filtration of fluid into the tissues. The steady reabsorption of this fluid by the venous circulation cannot occur in most organs, necessitating a lymphatic vasculature that absorbs and transports this fluid. Thus, the lymphatic vasculature serves a vital, rather than accessory, role in preventing tissue oedema (Levick & Michel, 2010). Lymphatic vessels have widely been regarded as 'impermeable' to fluid and

solute, which would suggest that these vessels must have special junction adhesion proteins to constitute a very tight barrier. However, detailed analyses of collecting lymphatic endothelial junction proteins reveal no major differences from those of blood vessels (Baluk *et al.* 2007). Recent studies designed to directly quantify solute flux across the vessel wall of intact lymphatic vessels *in vivo* (Scallan & Huxley, 2010) and *ex vivo* (Scallan *et al.* 2015) demonstrate that collecting lymphatics are not only permeable to solute and fluid, but that their albumin permeability is comparable to that of postcapillary venules; further, lymphatic permeability is actively regulated because it can be modified by several signalling pathways, including nitric oxide (Scallan *et al.* 2013, 2015).

Lymphatic capillaries are an order of magnitude more permeable than collecting lymphatic vessels (Scallan & Huxley, 2010), most likely due to their discontinuous pattern of junctional adhesion proteins (Leak & Burke, 1968; Baluk *et al.* 2007), facilitating fluid and solute absorption from the interstitium. Although permeable collecting vessels may seem inefficient compared to idealized vessels that would retain all fluid and solute, the basal permeability leads to several interesting consequences. For example, it has been hypothesized that this extravasation allows antigen transported by the collecting lymphatics to reach local immune cells to mediate immune responses (Kuan *et al.* 2015). The basal permeability may therefore serve as the means of communication between lymph contents and regulation of vessel contraction through the recruitment and activation of immune cells. Activation of these immune cells may then lead to the production of nitric oxide through inducible nitric oxide synthase (iNOS) or other vasoactive molecules, which would inhibit lymphatic contractions and reduce lymph flow. Another consequence of having permeable collecting lymphatics is that lipids carried by these vessels will be distributed to the tissues, which may help explain why there is always adipose tissue located adjacent to collecting lymphatics and lymph nodes (Harvey, 2008). Indeed, this supports the finding that mice heterozygous for the lymphatic identity transcription factor, *Prox1*, develop late onset obesity as

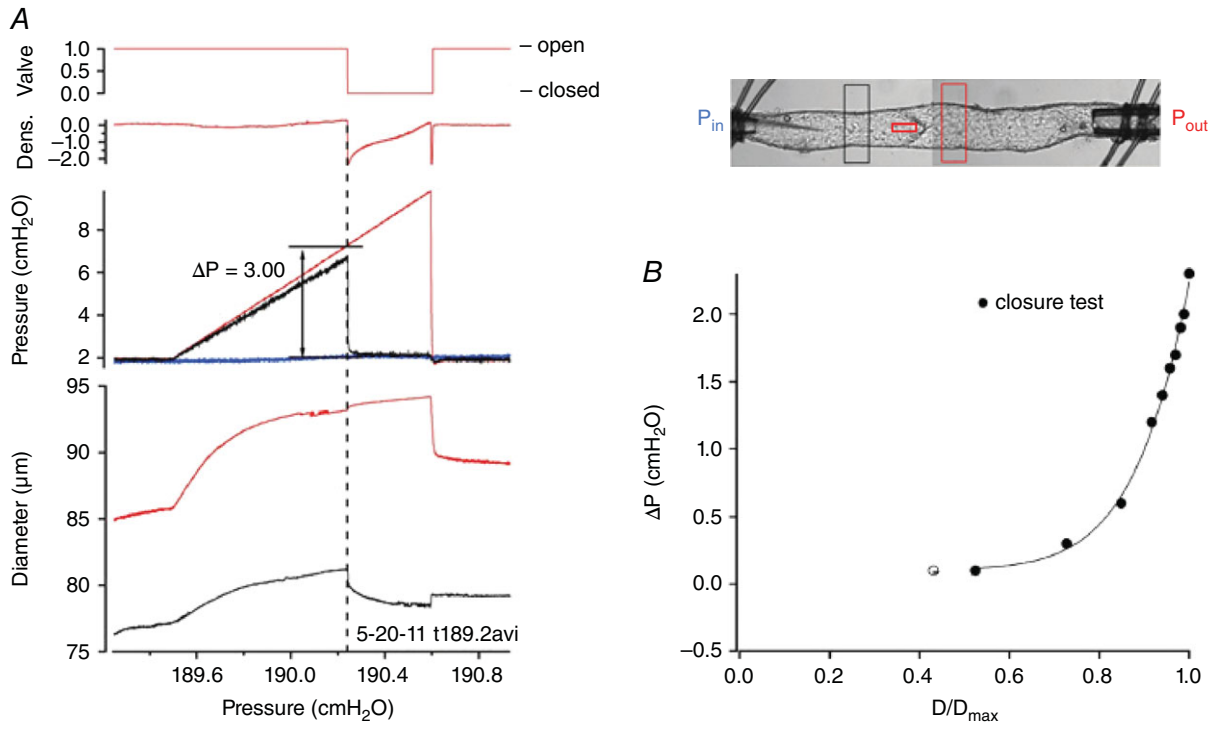

Figure 5. Ex vivo test for valve closure
A, valve closure test for a 1-valve mouse popliteal lymphatic. An image of the mouse vessel with diameter tracking windows and position of the servo-nulling pipette from which the black pressure trace in *A* was obtained is shown to the right. To measure the adverse pressure gradient required for valve closure, both pressures are set equal (valve is open), then P_{out} is elevated ramp-wise (red pressure trace) until the valve snaps closed, as indicated by a rapid drop in the black trace; after a few more seconds, the P_{out} ramp is terminated. Dotted line shows alignment of changes in densitometer window positioned upstream from leaflets, servo-null pressure and diameter on the input side of the valve at the point of closure. The ΔP for closure is the difference between P_{out} and P_{in} at the moment of valve closure. *B*, summary data for a single rat mesenteric lymphatic valve. ΔP for closure is <0.3 cmH_2O at low vessel diameters but rises to 2.2 cmH_2O when the vessel is near-maximally distended. *D* represents diameter. Panel *B* modified from Davis *et al.* (2011).

a result of pathological lymphatic leakage (Harvey *et al.* 2005).

Lymphatic pump dysfunction in pathological states

Lymphoedema can result from inherited (primary) or acquired (secondary) defects of the lymphatic system. Mutations in a number of genes including *PROX1, GATA2, FOXC2* and *VEGFR3/FLT4* lead to malformations in lymphatic vessels and/or valves, resulting in primary lymphoedema in humans (Brice *et al.* 2005; Ferrell *et al.* 2010; Mellor *et al.* 2011; Finegold *et al.* 2012; Sabine & Petrova, 2014). However, the majority of lymphoedema cases in developed countries occur secondary to other pathologies, for example breast cancer radiation/surgery (Szuba *et al.* 2003), where 30–50% of breast cancer survivors undergoing axillary node dissection ultimately suffer from lymphoedema (Rockson, 1998; Armer, 2005). Lymphatic pump dysfunction in this context could result from a number of factors, including contractile dysfunction, abnormal lymphangiogenesis, barrier dysfunction and valve defects. Each is discussed in the next section.

Contractile dysfunction

Contractile dysfunction is a common theme among several pathologies that involve the lymphatic system. Pioneering studies of human patients with chronic lymphoedema provide evidence of lymphatic contractile dysfunction. Olszewski and colleagues cannulated collecting lymphatic vessels in the dependent extremities of such patients and recorded pressures, either end-on or side-on, when the limbs were placed in various positions (Olszewski *et al.* 1968; Olszewski, 2002). Vessels in patients with lymphoedema were enlarged, exhibited weak contractions and elevated lymphatic diastolic pressures with the limbs in a dependent position. More recently, another group studying lymphatic transport in humans with breast cancer lymphoedema using lymphoscintigraphy has demonstrated that a component of the lymphatic dysfunction observed in these patients is lymphatic pump failure (Modi *et al.* 2007). That group is now employing genetic screening of human patients presenting with various forms of lymphoedema to identify new genetic mutations (Fotiou *et al.* 2015), some of which may regulate lymphatic contractile function.

Whether lymphatic pump dysfunction precedes the development of lymphoedema, or lymphoedema overloads the capacity of the lymphatics to precipitate contractile dysfunction, is difficult to determine. This question is particularly challenging in humans as patients are not seen in the clinic until lymphoedema is already established. Animal models of lymphoedema can, in principle, be used to distinguish between cause and effect, but the severity of lymphoedema produced in rodent models is usually quite limited (Shin *et al.* 2003). More recent rodent models (Mendez *et al.* 2012*a*,*b*) consistently produce lymphoedema, and those models might be useful particularly if combined with newly developed imaging approaches to assess lymphatic contractile function *in vivo* (Liao *et al.* 2011; Proulx *et al.* 2013; Kwon *et al.* 2014). Likewise, *ex vivo* methods for quantifying murine lymphatic contractile function (Scallan & Davis, 2013) could be used in conjunction with non-invasive, *in vivo* imaging techniques to assess collecting lymphatic contractile function at various time points during and after the development of lymphoedema (Dongaonkar *et al.* 2013).

Inflammation. Lymphatic contractile dysfunction is often contingent with tissue inflammation, and post-surgical infection is a significant risk factor for developing secondary lymphoedema. Histological changes in lymphatic capillary size and density are widely assessed in both human disease states, yet experimental animal models often fail to address the pump function of the collecting vessels. The lymphatic expansion seen in disease states may actually suggest a deficit in lymphatic pumping, resulting in an activation of a compensatory lymphangiogenesis that nevertheless fails to resolve the pumping insufficiency (Tammela *et al.* 2007). Inflammatory modulation of lymphatic pump function was first described in the 1980s as a consequence of endotoxin infusion in sheep (Elias *et al.* 1987), where the actions of prostaglandins (Johnston *et al.* 1983; Ohhashi & Azuma, 1984) were hypothesized to contribute to the oedema observed in sepsis. The formation of reactive oxygen species (ROS), a hallmark of inflammation, also negatively influences lymphatic contractions (Zawieja *et al.* 1991) and ROS production as a result of fluorescent dye excitation for prolonged periods during *in vivo* imaging sessions could become a significant confounding variable when assessing lymphatic contractile function.

Current research on lymphatic contractile dysfunction in inflammation has focused heavily on the production of NO by regional iNOS positive immune cells (Liao *et al.* 2011). Recent studies have demonstrated the significant investiture of immune cells, primarily myeloid-derived cells, in the adventitia of lymphatic collecting vessels, and their presence appears to be dynamically regulated in disease states and associated with adverse lymphatic function (Cromer *et al.* 2015; Chakraborty *et al.* 2015*b*). Even after removal from the tissue, a typical collecting lymphatic vessel still retains significant populations of immune cells (neutrophils, macrophages, monocytes, dendritic cells, mast cells) that reside on and within the wall, and can modulate contraction (Chatterjee & Gashev, 2012, 2014; Chakraborty *et al.* 2015*b*). As described above, NO is a potent inhibitor of lymphatic contractions and is

produced in abundance by iNOS-positive murine myeloid cells such as monocytes, macrophages and dendritic cells. Interleukin (IL)-1β increases iNOS expression in cultured LECs (Cromer *et al.* 2014) and impairs lymphatic pump function through PGE_2 production (Johnston *et al.* 1983; Hanley *et al.* 1989; Al-Kofahi *et al.* 2015). However, iNOS expression and NO production can vary between different mouse strains and this must be taken into consideration when using *in vivo* models to assess the consequences of inflammation and lymphatic pumping. Furthermore, the lack of iNOS expression or significant NO production by human macrophages (Schneemann *et al.* 1993; Gross *et al.* 2014) in response to classical inflammatory agents raises critical questions concerning the translation of lymphatic dysfunction in rodent inflammatory models to human disease.

Lymphatic dysfunction is also present in lymphatic networks of the ear in the apolipoprotein E knockout ($ApoE^{-/-}$) mouse model of hypercholesterolaemia (Lim *et al.* 2009). Dysfunction results in part from profound structural abnormalities in the lymphatic vasculature: initial lymphatic vessels are enlarged and collecting vessels assume an immature phenotype, with greatly decreased smooth muscle cell coverage, impaired valve formation or maintenance, and upregulation of the lymphatic capillary marker, Lyve1. It has yet to be demonstrated whether loss of mural cell coverage occurs in tissue beds outside of the ear, but if so, impairment of contractile function and lymph transport would certainly be expected.

Rheumatoid arthritis is a chronic inflammatory joint disease. In tumour necrosis factor (TNF)α-overexpressing mice, the popliteal lymph node enlarges during the pre-arthritic 'expanding' phase, and subsequently 'collapses'. The collapsed phase is associated with the loss of the intrinsic lymphatic pulse *in vivo* (which may represent pumping strength, contraction frequency, or both) as assessed using a pressurized occluding cuff in conjunction with near infrared fluorescence (NIRF) imaging (Bouta *et al.* 2014). Consistent with contractile dysfunction, the pumping pressure of popliteal afferent lymphatics in those mice was found to be significantly elevated compared to the pumping pressure in wild-type (WT) control mice in the expanding phase; however, pressure decreased below that of WT vessels in the collapsed phase. Interestingly, lymph node pressure followed the inverse pattern. Altogether, these changes result in a severely compromised lymph flow associated with collapsed lymph nodes in this mouse model.

Crohn's disease and inflammatory bowel disease (IBD). The earliest descriptions of Crohn's disease or 'regional ileitis' by prominent clinical pathologists involved observations of lymphangectasia, oedema and lymphatic-associated granulomas. The regional heterogeneity of the disease was suggested to be due to obstruction or failure of the lymphatics draining that region; however, the majority of current IBD research fails to account for an underlying component of lymphatic dysfunction (Van Kruiningen & Colombel, 2008). Artificial sclerosing or damage of the mesenteric collecting lymphatic vessels is able to recapitulate many of the phenotypes of Crohn's disease (Kalima, 1970) and chemically induced rodent models of IBD exhibit lymphatic contractile dysfunction. Guinea pig mesenteric vessels isolated from a 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced model of ileitis exhibit a dramatic loss of spontaneous contractions and enlarged collecting vessels (Wu *et al.* 2006), both of which are linked to NO-dependent activation of K_{ATP} channels (Mathias & von der Weid, 2013). Mesenteric vessels from rats with TNBS-induced colitis also show impaired lymphatic contraction regulation despite being isolated from the ileum and remote from the active site of TNBS administration (Cromer *et al.* 2015). While IBD studies in mice replicate the increased lymphatic density and remodelling observed in human disease, there are currently no data available on mouse lymphatic contractile function in this disease (Alexander *et al.* 2010; Ganta *et al.* 2010; Rahier *et al.* 2011). This is in part due to the lack of propulsive lymphatic contractions of murine mesenteric collecting lymphatics, which raises important questions regarding the role of lymphatic contractile dysfunction in the pathology of these diseases in the mouse and the usefulness of that species to replicate this particular aspect of human pathology.

Obesity and metabolic syndrome. Despite being a historical risk factor for the development of post-surgical lymphoedema in cancer patient survivors (Ahmed *et al.* 2011), we know relatively little about the effects of obesity on lymphatic function (Chakraborty *et al.* 2010; Mehrara & Greene, 2014). Clinical data continue to accumulate, linking obesity to lymphatic dysfunction with the emergence of massive localized lymphoedema in the morbidly obese population, which appears to be functionally, but not structurally, related to the obesity (Vasileiou *et al.* 2011). As mentioned above, mice heterozygous for the lymphatic transcription factor *Prox1* consistently develop adult onset obesity (Harvey *et al.* 2005; Sabine *et al.* 2015). While functional characterization of the lymphatic pump has not been performed in $Prox1^{+/-}$ mice, those mice display significant structural abnormalities. However, an obese phenotype has not been observed in other models of disrupted lymphatic development (Harvey *et al.* 2005). In humans, massive localized lymphoedema appears to be largely predicated on body mass index, although obese patients probably also fall under the spectrum of the metabolic syndrome, an amalgamation of metabolic impairments that are largely driven by

obesity (Grundy, 2004). Mesenteric lymphatic vessels isolated from a high-fructose-fed, non-obese rat model of metabolic syndrome were shown to have a significant reduction in lymphatic pumping as a consequence of reduced contraction frequency (Zawieja *et al.* 2012). The high-fructose fed Sprague–Dawley rat does not gain weight and adiposity changes are controversial, despite consistent presentation with elevated serum insulin, glucose, cholesterol and triglycerides (Oron-Herman *et al.* 2008). However, a diet-induced obesity mouse model recapitulates the impaired pressure–FREQ response of lymphatic collecting vessels observed *in vivo* using NIRF imaging (Blum *et al.* 2014), and interstitial flow and lymphatic capillary recruitment may be impaired in the tissue remodelling associated with the disease (Weitman *et al.* 2013). The primary defect in contractile function in both of these cases appears to be a reduction in FREQ. This effect is similar to the lymphatic dysfunction observed in aged rats, which also display a reduced FREQ (Akl *et al.* 2011) associated with increased ROS production in the vessel wall (Thangaswamy *et al.* 2012). These observations may point to a common underlying mechanism of dysfunction under conditions of obesity or metabolic stress, perhaps suggesting a role for a metabolic sensor such as the K_{ATP} channel.

Fibrosis and chronic inflammation. A clinical hallmark in lymphoedema patients is a predisposition for, and the presence of, elevated extracellular matrix deposition and fibrosis (Rockson, 2013). Unsurprisingly, fibrosis is a significant factor in the aetiology of both human obesity (Henegar *et al.* 2008; Divoux *et al.* 2010) and Crohn's disease (Lockhart-Mummery & Morson, 1960), where fibrosis may contribute to the inhibition of fluid return and lymphatic function in those disease states. Fibrosis is often the result of chronic inflammation and typically is associated with the activation of the Th2 inflammatory paradigm and the production of IL-6, IL-13, IL-4 and transforming growth factor (TGF)β. These cytokines have profound influences on altered macrophage accumulation and polarization (Mosser & Edwards, 2008; Ghanta *et al.* 2015), fibroblast activation and muscle cell dedifferentiation into a secretory phenotype. Recent studies that have attempted to delineate the role of fibrosis in lymphoedema have relied heavily on gross observations using the mouse tail model and oxazolone-induced swelling in the rat axillary lymph node dissection model. The resolution of lymphoedema in the mouse tail model depends on the ability of the wound to fill in and for superficial lymphatic vessels to reform connections. This process is accelerated by the application of vascular endothelial growth factor C (VEGF-C), which promotes lymphangiogenesis (Yoon *et al.* 2003; Rutkowski *et al.* 2006), or the blockade of IL-4 and TGFβ, which otherwise inhibit lymphangiogenesis (Clavin *et al.* 2008; Avraham *et al.* 2010, 2013). Similar patterns emerge in experiments using the rat axillary node oxazolone/dissection model, suggesting that fibrosis plays an important role in inflammation-induced swelling in that preparation (Lynch *et al.* 2015).

These studies highlight the negative impact of fibrosis on lymphatic function and raise important questions for further research. What are the effects of Th2 cytokines on the lymphatic pump function and/or contractile activity of the remaining lymphatic vessels in these models? Is the LMC phenotype and/or contractile function impaired due to the exposure to these cytokines or vessel wall remodelling? To what extent do fibrosis-associated changes in the adventitia/matrix covering the collecting lymphatic vessels alter their function? As previously discussed, both initial and collecting lymphatic vessels are populated by multiple types of immune cells, and the influence of those cells and the cytokines they produce on LECs and LMCs are areas of active research.

Lymphatic network development/maturation/disruption

Defects in lymphangiogenesis occur in numerous mouse models deficient in genes responsible for lymphatic development, maturation, or valve formation/maintenance. Several of these models are characterized by a lack, or augmented recruitment, of smooth muscle cells to lymphatic capillaries or collecting lymphatic vessels. Aberrant LMC recruitment has been reported in mice deficient for the genes *Foxc2* (Petrova *et al.* 2004), *Reln* (Lutter *et al.* 2012), *Angpt2* (Makinen *et al.* 2005; Dellinger *et al.* 2008) and *Akt1* (Zhou *et al.* 2010). Additionally, platelet-specific deletion of *Clec2* disrupts lymph flow, which indirectly leads to enhanced smooth muscle cell (SMC) recruitment to collecting lymphatic vessels and the thoracic duct (Sweet *et al.* 2015). Whether enhanced SMC recruitment or decreased SMC investiture would lead to augmented or inhibited contractile function, respectively, has yet to be directly explored.

Lymphatic vessel dilatation and network hyperplasia have also been observed in some of these and other models (Lapinski *et al.* 2012; Sevick-Muraca & King, 2014) and might be predicted to have a negative impact on lymphatic pump function; however, that idea has yet to be tested. Additionally, as lymph is propelled against an adverse pressure gradient, failure of the lymphovenous valve(s) and/or collecting lymphatic valves may lead to elevation of intralymphatic pressures that the lymph pump system may be unable to overcome; this could result in regurgitation and lymph stasis in the initial lymphatic network. Prolonged exposure to elevated pressure could lead to proliferation, remodelling and contractile muscle phenotype loss. A combination of *in vivo* imaging

techniques and intra-lymphatic pressure recordings in intact animals will ultimately help answer these questions.

Barrier dysfunction

Collecting lymphatic permeability is regulated and maintained at a low level ($\sim 2 \times 10^{-7}$ cm s^{-1}), but in certain disease states the barrier properties change and filtration has been shown to increase dramatically. In mouse mesenteric lymphatics studied *ex vivo*, comparisons of WT and *eNOS*$^{-/-}$ vessels suggest that basal nitric oxide (NO) production increases lymphatic permeability. Exogenous NO donors increase lymphatic permeability further, while inhibitors of NO synthase decrease basal lymphatic permeability. Histamine, which elicits endogenous NO production, exerts a similar effect on lymphatic permeability (Scallan *et al.* 2015). Diabetic mice deficient in the leptin receptor exhibit a >100-fold increase in lymphatic permeability. Unexpectedly, this barrier dysfunction is rescued by exposing the vessels to L-arginine to augment NO production, indicating that severely impaired, as well as elevated, NO production can both lead to disrupted lymphatic barrier function. A plausible explanation for this result is that cAMP, which stabilizes and lowers permeability, may be reduced in LECs of diabetic animals because it is normally degraded by the enzyme phosphodiesterase 3, which is overactive in those animals due to impaired NO production. Thus, treating the diabetic lymphatic vessels with a chemical inhibitor specific for phosphodiesterase 3 rescues the barrier dysfunction in leptin receptor knockout (*db*/*db*) vessels in mice (Scallan *et al.* 2015).

Two other metabolic diseases in which lymphatic barrier function has been shown to be compromised are obesity, as discussed above, and hypercholesterolaemia. Injection of Evans Blue dye into the ears of *ApoE*$^{-/-}$ mice reveals gross leakage of dye out of the lymphatic vessels of the ear (Lim *et al.* 2009). This defect is not due to the loss of the *ApoE* gene itself, since it can be rescued by pharmacological block of cholesterol absorption from the gut (Lim *et al.* 2013). Whether lymphatic leakage occurs in other organs during hypercholesterolaemia, and the mechanisms behind the leakage, remain to be elucidated. In atherosclerotic *ApoE*$^{-/-}$ mice, the lymphatic vasculature is required for reverse cholesterol transport even from the aortic wall. Without a functional lymphatic network near the aorta, labelled cholesterol was retained within the aortic plaques, presumably due to a defect in macrophage egress (Martel *et al.* 2013).

Valve dysfunction

Normal lymphatic pumping requires adequate contractile strength and synchrony of lymphatic muscle as well as proper valve function. If a lymphangion is undergoing robust, large amplitude contractions, but either valve has abnormal reflux, or is completely incompetent, then forward lymph propulsion will be compromised. When lymphatic output is calculated from the product of EF and FREQ (i.e. FPF), as it is in rodent vessels (Gashev *et al.* 2002, 2004; Liao *et al.* 2011; Nagai *et al.* 2011), that calculation assumes normally functioning valves; output will be grossly overestimated if the valves are in any way dysfunctional.

Defects in valve development underlie multiple types of primary lymphoedema (Sabine *et al.* 2015). Mice engineered with complete loss of *Gata2* function develop lymphoedema due to defective lymphovenous and lymphatic valves (Kazenwadel *et al.* 2015). Deletion of integrin $\alpha 9$, which with fibronectin lines the core of the lymphatic valve leaflet, results in truncated leaflets and retrograde flow from collectors to precollectors (Bazigou *et al.* 2009). Likewise, loss of function mutations of one allele of *FOXC2* in humans result in lymphoedema distichiasis, which is characterized by lymphoedema in dependent extremities (Mellor *et al.* 2007). *Foxc2*$^{+/-}$ mice lack about 50% of lymphatic (and venous) valves and exhibit many of the same systemic defects (Petrova *et al.* 2004). Inducible *Foxc2* null (*Foxc2*$^{\text{lecKO}}$) mice have a more severe phenotype, dying within a few days if induced shortly after birth, or at ~5 months of age if induced at 4 weeks, providing evidence that *Foxc2* is critical for lymphatic valve maintenance (Sabine *et al.* 2015). We recently applied some of the functional tests described above to valves in *Foxc2*$^{\text{lecKO}}$ mice. The valves exhibited a 4- to 9-fold increase in pressure back-leak compared to WT mice when outflow pressure was raised (Sabine *et al.* 2015). Defects in the valve closure–diameter relationship were also noted (M. J. Davis, unpublished observations), which would predict that pump output should be severely compromised even in the absence of changes in lymphatic muscle contractile function. Other than this example, pump function tests have not been reported for any genetically altered mice with valve defects; however, the application of such tests to newly developed mouse models of human primary lymphoedema (Lapinski *et al.* 2012; Kazenwadel *et al.* 2015; Geng *et al.* 2016) will be important for assessing the mechanism and severity of valve and/or pump dysfunction in those models.

Conclusions

Lymphatic pump function is essential for normal lymph transport, particularly in dependent extremities. Pump function is determined by the intrinsic properties of lymphatic muscle, which is regulated by lymphatic pre-load, afterload, spontaneous contraction rate, contractility and neural influences. In addition, normal pump function

depends on a coordinated LMC contraction wave, and proper LEC valve and barrier function. Each of these parameters may be compromised to a different extent in inherited and acquired forms of lymphoedema, as well as in diseases that have a secondary component of lymphatic dysfunction, including inflammatory diseases such as IBD, Crohn's disease, rheumatoid arthritis, obesity, diabetes and metabolic syndrome. The tools for investigating the roles of each factor are becoming available and can be applied to the study of lymphatic vessels in genetically modified mice, offering hope for the development of therapeutic strategies to target lymphatic dysfunction in humans, even in diseases that were previously unknown to contain a lymphatic component.

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Additional information

Competing interests

None declared.

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Supporting information

The following supporting information has available in the online version of this article.

Movie S1. Several spontaneous contraction cycles of a popliteal afferent collecting lymphatic vessel (pressurized to 3 cm H_2O, ex vivo) from a Prox1GFP mouse under brightfield illumination.
Movie S2. Same vessel as in Movie S1 recorded under confocal fluorescence illumination, with excitation at 499 nm and emission at 530± 10 nm.
Movie S3. Confocal fluorescence recording of lymphatic valve leaflets from a Prox1GFP mouse during a contraction cycle.