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Lymphatic Vessel Network Structure and Physiology

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Abstract

The lymphatic system is comprised of a network of vessels interrelated with lymphoid tissue, which has the holistic function to maintain the local physiologic environment for every cell in all tissues of the body. The lymphatic system maintains extracellular fluid homeostasis favorable for optimal tissue function, removing substances that arise due to metabolism or cell death, and optimizing immunity against bacteria, viruses, parasites, and other antigens. This article provides a comprehensive review of important findings over the past century along with recent advances in the understanding of the anatomy and physiology of lymphatic vessels, including tissue/organ specificity, development, mechanisms of lymph formation and transport, lymphangiogenesis, and the roles of lymphatics in disease.

Keywords

microcirculation; endothelial cells; smooth muscle

Introduction and Historical Perspective

A functional lymphatic vasculature is a requirement for the closed, high-pressure system for blood circulation in vertebrates, which leaks plasma components from capillaries and postcapillary venules. Lymphatic vessels maintain normal tissue fluid volumes by returning the capillary ultrafiltrate and extravasated plasma proteins to the central circulation. Lymphatics also have a key role in the transport of lipids absorbed in the digestive tract. In addition, the widespread distribution of lymphatics throughout the body allows the rapid identification of antigens and immunological responses. Interest in the contribution of the lymphatic system to health and disease has grown rapidly in recent years, due in large part to: 1) the advent to specific genes and molecular markers to study lymphatic vessels; 2) evidence that lymphatic dysfunction contributes to cardiovascular diseases; and 3) increased awareness that lymphatic dysfunction in patients is a fairly common occurrence. For example, lymphedema is the most prevalent secondary disease found in patients who have

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undergone treatment for cancer (205, 915). In addition, lymphedema filariasis is one of the most prevalent infectious diseases in the world, affecting millions of people worldwide (176). Many of the basic aspects of lymphangiogenesis and lymphatic transport have only recently begun to be clarified. Several major gaps in knowledge about the cellular and molecular mechanisms underlying lymphatic physiology persist, and the role of lymphatics in disease remains poorly understood.

The earliest known accounts of lymphatic vessels are contained in writings from the 4th century B.C.E. by Hippocrates and Aristotle. Several centuries later, the Greek physician Claudius Galen described mesenteric lymph nodes filled with chyle, or lymph consisting of emulsified fats that originates from the small intestine (623). However, during the middle ages, this knowledge was largely forgotten until 1622 when Gasaro Aselli had the serendipitous finding of "lacteal vessels" while dissecting a live dog's abdomen. He went on to trace these vessels back to the gut, and observed lymphatic valves that he surmised would prevent backflow. Aselli's work was published in 1627, after his death, at a time when William Harvey's theory of the circulation of blood was still novel and revolutionary (28, 337, 623). Several decades later, Jean Pecquet successfully demonstrated that chyle originating from the gut travels through the cisterna chyli and thoracic duct to the right subclavian vein, debunking the popular theory at the time that chyle traveled directly from the intestine to the liver. Contemporaries of Pecquet, Olaus Rudbeck (926) and Thomas Bartholin, independently demonstrated that lymph flows from the liver into the thoracic duct, and that lymphatics vessels are widespread throughout the body. Thomas Bartholin's naming of these vessels as "vasae lymphaticae" led to our current term "lymphatic vessels" (28, 623). In the 18th century William Hewson observed the rhythmic contractions of collecting lymphatics and noted numerous intraluminal valves to prevent backflow, while his colleague William Hunter identified that lymphatics are an "absorbent system" (433). However, the question of how lymph formed was left unanswered until the mid-19th century when Carl Ludwig postulated that lymph was a filtrate of the blood. Later, Ernest Starling established that the balance between the hydrostatic and osmotic forces of the plasma and interstitium favored lymph formation. This hypothesis was proven correct by Cecil Drinker and Joseph Yoffey, who reported in 1941 that changes in the protein concentration in blood or tissues could alter the rate of lymph formation (623). Another notable contribution was by Arnold Heller in 1869, who provided the first description of lymph propulsion, describing the intrinsic phasic contractions observed in collecting lymphatic vessels in the guinea pig mesentery (44, 623). Advances in electron microscopy provided a surge in understanding of the structure of microlymphatics in the 20th century (964). This was accompanied by discoveries of how lymphatic vessels contribute to immune function and reverse cholesterol transport, and greater understanding of the essential role of lymphatics in fluid recycling (809, 1178). Subsequent advances combining cinematography and intravital microscopy (1127), and later development of isolated lymphatic techniques in the second half of the 20th century allowed major advances in the physiology of lymph formation and propulsion (1201). Likewise, the revolutionary discoveries in molecular biology and developmental biology laid the foundation for our understanding of lymphangiogenesis (1169). Today, rapid advances in immunology and cancer biology continue to extend the boundary of understanding of lymphatic physiology (153, 515, 886, 927).

Organization and Anatomy of the Lymphatic System

Overview

Like the blood circulation, the lymphatic system is a network of specialized vessels that perform exchange and transport functions. Unlike the blood circulation, which has the heart as a central pump, the propulsion of lymph through the lymphatic vessel network is mediated by the forces driving the initial formation of lymph in the tissues, the intrinsic pump mechanisms that propel lymph forward through the system, and tissue pressures extrinsic to the system that favor forward flow. In addition, unlike the blood, lymph does not circulate per se, but rather is a filtrate formed in the tissues that the network delivers to the central circulation. Another way of viewing this is to consider veins like a super highway, while lymphatics are slow, local roads eventually leading to the heart (Fig. 1). The venous system permits the rapid return of deoxygenated blood to the right heart for reoxygenation in the lungs to support metabolism throughout the body at a rate equal to the cardiac output, approximately 5 L of blood per minute in an average-sized human. In contrast, the lymph that enters the great veins accounts for less than 0.05% of venous return (1178). This much slower transport of lymph, approximately 8–12 L per day, half of which reenters the systemic circulation at lymph nodes, and the other half returning via the great veins (8, 545, 600, 906), makes the system to act as an additional reservoir for fluids generated by filtering of plasma through the microvascular walls and interstitial space. This reservoir is essentially a sample of filtered plasma available for the immune system surveillance for potential threats in local draining lymph nodes distributed throughout the system.

Lymphatic vessels have been found in almost every tissue. Exceptions have traditionally included bone marrow, cartilage, the cornea, and the central nervous system. However, even within these tissues there has been evidence of lymphatic drainage under normal conditions, or lymphangiogenesis under pathologic conditions. Tracers injected into long bones have been found to drain into local lymph nodes with both radiological and histological approaches (357, 815). Sprouting of lymphatics into cartilage of the trachea has been reported in a mouse model of *M. Pulmonis* infection (64). Likewise, growth of lymphatic vessels into the cornea can be elicited by injury (221, 611). Recent evaluation of the brain with advanced imaging and use of molecular markers confirmed the presence of lymphatics in the dura mater of the brain and the existence of prelymphatic channels that had been previously been not been acknowledged by certain research communities (39, 340, 479, 494, 624, 737). With more careful evaluation using more advanced techniques, additional controversy about the existence of lymphatics in certain is likely to be resolved.

Like many biological systems in nature that absorb and transport fluids to a central location, lymphatic networks have a largely fractal geometric organization (648). This type of distribution allows for the smallest, most distal, blind-ended vessels to cover a large surface area within tissues to absorb fluids, serving as the site of lymph formation. By different conventions, these vessels are referred to as initial lymphatics because they are where lymph initially forms, terminal lymphatics due to their blind-ended nature, or lymphatic capillaries because like blood capillaries, they serve as a site of fluid exchange. In current literature, the terms “lymphatic capillary” and “initial lymphatic” are more frequently used, and both are

used in this review. The initial lymphatics are located in close proximity to the microcirculation and consist of a single endothelial layer with a poorly defined basement membrane. These vessels may be like saccules, blind-ended, or they may form an interconnected network or plexus. The initial lymphatics drain into collecting lymphatics, which are distinguishable by the presence of a smooth muscle layer and one-way bicuspid valves to prevent retrograde fluid flow. In some cases, an intermediary lymphatic vessel type known as a precollector, lacking smooth muscle but having the one-way valves, is present between the initial and collecting lymphatics. The smooth muscle of collecting lymphatics establishes vessel tone and unlike vascular smooth muscle, it also contracts phasically. Intraluminal valves within the collecting lymphatics ensure that the phasic contractions propel lymph forward through the network. The prenodal collecting lymphatics, also called afferent lymphatics, transport lymph to the lymph nodes, where it comes into contact with a collective of antigen presenting cells, T cells and B cells. The lymph composition is modified in the lymph nodes due to the hydrostatic and osmotic interactions with lymph node capillaries, and due to the exiting and entrance of various immune cells. The lymph exits the lymph nodes through postnodal collecting lymphatics, also called efferent lymphatics, although it is worth noting that this definition is relative to a particular node, as in some parts of the system the lymph passes through multiple lymph nodes in series. Eventually the collecting lymphatics throughout the body coalesce into the larger lymph trunks, of which the largest, the thoracic duct and right lymph duct, empty directly into the subclavian veins.

General Initial Lymphatic Structure

The initial lymphatics are the site of lymph formation. These lymphatic capillaries are often blind-ended vessels (Fig. 2), but are also observed as a plexus of interconnected vessels. Lymph within an initial lymphatic network is free to flow in the directions imposed by local hydrostatic forces, and solutes may diffuse freely within the vessels. The exit point from an initial lymphatic vessel or network is an intraluminal valve composed of endothelial cells and connective tissue that defines the border between the initial lymphatic vessel or network and downstream precollectors or collecting lymphatic vessels (Fig. 2). The morphology of initial lymphatics can vary widely and depends upon tissue location and species. In the human skin for example, initial lymphatics are typically 35–70 μm in diameter and form interconnected networks (332). In contrast, the blind-ended lacteals in rat intestinal villi are only 15–30 μm in diameter (592, 1097). One exceptional example is the initial lymphatics of the bat wing, which are blind-ended saccules with diameters as high as 450 μm that also have a smooth muscle layer (1128). A more comprehensive summary of the interesting variety of initial lymphatic morphologies can be found in a previous articles written by Schmid-Schönbein (964) and Ohtani and Ohtani (795).

The initial lymphatics are composed of a single layer of endothelial cells, with a discontinuous and often indistinct basal lamina (413). In tissue sections, the endothelial cells of initial lymphatics largely resemble those of capillaries, and in classic histology lymphatic capillaries are distinguished from blood capillaries based on their different luminal dimensions, and also the contours of the cells often having an undulating course and cytoplasmic processes projecting both luminally and abluminally (413). Reports on the

morphology of initial lymphatics in the cat tongue, mouse trachea, rat mesentery, rat small intestine, guinea pig and rat uterus, and human tonsils have revealed specialized endothelial cells having a relatively flat, "oak leaf" shape (Fig. 3A) (61, 166, 531, 736, 792, 1220). These specialized endothelial cells have apparent functional significance for lymph formation, as adjacent overlapping cells form structures that appear to be flaps (166, 792, 996). These flaps are thought to act as the microscopic one-way valves hypothesized to permit the formation of lymph from interstitial fluid. Schmid-Schönbein and colleagues named these *"primary lymphatic valves"* to differentiate from the luminal valves observed in collecting lymphatics, which they termed *"secondary lymphatic valves"* (634, 686, 965, 1083). Observations from studies of the localization of junctional proteins at these flaps support that they are indeed primary valves that favor lymph formation. VE-cadherin, an adhesive protein required for establishing normal endothelial barrier integrity, and several tight junction proteins (Occludin, Claudin-5, ZO-1, ESAM, and JAM-A) localize intermittently at cell borders, acting as "buttons" holding adjacent initial lymphatic endothelial cells together (Fig. 3B). PECAM-1 and Lyve1 also localize intermittently, but in the flaps, where VE-cadherin and the tight junction proteins are absent. Because PECAM-1 has a known role in leukocyte diapedesis, these PECAM-1 rich areas between the VE-cadherin "buttons" are also thought to be sites where lymphocytes may enter initial lymphatics with little resistance (61, 861).

The initial lymphatics also feature anchoring filaments that protrude into the surrounding interstitial spaces (585, 586). The anchoring filaments of the initial lymphatics of human skin were reported to be composed primarily of fibrillin, and connect from the extracellular matrix to the cytoskeleton via focal adhesions containing focal adhesion kinase (FAK) and $\alpha 3\beta 1$ integrin (375). It was hypothesized several years ago that these filaments transmit forces originating in the interstitium to the lymphatic endothelial cells as part of a physical mechanism of lymph formation. The idea was that if interstitial pressure became high, causing the tissue to expand, then the filaments would pull or push on the endothelial cells, facilitating opening of the primary valves to allow fluid entry (809). However, functional data to support this hypothesis are lacking. There is also the converse possibility that these filaments help maintain lymphatic vessel shape and patency in an environment where pressure gradients rapidly change. Another possibility is that the anchoring filaments allow for detection of local forces in the local tissue environment, and that they could serve to transduce signals (375). Additional work in this area will be needed in order to make a firm conclusion about the functional significance of these anchoring filaments.

Precollector Structure

Precollectors are defined as lymphatic vessels composed of a single endothelial layer but also having secondary valves to prevent backflow into initial lymphatics. In the precollectors, oak leaf shaped-endothelial cells may still be still present in the distal regions near initial lymphatics, but in more proximal regions the endothelial cells have a more rhombic shape, similar to venous endothelial cells (1221). Accordingly, the junctions between endothelial cells begin to have continuous expression of PECAM-1 and VE-cadherin, suggesting that these vessels may act more as conduits than sites of lymph

formation (61). Because there is no smooth muscle layer, movement of lymph within precollectors depends highly upon the inflow and outflow pressures of individual segments.

General Collecting Lymphatic Structure

The collecting lymphatic vessel wall has an inner endothelium surrounded by a medial layer of circular smooth muscle cells. Fig. 4 shows an example of the rat mesenteric collecting lymphatic wall with the endothelial and smooth muscle cells labeled (568). In this example, the smooth muscle cells often form a single, continuous layer, however it is not uncommon for discontinuous areas to also be observed in other cases, particularly around secondary valves. This conformation can vary between species. For example, the mesenteric collecting lymphatics of guinea pigs have been described to have overlapping smooth muscle cells oriented in the circular direction (1109). Another interesting example was reported in the diaphragms of 6-week old rats, in which the collecting lymphatics had circular muscle where intraluminal valves were located, but primarily longitudinal smooth muscle between valves (798). As the collecting lymphatics coalesce and become larger, thicker medial layers can also occur. For example, in larger bovine mesenteric collecting lymphatics, a conformation with three layers of smooth muscle cells has been described, with inner and outer longitudinal layers and a middle circular layer (783). Other cell types can also be identified on the adventitia of the collecting lymphatic vessel wall, such as dendritic cells, macrophages, and neurons (133, 466, 560, 1178, 1201). In addition, collecting lymphatics may have supporting microcirculation in close proximity to the vessel exterior, or a vasa vasorum within the adventitia or smooth muscle layer, that provides oxygen and nutrients (784, 964, 1178).

Also prominent in collecting lymphatics are the periodic *secondary valves* that organize the vessels into a chain of chambers in series (Fig. 5). These are in most cases bicuspid valves that prevent backflow of lymph (196, 665, 964). Each valve leaflet is composed of a folded bilayer of endothelial cells with their apical sides facing away from each other. The basal sides of the intraluminal valve endothelial cells are separated by an inner supporting extracellular matrix containing elastin fibers (665, 881). In some cases, single leaflet valves have been described. These may represent developing or regressing valves. In early studies of sectioned pulmonary lymphatics, a funnel-like structure was proposed (581). In early studies of sectioned dermal lymphatics, unicellular valves that may have been newly developing valves were described (235) and later confirmed (944). In any case, single leaflet valves appear to be rare with the exception of during development. The secondary valves are spaced along the length of a collecting lymphatic vessel at semi-regular intervals, forming chambers. Each chamber between two consecutive valves forms a functional contractile unit, called a *lymphangion*, meaning “lymph heart” (703). Each lymphangion is capable of contracting either independently or in conjunction with its upstream and downstream lymphangions (702). The coordinated phasic contractions of lymphangions, in concert with properly functioning secondary valves, allows for effective intrinsic pumping of lymph against the pressure gradient imposed by gravity in a standing individual. Details will be discussed in greater detail in a subsequent section.

Unlike initial lymphatics and precollectors, collecting lymphatics vessel networks are more consistently organized as a binary tree. At each bifurcation in of the network, one or two secondary valves are often present (665). Interconnected networks can be observed, but are less common. The networks of prenodal (afferent) collecting lymphatics lead to lymph nodes. Multiple prenodal collecting lymphatics may drain into a lymph node. Typically, but not in all cases, one postnodal (efferent) collecting lymphatic exits the node.

The larger lymphatic trunks also have the same generalized collecting lymphatic structure, but have functional differences with their smaller counterparts. The intestinal trunk and the lower lumbar lymphatic trunks drain into the cisterna chyli, at the base of the thoracic duct. Other peripheral lymphatic trunks from most parts of the body also drain into the thoracic duct. Lymphatics arising from the upper right thorax, right arm, the right side of the head and neck, and in some cases the lower lobe of the left lung drain into the right lymphatic duct (1178), although in some cases there are tributaries from intrathoracic organs into the thoracic duct (912). The thoracic duct drains into the left subclavian vein, while the right lymphatic duct drains into the right subclavian vein (1178).

Immunologic Markers of Lymphatic Endothelium

The discovery of several proteins that are generally found on lymphatic endothelial cells but not blood endothelial cells has aided in: 1) isolation of these two broad types of cells for study in culture, and 2) easier identification of lymphatic vessels by immunofluorescence labeling. These markers include a homolog of the *Drosophila melanogaster* homeobox gene prospero, known as prospero homeobox protein 1 (PROX1 or Prox1; abbreviations for human and rodent proteins, respectively), lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1 or Lyve1), vascular endothelial growth factor receptor-3 (VEGFR3 or Vegfr3), and podoplanin. All have proven useful markers, however are not exclusively selective for lymphatic endothelial cells. Therefore, they are usually used in combination with each other or with a pan-endothelial marker.

The *Prox1* gene encodes a nuclear transcription factor described as the master control gene allowing expression of lymphatic endothelial markers (1139). Prox1 is highly expressed in initial lymphatics and precollectors (Fig. 6). In collecting lymphatics, where the endothelial cell layer has a greater degree of basement membrane and is surrounded by an outer layer of smooth muscle cells, Prox1 expression is much lower (1169). However, Prox1 expression is very high in endothelial cells of the secondary valves in collecting lymphatics, where expression of FOXC2, an important regulator of valve cell identity (859), is also very high (944). Although a reliable marker of lymphatic endothelial cells, Prox1 can be detected in other non-endothelial cell types. These include heart valve endothelium, pancreatic epithelium, hepatocytes, bile duct cells, adrenal medullary neuroendocrine cells, megakaryocytes, cardiomyocytes, skeletal myocytes/satellite cells, and platelets (916, 1081).

LYVE1 is an integral membrane glycoprotein important for cell migration (65). Expression of LYVE1 is highest in initial lymphatics, while it is low or even undetectable in collecting lymphatics (633, 646, 1122, 1169). Recently, LYVE1 was successfully used as a marker for sorting and culturing rat dermal lymphatic endothelial cells (1068). While generally a good

marker for lymphatic endothelium, LYVE1 has also been detected on macrophages and in liver and spleen sinusoid endothelium (732).

VEGFR3, also known as FLT4, was the first antigen marker used to identify lymphatic endothelium (478, 502, 505). VEGFR3 is a tyrosine kinase receptor for both VEGF-C and VEGF-D that mediates lymphangiogenesis (518, 831, 832). It was widely used to identify lymphatic endothelial cells before the discovery of the other markers. Its expression is generally higher in initial lymphatics and lower in collecting lymphatics. During development, VEGFR3 can also be found on blood vessels.

Podoplanin was originally described on rat kidney podocytes as a mucin-type transmembrane glycoprotein (123), and was later found to reliably identify lymphatic capillaries (124). Podoplanin has proved as a useful target for sorting lymphatic endothelial cell populations (558) and is currently used commercially for producing cultures of lymphatic endothelial cells. Podoplanin expression persists throughout the lymphatic network (1122, 1169). However, one report suggests that there are endothelial cells with low podoplanin expression in precollectors that contribute to migration of CCR10+ T cells (1138).

The expression patterns of these proteins have led to the recent development of transgenic mice that are used as reporters or for selective deletion, mutation, or addition of genes in lymphatic endothelial cells (96, 188, 447, 475, 817). Along this line, two other noteworthy proteins should also be mentioned here. First, FOXC2, as mentioned above is required for valve maturation and is selectively expressed in lymphatic valve endothelium in the adult (944). Second, the expression of the extracellular glycoprotein reelin is very high in collecting lymphatics. Reelin-deficient mice have impaired maturation of the collecting lymphatic vessels and so retain high expression of Lyve1 with impaired smooth muscle coverage (633).

Lymphatic Networks in Different Organ Systems

Upper Gastrointestinal System—The gastrointestinal system is a specialized interface for digestion of food and absorption nutrients, vitamins, and water. The upper gastrointestinal tract, namely the mouth, esophagus, and stomach, perform the initial mechanical and chemical digestion and transport of ingested food and water. All are drained by lymphatic networks. Within the mouth alone, there are lymphatic networks within the oral mucosa, the tongue, salivary glands, and tonsils.

Considering the oral mucosa, one area of special interest is the gingiva due to the changes that occur to gingival lymphatic networks during periodontal disease (84, 85). Lyve1-positive vessels have been detected in the lamina propria in gingiva and in the free oral mucosa in mice (835, 1096). Prox1 expression has also been identified in equine gingival lymphatics (1021). Inflammation of the gingiva leads to formation of crevicular fluid and accumulation of CD45+ cells. During gingival inflammation, greater volumes of crevicular fluid and numbers of CD45+ cells accumulated in mice (K14-VEGFR3-Ig) lacking lymphatic vessels in the gingiva (713). Local injection of LPS from *P. gingivalis* caused an acceleration of local drainage of Alexa680-albumin also injected into the gingival mucosa,

assessed using near-infrared imaging over a 7-hour period (835). Local production of VEGF-C by CD45+ cells and lymphangiogenesis also occur over the longer term in response to local *P. gingivalis* infection in mice (712). Likewise, increased lymphatic vessel density, associated with an increase in the number of mast cells, has been reported in the gingiva of patients with periodontal disease (882).

The presence of lymphatics within the dental pulp has been a controversial topic over the past century. Martin *et al.* (654) summarized studies of the teeth of dogs dating back to 1894, which used dye tracking, light microscopy, or both, and overall the results were mixed. Their own findings showed a lack of Prox1-positive endothelial cells within the canine dental pulp (654). In cats, lymphatic vessels have been described within the subodontoblastic zone or more centrally within the pulp (97). In a study utilizing Lyve1 and Vegfr3 labeling in rats and mice, dense networks of blind-ended lymphatics were described in the coronal area of molars, while in incisors, lymphatics were only found in the apical region (83). Several studies of human teeth utilizing light microscopy, electron microscopy, and dye tracking have also been performed (reviewed by Martin *et al.*). Of these, the majority reported the presence of lymphatic vessels in the dental pulp (654). One of the more recent studies reported a low number of Vegfr3-positive vessels, but that these increase with inflammation within the dental pulp (863). In contrast, an additional recent study tested both human skin and dental pulp for the markers podoplanin, LYVE1, VEGFR3, and PROX1, and while these markers were all found on vessels in skin, they were not found within the dental pulp (374).

Lymphatics networks in the human tongue were initially characterized a century ago by Aagaard, who described networks both in the mucosa and the muscle (2). Initial lymphatics of the rat tongue were studied extensively by Castenholz using scanning electron microscopy. In corrosion cast preparations of the rat tongue, a dense network of lymphatics is apparent (Fig. 7). This work also showed the fine detail of oak leaf shaped endothelial cells with overlapping “button” junctions (Fig. 7) (165, 166). Lymphatics in different regions of the mouse tongue have also recently been identified using immunolabeling of Lyve1 (772).

Saliva plays roles in digestion, lubrication of the mouth and ingested food, and protection of the mucosa and teeth. In early studies of the salivary glands, initial lymphatics were described near the acini, and lymphatics containing valves were described located near the ducts (539, 840). Findings from recent studies of the mouse sublingual gland confirmed the presence of Lyve1-positive lymphatic vessel networks (772). In a study of local inflammatory challenge with TNF-α in the salivary glands, expression of ICAM-1 and VCAM-1 on lymphatic endothelium increased, which presumably facilitates entry of immune cells into the lymphatic networks (468).

The tonsils line the exit from the mouth through which ingested food must pass to reach the remainder of the GI tract. Strategically located at this gateway, they have an important surveillance role, and are composed mainly of mucosa-associated lymphoid tissue (MALT) that recognizes antigens and initiates immune responses when appropriate. Initial lymphatic networks shaped like flat sinuses surround the bases and lateral sides of follicles that contain immune cells (Fig. 8) (531, 795). These networks then drain to parafollicular collecting

lymphatics in the underlying connective tissue (356, 795), which lead to the deep cervical lymph nodes. In humans, lymph arising from the tonsils specifically drains to the jugulodigastric lymph node, which is often swollen during tonsillitis (663).

The esophagus and stomach have lymphatic vessel plexuses in the mucosal, submucosal, and muscular layers, with short vessels connecting these networks. Lymphatics containing valves are present in the deep submucosa and muscle layers, which drain into larger collecting lymphatics accompanying the left gastric artery (67, 483, 933). The networks have been reported to be denser in areas where gastric acid is secreted, with close contact to the glands, which may reflect a functional role related to metabolism in gastric parietal and chief cells (67, 483, 933). Connections between the gastric and duodenal lymphatics were found in humans, but not in dogs (67, 933). MALT is also present in the stomach, which is activated by bacteria such as *Helicobacter pylori* (718), and is also important for staging of stomach cancers, such as MALT lymphoma (931).

Small Intestine—The lymphatics of the small intestine have roles in dietary absorption, tolerance of symbiotic microflora, and immunity against pathogens in the gut. Lymph flow from the small intestine dramatically increases during absorption of nutrients, compared to nonabsorbent phases between meals (388). The lumen of the small intestine contains millions of villi that extend up to 1 mm from the surface of the mucosa. These villi, in combination with microvilli that compose the brush border of individual enterocytes, dramatically increase the surface area for absorption in the small intestine. In each villus, just beneath the absorptive surface of enterocytes, is a dense network of villus capillaries surrounding a lymph lacteal (843). Each lacteal is a blind-ended initial lymphatic. In most mammals studied, there is one lacteal per villus, although a notable exception is the rat, which has 1–10 lacteals per villus (Fig. 9) (792, 795, 796, 1094).

The lacteals play an important role in the transport and distribution of absorbed dietary lipids. Dietary triacylglycerol is digested in the gut lumen and absorbed by enterocytes in the form of free fatty acids and 2-monoacylglyceraol. Medium-chain fatty acids are absorbed by enterocytes and are transported away by the hepatic portal vein. Short-chain fatty acids (a product not of triglycerides but of gut microflora metabolism of dietary fiber) are absorbed by colonocytes and are metabolized. In contrast, long-chain fatty acids absorbed from the diet are transported away from the intestinal mucosa in the form of triacylglycerol by the lymphatic system (548, 809). Inside enterocytes, absorbed long-chain free fatty acids and 2-monoacylglycerol are re-esterified and packaged into chylomicrons in the endoplasmic reticulum. After further processing in the Golgi complex, mature chylomicron particles are released by exocytosis into the intercellular space of the lamina propria. Apolipoprotein (Apo) B48, which is packaged into chylomicrons, is selectively expressed in the small intestine, and appears to be important for the overall efficient delivery of lipids into the lacteals (619). From here, a rate-limiting step for chylomicron transport is crossing the basement membrane, prior to entry the lacteals (548). While the mechanism is unclear, junctional disruptions occur between the enterocytes at the tips of jejunal villi during lipid absorption (570), and poor tight junction integrity has also been reported due to a chronic high fat diet (458).

The mechanism for entry of chylomicrons into lymph lacteals appears to be a transcellular route through lymphatic endothelial cells. Reports from multiple histological studies identify chylomicrons inside lymphatic endothelial cells (52, 161, 273, 274), and data from a recent functional investigation with cultured lymphatic endothelial cells supports an active transport mechanism (268). This mechanism occurs during the enterocyte nutrient absorption-induced fictional hyperemia that helps establish favorable interstitial fluid pressures to drive the increase in lymph formation (388). Smooth muscle cells parallel to and coming into contact with lacteals have also been shown (198, 274, 690, 795). Their precise contribution is also undefined, but their function might be to contribute to the piston-like contractions that have been described to drive flow of lymph out of the villi and into the submucosal lymphatics (335, 336, 1150, 1151).

The lacteals connect to a submucosal lymphatic network. This has been visualized by injection of dye into the submucosa, including retrograde filling of the lacteals (1094). The rapid spread of the dye throughout the network indicated free flow of fluid within the network according to local pressure and osmotic gradients. Dye injection into the intestinal muscle layers was also absorbed into a lymphatic network, however this network is independent from the submucosal lymphatic network. The muscular layer lymphatic vessels were generally oriented parallel with the circular or longitudinal muscle fibers, and valves became apparent within the vessels near the border with the mesentery (1094). The lymphatics networks of the submucosa and muscular layers of intestine converge near the mesenteric border, where they empty into collecting lymphatics that enter the mesentery (792, 1094).

Several studies using genetically modified mice have generated results that highlight the importance of the normal development and function of the intestinal lymphatics to overall health (264). In *Prox1*$^{+/-}$ mice, the lymphatic vessels become leaky, allowing chyle to accumulate and promote adipose growth (418). Likewise, in mice with an inducible deletion of *T-Syn*, important for synthesis of core 1-derived O-glycans in endothelial cells, misconnections between blood and lymphatic vessels develop allowing chylomicrons to enter directly into the portal circulation, causing development of fatty liver (355). From these studies it is clear that disrupting the normal partitioning of dietary lipids out of the central circulation by intestinal lymphatics has an important impact on overall metabolism.

In addition to lipid absorption and distribution, the intestinal lymphatics also appear to have an important function actin as an endocrine conduit for incretins. Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like-peptide-1 (GLP-1) are incretins secreted by enteroendocrine K and L cells, respectively. However, both GIP and GLP-1 are rapidly inactivated by the enzyme dipeptidyl peptidease-4 (DPP-4), present in the intestine and in the blood. In the blood these incretins have a half-life of only 2–3 min. However, DPP-4 activity is relatively low in the lymph, allowing more distant delivery of these incretins at higher concentrations than in the blood (627, 790, 1077, 1177). A functional implication is that lymphatic vessels may serve as an important route for certain endocrine signals, such as incretins.

Lastly a major function that the lymphatics and lymphoid tissues in the gut mucosa is immunity. The gut wall encounters a complex mixture of water and nutrients combined with both helpful and potentially harmful microbes. In addition, the gut wall itself, a highly proliferative, constantly renewing layer of epithelium, must be continuously monitored for transformed cells. Specialized areas named gut-associated lymphoid tissues (GALT) perform these functions (50). In particular, Peyer's patches in the small intestine sample contents from the gut lumen to discriminate between antigens and non-antigenic material. Specific enterocytes, M cells, sample antigens, which are then processed in dendritic cells and presented to lymphocytes. Data obtained from rat tissue show that lacteals are present in the villi projecting parafollicular regions. These lacteals drain into a submucosal plexus that interconnect and form networks with many blind-ended lymphatics in the parafollicular areas, with many high endothelial venules in close proximity. These networks form the sinuses around each follicle (795). The perifollicular lymphatic sinuses then drain into submucosal collecting lymphatic vessels (795).

Large Intestine—The main functions of the large intestine are to absorb water from the remaining unabsorbed food matter, to transport and store the resulting feces prior to elimination from the body, and to protect the internal milieu of the body from potential pathogens that may be harbored within the intestinal lumen. Initial lymphatic vessels can be found in the superficial mucosa, about 50 μm below the basement membrane of the luminal epithelium (Fig. 10). These connect to larger, polygonal submucosal lymphatic networks, which in the rat cecum were described to contain valves and have circular smooth muscle (791). These in turn drain to collecting lymphatics between the inner and outer muscle layers of the colon, leading to mesenteric collecting lymphatics (791, 795). Like in the small intestine, GALT referred to as the cecal patch has been described (104, 353, 828), with lymphoid follicles surrounded by a dense plexus of initial lymphatics (795).

Mesentery—The intestinal mesentery supports the splanchnic circulation to the intestines, including the lymphatic network for draining intestinal lymph toward the mesenteric lymph nodes. For functional investigations of lymphatic physiology, perhaps the most well studied lymphatic vessels are those located in the intestinal mesentery, a thin, translucent tissue amenable for intravital microscopic observation. Lymphatics that drain both the intestine and the mesentery itself are present in the mesentery. The collecting lymphatics draining the intestine are primarily bundled with the mesenteric arteries and veins that connect to the intestinal wall, and have many adipose cells in close proximity. The sheets of translucent connective tissue between these conduit vessel bundles contain primarily initial lymphatics, and these lead to collecting lymphatics that may or may not be distinct from those draining the intestine depending on species. The distinction was obtained from a study mapping of the lymphatic networks of the cat mesentery by micropipette injection of a carbon suspension tracer to fill the initial lymphatic network of a mesenteric window (964, 995).

The initial lymphatics found in the translucent connective tissue between the arterial arcades form a network that features both blind-ended and interconnected vessels. In the rat mesentery, these networks can easily be observed by immunolabeling with selective markers for lymphatic endothelial cells, such as Lyve1 or Prox1 (1034). The endothelial cells of

these initial lymphatics have the oak leaf shape and discontinuous expression of VE-cadherin and PECAM-1 at their intercellular junctions (736). The mesenteric initial lymphatics coalesce into collecting lymphatics that along with the collecting lymphatics from the intestine, drain toward lymph nodes (964). The collecting lymphatics of the mesentery typically follow the cascading arteries, although in the rat mesentery can sometimes be observed traversing the mesenteric windows of translucent connective tissue. Phasic contractions and valve opening/closing can easily be observed in the rat mesentery with intravital microscopy (Fig. 11) (128).

It is important to note that the different regions of the intestine drain to distinct lymph nodes. A series of lymph nodes known as the superior mesenteric lymph nodes lie in close proximity to the superior mesenteric artery, whereas a single lymph node is located near the origin of the inferior mesenteric artery, known as the inferior mesenteric lymph node or the caudal lymph node (733, 1100). Duodenal lymph primarily drains to the proximal superior mesenteric lymph nodes, lymph from the jejunum drains into the middle superior mesenteric lymph nodes, and lymph from the ileum, cecum, and ascending colon drain into the distal superior mesenteric lymph nodes. For the transverse colon of the mouse, lymph drains to nodes buried in the pancreas, whereas in rats, drainage to the superior mesenteric lymph nodes has been described (156, 1072). Lymphatics draining the descending colon lead to the inferior mesenteric lymph node Similar regional patterns are also described in humans, although in the duodenum the lymph drainage parallels the more complex blood supply arising from both the gastroduodenal and superior mesenteric arteries. The anterior duodenal lymphatics drain to the duodenopancreatic lymph nodes along the superior and inferior pancreatoduodenal arteries branching from the gastroduodenal artery, while the posterior duodenal lymphatics drain to the superior mesenteric lymph nodes (1204). The distinct lymph nodes assigned to different functional parts of the small and large intestine, which likely have differences in lymph composition, may confer specialized immunological properties for each lymph node (733).

Pancreas, Liver, and Gall Bladder—The pancreas, liver, and gall bladder collectively perform an important exocrine function in the gastrointestinal system, delivering a mixture of digestive enzymes and bile to the duodenum. The pancreas and liver also have important endocrine and metabolic functions. These organs have complex networks of lymphatic vessels that drain along with most of the intestinal/mesenteric lymphatics into the thoracic duct.

The pancreas lies between the duodenum and liver, adjacent to the gall bladder. Its lymphatics drain to the thoracic duct, and blockade at the cisterna chyli causes pancreatic lymphedema (100). Initial lymphatics were reported by Klein in 1882 to be located around the exocrine acini, with additional lymphatics containing valves around pancreatic ducts and in the interlobular connective tissue near blood vessels (539). Subsequent reports have largely confirmed these findings (91, 482, 752, 753, 923). Lymphatic vessel distribution is similar among the head, body, and tail of the rat pancreas. Most lymphatics are found in connective tissue between lobules, in close relation to arteries and veins. However, about 19% of the lymphatics observed in a histological study could be found in thin connective tissue septa that enter lobules and provide support for acini and intralobular ducts (752).

Valves were observed in both intralobular and interlobular lymphatics, although the lymphatics within the lobules were smaller in caliber (753). The interlobular lymphatics have also been successfully identified using combined LYVE1, podoplanin, and PECAM-1 labeling (923). Generally no lymphatics are observed accompanying the microcirculation within the Islets of Langerhans (91, 752, 753, 923).

The pancreatic collecting lymphatics drain to five main groups of lymph nodes on the superior, inferior, anterior, posterior, and splenic regions of the pancreas (173). The hormone secretin, which is known to elicit release of pancreatic enzymes, has been suggested in some reports to elevate thoracic duct lymph flow and increase pancreatic enzyme concentrations in thoracic duct lymph due to increased pancreatic lymph output (59, 69, 286). However, other reports dispute this and note that the daily lymph output from the pancreas is relatively small (70, 839, 841). Considering the complexity of the duodenal cluster unit, lymph flow elevations from the pancreas during enzyme secretion to the gut are likely accompanied by elevations in blood flow to the other gastrointestinal organs, plus increased absorption of water and fats from the gut that would also contribute to the elevated lymph flow in the thoracic duct.

Unlike the pancreas, the liver produces a substantial amount of lymph, estimated to be 25–50% of the thoracic duct lymph (794). The liver has long known to have an extensive lymphatic network, documented in the early-to-mid-1800s with uptake of dyes into the vessels (1178). While primarily a metabolic organ required for maintaining energy homeostasis, the liver can also be considered a lymphoid organ, due to its large population of antigen-presenting cells and resident immune cells (212, 630). The portal blood that arrives to the liver contains nutrients and also potential toxins, toxicants, and antigens recently absorbed from the gut. A relatively permeable sinusoidal endothelium allows for rapid interstitial flow for the formation of lymph that is delivered to an extensive network of lymph node groups for immune surveillance.

The lymphatics of the liver are located within three general regions: the portal tracts, along the sublobular veins, and superficially within capsule and underlying subserosa (641, 794). The portal tract lymphatics are thought to contribute up to 80% of the hepatic lymph (794, 1178). The best detail of these networks has come from observations using scanning electron microscopy of corrosion casts of the lymphatic networks within rabbit liver (1164). A tree structure is apparent with short blind-ended initial lymphatics that coalesce into a network of longer lymphatics with valves following the path of the portal tracts, alongside the bile ducts and portal circulation (Fig. 12). In smaller portal tracts, 2–3 lymphatics run in parallel with the tract, with occasional side branches between vessels. In the larger portal tracts the number of lymphatics increases, with 6–10 lymphatics running in parallel (1164). It is also worth noting that the lymphatics of the liver have been positively labeled with the markers podoplanin, PROX1, and LYVE1 (732, 923). It is worth noting, however, that LYVE1 can also be found on sinusoidal endothelial cells (732, 923).

Lymph formation in the liver arises from filtration of at the sinusoids, which produces interstitial fluid in the perisinusoidal space of Disse (574, 800). This fluid crosses through prelymphatic channels to reach the perilobular space and then the periportal space where it

can enter into the initial lymphatics (Fig. 13)(428, 794, 800). Similar pathways between hepatocytes allow interstitial fluid to enter the perihepatic interstitial tissue to enter sublobular and superficial lymphatic networks (868). For the superficial lymphatics, the deepest superficial lymphatic plexus represents the initial lymphatics that serve as the site of lymph formation, with a middle precollector layer, and an outer capsular layer of collecting lymphatics.

Collecting lymphatics from the liver drain into multiple locations, each with its own set of lymph nodes. The portal tract lymphatics descend from the liver with the hepatic arteries and bile ducts and descend to up to three different locations: 1) the posterior head of the pancreas, 2) the hepatic artery and celiac trunk, and 3) the origin of the superior mesenteric artery. The different lymph node networks associated with these locations coalesce into para-aortic lymph nodes (1183). The sublobular lymphatics ascend with the hepatic veins and drain into the mediastinum, eventually reaching the pericaval and esophageal lymph nodes (1183). The superficial lymphatics drain into a variety of lymph node groups, including: the falciform pericardiac, superior phrenic, and juxtaesophageal nodes that lead into xiphisternal nodes; latero-aortic and pericaval nodes, leading to pancreatico-lineal lymph nodes; hepatic nodes, leading to celiac lymph nodes; and routes that go directly to the para-aortic, left gastric, or precaval lymph nodes (794, 1178, 1183). This elaborate network of liver lymphatics and lymph nodes plays important roles in preventing injury to the liver, but also serves as a pathway for metastasis of hepatic tumors. Importantly, lymphangiogenesis accompanies inflammation in the liver, and the study of this process may help clarify the role of lymphatics in the pathophysiology of liver diseases (630).

The gallbladder stores bile secreted by the liver until it receives signals to contract and release the stored bile into the duodenum. A key mechanism of bile storage within the limited size of the gallbladder lumen is the concentration of bile thorough reabsorption of water. Presumably, the microcirculation and lymphatics contribute to this mechanism. Scanning electron microscopy of corrosion casts of guinea pig gallbladder have been performed, and show an initial lymphatic network in the subserosal layer (793). Interdigitating junctions were observed (793) that resemble the more recently described button junctions on lymphatic capillaries of the trachea (61). Older reports identified submucosal, intramuscular, and serosal plexuses of lymphatics (1178). The lymphatics drain toward to the gallbladder neck, where in humans a lymph node may or may not be present, after which they may exit the gallbladder by three pathways. First, there are collecting lymphatics that follow the cystic and hepatic arteries, leading to the celiac lymph nodes. A second pathway is along the cystic duct and common bile duct to the pancreas head, leading to the retropancreaticoduodenal lymph nodes. A third pathway, currently thought to be of minor importance, leads to a lymph node on the portal vein and then nodes around the superior mesenteric vein (954).

Thyroid Gland—The thyroid gland plays an important role in growth and development. A plexus of anastomosing lymphatic vessels has been described as enshrouding the thyroid glands of dogs and cats, and to a lesser extent in rabbits and monkeys (232, 825, 909). Lymphatic vessels can also be found within the interlobular spaces passing between groups of follicles. Unlike capillary networks, the lymphatic capillaries generally do not come into

direct contact with the follicles (745, 795, 909). The thyroid lymphatics have been labeled positive for Lyve1, Prox1, and podoplanin in the mouse (358) and for Lyve1 in the rat (795). The lymphatic capillary networks coalesce into larger lymphatics that follow the arterial circulation, and some emerge onto the thyroid capsule. The latter lymphatics have been reported to have intraluminal valves (745). Of note, lymphatics do not appear to extend into the parathyroid glands. There are lymphatics located on the parathyroid capsule, but these appear to be part of the thyroid capsule lymphatic network (745). This is also evident in parathyroid tumor specimens, in which LYVE1-positive vessels have only been found in the peripheral areas (359).

The thyroid gland is a good example of an endocrine organ in which its hormones are released into the lymph at a higher concentration than into the venous blood draining the gland (232). Several early observations of material from the follicular colloid present in the lymph draining the thyroid gland (341, 909) were followed by data showing that the concentrations of both triiodothyronine (T_3) and thyroxine (T_4), along with the other iodinated tyrosine residues monoiodotyrosine and diiodotyrosine are all detectable in the lymph draining the thyroid (232, 301, 858). In addition, thyroglobulin, the protein secreted by follicular cells as part of the thyroid hormone synthesis pathway, also appears selectively in lymph but not venous blood originating from the thyroid gland (233, 234, 553). These findings suggest that the lymphatics represent an additional reservoir for thyroid hormone circulation, however it is worth noting that the release of thyroid hormones into the blood is still considered of primary importance for endocrine function due to the much higher flow rate through blood vessels (232).

In autoimmune thyroiditis, tertiary lymphoid structures and the generation of new lymphatic vessels have been shown to be present (358). Data obtained from studies with a mouse model in which CCL21 is overexpressed in the thyroid show similar lymphoid aggregates in the thyroid (358, 735). The new lymphatic vessel formation appears to be dependent upon $CD11c^+$ dendritic cells (735).

Ovary—The ovaries play the dual role of producing hormones and periodically releasing ova, approximately one per month in humans. Across several mammalian species, lymphatic vessel networks are primarily associated with the thecal layer around growing follicles and the peripheral zones of the corpora lutea (141, 455, 824, 1031, 1157, 1178). The ovarian lymphatics appear relatively late in development of the mouse ovary, after birth and prior to puberty (141, 1031). In active adult ovaries, the networks are profuse (Fig. 14), while in inactive ovaries, the networks have small and poorly developed lymphatic vessels (728). These lymphatics label positively for Lyve1 (1099, 1157). In adult mice, the ovarian lymphatics undergo nonpathologic remodeling corresponding to folliculogenesis, with local upregulation of the *Vegfc, Vegfd, and Vegfr3* genes (141). Steroid hormones produced by the ovaries are present in the draining lymph at levels higher than in peripheral blood, but lower than the ovarian venous blood (231, 610).

Observations from early studies indicated that the ovarian lymphatic networks coalesce at the hilum of the ovary into a dense plexus, and ultimately into 4–6 larger lymphatic vessels that anastomose with other lymphatics arising from the uterus and Fallopian tube and drain

into the lumbo-aortic nodes (68, 865–867). Recent work suggests two major drainage pathways, and one additional minor route in humans (541). One of the major drainage routes, from the cranial side of the ovary, follows along with the ovarian artery in the infundibulopelvic ligament toward the para-aortic and paracaval nodes (541). This route probably accounts for the metastases to these nodes commonly observed in ovarian epithelial cancers (145). The second major pathway includes lymphatics from the caudal side of the ovary that follow with a branch of the ovarian artery that anastomoses to the uterine artery in the ovarian ligament to the obrurator fossa and internal iliac lymph nodes. This pathway likely accounts for observed metastases to the pelvic region (642, 816). The minor pathway, with a smaller number of lymphatic vessels, follows the round ligament to the inguinal nodes. Inguinal metastasis is less common with ovarian cancers (541). Lymphatics may also participate in a feedback transport mechanism for regulation of ovarian hormone production (1024, 1025).

In the different stages of the estrous cycle of pigs and sheep, the ovarian lymphatic network varies in size and density (1178). Likewise, there are changes in the lymphatic network as a follicle develops, releases its ovum, and regresses into a corpus luteum. After the growth of new capillaries around a developing follicle, a wreath of lymphatics also develops. The fully developed lymphatic network includes channels with valves in the theca externa and a wreath of additional lymphatics in the theca interna and granulosa (30, 728). After ovulation, the follicle collapses and the theca interna and granulosa become corrugated, and 2–3 days later the lymphatics grow into the lutein tissue. An inner lymphatic network forms in a mature corpus luteum, connected by lymphatic capillaries to the outer, peripheral network. As a corpeus luteum regresses, the lymphatics rapidly degenerate and disappear (30, 728). Notably, lymph flow from the ovaries of conscious sheep was reported to be greatest during the luteal phase (728).

Testis—Like the ovaries, the testes also serve as both an endocrine gland and a site of gametogenesis. Unlike in ovaries of mice, in which lymphatics appear postnatally, lymphatics first appear in the testes 2–3 days prior to birth (Fig. 15) (141, 436, 1031). Also unlike the ovaries, there appears to be lymphatic network differences across mammalian species. In both large animals and rodents, a lymphatic network on the testes surface that penetrates into the tunica albuginea has been described (436, 921, 1031, 1178). In adult mice, the testes lymphatic networks identified either by Lyve1 labeling or using *Prox1-EGFP* transgenic reporter mice, do not penetrate further into the testes interstitium (436, 1031). Discrete lymphatic vessels were also not observed in the rat testes interstitium (192). However, in larger animals, lymphatic capillaries found within the testes interstitium were reported to be associated with seminiferous tubules. This lymphatic network drains via fibrous septa into an additional lymphatic network in the tunica albuginea and eventually into collecting lymphatics in the mediastinum testis (321, 322, 921, 1178).

In larger animals the testicular lymph drains into 3–8 larger collecting lymphatics that drain into the latero- or para-aortic lymph node groups (474, 731, 866). Interestingly, in some species the collecting lymphatics often drain to nodes on the opposite side, so that bilateral lymph node removal is used for testicular tumors (146, 862, 1023). In the rat, 11–15 collecting lymphatics in three groups (superior, middle, inferior groups) coalesce into a

larger testicular lymphatic trunk (857). The rat testicular lymphatics may lead to the renal or lumbar lymph nodes, or in some cases bypasses lymph nodes and goes directly to the thoracic duct (306). It is worth noting that the lymphatic networks of the testis are distinct from those of the scrotum (which drain to the inguinal lymph nodes), such that elephantiasis of the scrotum observed with lymphatic filariasis does not involve obstruction of the testes lymphatics (852).

Testosterone can be found in the lymphatics draining the testicles. Early studies using rams suggested that the relative concentration of testosterone is lower than in venous blood arising from the testicles, and that the lymphatics play an insignificant role in its transport due to the fact that their flow is 300–400 times lower than that of the blood (609). However, experimental observations in the pig and horse revealed that certain conjugated steroids (estrone sulfate, dehydroepiandrosetrone sulfate) in the testicular lymph have concentrations up to 10-fold higher than in the testicular venous blood, and that lymph returns the majority of these to the blood (970, 971). In the pig, lymph flow from the testes is reportedly higher than in the ram, and lymph drainage may account for a significant amount of free steroid release (about 10–20% of testosterone and dehydroepiandosterone) and the majority of conjugated steroid release (971). In addition, lymph flow may change in response to certain hormones, as human chorionic gonadotropin (HCG) infusion into the testes can cause higher testosterone concentrations in the lymph versus the venous blood draining the testes (640, 970). Based on all the current evidence, it appears that the relative importance of the testicular lymphatics for distribution of hormones varies from species to species.

Mammary Glands—The lymphatic networks draining the mammary glands are extensive, complex, and are intertwined with the outer dermal lymphatic networks. The mammary glands originate from the ectoderm and are situated between the outer dermis of the skin and the underlying fascia (651). As the number of sets of mammary glands varies across mammalian species, the lymphatic drainage pathways also differ. Our focus will be on humans, common domesticated animals, and laboratory rodents.

The initial lymphatic networks of the mammary glands are found in the interlobular connective tissue surrounding the individual secretory alveoli. Unlike the blood capillary networks, the lymphatics do not penetrate into the alveolar lobules (615, 797). The distribution and ultrastructure of lymphatics in the rat mammary gland was compared between the virgin, pregnant, lactating, and post-weaning periods. Lymphatic vessels were most abundant in the interlobular connective tissue during lactation, with few being found during other stages (797). Differences in ultrastructure included an increase in junctional gaps between endothelial cells during lactation, versus tighter junctions and a greater number and size of vesicles present in the endothelium during the virgin period (797).

The path of lymph flow from the mammary glands differs between species, and even within species there can be much variability. In humans, the larger collecting lymphatics arising from the mammary glands follow the same paths as the arterial branches to the breast, namely the axillary and internal thoracic arteries, and to a much lesser extent the perforating branches of the intercostal arteries. The lymph drainage along these pathways is proportional to the blood supply, with most lymph draining to the axillary lymph nodes, and a significant

amount to the parasternal lymph nodes (internal mammary chain). In some patients a small amount of lymph flows to the posterior intercostal lymph nodes. Normally all drainage goes to nodes on the ipsilateral side (315, 651, 1088). Lymphatics that arise from the posterior side of the breast lead to the interpectoral lymph nodes, and subsequently to the upper axillary lymph nodes. Some of the lobular lymphatics of the mammary glands coalesce into a network that follows the lactiferous ducts toward the subareolar plexus, which is interconnected with the dermal lymphatic networks (651) however whether this is a major site of lymph drainage is controversial (1088).

In domesticated animals, with dugs or udders, there is even more variety in draining patterns. Generally thoracic mammary glands drain to axillary lymph nodes, while abdominal mammary glands drain to caudal epigastric lymph nodes and superficial inguinal lymph nodes (429, 856, 879, 924). Lymphatic interconnections between adjacent mammary glands on the same side of the body have been observed in the dog, but not in the cat (879, 924). Lymphatic interconnections are generally not observed between glands of the left and right side of most species studied but may form in the case of a mammary tumor (856, 879, 924).

The mammary lymphatics participate in both physiological and pathological events in the mammary glands. For example, there is an increase in both blood and lymphatic vessel abundance during lactation (33, 797). The increased blood flow accommodates the increased exocrine function of the mammary gland, and also produces increased lymph flow (579, 613, 614). On the other hand, pathologic lymphangiogenesis is known to accompany breast tumors and facilitate metastasis (651). Understanding the molecular and physiological processes that promote lymphatic regression as the mammary gland transforms from lactation to a more dormant phase may help provide future therapeutic targets to combat breast cancers.

Uterus—The uterus has the essential gestational function of supporting the embryo/fetus, and structurally consists of three layers: the endometrium, myometrium, and serosa. During nongestational phases, growth and regression of the endometrium accompany the cyclic release of sex hormones under hypothalamic-pituitary control and ovulation. Mammals that undergo an estrous cycle reabsorb the endometrium if conception does not occur, while those with a menstrual cycle shed the endometrium through menstruation. Angiogenesis and lymphangiogenesis accompany the cyclical growth and regression during the estrous/menstrual cycle (1090).

Historically, the lymphatic networks have been easily detected in the muscular layers of the uterus but there has been controversy about their presence in the endometrium. Early on, this was based in large part to the limitations with dye uptake studies, which provided evidence of lymphatic plexuses located between the inner circular and outer longitudinal muscle layers, but endometrial and subserosal lymphatics were not consistently visible (443). Currently, some controversy still remains, as myometrial lymphatics are consistently observed (1090, 1091) and are LYVE1-positive (554) while endometrial lymphatics are more elusive and do not express detectible amounts of LYVE1 (554, 894). Still, there has been evidence from the monkey uterus that lymphatic networks exist in the deeper three-

quarters of the endometrium and that they coalesce into a plexus between endometrium and muscle layers, which drains into the plexuses of the myometrium (1147). Similar findings were also reported in sheep, with subsequent draining to subserosal lymphatics and the lumbar lymph nodes (1178). In serial sections of human endometrium, lymphatic networks were found in both the functionalis and basalis regions (99) layers (434). The lymphatics in the basalis region are in close contact with the spiral arteries, which are known to have a key role in menstruation and placentation (99, 276, 1090, 1107). Lymphatics were quantified in a study of uterine samples collected from women with menorrhagia or prolapse, and based on podoplanin and PECAM-1 labeling, 13% of vessels in the functionalis were identified as lymphatics, with 43% in the basalis region and 28% in the myometrium being lymphatic vessels (276, 917).

Lymphatic networks undergo structural and functional changes with the uterine cycle and in response to hormone-based drugs. For normal changes with the uterine cycle, VEGF-C expression increases during the proliferative phase compared to the secretory phase (276). In women treated with progestin prior to hysterectomy for heavy menstrual bleeding, podoplanin-positive lymphatic vessels were enlarged in the endometrium compared to controls (Fig. 16) (277).

During pregnancy, the lymphatics in the muscle layers were reported to increase in size, but not very much in number (1147). In contrast, there have been conflicting reports about the growth or regression of endometrial lymphatic vessels during decidualization of the endometrial stromal cells that occurs during the early stages of pregnancy. Increased lymphangiogenesis the decidua was reported from a study utilizing a mouse model of human placentation (893, 894). However, regression of the lymphatic vessels that accompany the spiral arteries was clearly observed in a study investigating human endometrial samples (1107). The reasons for the difference in conclusions might be attributable to the different experimental models, but also may depend on the precision of terms used to describe the decidua. In terms of the latter, within the same study reporting lymphatic regression in the decidua, an increase in size and elongation of lymphatic vessels was reported in nondecidualized hypersecretory endometrium (1107). Functionally, in a study utilizing pregnant ewes, lymph flow from the uterus does not increase significantly until the third trimester, is virtually absent during parturition, and returns 2–3 days post-parturition (1178).

Changes in lymphatic networks are also evident with uterine tumors. Expression of VEGF-C and VEGF-D, and lymphatic vessel density are significantly higher within and around grade I and II adenocarcinomas when compared to the endometrial functionalis (276). Proliferating lymphatic endothelial cells are detected at the invading front of endometrial carcinomas, however these vessels were, although initially incorporated into the tumor mass, appeared to undergo regression and breakdown (554).

Skin—The skin protects the body's internal milieu from the outside environment. Dermal lymphatic networks play a critical role in this function. Findings from early investigations utilizing locally injected dyes in the skin in by Teichmann (1057) and Neumann (762), and later in 1908 by Unna (1093), described the reticular networks of dermal lymphatics. Results from these early studies identified a polygonal initial lymphatic network just below the most

superficial capillaries in the dermis, and a deeper plexus of lymphatics that included intraluminal valves. Several subsequent observations provided additional details of the dermal lymphatic networks, including variability of network structure depending on thickness or tightness of the skin (561, 562, 628, 1137, 1165, 1217).

A two-dimensional polygonal network of initial lymphatics is apparent in the skin of humans and mice (Fig. 17A–B) (108, 562, 597, 1122). In the dorsal skin of the human foot, this network was found between the papillary and reticular layers of the dermis. The vessels found in this network lack valves, are individually 10–30 μm in diameter but can reach 70 μm diameters at connecting nodes, and the width of the polygons of the network are 400–600 μm (562). In a more recent study using skin of the human breast, the tips of initial lymphatics were found to appear starting at 25 μm beneath the surface of the dermoepidermal junction, connecting to the networks just beneath the superficial capillary networks of the papillary layer of the dermis (Fig. 17C–F) (1122). Precollectors arise from the initial lymphatic network and travel more deeply into the reticular layer of the dermis, forming a three-dimensional network. These vessels have one-way valves to prevent retrograde flow and connect to collecting lymphatics that either appear in the deeper dermis (>500 μm beneath the dermoepidermal junction) or in the subcutaneous layer (562, 1122). The deeper into the dermis, the lymphatic vessels in these networks become larger in diameter, and the density of the networks decreases (Fig. 17C) (562). In addition, there is a distinct loss of Lyve1 labeling on lymphatic endothelial cells, and the appearance of smooth muscle layer at the point where collecting lymphatics appear in the deep dermis (Fig. 17G–J) (1122). While the initial lymphatics of the skin do not appear to be associated with arterioles (938), the larger collecting lymphatics that drain these networks were observed to follow alongside arteries in the rabbit ear (78). A block diagram detailing the lymphatic networks of the skin is shown in Fig. 17C.

The skin is rich in immune cells that serve to protect against potentially harmful antigens or pathogens that may infect the skin. Upon activation, these immune cells may enter lymphatics and migrate to lymph nodes. A recent study (1122) utilizing whole-mount dermal sheet microscopy assessed the architecture of immune cells integration with lymphatic vessel networks. From the results it was estimated that in the first 30 μm below the dermoepidermal junction, for an average human with 1.8 m^2 of skin surface area, there are approximately 7.62×10^8 dendritic cells and 2.08×10^9 T cells. Throughout the dermis, dendritic cells, T cells, and macrophages were either located close to blood vessels are in the interstitial spaces (defined in this study as >15 μm from a vessel), but did not have a particular pattern in relation to lymphatic vessels. Despite the lack of an appearance of a pattern, dendritic cells functionally migrate toward, and enter initial lymphatics, while macrophages remain largely resident in the skin (1122).

Skeletal Muscle—In healthy individuals, skeletal muscle is the body's most abundant tissue. At rest, blood flow to muscle tissue is minimal, with many collapsed capillaries. During exercise, blood flow is increased due to a functional hyperemia characterized by recruitment of inactive capillaries and a marked net increase of capillary filtration. These changes dramatically increase lymph flow, as documented in both animal models and humans (57, 197, 424, 425, 469, 814, 897).

While there may be some variability in the three-dimensional lymphatic networks depending on the arrangement of fascicles in different skeletal muscles, observations to date suggest that the lymphatic networks generally follow the branching of arteriolar networks. For example, tracers such as Evans Blue dye or fluorescent microspheres injected into the rat cremaster muscle are rapidly taken up into lymphatic vessels, which closely follow along the arterial networks (1083, 1175). Observations from histological studies have also show that skeletal muscle lymphatics can be found wrapping around the arcading and transverse arterioles and occasionally venules (964). The origin of the lymphatic networks may be species dependent. Blind-ended lymphatic capillaries were reported to originate near venules in rat skeletal muscle (1027). However, small LYVE1-positive vessels were found in the capillary beds between muscle fibers in mouse and human skeletal muscle (538). The entire lymphatic network within the muscle appears to consist of lymphatic capillaries and precollectors, as no smooth muscle layer has been reported in histological studies, yet intraluminal valves can be found (665, 996, 1027). Lymphatics have a fairly even distribution within skeletal muscle, not favoring fast-twitch or slow-twitch fibers (372). The largest skeletal muscle lymphatic vessels are found in the perimysium (538).

Because the lymphatic vessels within skeletal muscle lack a smooth muscle layer, and intrinsic pumping has not been observed, the increase in lymph flow from the skeletal muscle during muscle contractions is likely governed by milking action of fibers around vessels. The continued elevation in lymph flow following exercise may involve pulse pressure from adjacent arterioles as a driving force, with the intraluminal one-way valves regulating direction of flow (996).

There is also evidence that lymphatic vessels in skeletal muscle respond to exercise training with a change in phenotype. In a report in which muscle biopsies were obtained from the vastus lateralis of male cyclists before and over the course of a period of training, LYVE1-positive lymphatics decreased over time, without an apparent change in podoplanin-positive lymphatics (372). While the mechanism for such changes are currently not clear, it has been observed that VEGF-D is expressed within some muscle fibers, large vessel endothelia, and in fibroblasts (538). In addition, VEGF-C has been localized to nerves, muscle spindles, fibroblasts, and connective tissue (538). Much additional work remains to be done for better understanding of how skeletal muscle lymphatic networks respond to changes in tissue demand with increased muscle mass that accompanies training, or atrophy due to lack of training or disease.

Diaphragm—Although a skeletal muscle, the diaphragm will be considered separately here due to the special role of its lymphatic networks in draining both the pleural and peritoneal spaces, in addition to absorbing any excess interstitial fluid within the skeletal muscle of the diaphragm itself. In the pleural space, fluid removal is highest at the base of the lungs in a standing mammal (754). The lymphatics on the pleural side of the diaphragm maintain the subatmospheric pleural fluid pressure that couples the lungs to the chest wall (759). On the peritoneal side of the diaphragm, the lymphatics serve as the primary site for absorption of peritoneal fluid (5, 744, 1084). These lymphatics also have a major defensive role against bacterial infections and inflammation in the peritoneal space, removing any pathogens or circulating immune cells due to infection or injury in the gastrointestinal or urogenitary

systems (137, 406, 439, 682). Lastly, the diaphragmatic lymphatic network, with subatmospheric luminal pressures lower than those in both the pleural and peritoneal spaces (discussed below), also prevents the flow of fluid from the peritoneal cavity into the pleural space (395).

The diaphragmatic lymphatics of the rat include a superficial, submesothelial plexus connected by transverse vessels to large collecting lymphatics centrally located in the diaphragm that drain both the muscle and the pleural and peritoneal spaces (395). The networks draining the pleural and peritoneal sides are connected, yet under physiological conditions act in a functionally distinct manner, i.e. the lymph formed on pleural side does not typically appear within the network originating on the peritoneal side of the muscle, and vice versa (721). On the pleural side (Fig. 18A), the initial lymphatics are tubular, have many blind ends and form an interconnected network (48, 973). The initial lymphatics of the peritoneal side are flattened with broad luminae (Fig. 18B), also called lacunae (756, 1084, 1085). Pleural and peritoneal fluid can enter through mesothelial stomata that open and close with the breathing cycle, and travel through the submesothelial lacunae to reach the initial lymphatics just beneath the mesothelium (48, 395, 799). Interstitial fluid within the skeletal muscle of the diaphragm also enters the network, directly into initial lymphatics along the perimeter of the skeletal myocytes (395). The diaphragmatic lymphatics primarily drain to the anterior mediastinal lymph nodes near the thymus, and eventually the right lymph duct, although about 20% of diaphragmatic lymph appears in the thoracic duct (1178).

Investigations of the physiology of the diaphragm lymphatics are technically challenging and have largely been limited to the pleural side, which can be more readily accessed for imaging studies and measurements of intralymphatic pressures. The pleural diaphragmatic lymphatics are primarily linear vessels located in the tendon and medial muscular area of the diaphragm. These lymphatics run both parallel and perpendicular to the muscle fibers, and interconnect, forming networks of vessel loops above the diaphragmatic muscular plane (719). In the central part of the diaphragm, the pleural lymphatics typically lack smooth muscle and are thus non-contractile, but are tightly linked to the surrounding tissue and easily deformed by contraction of the skeletal muscle fibers during breathing (723). In contrast, the pleural lymphatics located in the more peripheral areas of the diaphragm, near the costal medium, do possess a smooth muscle layer and display intrinsic contractile activity (725, 758). Interestingly, the strength of contraction appears to be weak and unevenly distributed in these peripheral pleural lymphatic networks, possibly to optimize lymph flow through the network (724, 725). Based on the current data, an integrated mechanism of lymph flow is proposed, combining both extrinsic forces from skeletal muscle contraction on an optimal morphology of the vessels and their network geometry in central parts of the diaphragm, with aid by intrinsic lymphatic contractions in peripheral regions to move lymph to these central regions (724). Such an optimization of lymph formation and flow is thought to guarantee fluid gradients that permit continuous removal of pleural fluid throughout the respiratory cycle (395).

Heart—The lymphatic networks of the heart have been recognized for many centuries, and have an important role in maintaining fluid homeostasis for optimal electrical conduction and force generation by the cardiac myocytes. Olaus Rudbeck provided the first description

of subepicardial lymph vessels in 1653 (926), followed many years later by Aagaard, who detailed a much more extensive cardiac lymphatic vessel network throughout the epicardial, myocardial and subendocardial regions using injection of dye into the heart muscle (1). Kampmeier later contributed the first observation of intraluminal valves in cardiac lymphatic system of humans (512), shortly after which Patek (850) detailed the lymph drainage pathways and confirmed lymph flow (693). He demonstrated that the left ventricle had the most extensive lymphatic system and lymph flow in cardiac chambers was from endocardium to epicardium (693). Despite these findings characterizing lymphatics in the heart (113, 589, 695), relatively few studies have given cardiac lymphatics much attention over the past few decades and their potential role in cardiac diseases has only recently regained interest.

Cardiac lymphatics arise in the embryo along with the development of coronary blood vessels (333, 503, 512, 524, 941, 1145). The mouse heart comprises a heterogeneous make-up of cell populations with contributions derived from both extra cardiac venous endothelium and progenitors from the yolk sac hemogenic endothelium (333, 543). Conditional knockout of Prox1 in Tie2+ and Vav1+ blood endothelial cells resulted in loss of cardiac lymphatic endothelial cells supporting a role of Prox1 for LEC identity and development (543).

The anatomy of adult cardiac lymphatic system of pigs, dogs and humans has been studied by topical application and injection techniques (491). Lymphatic vessels in the heart can be divided into three categories based on size and structure: small, initial lymphatics; mediumsized precollectors/collecting vessels; and larger, valve-containing lymphatic trunks (542). This can be observed using dye injection, which shows a plexus of initial lymphatics and the larger, precollectors and collecting lymphatic vessels. The capillary plexus is seen in myocardium while subendocardial initial lymphatic plexus lies parallel to the surface of the endocardium. The collecting vessels can be seen in subepicardium as they coalesce into lymphatic trunks that drain the entire heart and subsequently proceed to the medastinal lymphatic trunks (26, 218). Valves are most numerous in the subepicardial collecting vessels (172, 512, 850, 1076), which were reported to also possess a smooth muscle layer (407, 512). Lymph flows from the subendocardium outward to the subepicardial lymphatic plexus which feed into larger lymphatic vessels that follow the path of the main coronary blood vessels in the anterior and posterior interventricular grooves and the coronary sulcus (542). In general, these extensive subepicardial and subendocardial collecting vessels networks lead to toward the larger trunks in the AV sulcus, continuous with the main cardiac lymph duct, (491). Here the lymph drains toward lymph nodes and eventually outflows into the right lymphatic duct and thoracic duct (512, 850, 941). Cardiac lymph flow is likely driven entirely by myocardial contractions, producing extrinsic forces on the lymphatic vessel network (218, 361, 703, 1197). During diastole the pressure of the blood in the ventricles drives lymph from the subendocardial to the myocardial lymphatics and during systole myocardial contraction forces lymph from the myocardial lymphatics to the subepicardial lymphatics (218, 890, 1200).

Recently, interest in the understanding the function in the maintenance of intramyocardial pressure and preventing tissue edema has developed. Blockade of cardiac lymph flow causes

coronary blood vessel injury (1004) and it can also result in subendothelial edema and endothelial injury (218). By providing a margin of safety against edema, normal function of cardiac lymphatics likely have an important role in preventing many cardiac pathologies such as coronary atherosclerosis and interstitial fibrosis (693). Lymphatics in the cardiac mitral valve of the human are also thought to play an important role in the consequences of rheumatic fever because the disease has an impact on adjacent tissues lined with endothelium (697). Adventitial lymphatics are extensive along the aorta and coronary arteries (476, 503, 890) and lymphatics in the adventitial layer of atherosclerotic vessels drain fluid, inflammatory molecules and cells to local nodes (1160). Impairment of lymph flow has been associated with coronary artery injury as well as enhanced tissue damage followed by necrosis and increased interstitial fibrosis (218, 476, 696, 988). Additionally, surgery-induced damage to fat pad lymphatics located at aortic roots was reported to lead to atrial fibrillation and heart dysfunction (631).

Heart disease remains the leading cause of death, and myocardial infarction is the leading cause of non-accidental sudden death. Experimental myocardial infarction of the mouse heart stimulates both angiogenesis lymphangiogenesis, further suggesting an important role of lymphatics in overall tissue homeostasis in the heart (543). The peptide apelin appears to have an important role in regulating lymphangiogenesis under these circumstances (1052). In humans, an increase in new lymphatic vessel formation was reported near the edges of necrotic tissue following myocardial infarction, in scars, and in areas of reactive pericarditis (534). Lymphangiogenesis also occurs with heart valve disease, in association with elevated levels of VEGF-C, VEGF-D, VEGFR2, and VEGFR3 (1036). Lymphangiogenesis is particularly in areas rich in extracellular matrix, as opposed to inflammatory cell-rich areas that are prone to angiogenesis. In infective endocarditis, in some areas lymphatic vessels account for nearly 100% of all vessels present (534). Currently much effort has is being devoted to stem cell or progenitor cell therapies to repair and regenerate heart tissue lost during a myocardial infarction. Understanding how to optimize lymphatic function in the healing heart may represent a key factor for the successful development of effective regenerative medicine therapies.

Upper Respiratory Tract—The nostrils and subsequent structure of the upper airway network traps airborne particles and conduits air to the lungs for blood-gas exchange in the alveoli. The nasopharynx has a submucosal lymphatic plexus that has a prominent role in absorption of airborne particles that have been deposited onto the nasal mucosa (1179–1181).

Such particles include certain viruses and infectious bacteria, which although do not freely cross the nasal mucosa, can infect local cells, replicate, and then be found in the cervical lymph after several days (1178). In rodents there are nasopharyngeal-associated lymphoreticular tissues (NALT). However, NALT does not appear to be typical in humans, having only been described in a subset of children under 2 years old (252). Recent advances pertaining to nasal lymphatic networks deal mainly with its involvement to nasopharyngeal cancers. For example, in nasopharyngeal carcinoma biopsies from patients, an increase in podoplanin- and VEGFR3-positive lymphatic vessels, along with greater VEGF-C expression by tumor cells, was associated with advanced lymph node metastasis (1119).

The larynx and trachea also have rich lymphatic networks in the mucosa and submucosal tissues (1178). In the mouse trachea, arcades of blood and Lyve1-positive lymphatic vessels are apparent between the cartilage rings (Fig. 19) (64, 536), including initial lymphatics featuring button junctions (61). Experimental *M. pulmonis* infection has been shown to cause TNF-α-induced and Vegfr3 dependent lymphangiogenesis in this airway, with formation lymphatic sprouts crossing the cartilage rings (Fig. 19) (63, 64). IL-1β, which is also elevated during *M. pulmonis* infection, also causes Vegfr3-dependent lymphangiogenesis in the mouse trachea (62). These results suggest that inflammation in response to infection causes changes in the lymphatic network. The degree to how the remodeling of the lymphatic network in the trachea affects function remains to be determined.

Lung—The lung is a soft, highly deformable tissue that compresses and expands with airflow. Fluid pressures within the thin alveolar tissues are strongly influenced by vascular and alveolar pressures, and the active secretion of surfactant. The lung is also a unique organ because the entire volume of blood passes through it. To achieve its function of gas exchange between the alveolar space and blood, the lung has a dense microvascular network. The lymphatic network is more diffuse, yet the network has proven sufficient for estimation of the lung capillary permeability-surface area coefficients by measuring solute accumulation in lymph (295). The microvascular leakage in the lung appears to be rapid, as intravenously injected tracers like FITC-dextran can be found within minutes in the lung lymphatic capillary networks (795). The lymphatics thus appear to have a very important role in fluid balance within the lung tissues, keeping alveoli patent for optimal gas exchange.

Rudbeck provided the first description of pleural (superficial) lymphatics in the lungs of dogs (926). Later reports by Wywodzoff described a deeper, pulmonary network of lymphatics in the dog and horse (1154), also sometimes referred to as the intrapulmonary or parenchymatous lymphatics (583). The pleural and pulmonary lymphatic networks have now been observed in many species, although there is notable variation. Extensive pleural lymphatic networks have been shown in humans and larger animals (583, 587, 801), but these are moderate to sparse in rodents (587). In the rat, a pleural lymphatic capillary network with some longer, straighter lymphatics that are either precollectors or collecting lymphatics have been observed by scanning electron microscopy of corrosion casts (Fig. 20), and confocal microscopy of Lyve1-labeed lungs (17, 795). In mice, only sparse lymphatics have been reported extending to the pleura (60). In humans, lymphatic vessels with valves can be seen within this superficial lymphatic network (583). As for the deep lymphatics, these have been found in the hilum, in interlobular septa, around the bronchial tree and blood vessels, and within or below the visceral pleura (17, 21, 60, 587, 650, 795). While lymphatics can be observed in juxta-alveolar connective tissue, they have not been found within the thinner alveolar walls (17, 60, 587, 650). LYVE1-positive endothelial cells are typically observed within these lung lymphatic networks (795). As the smaller lymphatics coalesce into larger vessels, secondary valves become apparent (Fig. 21) (21, 580, 582, 583, 587, 650). In addition, circular smooth muscle can be found on the larger collecting lymphatics that are associated with arteries, veins, and bronchi (21, 583, 587).

While several histological studies using lung sections and corrosion casts have provided a detailed view of the lung lymphatics, this approach has the limitation of requiring fixation of tissues. The recent introduction of mice with Prox1-driven EGFP expression has allowed for detailed view of lymphatic networks in living tissues, and has allowed for a more detailed view of how the networks may adapt over time. In these mice, under pathogen-free conditions, many lymphatics are apparent around large bronchi and blood vessels in the hilum, but the number dramatically decreases with the branching into smaller airways and blood vessels. The lymphatics are typically associated with airways or blood vessels, even down to the level of alveoli and near the visceral pleura. The mean diameters of lymphatics in mouse lungs was reported as 60 ± 7 μm in the hilum, 38 ± 7 μm on medium-sized bronchi and vessels, and 9 ± 1 μm for the lymphatic capillaries that extended just beyond the terminal branches of pulmonary veins (60). As in the upper airways, local infection can cause remodeling of the lung lymphatic network. *M. pulmonis* infection in mice causes a progressive expansion of the lymphatic network in the lung, along with stronger CCL21 immunoreactivity in lymphatic endothelial cells. The lymphatic growth occurred in regions of bronchus-associated lymphoid tissue (BALT) and was dependent upon Vegfr2 and Vegfr3 (60).

Like other lymphatic networks, the lymphatic vessels of the lung have a key role in the clearance of excess fluids and the trafficking of immune cells. Measurements of microvascular pressures in the intact lungs of anesthetized rabbits suggest that the pulmonary microvasculature cannot effectively reabsorb fluid (757). Thus, the pulmonary lymphatics play a crucial role balancing normal microvascular leakage and ensuring a steady state interstitial fluid pressure and protecting against edema (136, 311, 391, 757). This concept is supported by data from several investigations indicating that the rate of lymph flow in the lung correlates to microvascular filtration (283, 511, 708, 846, 1022). Other factors that may affect lymph flow include breathing and depth of ventilation (282, 851), tissue hydrostatic pressure, intrinsic pumping of collecting lymphatics, and the systemic venous pressure (511).

Despite the role of lymphatics in maintaining fluid homeostasis in the lung, experimental evidence suggests that once the lung becomes edematous, its role in the clearance of edematous fluid is rather limited (371, 1022) (511, 661). A two-compartment model of the interstitial space, in which one compartment (perimicrovascular interstitium) is readily drained by the lymphatics, but another (peribronchial interstitium, farther away from lymphatic capillaries) has been used to explain this phenomenon (708). In addition, fluid that enters into the alveoli and airways would not be cleared by lymphatics, but rather by the combination of expiration into the air, and via the mucocilliary elevator and coughing.

Recent work utilizing Ccbe1- or Vegfr3-deficient mice, which lack lymphatic vessels, die at birth because they are unable to inflate their lungs after birth (472). While previously it was estimated that lymphatics remove about only about 11% of the fluid from fetal lungs at birth of lambs, at the same time it was also determined that lymph flow from the lung does not increase significantly due to birth (101). This work suggested that lymphatic clearance of fluid occurs prenatally. In fact, data from the study utilizing Ccbe1- and Vegfr3-deficient mice revealed that pulmonary lymphatic function is present prior to birth, and that it is a

prerequisite for increased lung compliance that occurs in late gestation (472). Other work in which VEGF-C was selectively overexpressed in the airways during embryonic day 15.5 to postnatal day 14 caused lymphatics near airways to take on an enlarged, sac-like morphology that was dysfunctional and produced respiratory distress and pulmonary lymphangiectasia (1172). Collectively, these findings implicate an important role for pulmonary lymphatics in respiratory distress syndrome in premature infants (472).

Lymphatics on Larger Vessels—Large blood and lymphatic vessels have been known for some time to possess a lymphatic network in addition to the vasa vasorum on the outer surface of the smooth muscle layer (442, 569, 591, 837, 972). Using ink uptake, lymphatic networks have been observed within the adventitia of the aorta, coronary arteries, pulmonary artery, pulmonary vein, and vena cava. While in veins the lymphatic networks may penetrate into the media layer, this was not generally observed in the aortic and arterial lymphatic networks. More recent studies utilizing antibodies to identify lymphatics have shown lymphatic vessels in both the adventitia and media of coronary arteries, with intimal lymphatics only present during the progression of atherosclerotic lesions (534). The large vessel lymphatics of the thoracic cavity drain to lymph nodes located to the para-aortic lymph nodes and the thoracic duct (490).

Over a half-century ago it was recognized that the arterial endothelium filters a certain degree of plasma proteins and lipoproteins. The pressure from the arterial lumen causes a gradient within the vessel wall that forces filtered plasma components to pass through the elastic lamina and media before reaching the lymphatics (12, 288–292, 802). Early on, the potential significance of this anatomical arrangement for the pathophysiology of atherosclerotic plaque development was recognized (830, 1056). At the same time, it was noted that endothelial injury can cause abnormally high permeability to macromolecules in the arterial wall (208, 209). Moreover, it was found that in rats fed atherogenic diets for 9 weeks, the perivascular lymphatics on the aorta become distended, lymphatic endothelial cells have increased vesicles, and lipid droplets are apparent within the endothelial cells (477), supporting the concept that lymphatic insufficiency may contribute to atherosclerotic plaque formation. An additional report of ingrowth of vasa vasorum in the arterial wall in response to elevated metabolic demand, without an accompanying expansion of the lymphatic network, suggested development of an imbalance in delivery and removal of lipid that might facilitate its accumulation to form intimal plaques (490).

If a certain degree of local lymphatic insufficiency leads to plaque formation, then evidence that loss of local lymphatic draining of a vessel could cause local abnormalities in remodeling or changes in mechanics. would be pertinent. Such evidence was found in a study in which lymphatics draining the femoral artery were occluded in dogs, which resulted in inward hypertrophic remodeling and a decrease in elastic modulus (742). In a separate study, ligation of lymphatics draining the aorta in dogs led to intimal thickening within three weeks (749). In addition, lymphstasis in the arterial wall was reported to typically accompany atherosclerosis in human patients (596).

A second line of evidence needed to support the role of local lymphatic insufficiency in atherosclerotic plaque development is that lymphatics actually clear components of

atherosclerotic plaques, such as cholesterol. In studies in which radiolabeled cholesterol esters in high density lipoprotein (HDL) and low density lipoprotein (LDL) particles were tracked crossing the porcine thoracic aorta, the HDL were found to pass through the media, easily cross the intima and enter the adventitia, and efflux from the vessel wall via vasa vasorum or lymphatics. In contrast, LDL particles did not go beyond the intima and were thought to efflux via the vessel lumen. Only 10–20% of cholesterol disappearance was explained by local hydrolysis in the aortic wall. These data suggest a specific mechanism to facilitate LDL passage across the vessel wall (438, 773). More recently, using both genetic and a surgical mouse models of local lymphatic insufficiency on the aorta, macrophage-mediated mobilization of cholesterol on HDL (called reverse cholesterol transport) was significantly reduced (653). Notably, macrophages and dendritic cells can depart a regressing atherosclerotic plaque through lymphatics (618).

The number of lymphatics present in arterial vessel walls, and how this changes with vessel remodeling or atherosclerosis, has been variable among studies. Data from a study of human aorta and common iliac artery specimens, in which LYVE1 and podoplanin labeling was used to identify lymphatics, suggested a correlation between the number of lymphatics in the adventitia and intimal thickness as well as age of the subjects (285). In human coronary arteries, lymphatics have either been not positively identified (305) or found in the adventitia (534, 747). In one study, in a subset of coronary arteries with calcified, fibrous plaques, lymphatics grew into acellular areas with cholesterol crystals and calcium deposits (534). In a recent study in apoE$^{-/-}$ mice, VEGF-C levels were higher in atherosclerotic aortic walls, however there was concomitant regression of the adventitial lymphatics (1045). Elevated VEGF-C and VEGF-D was also reported in atherosclerotic human coronary artery specimens, and it was proposed that the elevation these growth factors during atherosclerosis contributes to local angiogenesis rather than lymphangiogenesis (747).

Kidney—The kidneys are encapsulated organs that undergo relatively less deformation compared to the lungs, heart, and muscle, but can also be compressed by elevated abdominal pressure. The importance of the lymphatics in the kidney becomes apparent with kidney transplants in which the lymphatics are ligated, and infections develop (809). In experimental studies using rats, bilateral ligation of the renal lymphatic ducts led to severe proteinuria within one week, and the subsequent development of renal fibrosis and chronic renal failure (1212). Renal lymphangiogenesis has been reported to accompany renal fibrosis, proteinuria, and following transplantation, likely in response to a deficit in renal lymphatic function (1173).

Observations from anatomical studies indicate that lymphatic networks within the kidney originate mainly in the cortex, with some probably in the outer medulla, but very few to none draining the inner medulla or papilla (19, 559, 778). Data from studies investigating the uptake of tracers injected into the renal cortex and medulla also indicate that lymphatics drain interstitial proteins from the cortex, but that the vasa recta primarily is responsible for uptake of protein in the renal medulla (638, 1065). The lymphatic networks are distributed alongside arteries, within the adventitial collagen bundles of renal arterioles, and filling tissue cavities between the blood vasculature and renal tubules (778). The networks within the kidney are made up of initial lymphatics originating adjacent to the renal capsule, with

an arcuate precollector network leading to an intralobular precollector network (Fig. 22) (19, 20, 1167). In some mammalian species, there are also capsular lymphatics and lymphatic vessels that perforate the capsule and connect to the cortical lymphatic network. Outside the renal capsule, collecting lymphatic lymphangions have been observed (778).

While lymphatic networks in the kidney have a clear role in metastasis of tumors originating in the kidney and in the protection against infection and cancer metastasis, much still remains unclear about the contribution of lymphatics to various pathologic processes in the kidney. Lymphocele remains a common complication with kidney transplantation (699). The types of signals that are transmitted through lymph from the kidneys to the hilar lymph nodes are also only now being uncovered. Concentrations of angiotensin II, IL-1β, and IL-6 in renal hilar lymph have been reported to be higher than concentrations in the circulating plasma in rats (98). This suggests that although the blood circulation may be the primary route for most endocrine and inflammatory signals arising from the kidney, that signals also are also delivered directly to the draining lymph node, and how they collectively affect hilar lymph node cells remains to be determined. Another factor mentioned above is the increase in lymphangiogenesis associated with kidney injury or disease. One interesting function of the kidney worth noting here is that it is the major site of activation of vitamin D by parathyroid hormone. Activated vitamin D (calcitriol) was recently shown to inhibit lymphangiogenesis in the kidney through activation of Vitamin D receptors on lymphatic endothelial cells (1174). This finding shows that lymphatic vessel networks may adapt in the kidney according to the functional demands. Lastly, in spontaneously hypertensive rats that exhibit hypertension combined with renal injury (SHR-A3 strain), lymphatic vessel density and macrophage infiltration into the kidney were both significantly elevated compared to normotensive Wistar-Kyoto (WKY) background controls (544), suggesting lymphatic network remodeling in response associated with renal diseases. Similar observations were also reported when comparing aged Fischer rats (20–24 months old), along with elevated inflammation and Vegfc/Vegfr3 expression, compared to 4-month old controls (544). Increased lymphatic vessel density has also been observed in mice that have hypertension due to salt sensitivity or inhibition of nitric oxide synthase activity. These mice also display significant increases in renal infiltration of immune cells. When lymphangiogenesis was genetically induced only within the kidney in these mice, the immune cell accumulation was reduced and blood pressure returned to normal, suggesting that renal lymphatics have an important role in the trafficking of immune cells and regulation of blood pressure (622). Collectively these findings suggest lymphatic network functionality in the kidney contributes to overall renal function. Finding ways to improve disrupted renal lymph flow following transplantation, injury, or in renal disease represent important, understudied topics pertinent to development of better treatments for kidney disorders.

Lymphatics Draining the Central Nervous System—Lymphatic vessels have been observed within the dura mater of the dog, mouse, and human brain (39, 340, 624). These lymphatics absorb cerebrospinal fluid (CSF), formed primarily in the choroid plexus in the lateral, third, and fourth ventricles, which has a net movement through a network of prelymphatic channels toward the arachnoid space (668). Lymphatics have been reported exiting the skull via the cribriform plate, jugular foramen, foramen lacerum, and the petrosal

section of the internal carotid artery (338, 873). These vessels coalesce into the deep cervical lymph nodes (39, 624).

Lymphatic drainage from the brain was first described by Schwalbe in 1869, who found that Berlin blue injected into the cranial sub-arachnoid spaces of exsanguinated rabbits and dogs entered the lymphatic vessels and lymph nodes of the head and neck (967). Subsequently, results from studies by Key and Retzius (533), Zwillinger (1226), and Weed (1130) suggested that the cribriform plate as the main exit point by which cerebrospinal fluid (CSF) reaches the cervical lymphatics. Additional work by Brierley and Field clarified that macromolecule tracers could easily pass from the sub-arachnoid space into the cervical lymph nodes and subsequently to more distant lymph nodes in the front of the vertebral column (134). There were also significant contributions by Foldi and Csanda, who described both initial and collecting lymphatics in the dura mater in cranial sections from dogs (340), and detailed connections between the subarachnoid space and the cervical lymph system in the nasal cavity, orbita, and jugular foramen (338).

Despite these early advances and functional evidence pointing to lymphatics leading to cervical lymph nodes as major participants in CSF drainage, this route was dropped from medical textbooks for several decades in favor of the opinion that CSF escape occurred through the arachnoid villi into venous blood (119). The underlying principle for this opinion was that a pressure gradient between the CSF and venous sinuses favored fluid transport at this site, but there has been virtually no evidence supporting this as a sole route of CSF escape. Given the large amount of functional data showing appearance of tracers into cervical lymph after injection into the subarachnoid space, ventricles, or other regions of the brain, in multiple mammalian species (114–116, 120, 121, 134, 216, 535, 1163), plus the fact that lymphatic vessels are present within the dura mater (39, 340, 624), the absence of a lymphatic mechanism is simply impossible.

Most data supporting a role for lymphatics in draining CSF were from studies performed prior to the advent of the identification of specific markers for identification of lymphatic vessels by immunofluorescence methods, or production of mice with reporter proteins that identify lymphatics. Findings from several studies using tracers injected into the brain primarily show escape of CSF through the cribriform plate, and that movement of CSF through the arachnoid villi into the bloodstream is actually a secondary route that only becomes important when intracranial pressures become very high (114, 115, 985, 1190). This was supported by findings that the arachnoid villi are absent in the fetus and are only found in abundance in adults (492, 493, 820), yet rates of CSF formation are similar between the fetus and adult mammals (717). Also, experimental occlusion of the cribriform plate with glue or bone wax reduced CSF escape when intracranial pressure was elevated (984). Other lymphatics exiting at the base of the skull likely account for additional drainage of CSF (338, 873). Additionally, data from several studies suggest that intracerebral injection of cells, including erythrocytes, macrophages, T cells, and dendritic cells, also reach the cervical lymph nodes (13, 155, 377, 420, 520, 780), and production of a deviant immune response in mice evoked from injection of ovalbumin in the brain could be adoptively transferred to naïve mice using cervical lymph node cells (1136). Collectively, these results

provided a strong case that lymphatics are an exit pathway for CSF and immune cells in the brain.

Where CSF precisely is absorbed by lymphatics has been less clear, and only recently using lymphatic-specific markers and transgenic mice have visualization of complete lymphatic vessel networks in the dura mater have been observed (39, 624). Intracerebrally injected tracers and GFP+ CD4+ T cells have been found to travel along the olfactory nerves, through the cribriform plate en route to the deep cervical lymph nodes (217, 377). It is not currently known if this path consists of lymphatic vessels or prelymphatic channels, however it is known that this path is consistent among species. In reports by Johnston and colleagues, who utilized Microfil injection into the subarachnoid compartment, they delineated a clear path from the subarachnoid spaces to the cribriform plate in multiple species, including primates, (494, 836, 1188). While there has been debate about the importance of this pathway in humans, data obtained from human autopsies revealed that injection of India ink into CSF fills into the perineurial spaces of the olfactory nerve branches and appear in the nasal submucosal tissue (170, 1135). Lymphatic vessels can be found penetrating the cribriform plate and coming into close contact with olfactory nerve fibers (1190). Here, lymphatics may encircle the olfactory nerves and attach tightly to perineurial cells and fibroblasts to form a seal that prevents CSF from entering the interstitium in significant amounts (547, 1189). Alternatively, these lymphatics previously through to originate at the cribriform plate may actually be part of a continuous network with the dura mater lymphatic vessels (39). It remains to be seen whether previously described paths of CSF flow along the olfactory and optic nerves (312, 313) may include dura mater lymphatic vessels.

An additional region where CSF escapes from the human central nervous system is the spinal subarachnoid space, and the rate is higher during physical activity than during rest (298). In a rat model of cisternal kaolin injection-induced hydrocephalus, CSF was observed to escape along lumbosacral rootlets (629). Tracings of the route of CSF escape, using high-molecular weight ferritin, showed passage from the central canal, through ruptured ependymal and dorsal columns, along spinal nerves and then into lymphatic vessels (1106). CSF escape from the spinal column may be of greater significance in humans, standing upright, compared to animal models (485).

The “Glymphatic” System—Within the brain, a series of perivascular prelymphatic channels (also called the Virchow-Robin space or the paralymphatic system), was proposed by Foldi and others to be responsible for the aforementioned movement of CSF toward the lymphatic system (163, 214, 215, 338, 339, 535, 1135, 1205). This network of perivascular, prelymphatic channels was recently dubbed the “glymphatic system” due to its drainage function dependent in part on convective water movement facilitated by glial cells to promote efficient elimination of soluble proteins and metabolites from the CNS (479). These prelymphatic channel networks may be up to 20 cm long in larger mammals (809). This system also appears to be limited to fluid movement, as the channels are not large enough to accommodate immune cell traffic and also restrict large particles (151, 460, 575).

Recent work using two-photon microscopy of closed cranial windows in anesthetized mice identified that intracisternally injected tracers enter the brain through perivascular spaces on

pial arteries. Subsequently the tracer flows along perivascular, prelymphatic channels in penetrating arteries and arterioles. These channels are between the vascular smooth muscle and the perivascular astrocyte end-feet (460). This highly polarized macroscopic system of convective fluid fluxes with rapid continuous interchange and exchange of CSF and ISF is facilitated by glial aquaporin-4 (AQP4) water channels (460, 479). AQP4 channels are expressed on the astroglial end-feet abutting the vessel wall, and the endothelial barrier is devoid of these channels, and genetic deletion of AQP4, decreases fluid movement through this prelymphatic channel network by more than 60% (460). CSF movement into the parenchyma drives convective interstitial fluid fluxes within the tissue toward the perivenous spaces surrounding the large deep veins. Local arterial/arteriolar pulsations are help drive the fluid flux through the perivascular channels (401, 908). The CSF is collected in the perivenous space from where it drains out of the brain toward the cervical lymphatics (494, 737).

Another important function of the glymphatic system is lipid transport. An influx of lipids and lipoproteins into the brain is prevented by blood brain barrier so the brain synthesizes all of its cholesterol de novo. It was demonstrated that injection of lipophilic molecules <1 kDa and >3 kDa entered the brain via periarterial routes and exited perivenously (888), in similar fashion as hydrophilic molecules (459), suggesting that the glymphatic system plays a central role in macroscopic distribution of lipids in the brain.

In addition, recent data suggest that the glymphatic system is enhanced during sleep and suppressed during wakefulness (1155). This finding suggests a possible role of convective fluid fluxes and clearance of metabolite, and implies that sleep facilitates clearance of neurotoxic waste products produced during wakefulness (1155).

Fluid movements in the glymphatic system decrease with aging (556), which is associated with loss of perivascular AQP4 polarization, likely leading to dysregulation of astroglial water transport (556). Clearance of extracellular β-amyloid has been attributed to CSF bulk flow, and failure in adequate CSF bulk permits β-amyloid accumulation (151, 152, 556). Therefore, impairment of either the glymphatic channels or dura mater and cervical lymphatics may be as a risk factor for neurodegenerative disease (1133, 1134). This issue has also been raised as a possible contributing factor in recovery from traumatic brain injury (460).

Eye—Until recently, the lymphatics of the eye were poorly characterized. This is probably in large part to the belief for many years that the eye was an “immunoprivileged” site because antigens were found to drain to the venous blood directly (1028). However, this concept has been revised in light of observations that antigen from the anterior eye can reach lymph nodes of the neck at least in part through lymphatic vessels (147). Also, considering the discovery of a symbiotic ocular microbiome (625) that contributes to immunologic protection, local lymphatics in the eye likely have the same important roles on the surface of the eye as in the skin, gut, and other areas exposed to potential pathogens for immune surveillance and tolerance.

Eye lymphatics also contribute to the drainage of the aqueous humor continually produced by the ciliary body epithelium. Maintenance of normal pressure within the eyes is important for prevention of glaucoma. The aqueous humor drains through at least two pathways. First, the conventional outflow pathway, consisting of the trabecular meshwork into Schlemm's canal, accounts for approximately 80% of aqueous humor outflow. In addition, a more recently described uveoscleral pathway through interstitial spaces of the ciliary body accounts for the remainder (523, 1187).

Lymphatics of the anterior eye have been observed in tissue sections of the limbus and conjunctiva, are often partly collapsed, and located immediately underneath the conjunctiva (86, 370). The endothelial cells in these vessels are positive for podoplanin and LYVE1 (222, 369, 1187). In addition, the endothelial cells of Schlemm's canal were recently found to be positive for Prox1 (1082). Independent investigations utilizing lineage tracing studies and serial imaging of Schlemm's canal in *Prox1-GFP* reporter mice suggest that the endothelial cells of Schlemm's canal arise from the blood vasculature and acquire lymphatic-like properties during development (41, 845).

There are both superficial lymphatics networks within the loose adenoid layer, and also within the deeper fibrous layer to a lesser extent. Under the limbus, the lymphatics form a structure known as the circulus lymphaticus, also called the lymphatic circle of Teichmann (199, 1029). The lymphatic vessels are typically located near capillaries and venules. In the limbus, they are located within the palisades of Vogt, reaching the extreme periphery of the cornea (398, 930). They may also extend into the cornea when neovascularization of the cornea develops (223). No lymphatics are located within the globe of the eye, which may be due to the presence of anti-angiogenic factors present in the aqueous humor like vasoactive intestinal peptide and α-melanocyte stimulating hormone (103). Lymphatics only contact the globe at the limbus, following the pattern of the limbal loop blood vessels. Lymph from the anterior eye generally flows toward the lateral extremes of the eyelid. The superficial plexus of lymphatics in the adenoid layer drains on the temporal side of the eye to the parotid or preauricular lymph nodes. The plexus in the deep fibrous layer drains on the nasal side of the eye to the submandibular lymph nodes (86).

Initial lymphatics and precollectors have also been identified in the posterior eye within the choroid of humans (549). Other evidence also supports that choroid lymphatics or precursor cells are present, yet much remains unknown about these networks (251, 966, 1158). It is also worth noting that lymphatic vessels have also been found in some orbital tissues around the eye, such as the lacrimal gland and dura mater associated with the optic nerve (261, 370, 976).

The functional importance of lymphatics of the eye has also been demonstrated. As stated earlier, lymphatics were been shown to contribute to the delivery of antigen from the anterior eye to neck lymph nodes in rats (147). In a sheep model, selective uptake of ^{125}I human serum albumin after intercameral injection in the eye into the submandibular, retropharyngeal, preauricular and cervical lymph nodes was attributed to the uveolymphatic pathway in the ciliary body (1187). In addition, deletion of the gene that encodes the receptor for the lymphangiogenic peptide adrenomedullin led to corneal edema in mice

(448). Lymphatics in the eye can also respond adapt through lymphangiogenesis in response to stimuli such as injury or inflammation in the eye. This has been demonstrated in a mouse model in which newly formed lymphatic vessels protrude into the cornea in response to inflammation caused by ophthalmic sutures or burn injury (221, 611), or to angiogenic/lymphangiogenic factors such as VEGF-A, VEGF-C, or fibroblast growth factor-2 (149, 150).

Collectively these reported findings suggest a dynamic lymphatic network in the eye that supports both immune function and balancing intraocular pressure. The eye lymphatics may serve as a potential target for glaucoma therapy (1187), possibly using a VEGF-C therapy to expand drainage via Schlemm's canal proposed (41, 523, 845). Anti-angiogenic/lymphangiogenic strategies may prove useful for corneal transplants (1207). In addition, better understanding of the lymphatics of the eye and surrounding orbit may help our with identifying new pharmacologic targets for metastatic disease involving the eye, or autoimmune disease involving the eye such as Graves disease (261).

Concluding Thoughts on Lymphatic Network Structure

In our discussion of different organs that perform a variety of functions the body, there are commonalities in the lymphatic networks. While there were variations in the network structure, generally there is an initial lymphatic network (lymphatic capillary network) present that serves as the gateway for interstitial fluid uptake. The lymphatic networks are frequently located in close proximity to the local microvasculature, and often follow the arterial/arteriolar network. In many cases there are precollectors with one-way valves within the tissue, while collecting lymphatics are may be located within an organ or at exit points of an organ. Some organs like mesentery have many pumping collecting lymphatics, while organs that frequently contract (heart) or are distended (lungs) appear to require less intrinsic pumping of collecting lymphatics. Some organs, like the brain and eye, have relatively few lymphatics, while others, like endocrine glands, have extensive lymphatic networks. Despite organ-specific differences in network structure, the ultrastructure of lymphatic vessels and the protein markers that identify lymphatic endothelium are remarkably similar across the different organs studied.

Development of Lymphatic Vessel Networks

The formation of the lymphatic vasculature includes four important aspects: origination of lymphatic endothelial cells (LECs), specification and migration of LECs, maturation of lymphatic vasculature and smooth muscle cell (SMC) recruitment, and lymphatic valve formation. Here we cover these topics and discuss the latest progress in lymphatic vasculature development research.

Origination of LECs

Unlike the blood vasculature, the lymphatic vasculature is a translucent vasculature in the body, which until recently made it relatively difficult to study. Research on finding the origin of the developing lymphatic vasculature started in the 17th century. At the beginning of 20th century Florence Sabin first proposed that the lymphatic vasculature arises from the cardinal

veins (CVs) from her research on pig embryos (943). Much later, when the transcription factor *Prox1* was identified as a key regulator of lymphatic vasculature development (1140), lineage tracing using *Prox1* reporter mice revealed that indeed the venous endothelial cells in the cardinal veins gave rise to LECs (1011). Similar results were seen in the zebrafish embryos using live imaging techniques (1170).

Notably, the formation of the mouse lymphatic vasculature occurs very quickly during development. From embryonic day (E) 9.5, when the specification of Prox1$^+$ LEC progenitors first occurs in the CV, to E14.5 when the entire skin of the mouse embryos is covered by the lymphatic vasculature, the specification, proliferation and migration of millions of LECs needs to take place in a short period of time (Fig. 23A). This raises the question whether the CV is the sole source for LEC progenitors. In the past five years this question has been investigated in many different ways. First, the intersomitic veins (ISVs) and the superficial venous plexus were identified to be an additional source of LEC progenitors in mouse embryos. Prox1$^+$ LEC progenitors are specified not only in the CV but also in the ISVs and the superficial venous plexus (403, 1168). These findings are consistent with the previous observations that LEC progenitors are derived from the veins. More significantly, other studies have discovered a nonvenous origin for some parts of the lymphatic vasculature. For example, specialized angioblasts can differentiate into LECs within the CV in zebrafish embryos (765). Although George Huntington and Charles McClure suggested a nonvenous origin for at least some LECs in 1910 (454), it was the first time that mesenchymal cells were shown to contribute to the formation of the lymphatic vasculature during embryonic development (765). Meanwhile, in the mouse dermal lymphatic vasculature, while the lymphatic vasculature of the cervical and thoracic skin are still derived from the veins, the lymphatic vessels of the lumbar and dorsal midline skin were found to form from nonvenous-derived progenitors (Fig. 23B) (657).

While the origin of these nonvenous LECs in the dermal lymphatic vasculature remains unclear, lineage tracing has demonstrated that in the mouse mesentery hemogenic endothelium contributed to the mesenteric lymphatic vasculature in addition to the venous-derived mesenteric lymph sac (Fig. 23B) (1018). Independently, the gene encoding paired like homeodomain 2 (*Pitx2*) was found to be required for the formation of the mesenteric lymphatic vasculature, which indicated the existence of a separate population of lymphatic progenitors that is not dependent on *Prox1* (644).

Over the years, the diversity of the lymphatic vasculature in different tissues has drawn significant attention. In addition to the tissues that are well known to have a lymphatic vasculature such as the skin and the mesentery, lymphatic vessels have been discovered in other tissues previously thought to be immune privileged, including the brain and eyes (39, 41, 543, 624, 845). This raises the possibility that the concept of immune privilege may be an outdated one that does not apply to any tissues of the body. The origin of these tissue-specific lymphatic vessels is also diverse. For instance, while the main source of cardiac LECs is from extra-cardiac venous endothelium, the yolk sac hemogenic endothelium also contributes to a part of the cardiac lymphatic vasculature (Fig. 23B) (543). Nevertheless, the origin of the lymphatic vessels in the central nervous system and in the eyes is still not clear. Taken together, all these findings have improved our knowledge on the various origins of the

lymphatic vasculature in different organs, which also indicates the importance of trans-differentiation in both physiological and pathological conditions.

Specification and Migration of LECs

The stepwise model of how the lymphatic vasculature forms with regard to the main genes that regulate LEC specification, budding and migration during mouse embryonic development is well established and discussed in multiple reviews (40, 184, 803, 968, 1169). Briefly, $Prox1^+$ LEC progenitors are specified in the CVs and ISVs at E9.5–E9.75 and the majority of these cells bud off from the veins right after this specification. Immediately after LEC progenitors bud off the veins, they differentiate into mature LECs expressing an additional LEC protein Podoplanin (Pdpn) (351, 1168). These $Prox1^+Pdpn^+$ LECs proliferate, migrate, and assemble into a sac-like structure along the anterior and posterior axes of the embryo on the dorsal half of the CVs, called a lymph sac. As the main sources of LECs, lymph sacs give rise to the lymphatic vasculature all over the body, with the organ-specific exceptions noted above (Fig. 23A). Several reviews have summarized the function of the key factors that are involved in LEC specification including *ERK, Sox18, CoupTFII, Prox1, Flt4* and *Notch* (350, 1169). ERK signaling activates the expression of *Sox18* in the CVs (259), and then Sox18 and CoupTFII both bind to the promoter region of *Prox1* to activate its expression (349, 1013). The expression of Prox1 defines the specification of LECs and Prox1 activity is required in maintaining LEC identity throughout life (489). In addition to *Sox18, CoupTFII*, and *Prox1*, another main player in maintaining LEC identity is *Flt4*, which encodes Vegfr3. *Flt4* and *Prox1* interact with each other in a feedback loop wherein Prox1 binds to the *Flt4* promoter to maintain its expression and *Flt4* is also necessary for maintaining the expression of Prox1, thus helping to maintain LEC identity (1012). Contrary to *Flt4, Notch1* functions as a negative regulator of LEC specification. Decreased *Notch* activity results in an increased number of LEC progenitors in the veins, which leads to malformation of the lymphatic vasculature (738). Moreover, the expression of *Prox1* is modified by microRNAs. *miR-181a* and *miR-31* negatively regulate *Prox1* expression in LECs (528, 854). Intriguingly, *Wnt5b* was recently found to induce LEC specification through β-catenin signaling in the zebrafish (765). On the other hand, β-catenin signaling is not critical for the specification of LEC progenitors during mouse embryonic development, but is essential for lymphatic vascular morphogenesis (174).

It is critical for LECs to bud off the veins and migrate to form the lymph sacs and this process requires Vegfc/Vegfr3 signaling (517, 550, 979). This signaling pathway has also been discovered to be crucial for mesenteric lymphatic vessel formation (1018), intestinal lymphatic vessel (lacteal) formation (88, 776), cardiac lymphatic vessel formation (543), central nervous system lymphatic vessel formation (39, 624), and Schlemm's canal formation in the eyes (41, 845). Secreted factor collagen and calcium binding EGF domains 1 (*Ccbe1*) knockout mouse embryos had no lymph sac formation, which was similar to *Vegfc* knockout embryos and indicated a connection between Ccbe1 and Vegfc (111). Further study has revealed that Ccbe1 can form a complex with a disintegrin and metalloproteinase with thrombospondin motifs 3 (Adamts3) and convert Vegfc into an active ligand (144). Therefore, Ccbe1 appears to anchor Vegfc to the extracellular matrix in a concentration gradient, where it can be proteolytically cleaved and released by Adamts3.

Besides Vegfc/Vegfr3 signaling, many other signaling pathways affect LEC migration, such as the adrenomedullin signaling pathway (354), angiopoietin 2 (Angpt2)-Tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (Tie1) (Angpt2 receptor) signaling pathway (227, 874), *PU.1* in the macrophages (383), Nfatc1 signaling (563), and transcription factor *Gata2* mediated signaling (606). Here, we will mainly discuss the latest findings in the past three years since previous discoveries have been included in another review (1169). Loss of adrenomedullin signaling results in hypoplastic lymph sacs, which indicates the defect of LEC migration (354). Lately it was found that the dosage and signaling of adrenomedullin is tightly controlled by a decoy receptor *Cxcr7*. Deletion of *Cxcr7* results in an abnormally high level of adrenomedullin signaling in the LECs and leads to enlarged blood-filled lymphatic sacs and dilated dermal lymphatic vessels (540).

The metabolism of blood endothelial cells has been studied for years in health and cancers (299). A recent study focused on how the metabolism of LECs affects the formation of this vasculature. Deletion of *CPT1A*, a rate-controlling enzyme in fatty acid β-oxidation in the LECs, lead to impaired lymphatic morphogenesis. Mechanistically, Prox1 directly upregulates *CPT1A* expression, which increases acetyl coenzyme A production. Acetyl coenzyme A is used by the histone acetyltransferase p300 to acetylate histones at lymphangiogenic genes, such as Vegfr3 to regulate LEC migration and identity maintenance (1152).

As mentioned previously, Notch signaling is vital for the specification of LECs in the veins. It also regulates lymphatic vessel morphogenesis. LEC-specific deletion of *Notch1* resulted in substantial lymphatic overgrowth with dilated lymphatic vessels. Increased cell proliferation and filopodia formation, and decreased cell death of LECs were seen in the *Notch1* mutant mice (320). Thus, Notch signaling is an important negative regulator in balancing the number of LECs and their migration. In addition to *Notch1, Bmp2* also functions as a negative regulator during lymphangiogenesis. *Bmp2* represses Prox1 expression through Smad1/5/8 activity. *miR-181a* and *miR-31* function as downstream targets of Bmp2 signaling to negatively regulate *Prox1* expression (293).

Planar cell polarity (PCP) signaling plays a significant role in many developmental processes. The components of the PCP signaling pathway are involved in the development of lymphatics as well (1053). Polycystin 1 *(Pkd1)* is a newly identified gene that regulates lymphangiogenesis in two back-to-back studies. During lymphatic vessel formation, *Pkd1* is not involved in the budding of LECs off the veins, but controls LEC migration during vessel formation by regulating cell polarity (210). Loss of *Pkd1* leads to random migration of LECs and reduced lymphatic vessel density (826).

Maturation of Lymphatic Vasculature and Smooth Muscle Cell Recruitment

From E10.5 to E14.5 during mouse embryonic development, LECs proliferate and migrate to nearly everywhere in the body. After E14.5, LECs start to remodel into more specialized vessel types (Fig. 23C). As mentioned earlier, the two general types of lymphatic vessels are the collecting lymphatic vessels and lymphatic capillaries, which have distinct morphology and function. The collecting lymphatic vessels are covered by SMCs. The SMCs are responsible for the contraction of the collecting lymphatic vessels to push the lymph forward

(956). The collecting lymphatic vessels also have intraluminal valves to prevent lymph backflow (159, 947). The collecting lymphatic vessels and lymphatic capillaries bear different types of cell-cell junctions. The collecting lymphatic vessels have continuous zipper-like junctions similar to blood vessel endothelium, whereas the capillaries have discontinuous button-like junctions visualized by staining for the junction molecule VE-cadherin (*Cdh5*) (61). Buttons on the lymphatic capillaries are considered to act as primary valves for fluid and cell entry into the lymphatic vessels. Developmentally, the tracheal lymphatic vessels have LECs that are joined by zipper-like junctions before E16.5. However, the percentage of button-like junctions increased from 6% at E17.5 and 12% at E18.5 to 35% at birth, 50% at postnatal day P7, 90% at P28, and 100% at P70, which indicated the maturation of lymphatic capillary junctions. Similar values were seen in the lymphatic capillaries of the diaphragm. During this period, the zipper-like junctions in the collecting lymphatic vessels remained unchanged (1171). Although the forces involved in the transformation of zippers into buttons are unclear, the increasing amount of fluid that needs to be cleared might have an effect on the junctional remodeling in the lymphatic capillaries. Angiopoietin 2 *(Angpt2)* is indispensable for the maturation of cell-cell junctions in the lymphatic capillaries since genetic deletion of *Angpt2* suppresses the zipper-to-button junctional transformation (1218).

Relative to lymphatic capillaries, however, more studies have been performed on the maturation of collecting lymphatic vessels. Down-regulation of the capillary genes in the collecting lymphatic vessels, SMC recruitment, and valve formation are important steps in the maturation of collecting lymphatic vessels. Forkhead box c2 (Foxc2)/calcineurin/Nfatc1 signaling is a well-studied signaling pathway that is essential for the maturation of collecting lymphatic vessels. Foxc2 and Nfatc1 cooperatively bind to the regulatory elements of lymphatic genes to control their expression. In the collecting lymphatic vessel-rich mesentery, loss of *Foxc2* leads to the retention of high expression levels of genes *Prox1, Flt4* and *Lyve1* in the primitive lymphatic vessel plexus and these lymphatic vessels have excessive SMC coverage. Platelet-derived growth factor receptor-beta (*Pdgfrb*) is essential for the recruitment of pericytes by developing blood capillaries (430, 607). *Foxc2* knockout collecting lymphatic vessels exhibit unregulated expression of *Pdgfrb*. These vessels are not able to differentiate into functional collecting lymphatic vessels and lack valves, indicated by the backflow of lymph (774, 859). *Flt4* is a potential candidate to regulate *Foxc2* expression during the formation of collecting lymphatic vessels. In the *Flt4* heterozygous mice, *Foxc2* expression is decreased (859). Foxc2 activity is also regulated by cyclin-dependent kinase 5 *(Cdk5)* by phosphorylation at serine/threonine residues. Endothelial cell specific deletion of *Cdk5* leads to blood-filled dilated lymphatic vessels and edema (603). Besides *Foxc2*, the *Reln* gene that encodes the protein reelin is also critical for the maturation of collecting lymphatic vessels. In the *Reln* mutant mice, the collecting lymphatic vessels retained a high expression of Lyve1 and had a reduced amount of SMC coverage. Consistent with the low SMC coverage, the lymph flow rate was also reduced in the collecting lymphatic vessels, indicated by indocyanine green (ICG) dye uptake assay. Mechanistically, secreted reelin protein could act as a communicator in the adherence of SMCs to collecting lymphatic vessels (633). In addition, Angpt2-Tie1 signaling was recently published to play a role in collecting lymphatic maturation. First, inhibition of Angpt2 by

using an anti-Angpt2 antibody results in immature collecting lymphatic vessels with high Lyve1 expression and lymph transport is defective in those vessels. Meanwhile, lymphatic capillaries had ectopic SMC coverage in mice deficient for *Angpt2* (1218). Similarly, deletion of *Tie1* revealed persistent expression of the lymphatic capillary marker Lyve1 in the collecting lymphatic vessels, indicating attenuation of Tie1 prevents the formation of collecting lymphatic vessels. Moreover, abnormal coverage of SMCs in the lymphatic capillaries was also seen in *Tie1* knockout mice (875, 975). Together, Angpt2-Tie1 signaling is critical for the maturation of both collecting lymphatic vessels and lymphatic capillaries.

Lymphatic Valve Formation

Lymphatic valves are an indispensible part of the lymphatic vasculature. They are crucial for maintaining lymph transport in one direction. They are comprised of two valve leaflets, each with an extracellular matrix (ECM) core that is lined by a layer of LECs on each side of the leaflets (582, 753, 939, 945). How lymphatic valves form has become a hot topic in the past few years. Several signaling processes are important for the formation of the lymphatic valves, including transcription factors, junction proteins, ECM, and axonal guidance genes (1169). Here we will introduce the latest understanding of the formation of valves throughout the lymphatic vasculature.

Collecting Lymphatic Vessel Valves—The valves in the mesenteric collecting lymphatic vessels start to form in the embryo around E15.5. Lymphatic valve formation has at least four defined steps. Step one (initiation, E15.5–E16.5): a cluster of LECs forms on one side of the collecting lymphatic vessel; Step two (condensation, E16.5–E17.5): the LEC cluster gives rise to a ring-like constriction; Step three (elongation, E17.5-E18.5): the LEC ring-like constriction grows into the vessel lumen along with ECM deposition; Step four (maturation, E18.5-): two leaflets form. *Foxc2* is a key regulator of lymphatic valve formation. *Foxc2* global knockout mice do not develop any lymphatic valves and die perinatally (859). During embryonic development, increased fluid shear stress in the collecting lymphatic vessels and regionally increased levels of Foxc2 and Prox1 are responsible for the specification of valve cells (944). Foxc2 and Prox1 regulate the expression of connexin-37 (*Cx37*) and calcineurin/Nfatc signaling in response to flow to facilitate the emergence of lymphatic valve territory (513, 944). *Cx37* regulates the transition from the early valve forming cell cluster to a ring-like lymphatic valve territory. Loss of *Cx37* inhibits the formation of lymphatic valves and leads to a severely abnormal lymphatic drainage (944). Upon oscillatory flow, Foxc2 and Prox1 control cytoskeletal reorganization and cell alignment of the valve-forming cell to accelerate valve morphogenesis (944). *Foxc2* is not only critical for lymphatic valve formation but also for postnatal valve maintenance. Deletion of *Foxc2* in postnatal pups leads to chylous ascites and chylothorax. Collapse of vascular lumen and degeneration of the lymphatic valves was seen in these pups, indicated by the fact that both the total number of valves and the number of the valves with two normal leaflets were significantly decreased. Mechanistically, *Foxc2* inactivation caused abnormal shear stress sensing in cell culture and defects in cell-cell junctions shown by VE-cadherin staining (945). These results revealed that oscillatory shear stress plays a critical role in the induction of valve formation. Meanwhile, the requirement for lymph flow in valve formation was directly examined in a mouse model. Platelet-specific receptor C-type

lectin-like receptor 2 (*Clec2*) is the only known receptor for podoplanin. Recent studies have shown that platelet activation by the ligation of Clec2 and podoplanin is required to prevent blood from entering the lymphatic vascular network and *Clec2*-deficient mice expectedly exhibit blood-filled lymphatic vessels (92, 331, 1092). By taking the advantage of the blood backfill in the lymphatic vessels, which blocks the lymph flow in the mesentery, the authors were able to show that the *Clec2* deficient mice exhibited normal growth of the primary mesenteric lymphatic plexus but failed to form valves in these vessels. SMC coverage of *Clec2* deficient lymphatic vessels was also abnormally excessive (1035). Thus, the phenotype of *Clec2* deficient mice is similar to the *Foxc2* deficient mice. This is strong evidence that lymph flow directly regulates valve formation.

Moreover, *in vitro* experiments on the cultured LECs revealed that oscillatory shear stress induced the expression of the genes required for lymphatic valve development, such as *Foxc2, Cx37, integrin α9 (Itga9)* and *Gata2* (944, 1035). *Gata2* is a transcription factor that is highly expressed in the valve cells (529) and is required for the upregulation of *Foxc2, Cx37* and *Itga9* in the cultured LECs upon oscillatory shear stress. It indicates that *Gata2* is an upstream regulator of these lymphatic valve genes (527, 1035). Gata2 has been shown to regulate *Prox1* expression by binding to a putative enhancer element upstream of *Prox1*. Deletion of *Gata2* in mouse embryos leads to severe edema. No valves were formed in the mesenteric collecting lymphatic vessels. Postnatal deletion of *Gata2* results in disorganized valves and bulbous lymphatic vessels (527). These data illustrate that *Gata2* is essential for both initiation and maintenance of lymphatic valves.

In addition, the signals from ECM are critical for lymphatic valve formation. It was shown that *Itga9*-deficient mice have a reduced number of lymphatic valves, with the remaining valves appearing abnormal morphologically (72, 73, 432). Interestingly, *Itga9* ligand *polydom* (also named *Svep1*) knockout mice exhibited severe edema. Polydom was secreted from mesenchymal cells and deposited around the lymphatic vessels. *Foxc2* expression was decreased in the *polydom*$^{-/-}$ mice. Those animals lacked maturation of collecting lymphatic vessels and lymphatic valves (522, 727). It was mentioned previously that *Notch1* negatively regulates *Prox1* expression during LEC specification. *Notch1* also plays a role in lymphatic valve development by regulating the key factors of valve formation, *Cx37, Itga9*, and fibronectin (*FNEIIIA*). Lymphatic endothelial-specific loss of *Notch* signaling resulted in defective valve formation and decreased FNEIIIA and Itga9 expression (739). Taken together, these data demonstrated that oscillatory shear stress, junction proteins, and ECM proteins play critical roles in valve formation.

During valve leaflet formation, LECs undergo elongation, reorientation, and migration into the vessel lumen. Planar cell polarity proteins *Celsr1* and *Vangl2* regulate the rearrangement and reorientation of the valve-forming cells, which is important for lymphatic valve formation. Valve formation is abolished in the *Celsr1*- or *Vangl2-null* mice (1053). Transmembrane (type I) heparan sulfate proteoglycan Syndecan-4 (*Sdc4*) was shown to control LEC polarization via regulation of *Vangl2* expression recently. *Sdc4* deletion in mice results in abnormal valve morphogenesis (1123). Previously, it was reported that axon guidance genes semaphorin 3A (*Sema3a*), its receptors neuropilin-1 (*Nrp1*), plexin A1 (*Plxna1*) and *ephrin B2*, a member of the Eph receptor tyrosine kinase family are required

for lymphatic valve formation (72, 117, 501, 646). Recently, one study revealed a new role for Eph receptor B4 (*Ephb4*) in lymphatic valve development. Blockade of EphB4 using an anti-EphB4 antibody caused clear lymphatic defects, including chylothorax, dilated lymphatic vessels, and loss of lymphatic valves (1206). Unexpectedly, ubiquitin-binding adaptor proteins Epsin 1 and 2 regulate lymphatic valve formation through Vegfr3 signaling. It was observed that activation of Vegfr3 after binding to its ligand Vegfc is followed by internalization of the receptor in the sprouting lymphatic capillaries (1146). Epsins physically interacted with Vegfr3 by the ubiquitin-interacting motif and are required for Vegfr3 internalization after binding to Vegfc. Loss of Epsins resulted in the maintenance of high Vegfr3 expression in collecting lymphatic vessels, which blocked their maturation and the formation of the valves, which caused defective lymphatic drainage. Interestingly, decreased activity of Vegfr3 in these mice restored normal lymphatic valve formation and improved lymphatic function (617). This study indeed revealed that the posttranscriptional modification of the lymphatic genes affects lymphatic valve development.

Lymphovenous Valves—Lymphovenous valves are the specialized valves where the thoracic duct empties into the blood vasculature at the subclavian veins. During lymph sac formation, most of the LECs bud off the veins and differentiate into more mature LECs to form lymphatic vessels. However, a small amount of LECs remain in the CVs and become the leaflets of the lymphovenous valves that make a junction between the jugular lymph sac and the jugular and subclavian veins during embryonic development (1101). One lymphovenous valve is located between the subclavian and external jugular veins. Another lymphovenous valve is located between the external jugular vein and internal jugular vein. The lymphovenous valves regulate the return of lymph fluid to the blood circulation and prevent blood from entering the lymphatic vasculature.

When the lymphovenous valves are missing or malformed, it leads to blood-filled lymphatic vessels (1014). It has been reported that platelets are required for the separation of the lymph sacs from the veins at their connecting points - the lymphovenous valves (92, 154, 331, 819, 1092). The binding between lymphovenous valve LECs expressing podoplanin and the platelet receptor Clec2 is crucial for the initiation of platelet aggregation. In the *Clec2*-deficient mice, fibrin-containing platelet thrombi are missing at the lymphovenous valves, which leads to blood-filled lymphatic vessels. Furthermore, platelet thrombi are much larger in animals that lack lymphovenous valves and lymphatic valves, indicating that platelet thrombi can compensate for the loss of lymphovenous and lymphatic valves to maintain blood-lymphatic separation (432). Similar to the formation of collecting lymphatic valves, the formation of lymphovenous valves contains three main transformation steps of the LECs: Delamination (E12.0), Aggregation (E12.5) and Maturation (E14.5-E16.5). Genetically, the many genes that are important for the formation of collecting lymphatic valves are also critical for lymphovenous valve formation. The transcription factors Prox1, Foxc2 and Gata2 are highly expressed in the lymphovenous valves in addition to the lymphatic valves (373). *Prox1* heterozygous animals completely lack lymphovenous valves (1014). *Foxc2* heterozygous animals have a variable phenotype. Half of the animals develop one lymphovenous valve instead of two valves. And in *Gata2* knockout embryos, properly differentiated lymphovenous valves are absent (373, 527).

Interestingly, β-catenin was recently reported to bind directly to the promoter region of *Foxc2* and *Prox1* to regulate lymphovenous valve formation. β-catenin is highly expressed in both collecting lymphatic vessel valves and lymphovenous valves. Loss of the gene that encodes β-catenin, *Ctnnb1*, resulted in the absence of lymphatic valves in the mesentery and the absence of lymphovenous valves. In cultured LEC, β-catenin was shown to be necessary for the upregulation of the valve formation genes *Foxc2* and *Gata2* in response to oscillatory shear stress. Therefore, β-catenin signaling is a critical regulator of valve formation (174). In addition, *Ephb4* is not only important for collecting lymphatic vessel valve formation but also critical for lymphovenous valve formation.

Inactivation of *Ephb4* in LECs leads to defective lymphovenous valve formation and consequent subcutaneous edema (655). As a main component of the valve, ECM also plays an important role in the lymphovenous valve formation. It was mentioned previously that *Itga9*-deficient mice develop defective lymphatic valves. But the lymphovenous valves have been reported as normal in these mice (72, 73, 432). Instead, *Itga5* mutants display defects in the formation of the lymphovenous valves. These mice develop dilated, blood-filled lymphatic vessels and the lymphatic capillaries are ectopically covered with SMCs (1087). It indicates that although the lymphatic and lymphovenous valves share many similarities, the formation of the lymphatic and lymphovenous valves have their own distinct mechanisms.

Lymphatic Development and Diseases

So far we have discussed the developmental processes of the lymphatic vasculature and their respective genes. Intriguingly, mutations in many of those genes cause lymphatic diseases in humans. Primary lymphedema is a major lymphatic disease in humans. Mutations in the LEC specification gene *SOX18* have been identified as the cause of hypotrichosislymphedema-telangiectasia in humans (464). In the LEC specification-signaling pathway, mutations in *RAF1* constantly activate MAPK/ERK signaling and are associated with Noonan syndrome, a disease that includes lymphangiectasia (36, 1071). Heterozygous mutations in human *FLT4* that affect the VEGFR3 tyrosine kinase domains lead to lymphatic vascular defects and primary lymphedema (Milroy's disease) (385, 465, 516, 519). VEGF-C/VEGFR3 signaling acts through Akt (protein kinase B) pathway (260). Akt is required for lymphatic vasculature formation and valve development (1219). Mutations in class I phosphoinositide 3-kinases (*PI3K*) that lead to AKT hyperphosphorylation are associated with Lymphatic Malformations (LM) in humans (112). LEC migratory factor *CCBE1* mutations in humans cause a type of lymphatic dysplasia known as Hennekam syndrome (22, 201, 202). Point mutations in human *FOXC2* are associated with lymphedema-distichiasis (LD) syndrome (132, 317), in which lymphatic valves are defective. Mutations in valve formation transcription factor *GATA2* cause Emberger syndrome, myelodysplastic syndrome (MDS), acute myeloid leukemia (ALS), and "MonoMAC" syndrome with primary lymphedema (529, 822). Gap junction protein connexin-47 (Cx47) is highly expressed in the lymphatic valves (513). Mutations in *CX47* are linked with primary lymphedema in humans (327, 821). An inactivating mutation in PCP pathway gene *CELSR1* was recently found to be associated with hereditary lymphedema (382). Mutations that cause the loss of tyrosine kinase activity of *EPHB4* (crucial for valve formation) are associated with an autosomal dominant, inherited form of lymphatic-related

(nonimmune) hydrops fetalis (LRHF) (655). Similarly, congenital chylothorax in human fetuses is caused by mutations in the valve formation gene *ITGA9* (635). A nonreceptor tyrosine phosphatase (Ptpn14) can interact with Vegfr3 and affect lymphangiogenesis. Mutations in *PTPN14* are linked to an autosomal-recessive lymphedema-choanal atresia syndrome, which displays a lymphedema phenotype in humans (43). Ras GTPase-activating protein (*Rasa1*) deficient mice display loss of lymphatic valves, hyperplasia, dilation, and leakage of lymphatic vessels and chylothorax. In humans, *RASA1* mutations cause capillary malformation-arteriovenous malformation (CM-AVM) (577, 578). Transcription factor T-Box 1 (*Tbx1*) plays a critical role in lymphatic vessel development and regulates the expression of Vegfr3 by binding to an enhancer element in the *Flt4* gene (186). *Tbx1* mutations are associated with DiGeorge syndrome; however, lymphatic defects so far have been reported only once as abdominal lymphatic dysplasia in patients with DiGeorge syndrome (649, 1162). Although the developmental function of Kruppel Like Factor 11 (*KLF11*) remains unclear, its mutations cause microcephaly-lymphedema-chorioretinal dysplasia (MLCRD) syndrome (427, 823). More recently, homozygous and compound heterozygous mutations in *PIEZO1* (encodes a mechanically activated ion channel), resulting in an autosomal recessive form of generalized lymphatic dysplasia (GLD) with a high incidence of non-immune hydrops fetalis and childhood onset of facial and four limb lymphedema. It indicates the potential function of *PIEZO1* in lymphatic development (346).

Lymphangiogenesis and Relationships with Angiogenesis During Microvascular Network Remodeling

Lymphatic network growth and dysfunction associated with lymphedema, inflammation, cancer metastasis and other pathological conditions highlight the need to better understand the dynamic nature of lymphatic networks structure at the vessel, cellular, and molecular level (481, 521, 1017). In recent years, whole-mount tissue models have provided insight into the organization of lymphatic networks and the mechanisms involved in lymphatic vessel growth, i.e. lymphangiogenesis (61, 848, 978). Given the functional importance of lymphatic networks on tissue homeostasis and immune cell trafficking, the design of molecular based therapies aimed at manipulating the microcirculation requires understanding the relationships between lymphangiogenesis and angiogenesis, the analogous growth of new blood vessels. Critical questions remain unanswered: To what extent are angiogenesis and lymphangiogenesis related? Can lymphatic vessels become blood vessels, and vice versa? Can blood capillaries directly drain into initial lymphatic vessels? The focus on lymphangiogenesis and the interrelationships between lymphatic and blood vessels during microvascular network growth highlight this emergent research area (Fig. 24).

Lymphangiogenesis

Lymphangiogenesis is the process of lymphatic growth during which new initial lymphatic vessels are formed from pre-existing vessels. While modes of lymphangiogenesis might include intussusception (i.e. vessel splitting) or other less characterized dynamics such as endothelial cell migration and re-connection (481, 798, 936), the relative contributions of these modes to network growth are unclear. Currently, lymphatic endothelial cell sprouting is the most well described mode of growth. Lymphatic capillaries first form fine filopodia

which precede sprout formation (63, 80), similar those in the early process of angiogenesis from existing blood vessels. These sprouts proliferate and extend to form new mature, blind-ended lymphatic vessels. Current evidence suggests that lymphatic sprouting is primarily mediated through VEGFR3 expressed by lymphatic endothelial cells. VEGFR3 is a tyrosine kinase receptor activated by VEGF-C and VEGF-D (621). VEGF-C and -D also bind to neuropilin-2 (Nrp2), a transmembrane receptor that plays a role in developmental axon guidance. Nrp2 is thought to act as a co-receptor for VEGFR3 and a mediator of lymphatic sprouting (1161). Notably, in a recent study with an incomplete postnatal deletion of VEGFR3, resulting in a mosaic of VEGFR3+ and VEGFR3- lymphatic endothelial cells, lymphangiogenesis was actually enhanced, with the VEGFR3+ cells migrating to the tips of sprouting cells. The VEGFR3+ cells were hyperproliferative due to cell-cell contact-dependent downregulation of Notch by the adjacent VEGFR3- cells (1216). VEGF-C has also been shown to stimulate angiogenesis by activating VEGFR2 (621). Meanwhile, the list of primarily angiogenic growth factors that also participate in lymphangiogenesis is growing (621). These include VEGF-A (412) and bFGF (149). The overlaps suggest a fundamental relationship between the two processes, which has led to investigation of their interplay in microvascular networks (Fig. 25). The knowledge to be obtained from these ongoing studies is expected to improve targeting of therapeutics aimed at manipulating blood and lymphatic growth.

Relationships Between Lymphangiogenesis and Angiogenesis

Analyses of microvascular networks using intravital microscopy and contrast media have historically suggested that lymphatics networks remain distinct from blood microvascular networks in adult tissues (191, 421, 963, 1094). However, the growing list of common growth factors involved in both lymphangiogenesis and angiogenesis suggest that interactions at the capillary level are possible. The concept of lymphatic/blood vessel mispatterning is further supported by the phenotypic similarities between lymphatic and blood endothelial cells during quiescent and certain pathological conditions (521, 1017). More recent observations suggest that endothelial cells are in fact capable of forming connections, defined by the sub-micron continuous junctional labeling of endothelial cell adhesion molecules, between lymphatic and blood vessels at the capillary level in adult rat microvascular networks (914). While the functional significance of connections and whether they may provide sites for direct fluid conductance remains understudied, these descriptive observations emphasize the need to investigate the common cellular and molecular mechanisms involved in lymphatic and blood vessel growth.

The close coordination between blood and lymphatic vessels starts during development. As the blood vascular system is developing, a subpopulation of venous endothelial cells begins expressing the lymphatic specific transcription factor Prox1 and buds off of the cardinal vein, forming the first lymph sacs that later mature into the lymphatic vascular system (1140). The commonality of multiple growth factors also suggests greater physiological interactions may exist between these two systems. Evidence for this was given by Benest et al., who demonstrated that the presence of lymphatic vessels influenced VEGF-C-induced angiogenesis (80). Additionally, Nakao et al. suggested that VEGFR-2 expressed by angiogenic blood vessels impeded lymphangiogenesis by sequestering VEGF-C (748).

Indeed, angiogenic molecular signals such as VEGFs and the angiopoietins play similar roles in lymphangiogenesis (521). The intracellular domain of EphrinB2 was found to be critical for mouse lymphatic vessel patterning (646), suggesting that ephrins also represent a patterning cue shared by both the lymphatic and microvascular systems. EphrinB2 and its ligand EphB4 are important regulators of arterial and venous cell fate during embryonic development (15, 1121). In adults, the bidirectional signaling has been linked to vascular cell guidance and recruitment of perivascular support cells, yet the roles of these molecules in vessel identity remain unclear (343).

The existing dogma implicates that lymphatic and blood microvascular networks remain separated in the adult. However, in the past 15 years, a handful of observations have provided evidence for direct connections between these two systems. Injections of FITC-labeled dextran or BSI-lectin identified blood-to-lymphatic perfusion in mice lacking SLP-76 (4), Rac1 (226), O-glycan (355), or Fiaf (58). Similar results were documented in Prox1 conditional-mutant mice (489). Even direct lymphatic-blood vessel connections at the microvascular level have been discovered in adult rat mesenteric microvascular networks (914, 1020, 1034). As for evidence that local environmental factors can induce lymphatic blood perfusion, Jensen et al. demonstrated that nitric oxide and hypoxia were sufficient (229). Nitric oxide has been shown to permit hypoxia-induced lymphatic blood perfusion in zebrafish at sites of arterial-lymphatic anastomoses (229). The findings of this particular study implicate a molecular cue that is sufficient to induce conductance between the lymphatic and blood microvasculature. Increased nitric oxide production has also been directly linked to lymphatic metastasis implicated nitric oxide as a mediator of cell flux into lymphatic vessels (573). Anatomical mispatterning between the two systems is also documented by more historical reports in the literature. For example, lymphatic-venous connections at the large vessel level outside the entry points to the subclavian and thoracic ducts have been reported, particularly in situations of increased pressure due to vessel occlusion (694, 1178). Lymphatic-venous anastomoses at the large vessel level were also reported in the heart 7 and 14 days after lymphatic occlusion (304).

Endothelial cell phenotypic identity also provides an example of the interrelationships between blood and lymphatic systems. Immunolabeling of VEGFR3, LYVE1, and PECAM, offers evidence for both the similarity and delineation of lymphatic and blood endothelial cell types (481). During development, lymphatic endothelial cells originate and bud off the venous system. Phenotypic differences then emerge between lymphatic and venous endothelial cells during later stages of development, yet these differences are challenged in the adult during certain pathological scenarios. Even VEGFR3, a very common marker of lymphatic endothelial cells in quiescent adult tissues, is expressed along blood vessels during pathological angiogenesis (1148). Also, the generally accepted lymphatic marker LYVE1 in adult tissues is first expressed by a subset of cardinal vein endothelial cells involved in the initial lymphatic budding (470) and in the adult becomes down regulated in larger collecting vessels (646). In addition to marking lymphatic endothelial cells, LYVE1 expression also identifies activated macrophages in adult tissues (262, 775). Macrophages have indeed recently been implicated as paracrine regulators of both angiogenesis and lymphangiogenesis (526, 537, 602, 775). Their phenotypic overlap also highlights the emerging area of research focused on lymphatic endothelial cell lineage during

lymphangiogenesis in adult tissues and the and the potential for macrophages or circulating progenitor cells to differentiate into both blood and lymphatic endothelial cells (204, 303, 411, 658, 829, 844). Undoubtedly, the advances in our understanding of macrophages as a cell regulator and source for angiogenesis and lymphangiogenesis implicates the potential for new discoveries related to endothelial cell plasticity and the potential for blood to lymphatic endothelial cell phenotype plasticity, a concept which has already proven possible in vitro (203).

Interstitial Flow and Lymph Formation

The closed-loop circulation of the mammalian cardiovascular system, in order to fulfill its role delivering fluids and nutrients to tissues, continuously leaks plasma. This leak occurs within the microvasculature, particularly from the capillaries and postcapillary venules. For an average human with a 3 L plasma volume, approximately 8 L of plasma is filtered per day by these exchange microvessels throughout the body, meaning that the entire plasma volume is filtered about every 9 hours (600).

Lymphatic vessels have long been recognized for their role in fluid and macromolecule homeostasis, which serve to largely protect tissues against the formation of edema. Experimental impairment of the return of lymph (thoracic duct fistula) leads to severe dehydration within just a few days (983). The initial lymphatics serve as the entry point for this fluid to be returned to the central circulation. This function is critical to maintain sufficient volume of the central circulation and also prevent tissue swelling, ensuring homeostasis of the tissue environment. The physiological reserve of lymphatic vessels to remove leaked plasma is estimated to be 10-fold higher than the normal amount of plasma that leaks from the microcirculation, establishing a margin of safety which can protect against the formation of edema during periods when microvascular permeability is elevated by inflammatory stimuli (391). Thus, up to a certain limit defined by the margin of safety, when microvascular leakage increases at a particular site, lymph flow from that site also increases.

Microvascular Leakage

Leakage of plasma through the capillary and postcapillary venular wall establishes the gradient for interstitial flow and peripheral lymph formation. Microvascular leakage is determined by the permeability of capillaries and postcapillary venules, and by the overall combined hydrostatic and osmotic pressure imbalance, known as the Starling equation (Fig. 26), across the endothelium of these vessels (295).

It was pointed out several years ago that one problem with the Starling principle is the assumption that the microvasculature is impermeable to plasma proteins, when in fact postcapillary venules steadily allow protein plasma leakage (423). Several other data have accumulated that have led to a revision of the Starling principle (600). These include observations that were incompatible with, or significantly complicated the original concept: 1) Experimentally raising the tissue osmotic pressure ($\prod_t$) around frog mesenteric capillaries to the equivalent of the plasma osmotic pressure ($\prod_p$) caused no net change in filtration (452); 2) Changes in volume flux of fluid (J_v) are known to change in $\prod_t$ and tissue

hydrostatic pressure (P_t) (906, 907, 1055); 3) Findings from experimental and theoretic studies support that the subglycocalyx fluid immediately outside the semipermeable barrier has a significantly different composition compared to the interstitial fluid in the tissue (16, 453, 598, 687, 1131); 3) data obtained in studies evaluating the sum of the Starling forces did not support the traditional filtration/reabsorption model (71, 599, 1224); and 4) at capillary pressures below the plasma osmotic pressure, absorption is only observed transiently, but not in the steady state in mesenteric capillaries or using endothelial monolayers in Ussing chambers (689, 834, 1214).

In the revised model, changes in the balance of the hydrostatic and osmotic pressures of the microvessel lumen and local tissue environment may cause momentary changes the direction of fluid movement. However, under steady state conditions, filtration is generally expected throughout the length of continuous capillaries and postcapillary venules in most tissues. Notable exceptions include the intestinal mucosa, and the renal cortex and medulla, all which have local epithelial transport mechanisms that significantly influence $\prod_t$ and P_t. Additional details are available in the aforementioned review (600). A takeaway message from the revised Starling principle is that reabsorption of fluid into capillaries is not likely to account for much removal of fluid from the interstitial areas. Lymphatics primarily perform this function.

Intestinal Absorption of Fluid

In the small intestine, lymph formation is also driven in large part by absorption of ingested water or lipids into the interstitial space (1178). While functional hyperemia may also increase filtration of plasma following ingestion of a meal, when large quantities of hypotonic or isotonic fluids are administered, lymph flow rapidly increases and a significant portion of the ingested fluid appears in the lymph (66, 110, 986). In addition, in thoracic duct fistula experiments, oral administration of 0.6% or 0.8% NaCl in a 5% glucose solution significantly increased thoracic duct lymph flow, compared to 5% glucose solution without NaCl (376). Ingestion of carbohydrates and proteins do not appear to contribute to elevations in lymph flow (1178).

Interstitial Fluid and Lymph Formation

Interstitial fluid is generated by microvascular filtration of plasma. Because tissues and organs occupy limited space, the accumulation of interstitial fluid generates pressure and serves as a driving force for lymph formation. Initial lymphatics are often located in fairly close proximity to capillaries and postcapillary venules, often within hundreds of μm (964), so that pressure gradients within tissues are established, favoring movement of interstitial fluid from the sites of microvascular leakage toward initial lymphatics (89, 400, 441, 1213). Various prelymphatic pathways in the interstitium form routes of passage of fluid along connective tissue fibers, through spaces, and in some cases within longer prelymphatic channels (422). Thorough details of the physical and chemical mechanisms governing interstitial flow are provided in a recent review (1143).

Schmid-Schönbein pointed out that at the typical pressures encountered *in vivo*, fluids are incompressible, which leads to two general principles: 1) Changes in tissue volume are

directly affected by the amount of fluid that enters and leaves the tissue at any given moment. If the tissue is compressed, because the fluids are incompressible, either less fluid must enter the tissue or more must escape, or a combination of both; and 2) If an organ maintains constant volume, then if one component of the organ expands, a compression of other parts of the organ to an equal degree will occur (964). According to these principles, in healthy tissues with no absorptive or secretory function and no edema, lymph formation occurs at the same rate as leakage of fluids from the microcirculation. Experimental intravenous infusion of saline, which causes hemodilution, increased filtration in the microcirculation, and a gradual elevation of interstitial pressure over time (1141), yields increases in lymph flow evident in collecting lymphatics (82, 880). As stated above, the maximum rate at which interstitial fluid can be removed from a tissue (i.e. the maximum rate at which new lymph can be formed), defines a margin of safety that protects against edema formation (391). Taking this into consideration, along with the revised Starling principle, all forms of edema likely involve inadequate lymph drainage (730).

The forces that influence the movement of interstitial fluid are dynamic and strongly influenced by tissue movements and changes in local blood flow. Several decades ago, Guyton measured interstitial fluid pressure using perforated capsules that were implanted in various tissues of dogs for up to four weeks. His work revealed subatmospheric pressure values in normal tissues, but positive pressures in edematous tissues (399). The concept of average subatmospheric pressures, combined with later findings of intraluminal fluid pressures of lymphatics slightly above atmospheric pressure created a paradox as to how interstitial fluid could become lymph against an uphill pressure gradient (415, 1010, 1223, 1225).

To answer this, the nature of transport of interstitial fluid from the microcirculation to initial lymphatics must be considered. One possibility could be a steady flow mechanism that would not require compression or expansion of the tissues, and in which fluid would move at constant rate through the interstitial space. With average negative interstitial fluid pressure and positive intraluminal pressures in lymphatics a steady flow mechanism of lymph formation is not likely, and evidence has not accumulated to support this possibility. However, if there is periodic compression or expansion of tissues, this could produce unsteady transport of fluids, and there has been much evidence to support this (964, 1201). Another consideration is the relative contribution of osmotic versus hydrostatic pressure gradients for moving interstitial fluid across the initial lymphatic wall. An early hypothesis postulated that newly formed lymph could create cyclic osmotic gradients that favored additional lymph formation and overcome the hydrostatic pressure gradient (162, 964, 1143, 1201). However, subsequent analysis of protein concentrations in lymph from initial and collecting lymphatics did not support this hypothesis (1198).

An additional hypothesis that is more likely to be true is that hydrostatic pressure in the tissue fluctuates, particularly in organs with oscillatory movements like the heart or lungs, and that these momentary changes in hydrostatic pressure generate unsteady flow of interstitial fluid into the initial lymphatic vessels (964, 1201). For example, the contraction and relaxation of the diaphragm with inspiration and expiration, respectively, optimally stretches the compliant network of pleural lymphatics, keeping the intralymphatic fluid

pressure consistently lower than the pleural fluid pressure throughout the breathing cycle (395, 700, 701, 720–722, 755, 761). Notably, during paralysis and mechanical ventilation of the rat lungs, intralymphatic pressure does not decrease as significantly as with spontaneous inspiration, indicating the importance of the diaphragmatic contractions in the establishment of gradients favorable for lymph formation from the pleural space (722). Interestingly, forces transmitted through the thorax due to the cardiac cycle also contribute to the overall transmural pressure gradients that facilitate lymph formation in pleural lymphatics from the pleural fluid, revealed from studies that included simultaneous measurement of interstitial and intralymphatic pressures (722, 760). There are several other examples of tissue movements contributing to lymph flow. Moving limbs, walking, or gentle skin massage also promote lymph formation (469, 670, 805, 812), In the intestine, both the movements of the outer smooth muscle layers and the villous contractions stimulated during fluid absorption correlate increased lymph flow (1151).

Does the hydrostatic hypothesis also apply to tissues at rest? The concept that this may be the case was initially introduced based on observations in the rabbit ear, in which the lymphatic removal of subcutaneously injected tracer particles required local arterial pulsations (679, 847). Steady perfusion of arterioles led to edema, while pulsatile (unsteady) flow promoted lymphatic clearance of interstitial fluid (679) Subsequently, it was shown that arterial pulsations are transmitted to lymphatic vessels (211, 1126), which conceivably could generate momentary pressure gradients that promote ripples of fluid movement across the interstitial space toward initial lymphatics. Cyclic changes in pressure gradients were in fact later demonstrated in the bat wing, although the bat wing initial lymphatics are atypical as they are large bulbs with a phasically contracting smooth muscle layer (440, 441). While results from at least one study in the sheep hindlimb suggested that the arterial pulsation-driven pressure gradient mechanism does not drive lymph formation (669), the majority of data at this point provide evidence that in tissues at rest, there are momentary hydrostatic gradients that can facilitate interstitial fluid flow and lymph formation.

Paracellular Entry of Fluids into Initial Lymphatics

The endothelial cells that form the walls of initial lymphatics form a semi-permeable barrier that favors entry of excess interstitial fluid into the lymphatic lumen, while preventing escape of this newly formed lymph when intraluminal hydrostatic pressure exceeds the interstitial pressure. The primary lymphatic valves, also known as button junctions, (Fig. 3B) have a key role in allowing one-way paracellular fluid movement into the lymphatic lumen (69, 77, 107, 117). These valves or leaflets are formed by the unique junctional structure of oak leaf-shaped initial lymphatic endothelial cells, with alternating patterns of VE-cadherin and PECAM-1 labeling (5, 81). Similar flap structures have also been described at the tips of lymph lacteals (792), although data from at least one study has suggested that these structures may be more like pores than one-way valves (594).

The primary, one-way valve leaflets are thought to permit fluid entry due to hydrostatic oscillations in interstitial fluid pressure. When interstitial pressure exceeds that of the lymphatic lumen, the valves open to permit fluid entry. However, when the luminal pressure exceeds interstitial pressure, these valve leaflets are forced to close. In addition to the

primary valves, anchoring filaments projecting from the initial lymphatic endothelial cells into the interstitial space are thought to help to maintain the patency of the initial lymphatic lumen (69, 117).

In addition to the passive diffusive and convective aspects of transport, lymphatic endothelial cells also possess the ability to actively alter their shape and the barrier properties of their junctions in response to physical or chemical stimuli. Transmural flow through lymphatic endothelial cells acts as a cue for upregulation of aquaporin-2 expression, CCL21 secretion, reorganization of VE-cadherin and PECAM-1 localization, increased permeability and dendritic cell migration into lymphatics (704). In contrast, elevated fluid shear flow over the apical surface of cultured lymphatic endothelial cell monolayers enhances barrier function (129). Several chemical stimuli, including the inflammatory cytokines and mediators TNF-α, IL-6, IL-1β, IFN-γ, histamine, and thrombin, bacterial endotoxins, and the growth factor VEGF-C have been shown to disrupt barrier integrity of cultured lymphatic endothelial cells (127, 130, 213). Elevated permeability of lymphatic endothelial monolayers was accompanied by phosphorylation of myosin light chains, cytoskeletal activation and reduced expression of VE-cadherin, all of which could be prevented by pharmacological blockade of NO synthase (213). Such mechanisms may account in part for the opening of lymphatic endothelial junctions and elevated lymph flow previously reported to be associated with traumatic injury (157).

Contribution of Transcellular Transport Mechanisms to Lymph Formation

In addition to the diffusive permeability of fluids and solutes into initial lymphatics, there is evidence for active transport through lymphatic endothelial cells. Transport vesicles have long been postulated to serve as a transendothelial transport mechanism (273, 274, 584). It is worth noting, that there has been debate for several decades about paracellular versus transcellular mechanisms of chylomicron uptake into lacteals (265). For example, electron micrographs have featured chylomicrons both in the junctional space between adjacent lymphatic endothelial cells of lacteals and within vesicles inside lymphatic endothelium (157, 161, 198, 352, 572, 833, 842, 925, 942, 1089).

One potential concern with these studies has been potential trauma to the tissues during the fixation and sectioning process (55, 273). Taking this into consideration, others have reported that the opening of junctions between the endothelial cells of lymph lacteals is generally rare, even when lacteals were massively distended with fluid (51, 52, 274, 1075). Dobbins and colleagues presented the case that transport vesicles in endothelial cells, which take up 15% of the cytoplasmic volume, likely account for most chylomicron entry into lacteals (273, 274, 1075). The concept of active transport is also supported by evidence of selective uptake of chylomicrons based on their composition. Mice deficient in pleomorphic ademona gene-like2 ($Plag2^{-/-}$ mice) die from postnatal wasting (essentially starvation) due to poor chylomicron absorption into lymph. The chylomicrons in $Plag2^{-/-}$ mice appear to lack modifications required for uptake into lacteals (1102). Abetalipoproteinemia, the virtual absence of Apolipoprotein B (ApoB) in the lymph (and also plasma), is also caused by an abnormality in lipoprotein assembly caused by a recessive mutation in the microsomal triglyceride transfer protein (MTP) (319, 394). There is also recent functional evidence to

support active transport of chylomicrons, from a co-culture system with Caco-2 cells and human dermal lymphatic endothelial cell monolayers (268). In this system, intended as a model of nutrient transport through enterocytes and the lacteal wall, lipid transport was more rapid than dextran or albumin. In addition, an abundance of lipid-containing vesicles appeared to shuttle across endothelial cells, and basal-to-apical transport of chylomicrons was favored, supporting that active transport may contribute to lymph formation (268). This transport process was when ATP was inhibited with sodium azide (896). Selective uptake of subcutaneously injected fluorescently labeled albumin into dermal lymphatic endothelial cells has also been reported. In cultured lymphatic endothelial cells, the process involved uptake into caveolin-1+ and clathrin+ vesicles, and was attenuated by inhibition of dynamin (1079).

Cell Entry Across the Lymphatic Endothelium

The cells found within lymphatic vessels include mostly immune cells, including T- and B-lymphocytes, monocytes, macrophages, and dendritic cells, and some neutrophils and eosinophils. In some cases erythrocytes are also detected, and during an immune response, activated, antibody-secreting B lymphoblasts (plasma cells) are present. Tumor cells may also enter lymphatics. Immune cells enter both at initial lymphatics and at lymph nodes (809). Initial lymphatics serve as a site of entry into the afferent lymph for memory T cells and conventional $CD11c^{hi}$ MHC II^{+} dendritic cells, and to a lesser extent monocytes and macrophages from the peripheral tissues under non-inflamed, steady state conditions (487, 808, 883). In the lymph nodes, immune cells can exit the blood circulation via the high endothelial venules (HEVs). Cells that enter lymph nodes by this pathway include $CD11c^{lo}$ MCH II^{+} plasmacytoid dendritic cells and naïve T and B lymphocytes (53). The distinct populations of cells entering the lymphatic system at initial lymphatics and lymph nodes makes the cell composition of afferent lymph different from that of efferent lymph. The composition of cells in lymph is also dependent upon the presence of antigens and inflammation.

CC-chemokine receptor-7 (CCR7) regulates homing of immune cells in response to the chemokines CCL19 and CCL21. CCR7 is expressed by thymocytes during defined stages of development, naïve T and B cells, a subpopulation of memory T cells known as central memory T (T_{CM}) cells, T_{Reg} cells, semi-mature and mature dendritic cells, and a subpopulation of neutrophils (74, 344). Expression levels increase in dendritic cells or B cells following activation, facilitating migration toward T-cell areas in lymph nodes (344). CCR7 is also expressed by some non-immune cells, of which the most noteworthy are certain types of malignant tumor cells (977). CCR7-deficient mice have weakened or delayed immune responses and develop autoimmunity because normal migration of dendritic cells, T cells, and B cells is impaired and the normal functional microenvironments of lymph nodes are disrupted (240, 241, 345, 566).

CCR7 has two known ligands, the chemokines CCL19 and CCL21 (345). Cells expressing CCR7 directionally migrate toward CCL19 and CCL21 (344). Both CCL19 and CCL21 are produced by fibroblastic reticular cells of the T cell-rich area of lymph nodes (612, 632, 763, 1144). In addition, CCL21 is also produced by endothelial cells of HEVs, and the

endothelial cells of initial lymphatics (344, 396, 558). Initial lymphatic endothelial cells constitutively express CCL21, but expression can also be upregulated by the presence of dendritic cells or TNF-α (488, 656). On initial lymphatics, discrete patches of immobilized CCL21 can be observed on the endothelial cells (488, 1050). These patches are located near, but are not part of the primary valve leaflets (Fig. 27). Their function appears to be coordination of docking of dendritic cells and other CCR7-expressing cells to the lymphatic endothelium (1050).

Dendritic cells—Dendritic cells are highly motile, phagocytic cells that have important roles in both innate immunity and as antigen presenting cells in adaptive immunity. They are also the major antigen-presenting cell types found in afferent lymph, although very few enter efferent lymph (637, 871, 883, 1026). Dendritic cells arise from hematopoiesis in the bone marrow, initially as immature cells that continuously sample for pathogens, with diverse subpopulations that are resident in tissues or recruited from the blood (883). Under non-inflammatory conditions, a relatively low yet steady number of dendritic cells enter into afferent lymph. This steady-state entry appears to have a role in the development and maintenance of immune tolerance to self-antigens (789). Inflammatory signals cause the number of dendritic cells in afferent lymph to increase by an order of magnitude. This inflammation-induced increase involves both activation of the dendritic cells and lymphatic endothelium to coordinate dendritic cell entry into lymph (471, 639, 656).

Dendritic cells become activated and matured upon phagocytosis of pathogens. This involves degradation of the pathogenic proteins into presentable antigens, MHC-dependent presentation of antigens on the plasma membrane, and upregulation of CCR7 and co-receptors for T-cell activation (656). To reach the T-cell-rich lymph nodes, dendritic cells must migrate from peripheral tissues into the draining lymphatics. The actin filament bundling protein fascin, which is important for filopodia formation and migration, becomes upregulated in mature dendritic cells (565, 920, 1122). As mentioned above, the attractive gradient of the CCR7 ligand, CCL21, produced by podoplanin-rich lymphatic endothelial cells is one key factor. This is evidenced by defective dendritic cell migration to lymph nodes in CCR7-deficient mice (345, 789), impaired migration of CCR7-deficient dendritic cells adoptively transferred into normal CCR7+ hosts (656), and the observation that dendritic cells injected into footpads of mice fail to accumulate in lymph nodes when co-administered with CCL21-neutralizing antibodies (948). The CCR7-mediated directional migration of activated dendritic cells through the interstitium is thought to be in an adhesion-independent, amoeboid fashion, autonomous from the tissue context, allowing rapid transfer to the lymphatics, based on results from studies utilizing CCL19 gradients (576). Long distance gradients of CCL21 in tissues are also established due to its immobilization on heparan sulfate, making it difficult to wash out (1129). Activated dendritic cells have also been reported to secrete CCL19 (344, 949), which may act in an autocrine fashion or promote recruitment of additional cells to migrate toward initial lymphatics. Another key factor is inflammatory stimuli, such as cysteinyl leukotrienes and prostaglandin E, which sensitize CCR7 to CCL19 and CCL21 (887). The exoenzyme CD38, an ADP-ribosyl cyclase, also appears to sensitize CCR7 to CCL19 and CCL21 (849). Inflammatory mediators such as TNF-α and IL-1β also stimulate production of chemokines and

chemokine receptors to mobilize and direct dendritic cells (310). Type I interferons also promote migration toward, and binding to lymphatic endothelial cells (922).

Upon reaching the initial lymphatic endothelium, dendritic cells (and other types of cells that enter here) encounter a discontinuous basement membrane containing collagen IV, laminins, perlecan and nidogen. Dendritic cells may pass through the clefts between button junctions (paracellular entry) or through pores in the cytoplasm of endothelial cells (transcellular entry). Microvilli extending from the endothelial cells containing additional adhesion molecules, and patches of CCL21 likely facilitate docking and transendothelial migration into the initial lymphatic lumen (488, 1050). Dendritic cells have been observed to enter in an integrin-independent process under resting non-inflammatory conditions (576, 861). However, under inflammatory conditions, dendritic cells express the integrin ligand ICAM-1, and to a lesser extent, VCAM-1 on their surface, and bind to β-integrins on lymphatic endothelial cells (486, 1058, 1066). In addition, CCL21 released by LECs also produces active $\alpha_1\beta_2$ (LFA-1) on human dendritic cells (300). Several other lines of evidence support the important roles of ICAM-1 and LFA-1 in this process: ICAM-1-deficient mice display defective recruitment of dendritic cells to lymph nodes (998, 1159). Dendritic cell migration to lymph nodes is also inhibited when ICAM-1 is blocked using specific antibodies (636). Dendritic cells lacking β_2 integrin also have impaired migration (1159), and migration is inhibited by anti-LFA-1 antibodies (636, 922). Combined blockade of both ICAM-1 and LFA-1 with antibodies completely blocked migration of dendritic cells to lymph nodes (636).

Additional chemokine-induced changes in dendritic cell shape facilitate passage through the initial lymphatic endothelium (27, 487, 883). Non-muscle myosin II-driven changes in the dendritic cytoskeleton play a key role in producing the changes in cell shape to pass through narrow gaps between initial lymphatic endothelial cells or through transcellular pores (576). Recently, the semaphorin receptor plexin-A1 was shown to be crucial for dendritic cell entry into initial lymphatics by activating this process. Sema-3A produced by lymphatic vessels functions as the ligand for plexin A1-Neuropilin 1 (Nrp1) receptors on dendritic cells (1048). Activation of plexin A1-Nrp1 by Sema-3A elevates phosphorylation of myosin light chains (MLC), which promotes actomyosin contraction and orchestrates the necessary shape changes within dendritic cells to squeeze through narrow gaps or pores in the lymphatic endothelium (1048).

Upon entry into lymphatics, dendritic cells then ferry their antigenic cargo as they are carried in the afferent lymph toward the lymph nodes (883). In the initial lymphatics, crawling of dendritic cells on the endothelium, in the direction of flow, has been reported (768, 1050). Upon reaching collecting lymphatics, dendritic cells are carried away by the higher velocity lymph flow en route to the lymph nodes (1050).

Lymphocytes—Memory T cells also enter initial lymphatics (487, 883). In rat lymph lacteals, the route of entry for T lymphocytes has been described as paracellular, while other cells like macrophages were observed passing through cytoplasmic pores (49). Tissue-resident memory cells lack CCR7, but migrating memory T cells express CCR7 and enter initial lymphatic vessels, following the CCL21 gradient (138, 253, 1153). T cell entry into

afferent lymphatics is regulated by the bioactive lipid sphingosine-1-phosphate (S1P), which activates specific S1P receptors on T lymphocytes to direct migration (590). S1P can antagonize CCL21-CCR7-mediated T cell migration during an immunologic challenge, holding T cells in the periphery until inflammation resolves (487).

There are less B lymphocytes than T lymphocytes present in both afferent and efferent lymph (809). In adult sheep resting lymph nodes, afferent lymph typically delivers 2–5 million cells per hour, composed of approximately 85% T cells, 5% B cells, and 10% dendritic cells. Efferent lymph for a resting lymph node contains 10-fold the number of cells as afferent lymph (20–50 million per hour), with about 75% T cells and 25% B cells (405). Upon stimulation, the number of cells in afferent lymph doubles, with many more dendritic cells and blast-transformed T cells. Efferent lymph from an activated lymph node contains 100–500 million cells per hour, with both blast-transformed T cells and plasma cells producing high levels of antibodies (405). Maximal numbers of plasma cells (15–30% of total cells in efferent lymph) are apparent 80–120 h after initiation of immune response (409).

Neutrophils—At the onset of injury or infection, neutrophils are the first leukocyte population to extravasate from the circulation. While traditionally thought of as having a role limited to innate immunity, neutrophils have more recently shown to capture antigens and migrate to lymph nodes, present antigen in an MHC-II-dependent manner, and activate T cells (3, 189, 219, 456, 457, 647). In mice immunized with *Myobacterium Bovis* bacillus Calmette-Guérin (BCG), the only currently effective vaccine against tuberculosis, neutrophils are rapidly recruited at the site of inoculation. However, neutrophils harboring the bacteria are also found in the draining lymph nodes (3).

For neutrophils, the route to lymph nodes could potentially be either via afferent lymphatic vessels or by transmigration across high endothelial venules. Some of the ligands for transmigration appear to be shared, such as the LFA-1 integrins, macrophage-1 AG, and CXCR-4, whereas L-selectin and P-selectin glycoprotein ligand-1 are specific for transmigration across high endothelial venules (386). For entry into lymphatics, recent evidence suggests that a subset of neutrophils express CCR7 (74). Neutrophil β_2 integrin also appears to be a key ligand for entry into lymphatics. Blocking antibodies for β_2 integrin prevented >80% of neutrophil migration to lymph nodes in GFP*lysM* reporter mice infected with *M. Bovis* BCG (911); vaccine against tuberculosis). Blocking antibodies against ICAM-1 reduced neutrophil appearance in lymph nodes by about 50% in the same model. To account for the possibility that β_2-integrin blockade may have blocked neutrophil extravasation through high endothelial venules at lymph nodes, studies were also performed to examine the entry of injected CellTracker Green-labeled murine neutrophils into dermal lymphatic vessels. The results showed that blocking antibodies against β_2-integrin or ICAM-1 caused the neutrophils to accumulate locally, compared to treatment with isotype control antibodies (911).

Considering that neutrophils are the first leukocytes to enter tissues in response to injury or infection, it is reasonable to speculate that their transmigration across the lymphatic endothelium is also a very rapid event. This notion is supported by findings using cultured

neutrophils and lymphatic endothelial cell monolayers grown in Transwell inserts (911). Neutrophils appear to require the lymphatic endothelium to be activated with an inflammatory mediator, such as TNF-α, for transmigration to occur. In addition, blockade of β2 or α4 integrin on the neutrophils, or E-selectin, ICAM-1, or VCAM-1 on the endothelium substantially reduced transmigration across lymphatic endothelial cell monolayers (911). It is also worth noting that upon activation of the lymphatic endothelium, neutrophil transmigration had a very rapid onset and time to cross the monolayer when compared to dendritic cells (911).

Neutrophil transmigration also involves chemotaxis to ELR+ CXC chemokines, particularly CXCL8, secreted from the luminal surface of lymphatic endothelial cells. The process also appears to involve neutrophil-induced endothelial retraction mediated in part by serine proteases, MMPs, and the 15-lipoxygenase-1 derived metabolite 12(S)-HETE (911). Contact with immune complexes may also enhance migration of neutrophils into initial lymphatics. In ovalbumin antigen (OVA Ag)-immunized mice, OVA+ neutrophils appear both at the injection site and in the draining lymph nodes. Neutrophils that come into contact with the OVA/anti-OVA immune complexes migrate more efficiently (647). Interestingly, immunocomplexes can stimulate higher expression of S1PR4 in bone marrow neutrophils in vitro. Activation of S1PRs with FTY720 or FTY720-P reduces entry of neutrophils into lymph nodes through both pathways (386).

Macrophages—While there has been much recent new information about the roles of macrophages in the promotion of lymphangiogenesis, antigen clearance, and resolution of inflammation (526), relatively little is known about their transport within lymph. Some papers have described the entry of macrophages into initial lymphatics (49, 54). The mechanisms of entry have not been well studied, yet probably have some common steps as seen with other immune cells.

Tumor Cells: Chemokines produced by the lymphatic endothelium can also attract the entry of tumor cells into the lymphatic system and also attraction of tumor cells to lymph nodes (79, 237). CCR7 and CXCR4 were both reported to be highly expressed in malignant breast cancer tumors, and a high degree of expression of the receptive ligands, CCL21 and CXCL12 were found in lymph nodes (734). The CCL1-CCR8 axis, implicated in leukemia and lymphoma, and more recently in human malignant melanoma, was also shown to control the egress of tumor cells across the lymphatic endothelium within lymph nodes (236). Strong expression of CCL1 protein was detected in lymph node lymphatic sinuses of both humans and mice, but not in peripheral lymphatic vessels. Blockade of either CCL1 or CCR8 prevented entry of tumor cells into the lymph node (236). In addition to these homing mechanisms, tumor cells cause disruption of the lymphatic endothelial wall. Tumor cell-derived 12S-HETE following lipoxygenase-1 (ALOX15) catalysis can impair the lymphatic endothelial wall, facilitating tumor cell entry, (532). The susceptibility of the lymphatic endothelium within lymph nodes to invasion thus depends both upon homing mechanisms that facilitate interactions of tumor cells with the lymphatic wall, and also the degree of aggressive/malignant activity presented by the tumor cells.

Water and Electrolytes in Lymph

The water and electrolyte contents of newly formed peripheral lymph are assumed to be very similar to that of interstitial fluid. The ionic composition of lymph is not markedly different from plasma, although the cations Na^+, K^+, Ca^{2+}, and Mg^{2+} are slightly lower, while the anions CI^- and HCO_3^- are slightly higher in lymph (1178). Glucose and other small molecules likely also have fairly similar concentrations in lymph, considering that intravenously injected sucrose accumulates in lymph and eventually forms a steady-state concentration equivalent to the plasma concentration (360). Other small molecules secreted by endothelial cells, such as NO, would also be expected to be present in lymph.

In the small intestine, absorption of nutrients and electrolytes can make the concentration of electrolyte constituents within initial lymph formed in lacteals vary. The osmolarity of lymph obtained from the lacteals of rat jejunum was reported to be about 400 mOsm, hypertonic with respect to plasma, with further increases in both osmolarity and the rate of lymph flow in response to nutrients in the gut lumen. The osmolarity becomes isotonic as the lymph enters the submucosal plexus, probably due to sodium efflux (106).

Proteins in Lymph

The proteins in lymph come multiple sources: 1) the plasma ultrafiltrate; 2) cells in local tissues; 3) lymphatic endothelium; and 4) release from cells present within the lymph such as lymphocytes, dendritic cells, neutrophils, and others. A portion of these proteins may be derived from damaged cells, especially during inflammation. During infection, significant amounts of antigenic proteins or fragments will be present, plus antibodies and inflammatory cytokines.

The concentration of proteins in lymph is lower than in the plasma, and the largest macromolecules make up a smaller proportion of the lymph protein content compared to that of plasma (87, 810, 811, 813). Lymph within initial lymphatics generally has the same protein concentration as the local interstitial fluid. This statement is supported by results from several studies that were performed a few decades ago, which compared protein concentrations between interstitial fluid and lymph from lymphatics either draining the local tissue or much further downstream (316, 360, 869, 935, 1054). Garlick and Renkin reported that the lymph:plasma ratio (using prenodal popliteal lymph from dogs) for endogenous albumin was 0.16, and they also obtained similar results after measuring plasma and lymph concentrations of intravenously injected dextrans with molecular weights averaging 20 kDa (360). Prenodal peripheral lymph collected from rabbit subcutaneous tissue by lymphatic micropuncture displayed equivalent amounts of total protein, albumin, globulin, and transferrin as the interstitial fluid (935). Findings from some studies during this time period suggested that lymph in initial lymphatics had higher concentrations of protein than interstitial fluid, with subsequent dilution as lymph entered collecting lymphatics (158, 160, 164). However this concept was not supported by subsequent, direct measurements of protein concentrations in initial lymphatics (1198).

Some findings have suggested that lymph protein increases as lymph travels downstream through the network. Data from an investigation of rabbit and cat mesenteric lymph obtained

from micropuncture of initial lymphatics, prenodal collecting lymphatics, and postnodal collecting lymphatics indicated a significant increase in protein concentration (416). Results from a different study, investigating the concentrations of a 149 kDa FITC-dextran tracer in rat mesenteric lymphatics by intravital microscopy with a slit-laser epifluorescence system demonstrated increased concentrations of the tracer at downstream sites along the length of vessels (1047). Lymph nodes appear to be a key site where lymph protein concentrations are increased (76), due in part to the loss of water to the bloodstream (7). It is important to note that the increase in lymph protein concentration may not be universal throughout all lymphatic networks. Analysis of lymph obtained from rat intestinal villi and mesenteric collecting lymphatic vessels showed no significant difference in protein concentrations (1198). In the mouse lungs, no change in the concentration of lymph protein was detected (766, 767).

Protein concentration can be affected by changes in lymph flow. General anesthesia was reported to increase protein concentration, while gentle massage increases local lymph flow and reduces concentration (118). In the small intestine, increases in lymph flow due to elevated fluid absorption result in a decrease in protein concentration (390). Similar findings were reported when determining the colloid osmotic pressure of lymph originating in ileum, which was 10 mmHg at rest and fell to 3 mmHg during active absorption (389).

Complement and inflammatory proteins that contribute to innate immunity, and immunoglobulins involved in adaptive immunity, are generally present in lower concentrations in lymph than in the plasma (810, 811, 813). Immunoglobulins, having much higher molecular weights than albumin, are present in the lymph at a lower proportion to albumin compared to the plasma (87). While this might be considered as evidence of size-exclusion and filtration from the plasma, significant immunoglobulin protein may also be synthesized in the local tissue. B-lymphocytes or plasma cells within lymph nodes or the lymph itself also contribute. In intestinal lymphatics, IgA and IgG were reported to appear in lymph at a higher rate than albumin, which was attributed to local production by immune cells in the lamina propria of the gut mucosa for at least part of the IgG and for nearly all of the IgA (878). Polymerization of various immunoglobulins can also affect the relative amount that appears in blood or lymph. In the case of IgA, its monomeric form generally appears in the blood while its oligomeric form linked with J-chains is secreted into the gastrointestinal lumen but also appears in the lymph (504). For this reason, secretory IgA is generally higher in mesenteric and thoracic lymph than in plasma for most species studied (504, 1098). Humans, with a higher degree of monomeric, serum IgA, are a notable exception to this trend (504).

Immunoglobulins and inflammatory proteins can be expected to become elevated within the lymph during inflammation or when the tissues that they drain come into contact with antigens and an active immune response is initiated (809, 811). Results from studies with experimental *Brucella abortus* infection in the leg revealed that while the overall protein concentration and immunoglobulin levels in prenodal lymph did not change, a small amount of IgG and up to 60% of the IgM present in the lymph was due to synthesis in the draining (popliteal) lymph node (877). The appearance of numerous antibody-producing B-immunoblasts (plasma cells), up to 15–30% of cells present in postnodal lymph in sheep,

was reported to coincide with peak production of IgG and IgM in response to a variety of antigens (409, 410). Exposure of sheep to bacterial lipopolysaccharide (LPS) also resulted in elevated production of IgG and IgM by B lymphoblasts both within lymph nodes and free-floating in lymph, for up to 20 days (307–309).

Concentrations of enzymes (and activities, which are typically related to concentrations) are generally lower in lymph than in plasma/serum, but there are exceptions depending upon region (1040). The types of enzymes present can be classified as those that are actively secreted and those that are normally cytoplasmic and released from damaged cells. Clotting factors present in the blood are also present in lymph but at lower concentrations (435, 809). For lymphatics draining exocrine organs, the concentrations of the secreted enzymes in lymph may exceed that of the plasma (809, 1037). For example, pancreatic enzymes and bicarbonate, although primarily secreted into the pancreatic duct, also enter the interstitial space and are absorbed into pancreatic lymph (70, 841). Concentrations of secreted enzymes in lymph will also become elevated if there are obstructions of secretory ducts that divert enzymes in the secretions to the plasma and lymph, such as with obstruction of the pancreatic ducts (841). Uptake of pancreatic enzymes from the intestinal lumen into lymphatics may also be possible, as evidenced by the presence of pancreatic lipase in duodenal lymph (838). In addition to secreted enzymes, cytoplasmic enzymes released by cell damage or turnover appear in lymph. A physiological example is release of cytoplasmic enzymes from skeletal myocytes during muscle activity, which was reported to increase the content of lactate dehydrogenase and other muscle-related enzymes in lymph more rapidly and to a higher degree than the venous plasma (608, 1038). Elevations in cytoplasmic enzymes in regional lymphatics draining damaged tissues have also been reported (1037). For example, following an experimental myocardial infarction, the cytoplasmic enzymes typically used as plasma markers of damaged cardiac myocytes appear more rapidly in cardiac lymph and at higher levels than in the plasma (1041). Similar findings were reported in studies of renal lymph following renal ischemia (1039) and leg lymph following ischemic shock (1042).

The proteins carried by lymph also include peptide hormones, and the levels vary according to the glands being drained. Positive levels of adipokines, cholecystokinin, growth hormone, insulin, prolactin, and incretins have been reported in lymph from various sources (627, 645, 698, 790, 809, 952, 1030, 1077, 1177). Thyroid hormones and steroid hormones can also be carried by proteins in lymph (232, 609, 858, 987).

Although protein concentration may generally be lower in lymph than in plasma, lymph does not simply reflect a plasma ultrafiltrate. Proteomics analysis of lymph has revealed that the composition of proteins in lymph, while having some overlap with plasma, is significantly diverse in protein composition. Quantitatively, there are proteins and peptides that have significantly different concentrations from those in plasma, or even have never before found in plasma (296, 463, 588, 616, 705, 855, 1185). Protease inhibitors, γ-fibrinogen, proteins related to lipid transport and metabolism, and proteins related to innate immunity were found to be more abundant in rat mesenteric lymph than in plasma (705, 1185). An example from one study of mesenteric lymph from rats is shown in Fig. 28. One notable functional group is a vast array of peptides that originate from several different

processing pathways, including ADAMs, calpains, caspases, cathespsins, granzymes, kallikreins, MMPs and others. These self-peptides have a role in immune tolerance, as they complex with MHCII and are loaded onto circulating dendritic cells (193–195).

Shock has been known to promote a proinflammatory bioactivity of mesenteric lymph (10, 659, 950), which has driven several proteomic investigations of post-shock lymph. In rat models of trauma/hemorrhagic shock, the mesenteric lymph becomes more enriched in pancreatic enzymes and normally intracellular proteins, suggesting cell damage (706, 707). Increased products of hemolysis, glycolytic enzymes, major urinary protein, lipid carriers, and modified albumin have also been reported elevated in post-shock mesenteric lymph (318, 506, 507, 855, 1222). Time dependent events in hemorrhagic shock models in rats are also evidence, such as an early decrease in Ser protease activity, a gradual increase in serpins, and progressive increase in activity of MMPs and ECM proteins, producing an overall protease/antiproteases impaired homeostasis (225). Assessment of human mesenteric lymph obtained after traumatic injury and shock has revealed a variety of proteins suggesting coagulopathy, lysis of cells including erythrocytes, inflammation, extracellular matrix remodeling, immunomodulation, and changes in energy and redox metabolism (296, 297). In a rat model of sepsis (cecal ligation and puncture), proteomic analysis of mesenteric lymph revealed 158 proteins significantly changed, including elevations in ApoE, annexin-1, S100A8/9, and the lipocalin NGAL (1208). Analysis of postdnodal lymph from rats subjected to LPS exposure revealed elevations in inflammatory cytokines and the disintegrin ADAMTS1 (827). Findings from these proteomic studies can be influenced by choices of models and methodology. In at least one case, namely the protein gelsolin, different methodological approaches to assess the proteins have led to distinct results, with a gel-based approach showing depletion after hemorrhagic shock, and HPLC-MS approaches showing an increase (296, 499, 706).

Lipids and Lipoproteins in Lymph

The role of intestinal lymphatics in the absorption of cholesterol and medium- and long-chain fatty acids from the intestinal mucosa is well known (175, 809). This role is highlighted in Prox1$^{+/-}$ mice, which have systemic lymphatic abnormalities, including impairment of transport of ingested lipid tracer (BODIPY FL C16) from the intestine to the thoracic duct (418). Absorbed long-chain fatty acids are packaged into lipoprotein fractions in lymph. Most appear in chylomicrons (>70%) and the VLDL fraction (15–20%), with the remainder appearing in HDL and LDL fractions (185). Cholesterol absorbed at the intestinal brush border is esterified within enterocytes by the enzyme ACAT2, packaged with ApoB into chylomicrons or VLDL, and delivered to lymph lacteals (242, 764, 928). Fat-soluble vitamins are also absorbed into the lacteals. The concentration of vitamin K absorbed into lymph was shown to be proportional to the amount present in the gut lumen (444).

In lymph from peripheral tissues, amounts of HDL and LDL closely reflect their levels in interstitial fluid, which are relatively low compared to plasma (1002). The HDL and LDL of the interstitial fluid and lymph derive from the plasma ultrafiltrate through passive mechanisms (688). However, they also have a distinct composition and physical properties when compared to plasma HDL and LDL (279, 918, 1002). Reichl and colleagues

performed the earliest studies of human peripheral lymph in normal and hyperlipidemic subjects, by cannulating lymphatics on the dorsum of the foot. They showed the first evidence that intravenously injected ApoA, ApoB, or ApoC could all reach the interstitial fluid, undergo modifications, and be taken up into the peripheral lymph (904). They also measured the level of ApoA1 in lymph, which was 9–16% of the level found in plasma (903), whereas ApoB levels were 5–10% of the plasma levels (901). All ApoB was essentially in the LDL fraction; no recognizable VLDL fraction was present (446). Interestingly, the mean size of HDL particles in lymph was larger than the corresponding plasma HDL, with more cholesterol relative to the ApoA1 (903, 929). Many of these observations were later confirmed by Miller and colleagues, who collected from a larger afferent lymphatic of the lower leg (751). Both groups reported that ApoA1 particles were generally larger, with a wider distribution of sizes than seen in plasma, and enriched in cholesterol (751, 905, 929). Reichl and colleagues postulated that free cholesterol was transferred to HDL and esterified by lecithin-cholesterol acyltransferase (LCAT) (902). Supporting this concept were findings of discoidal HDL in canine lymph not typically found in plasma, which were converted to cholesterol-ester-rich steroidal HDL when incubated *in vitro* with LCAT (278, 1000, 1001). In addition, lipoprotein particles containing ApoA1 but not ApoA2, also described as preβ-HDL which are primary acceptors of cell-derived cholesterol, were observed in human peripheral lymph (167, 750, 900). Collectively, the findings supported that the higher total cholesterol in lymph HDL is due to acquisition of cholesterol from cells in the tissues, and the total mean net reverse cholesterol transport in lymph was calculated to be 0.89 mmol/day or 344 mg/day (751). The importance of lymphatics as conduits for reverse cholesterol transport was also highlighted in recent studies in mice, in which surgical or genetic disruption of lymphatic function reduced delivery of labeled cholesterol originating from macrophages implanted in tissues (605, 652).

Few studies have examined the profiles of bioactive lipids mediators in lymph. The main interest in the lipidomics of lymph has arisen from the trauma field due to the known role of lymph in the development of systemic shock after injury (256). The lipid fraction of mesenteric lymph from rats that have undergone experimental hemorrhagic shock can inhibit apoptosis of neutrophils and enhance their surface molecule expression (378). These effects were shown to be inhibited by a nonspecific inhibitor of PLA_2 (380). Arachidonic acid was also reported to be elevated in post-hemorrhagic shock mesenteric lymph and could increase lung LTB_4 levels (500). More recently, a lipidomics study of post-hemorrhagic-shock and control rat mesenteric lymph utilizing liquid chromatography/electrospray ionization mass spectrometry was performed to identify potential mediators associated with the phospholipid and lysophospholipid species. The postshock lymph contained elevated levels of lysophosphatidylcholines and lysophosphatidylethanolamines that could induce priming of neutrophils (726). Collectively, the results of these studies indicate that lymph carries an important fraction of bioactive lipids that can significantly impact functions of tissues remote from sites of injury.

Lymph Transport in the Initial Lymphatic Network and Precollectors

Newly formed lymph flows freely within the lumen of an initial lymphatic vessel or network of initial lymphatics, with net movements largely dependent upon fluid pressure gradients. This can be observed by injecting a fluorescent tracer subcutaneously in the distal tail of the mouse and then following its uptake into, and movement within, the cutaneous lymphatic network. The tracer gradually moves proximally within the superficial initial lymphatics. This network has numerous connections with the deeper collecting lymphatics, which are thought to affect pressures within the initial lymphatic network, causing an overall proximal flow of lymph (402, 1032). Interconnected initial lymphatic and precollector networks allow for recruitment of additional, adjacent lymphatic vessels during periods where increased draining is required, as seen in the rat diaphragm. Under normal conditions, the initial lymphatics within the muscular region of the diaphragm drain both the pleural space and peritoneum. However, during pleural or peritoneal effusions, lymphatics in the tendinous region of the diaphragm are also recruited (721).

Fluid flow within the precollectors is restricted by the presence of luminal (secondary) valves that regulate the direction of fluid flow. The geometry of networks also contributes to the overall efficiency of flow. Based on the network geometry of initial lymphatics and precollectors in rat mesentery, computational models estimate that a downstream pressure drop in the range of 0.3 to nearly 3.0 mmHg is needed for reasonable flow through the network (999). While precollectors have traditionally been thought to be passive conduits that lead to collecting lymphatics, how such a gradient is established is poorly understood. There has been some evidence for the presence of discontinuous circular smooth muscle cells (1196) that may assist with movement of lymph. Another popular concept is that the downstream collecting lymphatics, through pumping, cause a suction effect (473, 999). Additional work is needed to elucidate these mechanisms.

Another difference between precollectors from initial lymphatics is the level of expression profiles of chemokines and their receptors. In addition to the relatively low expression of podoplanin compared to endothelial cells from initial lymphatics (1122, 1138), CCL27 is highly upregulated in precollector endothelium. CCL27 is a secreted chemoattractant for pathogenic CCR10+ T lymphocytes, which have been observed to cluster around and inside precollectors in human skin biopsies of inflammatory skin disease. These data suggest a specialized role in the trafficking of pathogenic CCR10+ T lymphocytes (1138).

Lymphangions and the Lymphatic Pump Mechanism

In a standing human being, for collecting lymphatics originating in the feet to deliver the lymph to the great veins in the upper thorax, they must overcome a significant hydrostatic pressure gradient. This is overcome by serial organization of individual lymphangions, the key collecting lymphatic pumping units, dividing the overall pressure gradient into a series of many smaller steps (805, 812). This was initially shown with measurements of pressures within lymphatic networks of exteriorized mesentery, which revealed that with each passage across a secondary valve to a downstream segment, intraluminal pressure increases gradually (415, 416, 1225). This concept was later confirmed in the rat tail with noninvasive near

infrared imaging and computational modeling (892). Each step can be overcome by the phasic constriction of the lymphangion by its lymphatic smooth muscle layer. Secondary valves between lymphangions prevent backflow of lymph. A constriction of a lymphangion sufficient to open the downstream valve will move lymph forward to the downstream lymphangion (415). The pumping process controlled by lymphangions represents the primary mechanism to propel lymph back to the central circulation (126, 408, 812, 1117, 1201). Both peristaltic and segmented contractions of lymphangions in series have been observed, and both can achieve forward flow of lymph (37, 1201).

It is worth noting that physical forces extrinsic to the lymphangion may also have profound influence on lymph flow. Skeletal muscle contractions, such as in the legs of a walking individual, typically are thought to aid lymph flow by compressing or stretching lymphatic vessels. However, it is also important to consider that increased muscle activity leads to elevations in blood flow to meet metabolic demand. Increased filtration of plasma and lymph formation will accompany this activity. Thus, the effects of extrinsic forces are complex in nature, and the sum of how extrinsic forces aid in lymph propulsion versus to what degree they affect the overall load of lymph formation must be considered (408).

Recall that collecting lymphatic vessels have an outer layer of adventitia and smooth muscle cells and an inner endothelial cell layer (Fig. 4), with an intermediate layer of extracellular matrix containing collagen and elastin. The two valves on either end of a lymphangion can be defined as having an “inflow” valve through which lymph from upstream in the network enters and “outflow” valve for downstream flow. An important feature of the lymphatic pump mechanism is its dynamic ability to optimize lymph flow in response to changes in intraluminal pressure or shear stress on the luminal wall. The following discussion will highlight known physiology of the lymphatic smooth muscle and endothelial layers in the generation and regulation of lymphatic contractions. In addition, influences from nerve cells in the adventitia and soluble mediators within the lymph or from the surrounding tissues will also be discussed.

Lymphatic Smooth Muscle

The smooth muscle layer of lymphatic vessels, often simply referred to as lymphatic muscle due to its unique properties, generates the lymphatic contractile cycle required for normal lymph flow. This cycle can be characterized in a similar fashion as the cardiac cycle, with systolic and diastolic phases. Lymphangion systole is produced by periodic phasic contractions of the lymphatic muscle. During diastole, the lymphatic muscle does not completely relax, maintaining a certain degree of tone. Lymphatic muscle function has been characterized by measurements of contractile force to determine length-tension relationships, or vessel diameter to study pump action. Because lymphatic muscle has functional properties resembling both cardiac and vascular smooth muscle, the parameters typically used to describe both cardiac and vascular smooth muscle function in arteries and arterioles have been adopted to describe lymphangion function. The parameters analogous to cardiac parameters include the phasic contraction frequency (CF), end diastolic diameter (EDD), end systolic diameter (ESD), and others derived from these measures (1201). Analogous to vascular function is tone, which for lymphatics is calculated by dividing the

EDD by the maximal passive diameter when the vessel is completely relaxed. Table 1 lists these parameters and how they are obtained or calculated. Due to the nature of the collecting lymphatic contractile cycle, both phasic contractions and vessel tone are generally studied simultaneously.

With its ability to produce both phasic contractions and maintain tone, it is not surprising that the biochemical characteristics of lymphatic muscle feature elements of both cardiac and vascular smooth muscle. As in cardiac muscle, action potentials and rapid influx of Ca^{2+} play a fundamental role in stimulating the phasic contractions of lymphatic muscle. Like vascular smooth muscle, cytoplasmic Ca^{2+} and the sensitivity of contractile molecules to Ca^{2+} are key factors. Collecting lymphatic vessels are very sensitive to changes in extracellular Ca^{2+}, such that lymphatic contractions cease fairly quickly in Ca^{2+}-free bathing solutions and also if the Ca^{2+} concentration is too high (245, 671, 1007, 1062). Voltage-gated L-type Ca^{2+} channels appear to have a predominant role in Ca^{2+} entry into lymphatic muscle cells, as drugs that block these channels inhibit the ability of lymphatics to contract (42, 595, 1005, 1062).

Electrical Properties of Lymphatic Muscle—Electrical recordings of human mesenteric collecting lymphatic vessels show a cycle with 1) a gradual depolarization that leads to a pre-potential with spontaneous transient depolarizations (STD) rapidly followed by, 2) an action potential with an initial fast repolarization, 3) a subsequent depolarization to a plateau phase, and then 4) a slower repolarization to the resting membrane potential (Fig. 29) (1062). There is a one-to-one relationship between action potentials and phasic contractions (25). The precise origin of the pacemaking electrical activity is unclear. The likely pacemakers are the smooth muscle cells themselves although associated cells expressing c-kit that are thought to be similar to the interstitial cells of Cajal of the intestine have also been proposed (135, 666, 951). Nerves can be found in the lymphatic vessel walls but their role is modulatory rather than to establish pacemaker action potentials.

Reported resting membrane potential (V_m) recordings of lymphatic smooth muscle typically lie on average between −65 and −45 mV. Individual recordings of guinea pig mesenteric lymphatic smooth muscle Vm ranged from −80 to −40 mV with a normal distribution and average ± SEM of −60.8 ± 1.1 mV (1116). In a different study utilizing small segments of guinea pig mesenteric lymphatic Vm a mean ± SEM of −65.1 ± 1.5 mV was recorded (1104). In rat mesenteric collecting lymphatics, the smooth muscle Vm was reported to be −48 ± 2 mV in nonstretched vessels, and −36 ± 1 mV when stretched (1111). A separate paper from the same group reported that upstretched rat mesenteric lymphatic vessels typically had a Vm between −65 to −55 mV (mean ± SEM 61 ± 2 mV) while the range for vessels on a wire myograph was from −45 to −35 mV, with an average ± SEM of −39 ± 2 mV (595). In stretched human thoracic duct and mesenteric collecting lymphatics the V_m was reported to typically be at or near −45 mV (1062). For comparison, lymphatic endothelium in guinea pig mesenteric collecting lymphatics was reported to be more negative, with an average ± SEM of −71.5 ± 0.5 mV (1116).

Between action potentials, lymphatic muscle Vm gradually depolarizes. A few ionic mechanisms have been proposed, and are all probably involved in the lymphatic pacemaking

mechanism. First, there is considerable evidence that Ca^{2+}-activated Cl^- (CaCl) channels significantly influence V_m of lymphatic smooth muscle (1112, 1116). Second, hyperpolarization-activated inward currents that are similar to the *If* in the sinoatrial node of the heart were shown to influence CF of sheep mesenteric lymphatic vessels (667). In agreement, HCN channel inhibitors reduced CF of rat diaphragmatic lymphatics, and all four members of the HCN channel family were detected at the mRNA level and by immunofluorescence in these vessels (758). Third, T-type voltage-gated Ca^{2+} channels, which have an established role in pacemaking in the heart (404), have been found in rat mesenteric lymphatic smooth muscle. Specifically, the $Ca_v3.2$ isoform has been found at both the mRNA level and by immunofluorescence labeling, and inhibition of these channels reduces CF without affecting contraction amplitude or force (595). Collectively, the current evidence suggests that two types of molecular "clocks" work in concert. The first is at the sarcolemma (plasma membrane) level and involves T-type voltage-gated Ca^{2+} channels, and possibly the HCN channels. The second oscillator involves IP3-mediated Ca^{2+} release from the sarcoplasmic reticulum and activation of CaCl channels. These oscillators are in communication across multiple cells through strong electrical coupling and weaker chemical coupling (461, 462, 595, 1103).

Of these mechanisms, the oscillatory Ca^{2+} release from internal stores and activation of CaCl channels has been most widely studied. This mechanism underlies spontaneous transient depolarizations (STDs) within lymphatic smooth muscle that precede action potentials (1112). STDs have been recorded just prior to action potentials in mesenteric collecting lymphatics of the guinea pig (1104), sheep (1074), and humans (1062). STDs have also been recorded between action potentials in mesenteric collecting lymphatics obtained from guinea pigs and sheep, but only rarely in lymphatic vessels from humans (1062, 1074, 1112). In guinea pig lymphatics, treatment with the L-type Ca^{2+} channel blocker nifedipine eliminates action potentials, while STDs remain (1112). STDs can be abolished by treatment with BAPTA-AM, which chelates intracellular free Ca^{2+} ($[Ca^{2+}]_i$), or by inhibition of Ca^{2+} reuptake into the sarcoplasmic reticulum with cyclopiazonic acid (329, 1104), suggesting the importance of internal Ca^{2+} stores. In addition, enhancement of IP_3 receptor-mediated Ca^{2+} release with thimerosal or $Bt_3(1,3,5)IP_3$-AM increased STD frequency and amplitude (1112). Moreover, pharmacological agents that increase CF through the IP_3-Ca^{2+} pathway also increase the frequency and amplitude of STDs (347, 462, 1104, 1109), while those that decrease CF lower the STD frequency and amplitude (180, 181)}(1109, 1115). CaCl channels have also been shown to have an important role in lymphatic pacemaking (75, 1074, 1112, 1116). Activation of CaCl channels by elevated $[Ca^{2+}]_i$ following IP_3-mediated release from internal stores appears to contribute to STDs (1112). The spatial and temporal summation of STDs leads to a sufficient depolarization to threshold to open voltage-gated channels responsible for action potentials.

Opening of ATP-sensitive K^+ (K_{ATP}) channels can hyperpolarize the membrane and thus regulate CF. Vasoactive inhibitor peptide, β-adrenergic stimulation, NO, ATP, calcitonin gene-related peptide have all been reported to decrease lymphatic CF through opening of K_{ATP} channels (451, 555, 899, 1108, 1114). The role of K_{ATP} channels in lymphatic pumping has also been shown by using the K_{ATP} blocker glibenclamide to prevent cessation of pumping in response nonselective K^+ channel opening with pinacidil (711). In addition,

selectively opening K_{ATP} channels with cromakalim can eliminate action potentials in guinea pig lymphatics (660). Recently, the expression of K_{ATP} channel subunits was observed to be upregulated in a rodent model of inflammatory bowel disease, in association with impaired lymphatic pumping. In addition, lymphatic pump function was restored after blockade of K_{ATP} channels with glibenclamide, further suggesting an important role for these channels in disease development (660).

Ca^{2+}-activated K^+ channels (BK Channels) were shown to produce outward currents in smooth muscle cells isolated from sheep mesenteric lymphatics (207). The potential role of BK channels in lymphatic pumping is unclear. In pinacidil-induced inhibition of lymphatic pumping in rat lymphatics, blockade of BK channels with Iberiotoxin had no effect (711). BK channels have a documented role in the control of arterial tone (122) and their role might be similar in lymphatics.

Both voltage-gated Na^+ and Ca^{2+} channels have been implicated in lymphatic smooth muscle action potentials, although there have been variable findings in different species. Using Tetrodotoxin (TTX), fast Na^+ channel inward current was shown to significantly contribute to the spontaneous action potentials initially in sheep mesenteric collecting lymphatics (445), and later in human mesenteric collecting lymphatics and thoracic duct, with $Na_v1.3$ as the predominantly expressed voltage-sensitive Na^+ channel (1062). However, in both humans and sheep there are some vessels that are resistant to TTX, in which voltage-gated Ca^{2+} channels may be sufficient to produce action potentials (445, 1062), as blockade of L-type voltage-gated Ca^{2+} channels can eliminate action potentials in collecting lymphatic vessels (1064, 1112). In bovine and guinea pig lymphatic vessels, TTX has not been found to block spontaneous contractions (47, 676, 1104). As stated above, nifedipine eliminates action potentials in guinea pig lymphatics, indicating the importance of L-type Ca^{2+} channels for inward current in these vessels (1112). The differences between species are not unexpected, as gross differences in contractile function in mesenteric collecting lymphatics have also been observed. This includes the extreme case in most strains of mice, in which phasic contractions are so weak as to appear absent. Notably, the regional differences in the strength of lymphatic contractions in mice is related to differences in the activity of L-type Ca^{2+} channels (1202).

Considering the shape of the lymphatic muscle action potential, the initial, rapid spike is likely due to the fast Na^+ channel current (1062). After the initial spike, there is a rapid repolarization with an undershoot (1062, 1111). The fast, initial repolarization may be due to an outward current caused by activation of K_v channels (1061). L-type voltage-gated Ca^{2+} channels, which open more slowly than the fast voltage-gated Na^+ channels, contribute to the plateau phase of cardiac action potentials, and are suspected to contribute to the depolarization and plateau phase in lymphatic smooth muscle (445, 1064). Recent work shows that the L-type voltage-gated Ca^{2+} channel isoform $Ca_v1.2$ is expressed in lymphatic smooth muscle from rats (595) and humans (1064). The $Ca_v1.2$ channel is the dominant Ca^{2+} channel in cardiac muscle contraction (168), and appears to have the same role in lymphatic muscle (595). Repolarization after the plateau phase presumably occurs due to the closing of the Ca^{2+} channels.

Lymphatic Contractile Mechanisms—Excitation-contraction coupling in lymphatic muscle is mediated mainly by $[Ca^{2+}]_i$. Phasic contractions of lymphatic vessels quickly follow the transient increases $[Ca^{2+}]_i$ that accompany action potentials of the lymphatic smooth muscle (462, 981, 1005, 1006, 1112). In between phasic contractions, a certain degree of basal vessel tone is maintained. In isolated collecting lymphatics mounted on glass micropipettes and subjected to physiological transmural pressures, the tonic contraction typically makes the luminal diameter 5–20% smaller than would be measured in a completely relaxed vessel. (245, 366, 368, 567, 568, 1007). Both vessel tone and phasic contractions are eliminated when isolated collecting lymphatics are subjected to Ca^{2+}-free bathing solutions.

Force generation in lymphatic muscle resembles aspects of both 1) cardiac muscle contractions and 2) tone in other smooth muscle types. First considering similarities to cardiac muscle, the shortening velocity of lymphatic phasic contractions is relatively close to that of striated muscle (82, 1211). This is likely attributable to expression of troponin C and I, smooth muscle B myosin heavy chain (MHC), and fetal cardiac/skeletal slow-twitch β-MHC, all of which are found in cardiac muscle (740, 741). Another aspect that in which lymphatic muscle resembles cardiac muscle is the ability of collecting lymphatics to intrinsically modulate CF in response to changes in transmural pressure. Within the physiological range, an increase in transmural pressure generally leads to an increase in the CF (82, 246, 302, 415, 674, 783, 895, 1210). Looking more closely, one can examine an individual lymphangion segment and study how preload and afterload affect lymphatic contractions, using analysis procedures developed for cardiac physiology. Preload, set by the end-diastolic pressure, can be elevated over time by increasing filling pressure, and for a certain range of pressures enhances pump output (Fig. 30), in a manner similar to Frank-Starling relationship described for the heart (961, 962). Elevations in afterload, the pressure against which the lymphangion must pump, which affects the axial load on the vessel wall (169), causes a combined positive chronotropic and inotropic response (Fig. 31), as evidenced by an increase in slope of the end systolic volume vs. pressure relationship (249, 962).

Considering the similarities of lymphatic muscle to vascular smooth muscle, elevation in transmural pressure elicits an increase in tonic constriction between phasic contractions (245, 1007). In addition, like blood vessels, lymphatic vessels possess a flow-induced endothelial-dependent relaxation. Increases in flow cause a combined decrease in pump activity and elevation in EDD (18, 361, 364). The mechanisms that control tone have similarities with vascular smooth muscle, such as the requirement of extracellular Ca^{2+} and the activation of myosin light chain (MLC) kinase (MLCK) by elevations in the basal $[Ca^{2+}]_i$ between phasic contractions in order to drive actin-myosin-mediated contraction (Wang et al., 2009). The tone is likely sustained by slow cycling latch bridges (281) in a similar fashion as described for vascular smooth muscle (263). There is also evidence that intracellular signals can alter the Ca^{2+}-sensitivity. PKC has been reported to increase Ca^{2+}-sensitivity by activating CPI-17-mediated inhibition of the myosin light chain phosphatase (MLCP), producing an increase in tone (280, 281). In addition, Rho kinase (ROCK), which

inactivates MLCP by phosphorylation of the MYPT-1 subunit, also promotes increased tone in lymphatic vessels (450, 568, 1008).

While phasic and tonic contractile mechanisms have been discussed separately above, there invariably is some overlap in the signals that control both types of contraction, such as the requirement of Ca^{2+}. These pathways are summarized in Fig. 32. Additionally, gap junctions (1200) may propagate phasic contractions or tonic signals to adjacent lymphangions. Lastly, as with any muscle type, there is a total tension produced during various contractile states that is the sum of the sum of the active tension generated by the muscle layer and the passive tension that is dependent upon the composition of the vessel wall (783, 1209). The passive tension is influenced by the connective tissue composition, which is rich in collagen and elastin fibers (881). Changes in the transluminal pressure (luminal minus outside pressure) affect overall wall tension, including components of both circumferential stress (hoop stress) and axial stress (264). Observations from a recent investigation of the passive properties of mesenteric lymphatics from rats show that at typical physiological transmural pressures (1–5 cm H_2O), these vessels are very compliant, but above this range they become much stiffer (881). Under normal conditions, a lymphangion senses when transmural pressure increases or decreases, and readjusts pump function to handle the load. Current understanding of this response and the associated molecular mechanisms will be discussed below.

Mechanisms Underlying the Responses to Changes in Transmural Pressure— As introduced above, the response of lymphangions to increased transmural pressure within a physiological range can be generally characterized as an increase in CF when preload is elevated, and a combined increase in CF and inotropy when afterload is elevated (249, 961, 962). Tone also increases when transmural pressure is increased within a lymphangion (245, 1007). However, above certain limits, contractions are not strong enough to eject lymph effectively, resulting in smaller stroke volume and reduced pump efficiency (674).

A stretch-induced response in the smooth muscle cells appears to underlie the lymphatic response to increased transmural pressure. The response is characterized by an increase in the STDs and the action potential frequency in rat mesenteric collecting lymphatics (1111). In addition, there is evidence of stretch-induced Ca^{2+} sensitization (981, 1006, 1008). When rat mesenteric lymphatics are mounted on a wire myograph or on glass micropipettes, they display phasic Ca^{2+} transients. When the lymphatic is stretched or the luminal pressure is elevated, the Ca^{2+} transients become more frequent, but the amplitude typically does not increase (Fig. 33). However, this is also accompanied by an increase the force of contractions (Fig. 33), suggesting an increase in Ca^{2+} sensitization of the contractile molecules within lymphatic smooth muscle (981).

Collecting Lymphatic Endothelium

Endothelial cells with continuous, zipper-like junctions constitute the inner lining of collecting lymphatic vessels. As in blood vessels, these cells form a very thin layer (~0.2 μm except where cell nuclei are present) and are connected by continuous belts of junctional proteins, including VE-cadherin and PECAM-1 (61). Functionally, collecting lymphatic endothelial cells are important for the formation and maintenance of secondary valves to prevent backflow and for sensing the chemical composition of lymph. However, probably

their most notable function is as sensors of fluid shear stress in the lymphatic flow-mediated vasodilator response. The endothelial cells integrate signals based on these inputs and subsequently signal to the smooth muscle layer to elicit changes in contractions. One very important function of the endothelium is its ability to modulate lymphatic contractions by release of NO as an endothelium-derived relaxing factor (324, 419, 898).

Responses to Changes in Fluid Shear Stress—As in the resistance vessels of the central circulation, lymphatics have a fairly well characterized vasodilator response to the elevations in fluid shear stress that accompany increases in lymph flow. This has been demonstrated in collecting lymphatics isolated from various tissues of rats (363, 364, 368). The vessels were mounted onto glass micropipettes connected to manometers to control inflow and outflow pressures. Changes in flow were imposed on the vessels by simultaneously raising the manometer feeding to the inflow pipette and lowering the manometer connected to the outflow pipette. By raising the input reservoir and lowering the output reservoir by equal heights, this maneuver can change the flow rate through the vessel while maintaining a constant average luminal pressure at the vessel midpoint. The general response to increases in flow with this protocol is a combination of decreased tone and frequency of phasic contractions (363, 364, 368). The interpretation of this shear-induced relaxation has been that it may serve as a mechanism to conserve energy during periods when pressure gradients are so favorable for forward lymph flow, that if the vessels were to continue pumping, they would actually increase resistance to downstream flow (363, 364).

The threshold of forward shear stress that can cause dilation of isolated rat thoracic ducts has been shown to be quite low, and is dependent upon the transmural pressure, with values of 0.97 dynes/cm^2 at 5 cm H2O vs. 0.64 dynes/cm^2 at 3 cmH_2O reported (551). In addition, when the shear stress was applied in an oscillatory manner, the phasic contractions became entrained to the flow (551), as predicted by computational modeling (564). It is also worth noting that composition of lymph contributes to the fluid shear stress. An example is the change with mesenteric lymph in the fasted vs. postprandial state (525).

While the shear stress sensing mechanisms are not well defined, it has been established that the elevated production of nitric oxide (NO) by endothelial nitric oxide synthase (eNOS) is a key part of the signaling cascade (364, 710, 982, 1110, 1118). The mechanism is presumed to involve activation of soluble guanylate cyclase in the smooth muscle layer, which would accelerate cGMP production and activate protein kinase G (366, 567), as this pathway has been shown to decrease STDs in other contexts (1108, 1117, 1118). It is worth noting that in rats of advanced age (22 months old), the roles of eNOS and NO in the shear stress-induced relaxation of lymphatic vessels appears to be impaired due to chronic inflammation. Histamine seems to also act as a vasodilator under these circumstances (743, 770). In addition, long-term increases in lymph flow that occur with chronic increases in pulmonary blood flow may impair the mechanism. This was shown in an ovine model of congenital heart disease that included increased pulmonary blood flow due to a KLF2-mediated reduction in PPAR-γ and accumulation of reactive oxygen species, which reduces bioavailability of NO in the lymphatic endothelium (238, 239, 729).

Unlike arteries and arterioles, in which pulsatile flow is always in the forward direction, in collecting lymphatics there can be periods of very slow flow, no-flow, or brief retrograde flow due to the phasic contractions and individual lymphangions and opening and closing of secondary valves (264, 266, 267, 269). Lymphatic endothelium senses these moment-to-moment changes, as demonstrated by placement of carbon electrodes that can sense NO next to pumping collecting lymphatics. The results of these experiments showed that the brief increase in fluid shear stress that accompanies each phasic contraction causes a local release of NO (107). This brief elevation in NO may contribute the overall timing of the contractile cycle of the lymphatic smooth muscle within a feedback loop (564), or may have importance in regional differences within a lymphangion, such as between near or far from the secondary valves.

There is evidence that the action of endothelial-derived NO may be context-specific. When lymph formation is very high and lymph flow is very rapid in the absence of lymphangion contractions, the inhibition of NO release can attenuate the forward flow of lymph. Similar findings have also been reported with eNOS knockout mice (402). Results from experiments utilizing perfused collecting lymphatic vessels from $eNOS^{-/-}$ mice suggest that under unstipulated conditions, the basal NO only impacts phasic contraction amplitude. However, under conditions in which NO production is stimulated by agonists, such as with acetylcholine, the elevated NO affects all other aspects of contractility (956).

Influence of Nerves in the Adventitial Layer

Several reports in the late 1800s and first half of the 20^{th} century provided circumstantial evidence that lymphatic vessel contractions could be influenced by the nervous system. These included observations of a neural plexus or nerve endings in lymphatics (571, 876), and constriction or relaxation of various lymphatic vessels in response to electrical stimulation of different nerves (6, 90, 148, 336, 934). In one study, no responses to nerve stimulation was observed, however epinephrine topically placed on prenodal popliteal lymphatics of rats, mice, and guinea pigs produced an increase in CF (1003). In anesthetized dogs, using an occluded limb technique, stimulation of the lumbar sympathetic chain caused an increase in intraluminal lymphatic pressure that was proportional to the degree of stimulus (142). While the lymphatic intraluminal pressure elevation followed more rapid increases in both arterial and venous pressure in the same limb, suggesting some degree of elevated plasma filtration and lymph formation, injection of saline into the arteries or veins in the same preparation to elicit elevated pressures in these vessels did not produce changes in lymphatic pressure, suggesting that elevated pumping may be responsible for the observed gradual increase in lymphatic pressure (142).

A different line of investigation was performed with bovine mesenteric collecting lymphatics, in which nonmyelinated nerve fibers were found to penetrate into the muscle layer, with varicosities containing small dense granular vesicles (782). Wire myograph studies of these lymphatics were performed to study the impact adrenergic agonists and antagonists on contractions. Generally, norepinephrine produced increased CF, which was blocked by the α-adrenergic antagonist phentolamine, while isoproterenol decreased CF and caused relaxation, which could be blocked by the β-adrenergic antagonist, propranolol (662,

785). Norepinephrine accelerates the frequency of action potentials, which can limit the ability of the vessels to generate a full contraction, reducing force (25). Electrical stimulation of quiescent bovine mesenteric collecting lymphatics elicited consistent contractions. These contractions were sensitive to TTX (786, 788) and could be completely blocked by α-adrenergic antagonists (788). In addition, the same stimulation caused a delayed relaxation in some lymphatics, which was abolished by blocking β-adrenergic receptors. These results suggested local intramural sympathetic nerve activity in the lymphatic wall (788). Data from a recent study with human thoracic ducts also show anatomical and functional innervation with adrenergic and cholinergic fibers that respond to electrical field stimulation and can be inhibited with phentolamine (1059). The functional contribution of this innervation appears to be relatively small compared to the intrinsic mechanisms that control pumping (1059).

Neurotransmitters and Collecting Lymphatic Contractions—A variety of neurotransmitters can elicit changes in the frequency and tone of collecting lymphatics and the thoracic duct. As mentioned above, norepinephrine generally increases CF and can also elevate tone through action on adrenergic α1 receptors (25, 81, 662, 672, 673, 675, 678, 785, 932, 1049, 1060, 1062, 1063, 1069) or in one case, α2 receptors (419). The α receptor stimulation leads to an increase in STDs (672, 1069, 1104). Stimulation of β adrenergic receptors has been reported to inhibit lymphatic contractions through outward K+ current {Allen, 1986 #2911).

Serotonin (5-hydroxytryptamine; 5-HT) has been demonstrated to promote contractions in bovine, canine, rat, and human collecting lymphatic vessels (270, 593, 785, 895, 991, 993), but decrease contractions in sheep and guinea pig collecting lymphatics (181, 677). The differences appear to be due to heterogeneity in the subpopulations of 5-HT receptors, with the 5-HT_2 receptor exerting excitatory and the 5-HT_4 and 5-HT_7 receptors exhibiting inhibitory effects (181, 677, 709). The context may also have an impact, as 5-HT was reported to relax porcine hepatic lymphatic vessels that were preconstricted with norepinephrine (419).

Substance P-positive nerve fibers have been observed widely distributed in and around the smooth muscle layer of human thoracic ducts (692), and are also found in the walls bovine mesenteric lymphatic vessels (946). Substance P was initially found to increase CF of bovine mesenteric collecting lymphatics (348). In guinea pig mesenteric collecting lymphatics, this positive chronotropic effect was found to require an intact endothelium, and was mediated by PLA_2 activation and production of thromboxane A_2 within the endothelium (891). In addition to increasing CF, Substance P has also been reported to increase tone in rat mesenteric collecting lymphatics (29, 247). The mechanism probably involves neurokinin receptors 1 and 3 on lymphatic smooth muscle cells, which results in phosphorylation of myosin light chains (177). Human mesenteric collecting lymphatics were reported to only respond to low concentrations of substance P, while only a high concentration (1 μM) evoked a response in human thoracic duct (1059, 1063).

Nerve fibers that are immunoreactive to calcitonin gene-related peptide (CGRP) have been described in bovine mesenteric collecting lymphatics (946). The response of guinea pig

mesenteric collecting lymphatics to CGRP was studied, and revealed an endothelium-dependent decrease in CF (451). At relatively low concentrations (100 nM), the mechanism involves activation of CGRP-1 receptors on endothelial cells and involves eNOS, cAMP/PKA, and K_{ATP} channels. At a slightly higher concentration (500 nM), CGRP also activated cAMP/PKA and K_{ATP} channels on smooth muscle cells (451).

Neuropeptide Y (NPY) is a marker for nonadrenergic postganglionic sympathetic fibers, which were observed in the human thoracic duct and mesenteric collecting lymphatics (228, 691, 692). Application of NPY to human thoracic duct rings increased the force of contraction and generally elicited phasic contractions (1059).

Cholinergic fibers have been reported in the walls of mesenteric collecting lymphatics of humans and guinea pigs and human thoracic duct (24, 228, 692, 1059). Initial studies of the impact of acetylcholine on collecting lymphatics suggested that it either had no direct effect on pumping, or could cause increased constriction when applied at supraphysiological concentrations (271, 662, 932, 991, 1069, 1073). However, when lymphatic vessels were preconstricted, acetylcholine elicited relaxation or cessation of phasic contractions (323, 419, 1046, 1060). The relaxation is endothelial-dependent and mediated an increase in endothelial $[Ca^{2+}]_i$, the production of NO (419), and a decrease in the size of STDs (1110). In endothelial tubes isolated from mouse popliteal collecting lymphatics, the acetylcholine-induced increase in $[Ca^{2+}]_i$ was evident. However, unlike arterial endothelial cells, which also hyperpolarize, the lymphatic endothelial cells depolarized, due to the absence of K_{Ca} channels (77). This lack of an endothelial hyperpolarization pathway in lymphatic endothelial cells may represent an adaptation to ensure efficient conduction of contraction waves in the adjacent smooth muscle for optimal propulsion of lymph (77).

Vasoactive intestinal peptide is also found in cholinergic nerve fibers. Bovine collecting mesenteric lymphatics were reported to contain nerve fibers that positively label with anti-VIP antibody (787). Infrequent labeling of VIP in nerve fibers was also reported in human thoracic ducts (692). Administration of VIP to precontracted bovine lymphatic vessels elicited relaxation (787). In isolated guinea pig mesenteric lymphatics, VIP was reported to reduce contraction frequency in a concentration-dependent manner (1114). The mechanism involves activation of the VPAC2 VIP receptor, activation of PKA, and opening of K_{ATP} channels, hyperpolarizing the membrane (1114).

NO can also be released from parasympathetic fibers. As noted above, NO causes a decrease in the frequency of phasic contractions by reducing pacemaker activity (1108, 1110, 1118). NO donors elicit a similar reduction in pumping activity and force of contraction (31, 567, 1110).

Influence of Inflammatory Cells and Soluble Mediators

Increased lymph flow is known to generally accompany inflammation. Application of peptides such as fMLP that evoke leukocyte extravasation also produces an increase in lymphatic pump activity and greater lymph flow {Benoit, 1992 #844}. However, the direct impact of immune cells on lymphatic pumping is unclear. Myeloid cells that express iNOS and are $CD11b^+$ and $GR\text{-}1^+$, which could represent monocytes, neutrophils, and eosinophils,

were reported to impair pumping of mouse popliteal lymphatics *in vivo* due to iNOS-mediated release of NO (601). In rats treated with LPS, decreased neutrophil association with mesenteric collecting lymphatics, and increased accumulation of $CD163^+$ $CD206^+$ macrophages (M2 phenotype) accompanied impaired lymphatic contractions, however the cause-effect relationships are not clear (178). In a rat model of metabolic syndrome, macrophages with the M1 phenotype ($CD163^+MHCII^+$) accumulated near mesenteric lymphatic vessels, and were associated with impaired pumping that could be partially restored with blockade of NOS or K_{ATP} channels (1203). Still, the direct impact of these cells on lymphatic contractile activity remains to be determined.

Much work has been done examining a role for mast cells, which are found at a relatively high density near collecting lymphatic vessels in the rat mesentery (182). Intestinal mucosal mast cells also influence downstream intestinal and mesenteric lymphatics when they are activated by certain nutrients (484, 953). In guinea pigs sensitized with cow's milk and then treated with beta-lactoglobulin to trigger mast cell degranulation, mesenteric lymphatic vessel contraction frequency increased, while the contraction amplitude decreased. This response could be reduced with a histamine H1 receptor antagonist, suggesting that histamine was a key mediator of the response (864). In a more recent set of studies, the role of mast cells in modulation of lymphatic pumping was evident with aging. These studies featured Fischer-344 rats, an established model of aging, in which 9-month old rats represent adulthood, and 24-month old rats represent the elderly. Lymphatic contractility and lymph flow are significantly impaired in the elderly rats due to increased inflammation (18, 362, 365, 367, 743, 1067). This includes a higher degree of activation of mast cells in the elderly rats (182). This increase in mast cell activation in the elderly rats is accompanied by decreased reactivity of mesenteric collecting lymphatic vessels to enhance contraction frequency and tone in response to LPS (769). The elevated degree of activation of mast cells in the older rats also correlates with increased baseline activation of NF-κB. This high degree of baseline activation essentially makes any difference due to additional LPS-induced stimulation of NF-κB negligible, which might impair the ability of the lymphatic vessels to react to acute inflammatory stimuli (769). In addition, mast cell degranulation appears to attract eosinophils and MHC class II positive cells to mesenteric collecting lymphatics, however this function is impaired in elderly rats (183). Based on the current data, it appears that mast cells impact lymphatic function through release of histamine and possibly other mediators.

There are some complexities pertaining to how histamine impacts lymphatic pumping. *In vivo*, histamine increases both microvascular filtration and permeability (179, 295), driving increased lymph formation (1109). The result can be seen when histamine is applied topically to the exteriorized rat mesentery, with an apparent elevation of lymph flow in collecting lymphatics having elevated diameter and stronger phasic contractions with no change in frequency (325). Increased lymphatic perfusion pressure in the canine forelimb in response to histamine has also been reported (271). *In vitro*, there have been different responses to histamine with different experimental preparations. Increased rhythmic contractions of isolated bovine lymphatic strips have been reported (497, 785). Concentration-dependent responses were also reported in bovine mesenteric lymphatics, with high concentrations (>5 μM) producing increased contraction frequency, and lower

concentrations (50 nM - 1 μM) lowering contraction frequency (1125), which may be attributable to the different histamine receptor subtypes (347, 1125). In tracheobronchial lymphatic vessel rings, histamine was reported to cause constriction that could be relaxed in an endothelium-dependent manner by bradykinin or acetylcholine (326). On the other hand, in canine thoracic duct, while histamine can elicit constriction on its own, it also can cause relaxation following preconstriction of the vessels by norepinephrine (1046). In porcine lymphatics, histamine also could cause constriction but also endothelial release of NO (898). In mesenteric lymphatics isolated from rats, histamine applied at low concentrations (1 – 10 nM) was reported to increase contraction frequency and amplitude, while application at higher concentrations (1 – 100 μM) elicits endothelium-dependent reduced contraction frequency and amplitude (860). Relaxation of isolated rat mesenteric lymphatic vessels by histamine can be inhibited by pharmacological blockade of NO synthesis with L-NAME or soluble guanylate cyclase activity (567). In addition, when histamine is applied at a relatively high concentration (100 mM), both H1 and H2 histamine receptor antagonists appear to be capable of blocking the histamine-induced lymphatic relaxation (567). Another interesting development has been the evidence of histamine production by endothelial cells of rat mesenteric lymphatic vessels from aged (24 month-old) rats, and the potential of histamine to substitute for NO as an endothelium-derived relaxation factor (770, 771).

Other inflammatory mediators like bradykinin also increase extravasation of protein-rich fluid, and an increase in lymph flow can be expected, as evidenced by the increase in lymphatic perfusion pressure in the canine forelimb after bradykinin infusion (272). In the rat mesentery, bradykinin causes an increase in collecting lymphatic contraction frequency without changes in the end systolic or diastolic diameters (1182) that probably reflect both the direct effect upon the lymphatic vessels and the elevated lymph formation secondary to microvascular leakage of plasma. The direct impact of bradykinin on lymphatic vessels includes both elevated contractions (46, 271, 1060) and relaxant effects through activation of the endothelium and release of NO (105, 323, 1060).

Additional work can also be found pertaining to how inflammatory mediators affect lymphatic pumping. Oxidative stress appears to have an inhibitory effect on lymphatic pumping (1199). Arachidonic acid metabolites appear to have differential impacts on lymphatic contractions. Leukotrienes B4, C4, and D4 have been reported to stimulate contractions (497). The prostaglandin PGH_2 or its analog U46619, which is a thromboxane A2 mimetic, stimulated lymphatic contractions (496, 497, 989, 992, 994) while prostaglandins PGE_1 and PGE_2 can inhibit contractions stimulated by U46619 (497). Cyclooxygenase inhibitors such as aspirin and indomethacin, or the combined cyclooxygenase/lipoxygenase inhibitor BW-755C can reduce spontaneous contractions of lymphatics (495, 496). The isoprostane 8-epi-PGF2α also potently stimulates lymphatic contraction (990). Ultimately, these individual signals comprise a larger, integrated mechanism that contributes to the overall lymphatic contractile cycle.

Gating of Secondary Valves in Collecting Lymphatics

The secondary valves are essential for lymph to flow forward throughout the network, as evidenced by the fact that certain genetic mutations, such as in the *Foxc2* gene, cause loss of

valves and lymphedema (132, 557, 685, 859). The valve leaflets act as passive gates that open or close in response to the trans-valve hydrostatic pressure gradient of the lymph. In addition, the structural properties of the leaflets, low Reynolds number, and highly viscous flow govern the gating position (964). The valve structure is essentially a folded endothelial layer, with the basal sides of the endothelial cells facing each other. In between the two sheets of endothelial cells, collagen forms a connective layer that adds stiffness. In addition, a network of collagen fibers also supports the base of each valve leaflet (665, 881).

The physiology of valve gating has recently been explored with isolated, cannulated lymphangions. To directly study gating in an environment in which the upstream and downstream hydrostatic pressures are tightly controlled, lymphatic vessel segments with a single valve are cannulated with inflow and outflow pipettes from which pressures can be imposed. The vessel may also be relaxed with Ca^{2+}-free bathing solution to prevent the influence of contractions. An additional servo null pipette that pierces the vessel wall and positioned near the upstream side of the valve allows for precise, local pressure changes that affect valve gating. A second configuration allows the study of how the upstream and downstream valves of a lymphangion open and close during the contractile cycle utilizes a two-valve vessel segment mounted on the inflow and outflow pipettes, with a third pipette piercing the wall between the two valves to measure the intraluminal pressure between valves (248, 945).

From investigations utilizing these models, it has become evident that valve leaflets tend favor the open position when there is no trans-valve pressure gradient. The functional implication may be to reduce resistance to flow under conditions in which there a very small pressure gradient, although this can also allow a certain degree of retrograde flow at certain points along the contraction cycle (248). This is also evident in a two-valve segment for much of diastole. However, when the outflow pressure exceeds the inflow pressure, the gating pattern is more like that seen for the ventricular valves of the heart (248). In addition, the ability of the valve to close is dependent in part on the vessel diameter. When diameter is relatively small, such as during systole, an adverse pressure gradient of 0.1 – 0.3 cm H_2O is sufficient for valve closure. However, when the diameter of the vessel approaches its maximum, then much higher trans-valve pressure gradients (several cm H_2O) are required for valve closure. The functional implication is that tone can impact valve gating, and that lymphatic vessels that remain dilated, such as in lymphedema, may have a tendency to have malfunction of their secondary valves (93–95, 248, 957). A “valve lock” mechanism, in which a valve locks open when the lymphatic smooth muscle layer at high outflow pressures becomes fatigued and can no longer drive lymph forward, has been described, and would essentially allow the outflow pressure to be transmitted backward through the lymphangion to its inflow valve (960). If this type of phenomenon were to repeat and be transmitted across several lymphangions, the normal function to prevent large pressure gradients would be lost, causing an overall failure of the vessel to propel lymph forward (960).

Permeability of Collecting Lymphatics

Lymphatic Vessels in Fluid and Macromolecule Balance

Historically, collecting lymphatic vessels were widely considered to be impermeable to substances that had gained entry into the lymphatic vessel lumen, including fluid and protein (664). However, this view has been overturned in the past decade by data from studies that have succeeded in directly quantifying the permeability of individual lymphatic vessels to macromolecules (957–959) and showing differential degrees of solute flux across the lymphatic wall in relation to molecular size (818). This revised understanding of lymphatic endothelial permeability has led to new proposed functions of lymph transport beyond merely maintaining fluid balance.

In the first study to directly determine permeability coefficients of lymphatic vessels, it was observed that both lymphatic capillaries and collecting lymphatic vessels are permeable to fluorescently labeled albumin perfused luminally (959). Because calculated permeability coefficients depend upon direct measurements of solute flux, vessel surface area, and the luminal and abluminal concentrations of a tracer at a constant hydrostatic pressure, to clarify the direction of *in vivo* albumin movement across the collecting lymphatic wall also required measurement of the albumin concentration of lymph, plasma, and interstitial fluid *in vivo*. The measurement of these parameters revealed that the normal gradient for albumin movement across collecting lymphatic vessels is from the lumen to the interstitium (959). In other words, the current evidence suggests that proteins normally “leak” out of collecting lymphatic vessels due to the concentration gradient and higher hydrostatic pressures of these vessels relative to lymphatic capillaries. The same study also showed that the permeability of collecting lymphatic vessels was not different from the permeability of venules, which represent the major site for microvascular leakage of plasma proteins. While at first glance this seems to indicate that the amount of albumin that leaks from collecting lymphatics and venules is the same, it is actually quite different. Collecting lymphatic vessels have much larger diameters than venules, thus higher surface area, and they operate at lower hydrostatic pressures, which reduces solute transport that is carried along with fluid (i.e. convective drag). Therefore, the amount of solute that moves across the lymphatic vessel wall (i.e. solute flux) must be higher than that of venules to maintain the same permeability. Indeed, when lymphatic vessel albumin flux is measured at the higher pressures observed in venules, it is much higher (958). The idea that collecting lymphatic vessels are permeable and that solute is lost from the lymphatic vessel lumen to the tissues seems to be at odds with its well-known role of ensuring fluid balance. However, only a small percentage of the luminal contents are filtered into the interstitium, while the majority is transported downstream to the lymph nodes, thoracic duct, and eventually to the bloodstream.

Early studies performed by Drinker and colleagues (284) demonstrated that another function of the lymphatic vasculature is the transport of large proteins from the tissues to the bloodstream, because the bloodstream typically does not reabsorb leaked macromolecules. It has also long been appreciated that lymphatic vessels have a key role in dietary and peripheral lipid transport to the bloodstream (605, 776). If we consider that collecting lymphatic vessels are not only permeable to protein, but also to lipid, then this helps to

explain why adipose tissue is always located around lymphatic vessels and lymph nodes (417). An interesting consequence of both transporting and leaking lipids into the tissue is that the lymphatic vasculature may play a causative role in obesity when permeability is elevated. Data from a single study to date provides support for this possibility, but remains controversial because it has not been replicated by other groups (418). Finally, lymphatic vessels have been shown to transport antigen derived from tissues, and their permeability enables nearby phagocytic immune cells to acquire antigen (560).

Methods to Determine Lymphatic Endothelial Permeability

Several methods exist for measuring the permeability of lymphatic vessels, including *in vivo, in vitro*, and *ex vivo* approaches. Here, the advantages and limitations of each will be discussed. The relative ease with which lymphatic endothelial cells (LEC) from various sources can be maintained *in vitro* has made cell culture a popular approach for the study of lymphatic permeability. The permeability of confluent cells may be determined by measuring the rate that a fluorescent molecule is able to cross a cell monolayer grown on a Transwell membrane insert, where the fluorescent molecule is added to the "luminal" or apical side fluid compartment and the rate of its appearance in the fluid on the "abluminal" or basal side is measured over time. With this rate, or solute flux, along with the surface area for diffusion, and luminal and abluminal compartment concentrations, the permeability coefficient can be calculated from Fick's First Law of Diffusion (295). Another method is to assess the barrier function of cell monolayers is by measuring the electrical resistance across the layer, which may better represent the permeability to small ions. There are many advantages of studying a single layer of LEC. First, with recent advances in cell culture methods to successfully isolate and grow LEC, the monolayer models can be easily learned and experiments can be performed quickly. In addition, this approach offers the advantages that come with cell transfection, such as gene knockdown or gene delivery (e.g. reporter fusion proteins) when experiments are to be done with human-derived cells, or for which genetically modified mice are not yet available. Additionally, subcellular dynamics that are usually difficult to see in complex *in vivo* environments can be visualized directly in real time (live cell imaging) or after fixation and immunolabeling. Lastly, when interpretations about specific intracellular mechanisms in LEC are needed, LEC monolayer studies offer simplicity with data interpretation. However, these advantages must be weighed against the many limitations of LEC monolayer models. LEC monoculture lacks other cell types known to contribute to a low basal permeability, including smooth muscle cells, and immune cells. The basement membrane matrix properties may also be significantly different. Because cells in culture are not in a round 3-dimensional tube, they may experience pressure and shear stress forces differently from a round vessel. In addition the LEC monolayers cannot develop complex valve structures. Local tissue microenvironment signals from lymph, circulating hormones, or nerves are also absent. Further, cultured LEC tend to have at least a 10-fold higher basal permeability to albumin than reported using *in vivo* or *ex vivo* methods; therefore the ability to measure their responses to various stimuli will likely be diminished or may have widely different time courses from the responses *in vivo* (220, 870, 919). This is most likely similar to the pro-inflammatory phenotype that is adopted by blood endothelial cells in culture (220). It is also unknown to what extent that LEC in culture reflect a lymphatic capillary or collecting vessel identity.

Another popular approach to assess lymphatic vessel integrity relies on the subcutaneous injection of an opaque dye into the mouse ear, where the lymphatic vessels rapidly absorb it, becoming blue in appearance. Most commonly, a 1% solution of Evans Blue dye is used. After some time has passed, images of the lymphatic vessels are obtained to show the extent to which the blue dye has leaked from the lumen into the surrounding tissues of the ear. The advantages of this method are that it enables investigation of lymphatic vessel leakage *in vivo* under physiologic conditions, it is relatively easy to perform, and it can be used in conjunction with knockout mice. However, one must be careful to avoid making the assumption that solute leakage is directly related to solute permeability. At best, solute leakage correlates with solute flux, i.e. the amount of solute that crosses the endothelial barrier over time, which is affected by both the permeability of the vessel and the degree of filtration due to hydrostatic and osmotic pressure gradients. The Evans Blue dye injection method also is limited in that it requires uptake of dye-labeled albumin from the interstitial space, for which the rates could vary, depending upon the interstitial fluid pressure. High concentrations of dye will likely result in most of the dye being unbound to albumin; thus the leakage better reflects the smaller molecular weight of the dye. The volume of dye injected into the ear from a syringe is usually not controlled very well, so interstitial pressure and more importantly lymphatic vessel pressure are unknown and uncontrolled, both of which greatly affect solute flux. Because of these limitations, it becomes difficult if at all possible to compare two different groups of knockout mice to determine which has a higher degree of leakage. Once dye has been injected into a mouse ear, it cannot be injected again after a treatment, so this technique is not repeatable.

To date, a single study has directly determined the permeability of individual lymphatic capillaries and collecting lymphatics *in vivo* (959). This approach involved cannulating single lymphatic vessels in the rat mesentery with a small glass micropipette and perfusing the vessel with a fluorescent solute. While the advantages are that this method is quantitative, provides direct measures of a known vessel type, and permits pharmacologic interrogation, it is relatively low throughput, technically challenging, and labor intensive. Only vessels not completely covered with adipose tissue can be easily cannulated, and rat mesenteric collecting vessels spontaneously contract, so their volumes and fluorescence are constantly changing. Finally, the hydrostatic pressure of the perfusate must be higher than the pressure in the vessel, so it lacks complete control over this variable.

Circumventing problems with the latter method, the same group developed a novel *ex vivo* assay similar to those used in the *in vivo* studies (958). With the *ex vivo* assay, collecting lymphatic vessels are surgically excised from the mouse mesentery and then cannulated on two glass micropipettes (Fig. 34). An advantage of using mouse mesenteric collecting lymphatic vessels is that these vessels lack robust, spontaneous contractions so the diameter, thus volume, of the vessel remains constant resulting in rapid measurements (< 5 min). Further advantages are that this method enables full control over hydrostatic pressure, osmotic pressure, flow gradient, and luminal and abluminal solution contents. This in turn facilitates the addition of pharmacologic agonists/antagonists, function-blocking antibodies, or other chemicals to investigate mechanisms. Because this approach is used with mice, it also allows the use of the abundant knockout or transgenic mice available to investigate the specific roles of selected genes in the control of permeability. The limitations of this model

are that it requires the study of vessels outside of their normal microenvironment, technical expertise in careful dissection and cannulation of small vessels, and only collecting lymphatic vessels can be studied because lymphatic capillaries are too thin and fragile with current cannulation methods.

Lymphatic Vessel Permeability in Disease

Recently, it has been recognized that there is a link between lymphatic valve development and the regulation of lymphatic permeability (945). Thus, it would be exciting to test whether valve dysfunction and/or severely increased lymphatic permeability are causative in primary lymphedema. Additional physiological mechanisms have been identified in the development of secondary lymphedema and include defective lymphatic smooth muscle contractions (1019) or T-cell mediated inflammation in the tissue secondary to lymph stasis (45). Considerably more is known regarding the pathophysiology of secondary lymphedema due to the development of several mouse models of secondary lymphedema, whereas there are currently no mouse models of primary lymphedema that recapitulate the severe swelling observed in human patients.

Aside from the obvious importance of the lymphatic networks in preventing edema, recent advances have uncovered new roles for lymphatic vessels in several major cardiovascular diseases, including obesity, hypercholesterolemia, atherosclerosis, and type 2 diabetes (418, 604, 605, 652, 958). Interestingly, in all of these metabolic diseases, the lymphatic vessels were shown to be excessively leaky.

In mice haploinsufficient for *Prox1*, a transcription factor that regulates cell fate in various organs, lymphatic vessels were shown to leak lipid-rich lymph into the tissues, suggestive that lymphatic permeability is increased (418). Lymph was then shown to stimulate adipogenesis *in* vitro as an explanation for the adult onset obesity. Because *Prox1* is expressed in many metabolically active organs, it leads to the question of whether or not the obesity in this mouse model is lymphatic-specific. Additionally, this is the only mouse model of lymphatic defects that develops obesity. However, it was recently shown that overexpression of *Prox1* in lymphatic endothelium on the *Prox1* global heterozygous background does rescue the obese phenotype (314). Others have suggested that obesity leads to chronic inflammation that can then lead to disrupted lymphatic barrier function (431). In combination, these two concepts suggest that there is potential for a positive feedback loop between lymphatic permeability and obesity, which may drive constitutive adipose deposition and inflammation.

Another metabolic state that leads to leaky lymphatic vessels is hypercholesterolemia (604, 605). Mice deficient in *ApoE* that are placed on an atherogenic diet become hypercholesterolemic. When an opaque dye is injected into the ear, the lymphatic vessels rapidly absorb, then leak this dye into the surrounding parenchyma, indicating severe leakage (604). In a later study, the same group demonstrated that the lymphatic leakage could be rescued by normalizing the plasma cholesterol levels, demonstrating that *ApoE* was dispensable for normal lymphatic function (605). Intriguingly, lymphatic vessels from hypercholesterolemic mice downregulated *Foxc2* expression and had excessive mural cell

coverage. Loss of *Foxc2* also leads to disrupted VE-cadherin at cell-cell junctions (945), so this may explain why lymphatic vessels become leaky in this model.

Because obesity might lead to leaky lymphatic vessels through chronic inflammation, and humans with obesity also develop type-2 diabetes, another study examined whether lymphatic vessels become dysfunctional in a mouse model of type-2 diabetes (958). In *db/db* mice that had been obese and hyperglycemic for 20–30 weeks, the permeability of collecting lymphatic vessels was drastically elevated in *ex vivo* and *in vivo* assays. Pharmacologic agonists that augmented nitric oxide production led to a significant rescue of the permeability, identifying lymphatic endothelial dysfunction as a new component of type-2 diabetes.

Regulation of Lymphatic Vessel Permeability by Immune Cells and Inflammatory Mediators

The lymphatic vasculature is crucial for the transport of immune cells to local lymph nodes for the initiation of immune responses (885). Like blood vessels that are affected by immune cell transport and transmigration, lymphatic vessel function is also regulated by nearby immune cells. A subset of cells expressing dendritic cell (DC) markers Cd11c and MHCII were found to congregate around collecting lymphatic vessels in perinodal adipose tissue (560). Upon closer examination these DC were actually identified to be within the lymphatic vessel wall between adjacent smooth muscle cells. These DC occasionally had pseudopodia extending into the lumen to acquire antigen, and upon activation could migrate to lymph nodes. Since this behavior would require interaction with the endothelium, it was hypothesized that DCs within the lymphatic vessel wall could regulate the permeability of these vessels (466). When $Cd11c^+$ cells were selectively ablated with diphtheria toxin for 48 h, collecting lymphatic vessel permeability was elevated approximately 10-fold. Further, when mice lacking either *Ccr7*, which encodes a receptor required for DC homing to lymphatic vessels, or *Irf4*, a transcription factor that regulates the differentiation of $Cd11b^+$ DCs, were examined, collecting lymphatic vessel permeability was similarly elevated. The results of these two reports suggest that DCs are situated within collecting lymphatic vessel walls to sample antigen, and once it is acquired DCs may collectively migrate to lymph nodes. This would leave the collecting lymphatic vessels relatively devoid of resident DCs, substantially elevating the permeability so that antigen would travel into the tissues faster. Such a mechanism may allow lymph-derived antigen to reach memory T cells in adipose tissue to stimulate a T cell recall response.

In another study, oral gavage of *Yersinia psuedotuberculosis* led to an acute immune response that resolved completely after 4 weeks, and the bacteria were cleared, but the collecting lymphatic vessels in the mesentery became persistently leaky (342) The authors concluded that the leaky lymphatic vessels may lead to impaired immune responses to subsequent pathogenic infections (342). A related finding is that local methicillin-resistant *Staphylococcus aureus* infection has been shown to damage the smooth muscle layer of the draining collecting lymphatics in mice (498). While this study focused on the impairment of pumping mechanisms, it is conceivable that the damage to the muscle layer and resulting reduction in lymph flow would favor increased leakage of lymph across the collecting lymphatic wall.

Immune cells are well known to produce a variety of cytokines and other factors that are known to modulate blood vessel permeability. Many of these cytokines are now being demonstrated to similarly target lymphatic endothelium (23, 127, 213, 958). The pro-inflammatory cytokines TNF-α, IL-6, IL-1β, and IFN-γ were shown to increase lymphatic endothelial permeability 1.5–3-fold in cell culture and increased leakiness *in vivo* (23, 213). Likewise, histamine has been shown to exert effects on lymphatic permeability, albeit transiently, in both cell culture and *ex vivo* systems (127, 958). While LPS has been shown to increase in LEC monolayer permeability, it only stimulated a 1.5-fold increase. Whether a response of this same magnitude would be obtained *in vivo* is unknown, but should be the subject of future work focused on these cytokines.

Mechanisms of Lymphatic Permeability Regulation—The cellular mechanisms regulating the permeability of postcapillary venules have been extensively studied and include signaling through various receptors, junctional proteins, cAMP/cGMP, Ca^{2+}, nitric oxide, MLCK, RhoA/ROCK, and shear stress pathways (294, 295, 1186). How these pathways affect lymphatic endothelium are likely to be quite different due to a lower hydrostatic pressure, higher sensitivity to shear stress that regulates intraluminal valve formation, and a lack of red blood cells that release sphingosine-1-phosphate.

Several junctional adhesion and associated proteins have been identified in lymphatic capillaries and collecting lymphatic vessels, including VE-cadherin, Pecam-1, β-catenin, claudin 5, ZO-1, Occludin, Jam-A, and Esam-1 (61). Proteins that are classified as belonging to more classical epithelial adherens and tight junction families are both present and form structures similar to those seen in blood vessel endothelial cell-cell connections. However, little is known about how each of these molecules contributes to lymphatic permeability regulation. Interestingly, mice with a lymphatic-specific deletion of β-catenin have severe defects in the formation of lymphatic valves and are embryonic lethal, providing another potential link between defects in valve formation and permeability regulation (174).

One possible explanation for the link between valve formation and the control of permeability is that lymphatic endothelium is sensitive to shear stress, and shear stress may directly regulate permeability. One study demonstrated that LEC change their permeability with exposure to different levels of shear stress (129). This change in barrier function was sensitive to inhibitors of actin cytoskeletal dynamics and to the small GTPase Rac1. In addition, activity of Rho kinase (ROCK), which is the downstream effector of the small GTPase RhoA, appears to be important for maintaining baseline LEC monolayer barrier integrity (127). Likewise, the Slit2/Robo4 pathway, which can activate the RhoA/ROCK pathway and also influence $\alpha_5\beta_1$ integrin localization, was recently shown to contribute to enhanced LEC monolayer barrier function (1215).

Lymphatic vessel permeability appears to be differentially regulated by nitric oxide, depending on the health state of the endothelium (958). Under healthy conditions, nitric oxide stimulates an increase in lymphatic vessel permeability through as of yet unknown mechanisms. In diseased states where nitric oxide production is impaired, stimulation of endogenous nitric oxide production leads to a lower permeability, suggesting that a basal level of nitric oxide signaling is needed for normal barrier function. In the same model,

inhibition of phosphodiesterase-3 reduced lymphatic permeability by preventing degradation of cAMP. In other contexts, direct administration of cAMP analogs has been shown to enhance barrier function of LEC monolayers or tubes (127, 870).

Much about the control of collecting lymphatic vessel permeability remains unknown, and its potential roles in the pathogenesis of many diseases represents an exciting new topic of study. Future efforts will be directed at determining how different neural, endocrine, paracellular, and immune signals impact collecting lymphatic permeability. In addition, future studies of how lymphatic endothelial cells actively participate in the control of collecting lymphatic barrier function is a growing area under examination.

Filtration at Lymph nodes

With few exceptions, lymph is delivered by collecting lymphatics to at least one lymph node prior to entry into the thoracic duct. Here, immune cells can sample the lymph contents for antigens (546). This biological filtration through phagocytosis and retention of certain cells in the nodes causes postnodal lymph to be dramatically different from prenodal lymph in terms of cell types present. In prenodal lymph, monocytes, macrophages, and dendritic cells are often present, but these are largely absent in postnodal lymph (426). In contrast, lymphocyte counts can be higher in postnodal lymph due to exiting from the lymph nodes into the postnodal lymphatic vessels (546).

In addition to biological filtration, lymph nodes also mechanically filter the lymph. The blood-lymph barrier within the nodes allows passage of protein-free fluid according to the Starling forces. The result is that fluid is lost from the prenodal lymph into the blood, which increases the protein concentration to be higher in postnodal lymph than in prenodal lymph (8, 9). Thus, combined biological and mechanical filtration form postnodal lymph that is different in both solute and cellular composition from prenodal lymph.

Lymphedema

Lymphatic vessels are required for maintaining tissue fluid balance and, consequently, failure of lymphatic networks to adequately perform this function manifests in lymphedema. Lymphedema is classified as primary if it results from inherited genetic mutations and secondary if it results from trauma, such as surgery, radiation, or obstruction (e.g. filarial parasites). While lymphedema is not typically life threatening, it is a lifelong disease that depending upon its severity can feature a range of mobility problems, pain, and susceptibility to skin infections. Treatment is through compression garments and therapy. Because the disease is life-long, management typically needs a close physician-patient relationship.

Classifications of primary lymphedema are traditionally based upon clinical features, stage of life at which it presents (birth, puberty, or adulthood), and lymphoscintography findings (143). As mentioned above in the section on lymphatic development, many of the gene mutations involved in lymphedema contribute the development of valves or lymphatic endothelial integrity (140, 577, 859). In the case of Milroy disease, caused by mutations of either VEGFR3 or its agonist VEGF-C (131, 328, 384, 516), both impaired fluid absorption

in the initial lymphatics was initially proposed as a disease mechanism (684). However, more recent findings that VEGFR3 contributes to lymphatic valve formation and function suggest that valve dysfunction may also be a critical factor (617). In lymphedema distichiasis syndrome, caused by mutation of FOXC2 (317), patients present with aberrant eyelashes at birth, but the onset of lymphedema typically occurs at puberty or later in life, sometimes as late as over 50 years of age (132). The mutation affects both lymphatic and venous valves and can also cause abnormal appearance of smooth muscle around initial lymphatics (683, 859). Mutations of these genes and the several others associated with other types primary lymphedema point to the critical physiological function of lymphatic valves for maintaining a normal "safety factor" against the development of tissue edema.

Secondary lymphedema is much more frequent than primary lymphedema, and is commonly observed after removal of axillary lymph nodes in association with breast cancer surgery. The result is severe arm swelling that develops months to years after the operation. The onset and degree of secondary lymphedema in breast cancer patients who have undergone surgery is highly variable, and the pathophysiology is an intense area of investigation. One major risk factor for the development of secondary lymphedema is obesity, (393). Genetic risk factors are only beginning to be investigated. The same genes involved in primary lymphedema, i.e. those involved in lymphatic valve development and maintenance may contribute to susceptibility. To date, gene variants of *FOXC2* and *FLT4* (encodes VEGFR3) have been identified in lymphatic filariasis, the most common form of secondary lymphedema worldwide, and caused by infection with filarial worms, primarily *Wuchereria bancrofti* (56, 974). In addition, SNPs in the genes that encode VEGFR3, MMP-2, and carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM-1), all of which have roles in lymphangiogenesis, have also been identified to have significance in lymphatic filariasis (254). It is plausible that these gene variants may have a moderate impact lymphatic wall integrity or lymphatic valve maintenance, lowering the "margin of safety" against edema with high fluid loads, while not impairing lymph flow under normal fluid loads.

Several changes in the local tissues occur with the development of lymphedema. The accumulation of fluid is associated with increased infiltration of neutrophils, macrophages, and dendritic cells (1044). The inflammatory environment causes a slow, progressive tissue remodeling. Fat deposition increases, and in the skin cellulitis and erysipelas can develop (139). These infections are associated with altered immunity due to slower lymph clearance and that may impair dendritic cell migration to lymph nodes (34). Other tissue remodeling including fibrosis, affects overall tissue function (45, 1044, 1192, 1195, 1212). The inflammatory environment also appears to affect interstitial pressure (1142) and normal collecting lymphatic pump responses to changes in body position are impaired (806, 807). In addition, inflammation can cause the presence of excessive VEGF-C, abnormal lymphatic remodeling into enlarged and dysfunctional lymphatic vessels, and additional immune cell infiltration (387, 937). In more advanced lymphedema, evidence of impaired contraction of collecting lymphatics has been reported (714, 1019). Women who displayed higher lymphatic pumping pressures and lymph transport in their arms prior to breast cancer surgery were found to more likely develop secondary lymphedema (190). Blockage of collecting lymphatic vessels due to improper overgrowth and invasion of the smooth muscle

layer into the lumen has also been reported in patients with leg secondary lymphedema (781). This “lymphaticosclerosis” in combination with other observations of impaired contraction suggests that lymphatic smooth may enter a proliferative phase during lymphedema, similar to the synthetic vascular smooth muscle phenotype in atherosclerosis following angioplasty (1080).

Several animal models of secondary lymphedema have been developed to better understand the pathophysiological changes in lymphedematous tissues. In earlier studies, secondary lymphedema was produced surgically in the dog and rat hindlimb or rabbit ear (414, 514, 804, 1043, 1120). These models involve excision of skin and subcutaneous tissue to disrupt lymph flow, and can cause significant tissue volume increases. While the larger animal models may have more relevance to human anatomy, mouse models offer the ability to utilize genetic approaches not readily available in other species. Thus, the skin excision approach has also been widely used with the proximal mouse tail, producing significant edema, lymphatic hyperplasia, and elevated expression of VEGF-C (936, 997, 1033). This model has remained quite popular and has recently been used in efforts to ameliorate lymphedema through anti-inflammatory approaches (746, 1070), discussed in more detail in the next section. Secondary lymphedema in the mouse hindlimb have also been developed and are considered more relevant anatomically. Initial success at producing secondary lymphedema was obtained from a protocol of irradiation of the groin combined with ligation of lymphatic vessels (779). Because of concerns of radiation-induced depletion of myeloid cells as a confounding factor, the model was refined to produce hindlimb lymphedema by surgically removing the inguinal and popliteal lymph nodes and surrounding fat pads. This model produced significant edema and expansion between the muscle and epidermis, increased skin thickness, dilated and abnormal lymphatics, and elevated VEGF-C, VEGFR-3, and Prox1 mRNA (467). Collectively, the ongoing refinement of these models represents an important step toward better understanding of the pathophysiology of lymphedema.

Obesity, Lymphatics, and Lymphedema

Obesity is an epidemic of the 21st century, with worldwide prevalence vastly increasing and most of the adult population either overweight or obese (334). Even more alarming is the increasing rate of pediatric obesity. There is growing evidence of a reciprocal connection between lymphatic dysfunction and obesity (681). Obesity has long been known to be a risk factor for secondary, but more recently it has also been shown that extreme obesity can cause lymphedema in the absence of other risk factors (393, 715, 1078). There is also increasing clinical evidence to suggest that obese patients are predisposed to secondary lymphedema and primary lymphedema can induce adult onset obesity (330).

Animal models also show a connection between obesity and lymphatic dysfunction. Obese mice were shown to have impaired lymphatic transport, and decreased dendritic cell migration to lymph nodes (1132). Additionally, in a mouse model of lymphedema obese mice were shown to have impaired lymphatic function associated with increased inflammation, fibrosis and adipose deposition (955). The association between obesity and inflammation has been known for decades. Obesity-induced inflammation likely begins in

the fat cells themselves. Lymph is a fluid rich in emulsified lipids and fat deposition develops first along the lymphatic structures, and all lymph nodes are embedded in adipose tissue. However, the causal relationship between fat deposition and lymph still remains unclear (1113). What is becoming more evident is that lymph stasis and/or fluid leakage from lymphatic vessels may promote fat accumulation (940). A disruption or malfunction in lymphatic drainage, that leads to lymphedema and increased interstitial fluid that is chronic, results in a predisposition to increased fibroblasts, adipocytes and keratinocytes in the tissue (980). Accumulation of this fluid leads to fibrosis as well as decreased oxygen tension and macrophage function (1184). Obesity is considered a predisposing condition to fibrosis. With lymphedema, the lymphatic stasis initiates a cycle of inflammation-progressive tissue fibrosis-worsening lymphatic function, which over time leads to end organ failure of the lymphatic system (45, 187).

While haploinsufficiency of Prox1 leads to adult-onset obesity in mice (418), a clinical observation was seen in patients with familial combined hyperlipidemia (FCHL) which have a deficiency in PROX1 and/or FOXC2, both necessary for the process of lymphatic vessel formation (1113). The data from this study suggest that alterations in lymphatic system can contribute to the FCHL phenotype (449).

Mouse models with deletion of *Flt4* (Vegfr3), an important receptor for normal lymphangiogenesis, develop abnormal subcutaneous fat deposition (519). Normal lymphangiogenesis involves a delicate balance between pro and anti lymphangiogenic cytokines, which induce lymphatic repair (1193). Yan et al. demonstrated that stimulation of adipose derived stem cells with VEGF-C or inhibition of TGF-β1 induces lymphangiogenesis (1166). Hypoxia and inflammation increase HIFIα expression and this can coordinate lymphangiogenesis (1194). Decreased expression of HIFIα decreases expression of VEGF-C (1194). Lymphangiogenesis results in decreased lymphatic fluid stasis. This is a recurring cycle since stasis leads to inflammation and antilymphangiogenic cytokine expression, which also leads to decreased lymphatic function and worsening edema. It has been shown that lymphedema and lymphatic stasis result in CD4+ cell inflammation and infiltration of mature T helper cells while loss of CD4+ cells decreases the pathological changes associated with lymphedema (1195). CD4+ cell depletion was also shown to inhibit fibrosis and fibrosis is a critical regulator of lymphatic function and regeneration and a hallmark of lymphedema (45, 1195).

Impaired lymphatic flow has been shown to cause inflammation and upregulation of adipocyte differentiation genes and expression of adipokines (38). In addition to fatty acids the largest secretory product of adipose tissue is adipokines, which are also involved in inflammation. Adipocytes are very important to metabolic function, small adipocytes promote metabolic homeostasis, whereas large adipocytes recruit macrophages and promote inflammation (392). IL-6 is a known regulator of adipose homeostasis in obesity, and is elevated in primary and secondary models of lymphedema. Results in another study demonstrated the expression of IL-6 was associated with adipose deposition and CD4+ inflammation and was markedly decreased in CD4KO mice (224). In addition, loss of IL-6 function resulted in significantly increased adipose deposition after tail lymphatic injury. The observations in these studies suggested that IL-6 is increased as a result of adipose

deposition and CD4+ cell inflammation in lymphedema and that IL-6 expression in lymphedema acts to limit adipose accumulation (224).

In addition to all the findings mentioned earlier, mice with hypercholesterolemia have been shown to exhibit lymphatic dysfunction and vessel degeneration (604). Collectively, these findings indicate an important, interrelationship between lymphatics and adipose tissue. We think that this relationship contributes significantly to the development of disease caused by obesity, and thus could also serve as a potential target to ameliorate or even reverse the progression of obesity-induced disease, and more efficiently bring obese individuals to a leaner phenotype.

Clinical treatment for lymphedema has long been limited to physical therapies including compression garments. However, there has been one recent key advance that may lead to a medicinal therapeutic. Ketoprofen, a non-steroidal anti-inflammatory drug has been shown to reverse histopathology in experimental lymphedema in mice (746). More recently, the beneficial effects of ketoprofen were attributed to its specific ability to antagonize LTB_4, which was found to be elevated in lymphatic fluid during lymphedema (1070). These results show promise for a future drug target for lymphedema.

Gastrointestinal Lymphatics and Inflammation

An additional topic pertaining to inflammation, injury, and disease processes is how lymphatic vessels and networks adapt over time to disease and trauma in the gut. Trauma and inflammatory bowel diseases have been widely studied and have important lymphatic components. Other conditions such as portal hypertension or alcohol intake also have apparent impacts on lymphatic vessel function.

Inflammatory bowel diseases include ulcerative colitis, an inflammatory disorder along the length of the colonocyte layer of the colon, and Crohn's disease, which can affect any part of the GI tract and involves inflammation of multiple tissue layers. Increased lymphatic vessel numbers, along with lymphangiectasia are observed in both ulcerative colitis and in ileal and colonic Crohn's disease (508, 853). Lymphocytic and granulomatous lymphangitis of the gut lymphatics was one of the earliest noted pathologies in Crohn's disease (109, 206, 552, 620, 680, 910, 1105, 1124). The leakage of lipids from damaged lymphatics was proposed to act as a chronic irritant that perpetuated the inflammation (1124). Such leakage may also be connected to the expansion of normal mesenteric fat beyond the mesentery and onto the intestinal wall, known as creeping fat and a hallmark of Crohn's disease affected areas (200, 886). In rats and pigs, experimental obstruction of regional intestinal lymphatics produced inflammation in the intestine akin to that seen in inflammatory bowel disease (509, 510). Recent work has also described that B cells and IL-33R+CD3- cells were found in the collecting lymphatic vessel walls in creeping fat and may contribute to collecting lymphatic vessel remodeling (884).

Another important role of gastrointestinal-derived lymph is its role in the development of systemic inflammation following traumatic injury. It has long been known that cannulating the thoracic duct and diverting its lymph out of the body can cause immunosuppression

(287, 1086). This finding was later used to demonstrated that lymph derived from the gut following shock could promote systemic inflammation, lung injury, and multiple organ failure (171, 230, 255, 256, 379, 381, 643, 969, 1149, 1176, 1191). Mesenteric lymph collected after experimental shock has been shown to cause increased inflammatory adhesion molecule surface expression on endothelial cells (11, 125), endothelial apoptosis or injury (243, 250, 257, 626, 1095), priming of neutrophils (10, 11, 14, 250, 1095), and suppression of hematopoiesis (35, 258). As reviewed in the previous section on proteins in lymph, the proteome of post-shock lymph has been an intense area of investigation for the identification of biomarkers and key cytokines that may trigger systemic inflammation (244, 397, 507, 659, 872). Other types of potential signals such as microRNAs, bioactive lipids, or metabolites have also been areas of investigation in gut injury or in pancreatitis (102, 500, 716).

Additional factors that can affect the content and transport of mesenteric lymph include oral consumption of alcohol and local changes in blood pressure. Acute alcohol intoxication has been shown to decrease myogenic responsiveness of mesenteric collecting lymphatics in association with changes in RhoA activity levels and Ca^{2+} storage (1005, 1007, 1008). Alcohol ingestion also promotes inflammatory signals in the mesenteric fat and lowers plasma adiponectin levels (1009). Portal hypertension may also detrimentally affect mesenteric lymph flow. Experimental venous hypertension for several days was reported to cause a decrease in bovine mesenteric lymphatic vessel contractions, in association with decreased $[Ca^{2+}]_i$ (275). A sizeable number of other scenarios that could influence lymph composition and flow from the gut remain to be studied, yet it is clear that lymphatic vessels have a key role in the development of either local or systemic inflammation in response to the conditions in the gut.

New Directions: Microparticles, Nanoparticles, and Immunotherapy

Cells are known to release membrane vesicles called exosomes or microparticles into the extracellular environment. These extracellular vesicles serve as a mode of intercellular communication through the transfer of proteins, lipids and RNA between cells. Exosomes from different cell types contain distinct endosome-associated proteins as well as mRNA and non-coding RNA (889). Extracellular vesicles are involved in numerous physiological processes and have an important role in immune regulation. Notably, they can mediate immune stimulation or suppression and drive inflammatory, autoimmune and infectious disease pathology (913). This property, and the fact that exosomes can carry small bits of genetic information make them potential therapeutic devices, possibly for future gene therapy approaches (913). Exosomes have been shown to preferentially enter lymphatic vessels and can be transported to lymph nodes within minutes (1016). The functional role may be to prime cells in the lymph node ahead of the arrival of antigen present cells or other immune cells (1016). Exosomes isolated from the conditioned media of HEY cells treated with the TLR3 agonist poly-IC were found to be retained in lymph nodes when compared to control exosomes, and enhanced lymph flow and neutrophil recruitment to the lymph nodes (1015).

Due to the importance of lymphatic vessels in immunity, they represent an important transport route in drug delivery. The optimum range for lymphatic uptake of subcutaneously injected particles is 10–80 nm in diameter (1156), which is very similar to the diameter range of extracellular vesicles, which is 30–100 nm (913). Thus, engineering safe injectable biomaterials that can be encapsulated into microparticles for delivery represents a new and important avenue of investigation.

Nanoparticles, microparticles/exosomes and biomaterials are advantageous in vaccination because of the opportunity to modulate specific characteristics of immune responses (32). Lymph nodes and lymphoid organs play a crucial role in coordinating the type and specificity of these responses (32). Intralymph node injection (i.LN) may potentially serve as a universal strategy to enhance vaccine potency. The injection of antigens/adjuvants directly into LNs has shown promise for improving the potency of DNA, RNA, peptide, protein and dendritic cell-based vaccines (480). Efficient delivery of vaccine components into LNs is critical for effective immune response because antigen that fails to reach lymphoid organs may be effectively ignored by the immune system (480). The LN is a microenvironment for therapeutic contexts such as cancer immunotherapy, immunomodulation for allergy or autoimmune disorders (480). Furthermore, the direction of cancer therapy will involve combining nanocarrier technologies with loco regional therapy to the lymphatics. This has capacity to reduce non-specific organ toxicities and increase chemotherapeutic does (1156).

An additional emerging topic worth noting is the role of lymphatic endothelial cells in antigen presentation. In mice, LECs within the lymph node have been found to actively scavenge injected fluorescently-tagged ovalbumin antigen (437, 1051). LECs also can present the antigen to $CD8^+$ T cells and induce their proliferation (437). Moreover, LECs within the lymph nodes have also been shown to archive antigens for an extended period after antigen challenge (1051). While LECs cannot present the archived antigens to $CD8^+$ T cells, they can exchange these antigens with migratory dendritic cells, either directly or by LEC apoptosis, which then present the archived antigens to T cells (530). These findings suggest that LECs participate in immune tolerance and memory, and open an exciting new area of investigation the field.

Conclusion

New discoveries are advancing knowledge of lymphatic physiology at a significant pace. This pace will likely accelerate with the continued development of genetically modified mice used in combination with new methods to study subcellular events concurrently with lymphatic functions (77). Recent advances in understanding of the physiology of lymphatic vessels are also informed in large part from the new insights into the development of lymphatic networks and their ultimate anatomical structures in the different organs of the body (795, 1169). Likewise, concurrent advances in immunology have provided the tools to investigate cellular entry into and transport within lymphatic vessels (885). In some cases, new molecular tools have facilitated the rediscovery of lymphatic vessels by certain fields that had forgotten about their importance (624).

Continuing investigation of lymphatic physiology is an important priority for continued understanding of disease. Several problems remain, such as the underlying genetic and environmental factors that cause such a wide degree of secondary lymphedema outcomes in breast cancer patients. New tools to investigate genomic differences, and confirmation of functional differences will help provide the answers to this daunting puzzle.

Ongoing study of the physiological mechanisms of lymphatic pumping, including contractile mechanisms, valve function, local inflammatory signals, and potential long-term remodeling must also remain a priority for build on recent success (1070) and foster new successes with the development of new pharmacological therapeutics for treatment of edema and lymphedema. With the tight connection between immunity and lymphatic function becoming more apparent, the physiology of lymphatic vessels and networks also has significance for broader topics such as microbiome-host interactions, wound healing and regenerative mechanisms in tissues, and drug delivery guided by nanoparticles or other novel systems. Such advances will be guided by teams of physiologists working with experts in microbiology, immunology, bioinformatics, chemistry, physics, bioengineering, and a wide variety of other fields.

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Didactic Synopsis

Major teaching points:

- The lymphatic system has critical roles in fluid homeostasis, immunity, and lipid absorption.
- Development of the lymphatic system is under tight control of specific genes.
- Specialized initial lymphatic vessels (lymphatic capillaries) absorb interstitial fluid to form lymph. The mechanism of lymph formation involves microscopic one-way valve leaflets located at the junctions between initial lymphatic endothelial cells.
- Larger, collecting lymphatic vessels that have a muscle layer propel lymph forward through the network. Action potentials in the smooth muscle elicit phasic contractions of these vessels. Intraluminal bicuspid valves within these vessels prevent backflow of lymph.
- Lymphedema, which is edema due to lymphatic insufficiency, has been associated with many gene mutations that affect the intraluminal valves in collecting lymphatics.

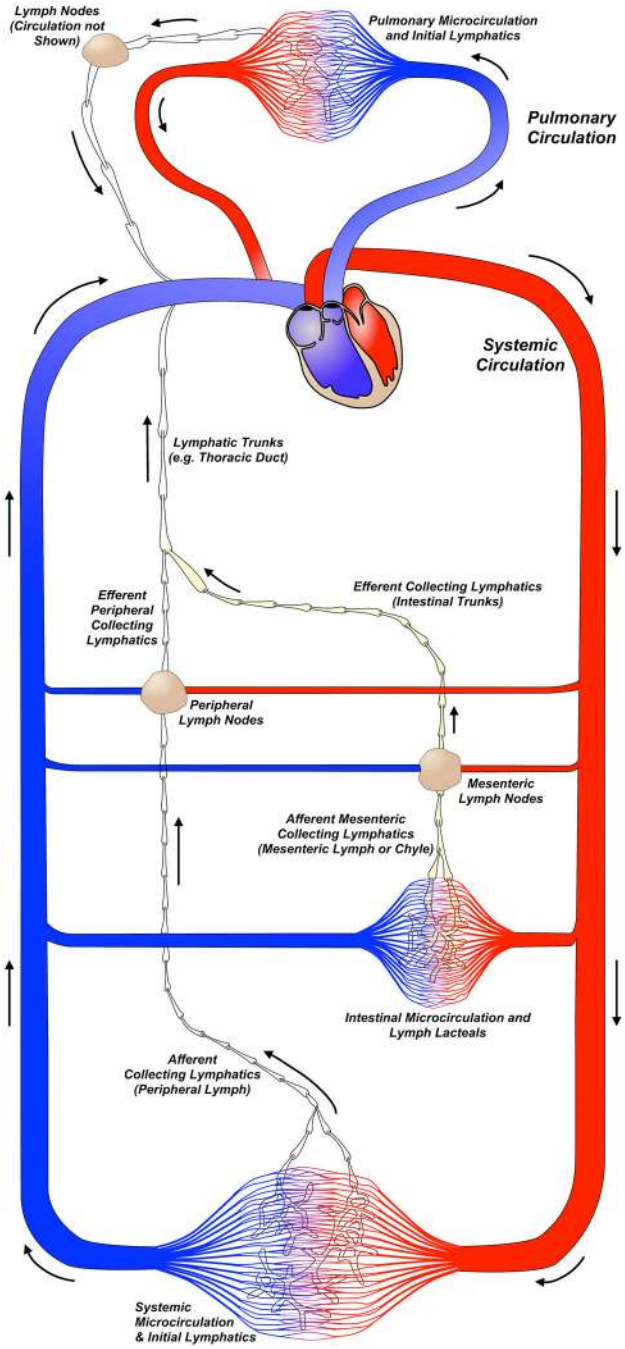

Fig. 1.

Diagram representing the blood and lymphatic circulation in mammals. Filtered plasma forms interstitial fluid that enters the initial lymphatics to become lymph. In the intestine, a significant amount of dietary lipids accompany the absorbed fluid, producing a milky lymph, or chyle. Lymph is transported through afferent collecting lymphatics to lymph nodes where for immune surveillance. Efferent collecting lymphatics then transport the postnodal lymph to larger trunks, which return it to the great veins. Arrows indicate the direction of transport.

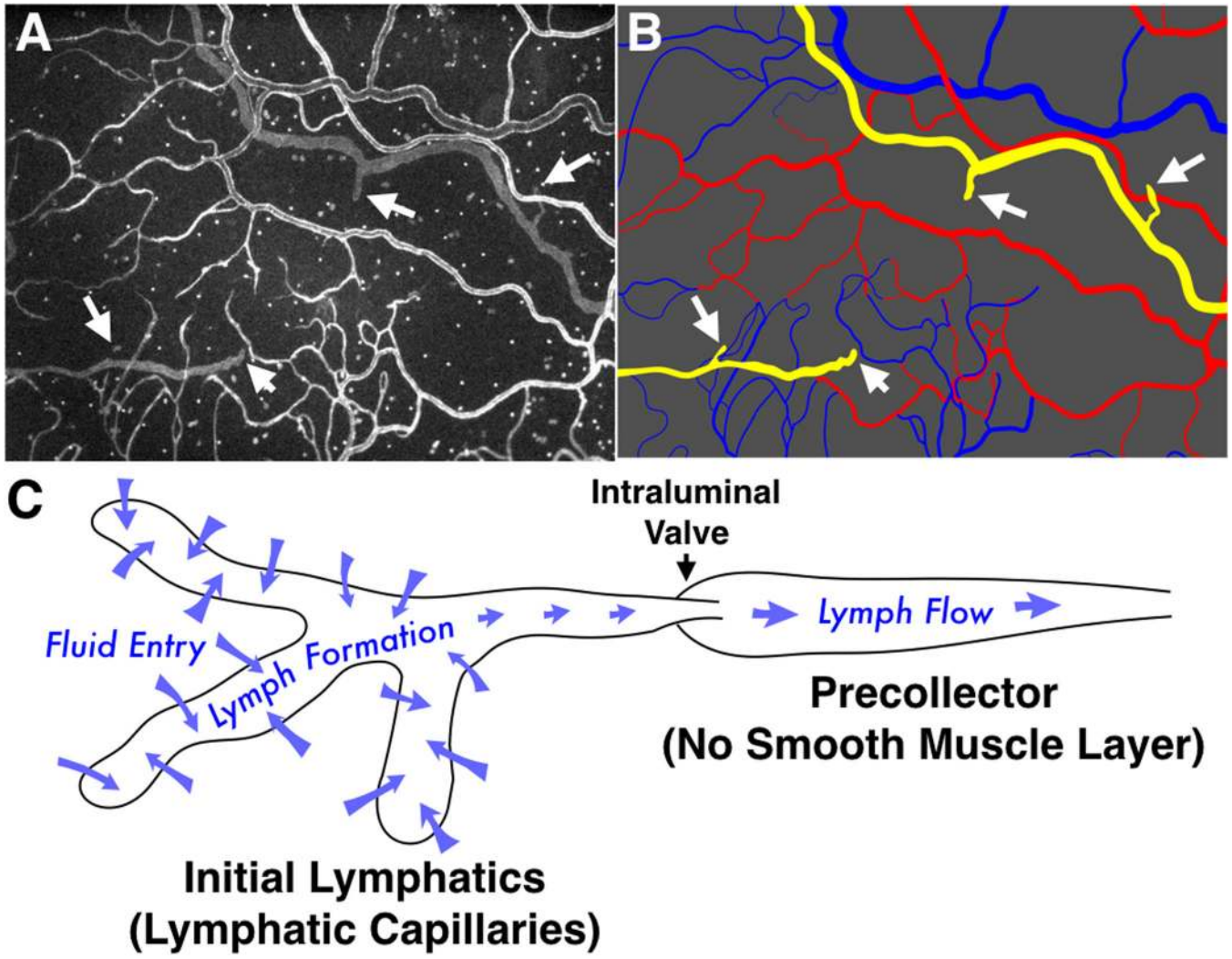

Fig. 2.

Initial lymphatics (lymphatic capillaries) as the site of lymph formation. *A*. The intravital microscopic image shows the rat mesenteric microcirculation and lymphatics labeled with topically applied FITC-BSI-Lectin, which allows for easy visualization of vascular structures. The arrows show blind ends of initial lymphatic vessels. *B*. A cartoon of the image in panel *A* shows color labeling of the arterioles (red), capillaries (red/blue), and venules (blue), and the initial lymphatics (yellow) for easier view. *C*. This cartoon depicts the entry of interstitial fluid (blue arrows) into initial lymphatics, forming lymph that is then transported toward a precollector. The precollector is separated from the initial lymphatic network by a one-way intraluminal valve.

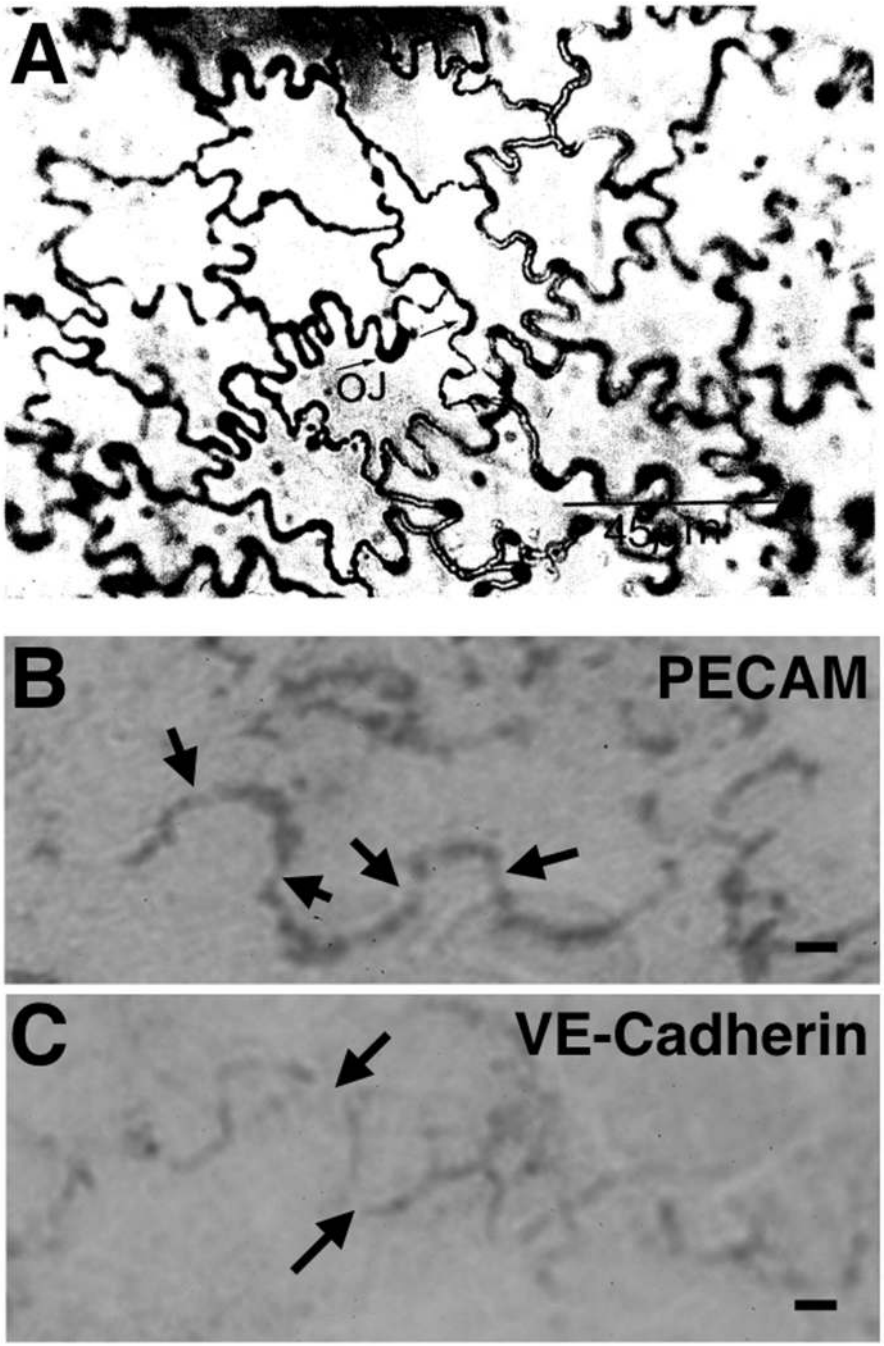

Fig. 3.

The oak leaf shape of endothelial cells of initial lymphatics. *A*. Silver nitrate labeling of the junctions between endothelial cells of initial lymphatics in the inner layer of the tunica vascularis from rat uterus. OJ = open junction formation. Scale bar = 45 μm. From reference (1220) with permission. *B*. Distribution of VE-cadherin and PECAM-1 at the junctions between initial lymphatic endothelial cells in the mouse trachea, identified by immunofluorescence microscopy. Scale bar = 5 μm. From reference (736) with permission.

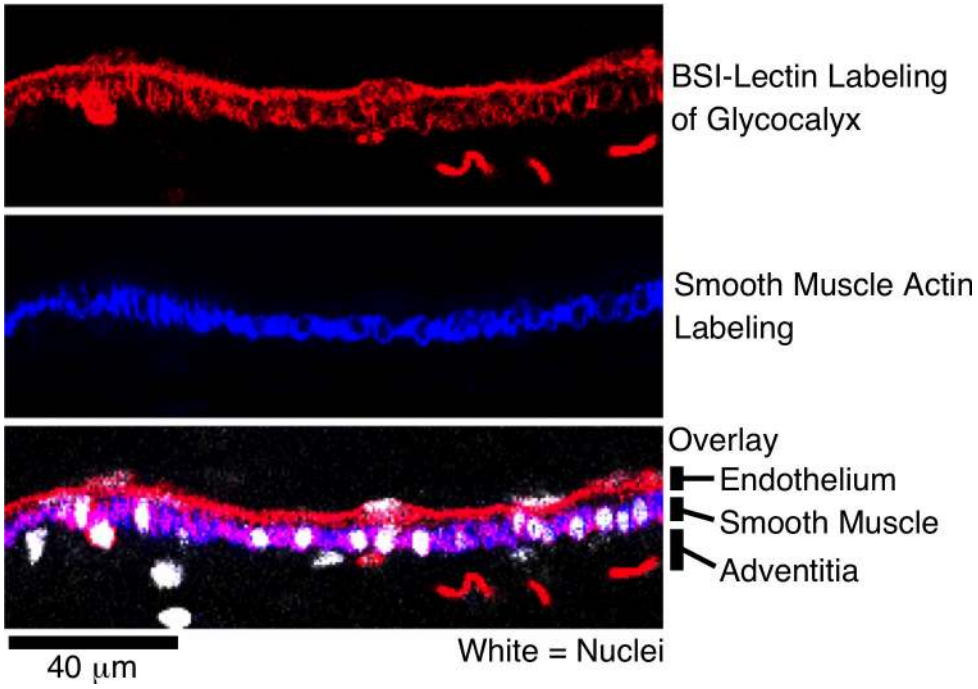

Fig. 4.

Confocal microscopy image demonstrating the endothelial, smooth muscle, and adventitial layers of a rat mesenteric lymphatic vessel. The top panel shows labeling of the glycocalyx (red) with BSI-Lectin-TRITC. The middle panel shows smooth muscle actin (blue). The bottom panel shows an overlay, plus the nuclei labeled in white. Note the longitudinal orientation of the endothelial nuclei, versus the circular smooth muscle cell nuclei. Additional cells, nerve fibers, and vasa vasorum are common in the adventitia. Images from reference (568) with permission.

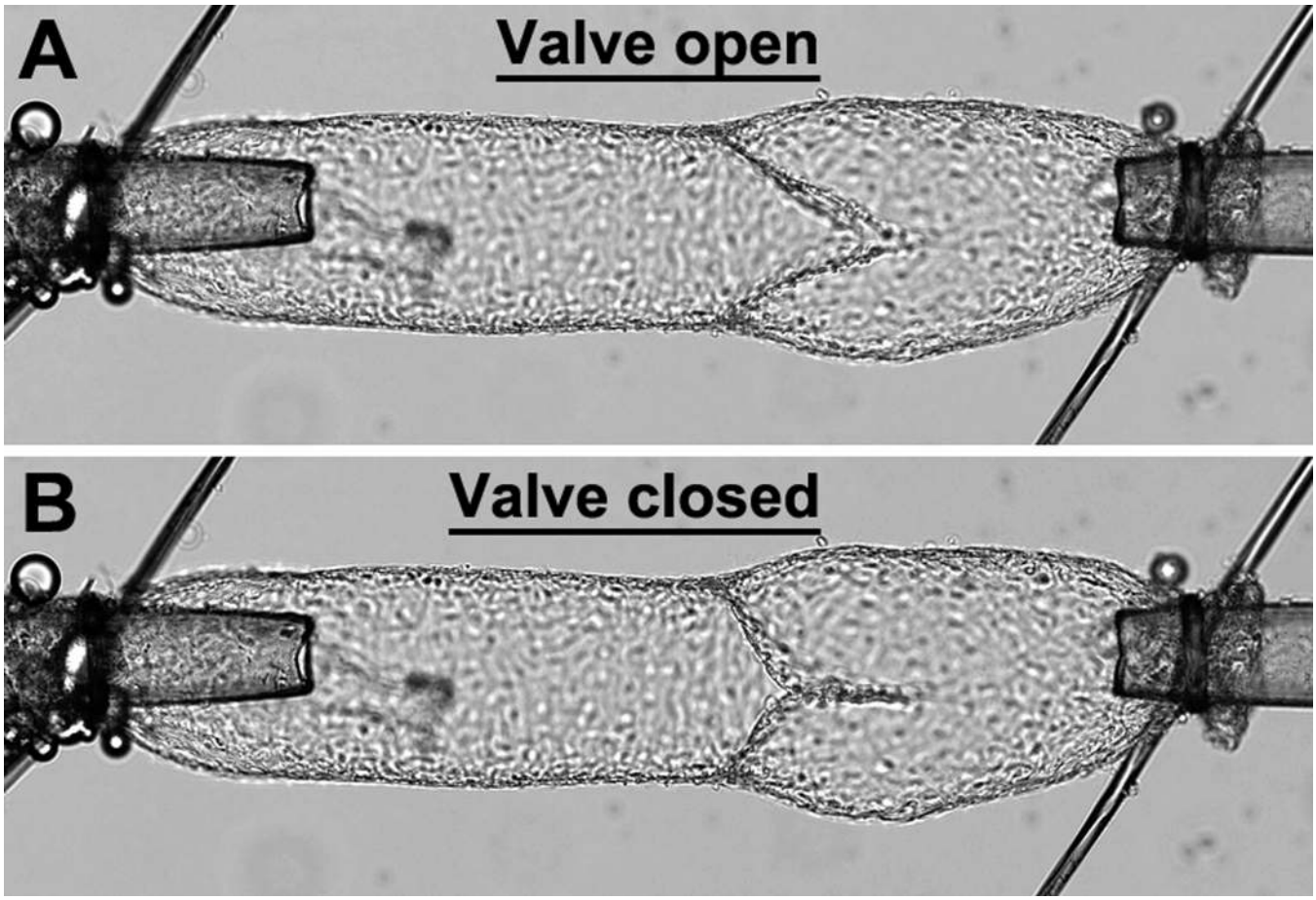

Fig. 5.

Secondary valve as seen in an isolated mouse mesenteric collecting lymphatic vessel. The vessel shown was cannulated on both ends and bathed in a Ca^{2+}-free solution so that it was completely relaxed. *A*. When the fluid pressures are the same in both pipettes, the valve is in an open position. *B*. When the fluid pressure in the outflow pipette (right side) is raised higher than that of the inflow pipette (left side), the valve closes. The images were obtained in Dr. Joshua Scallan's laboratory.

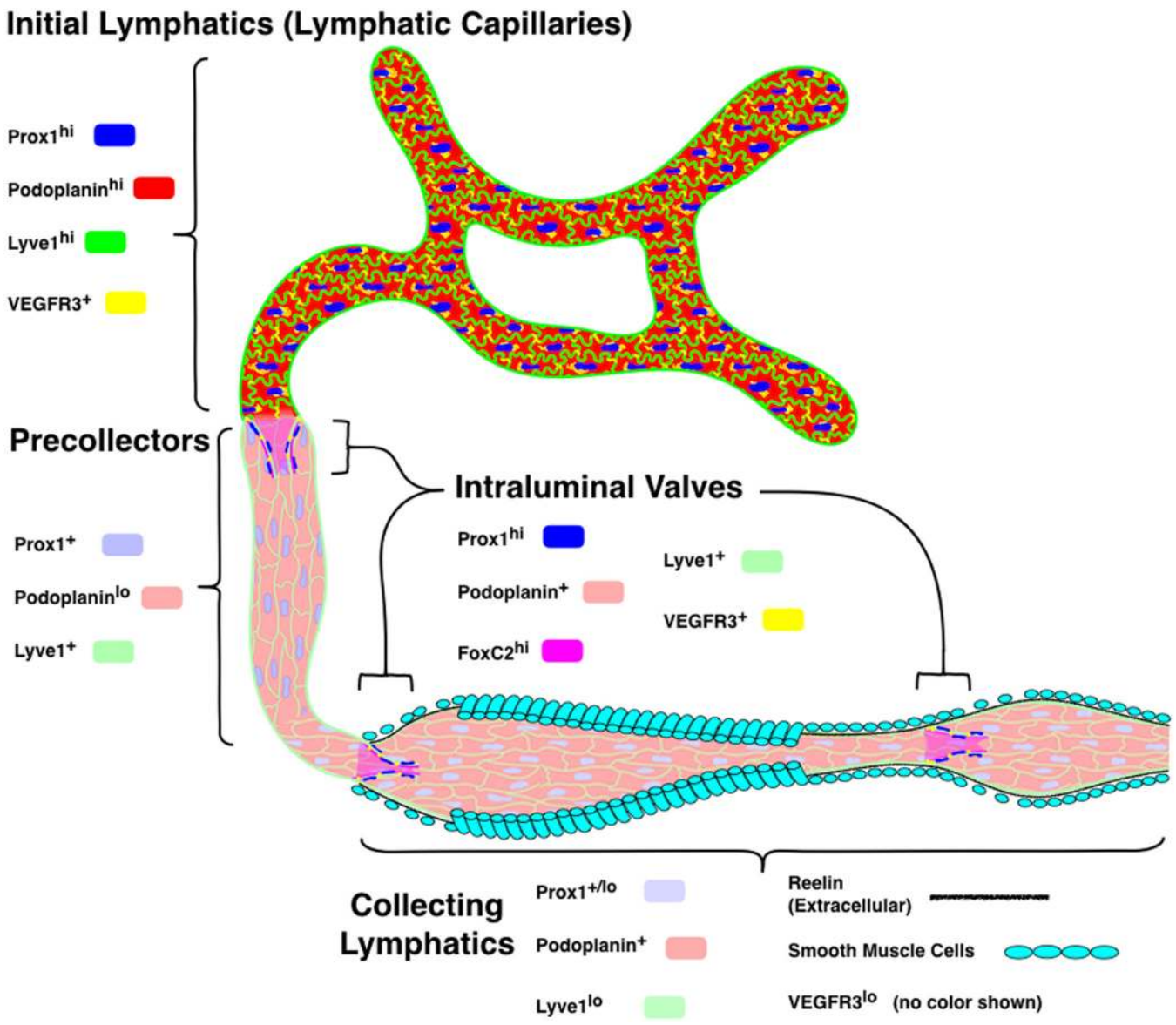

Fig. 6.

Antigen markers of the lymphatic endothelium have different labeling patterns in initial lymphatics, precollectors, collecting lymphatics, and intraluminal valves. In initial lymphatic networks (lymphatic capillaries), relatively high levels of Prox1, podoplanin, and Lyve1 are detected, plus these endothelial cells are also positive for Vegfr3. In Precollectors, the endothelial cells are positive for Prox1, Lyve1, and have relatively low levels of detectable podoplanin. Collecting lymphatic endothelium is positive for Prox1 and podoplanin, with extracellular reelin located between the endothelium and smooth muscle layer. The endothelium of intraluminal lymphatic valves (secondary valves) has high levels of Prox1 and FoxC2, and is also labels positively for podoplanin, Lyve1, and Vegfr3.

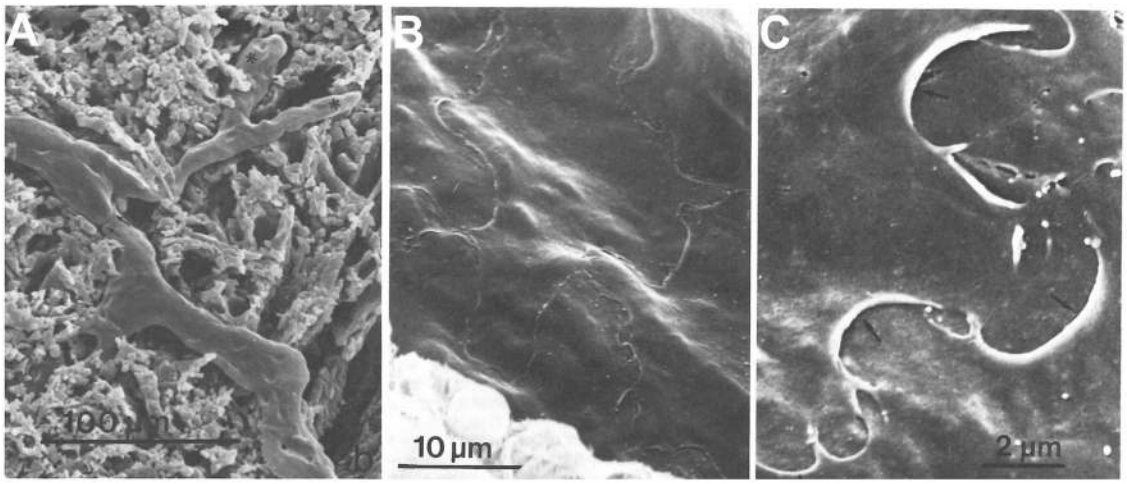

Fig. 7.

Lymphatic networks in the rat tongue. *A*. An image of a Mercox® corrosion cast of the rat tongue showing blind-ended initial lymphatics and impression patterns of "button" junctions and endothelial nuclei. *B*. The fine detail of oak leaf shaped endothelial cells, with (*C*) overlapping "button" junctions can also be observed in these images. Reproduced rom reference (165) with permission.

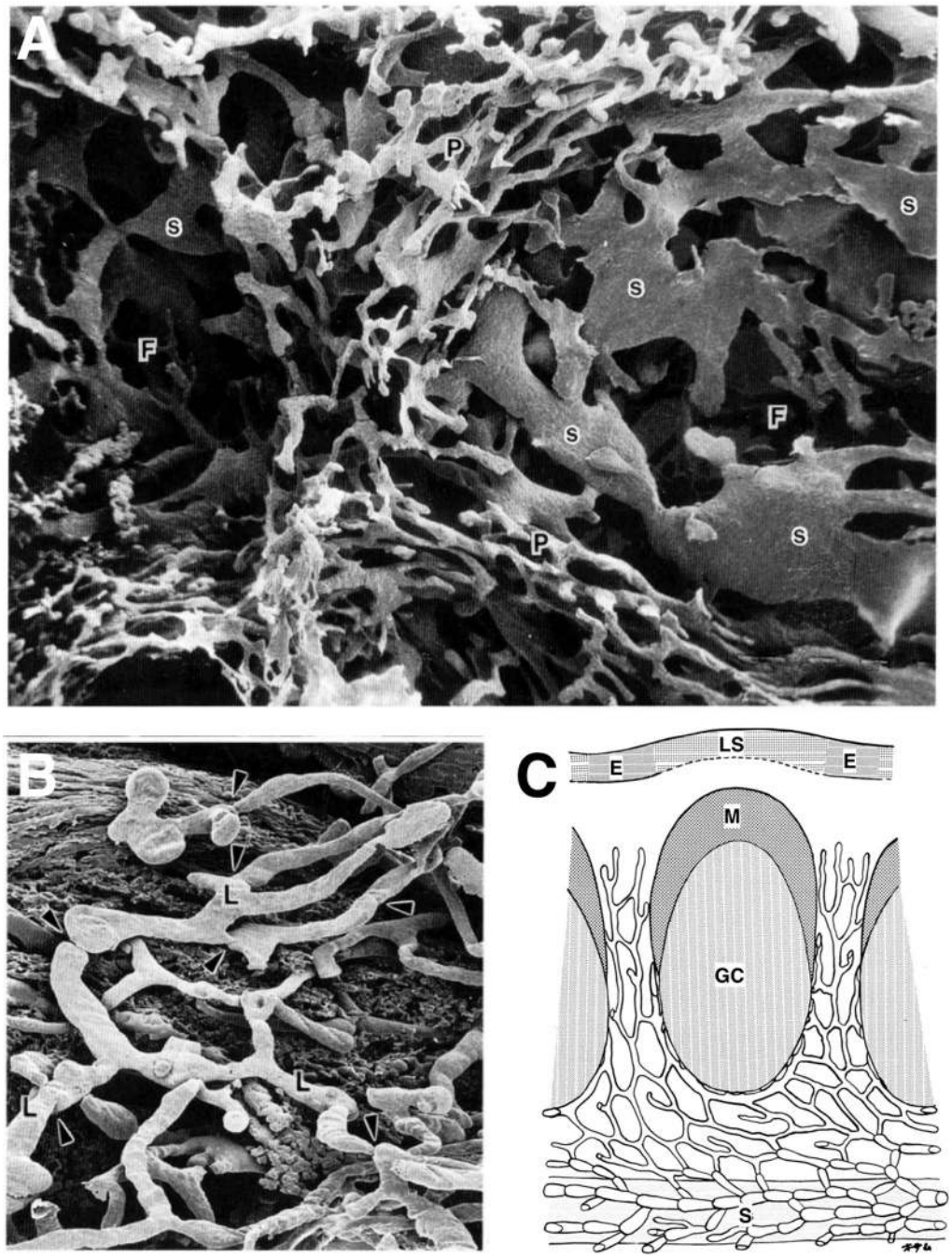

Fig. 8.

Lymphatic networks of the human tonsil. *A*. Lymphatic corrosion cast of human palatine tonsil viewed by scanning electron microscopy. Tubular lymphatic networks in the parafollicular area (P) connect to lymphatic sinuses (s) that surround the lower part of the follicle (110× magnification). *B*. Lymphatics (L) in the human palatine tonsil capsular region, with notches (arrowheads) showing locations of secondary valves (75× magnification). *C*. Schematic diagram of the organization of lymphatic networks in the human palatine tonsil, showing the epithelium(E) and epithelium infiltrated with

lymphocytes (LS), mantle zone (M), germinal center (GC), and septum (S). The images are from reference (356), with permission.

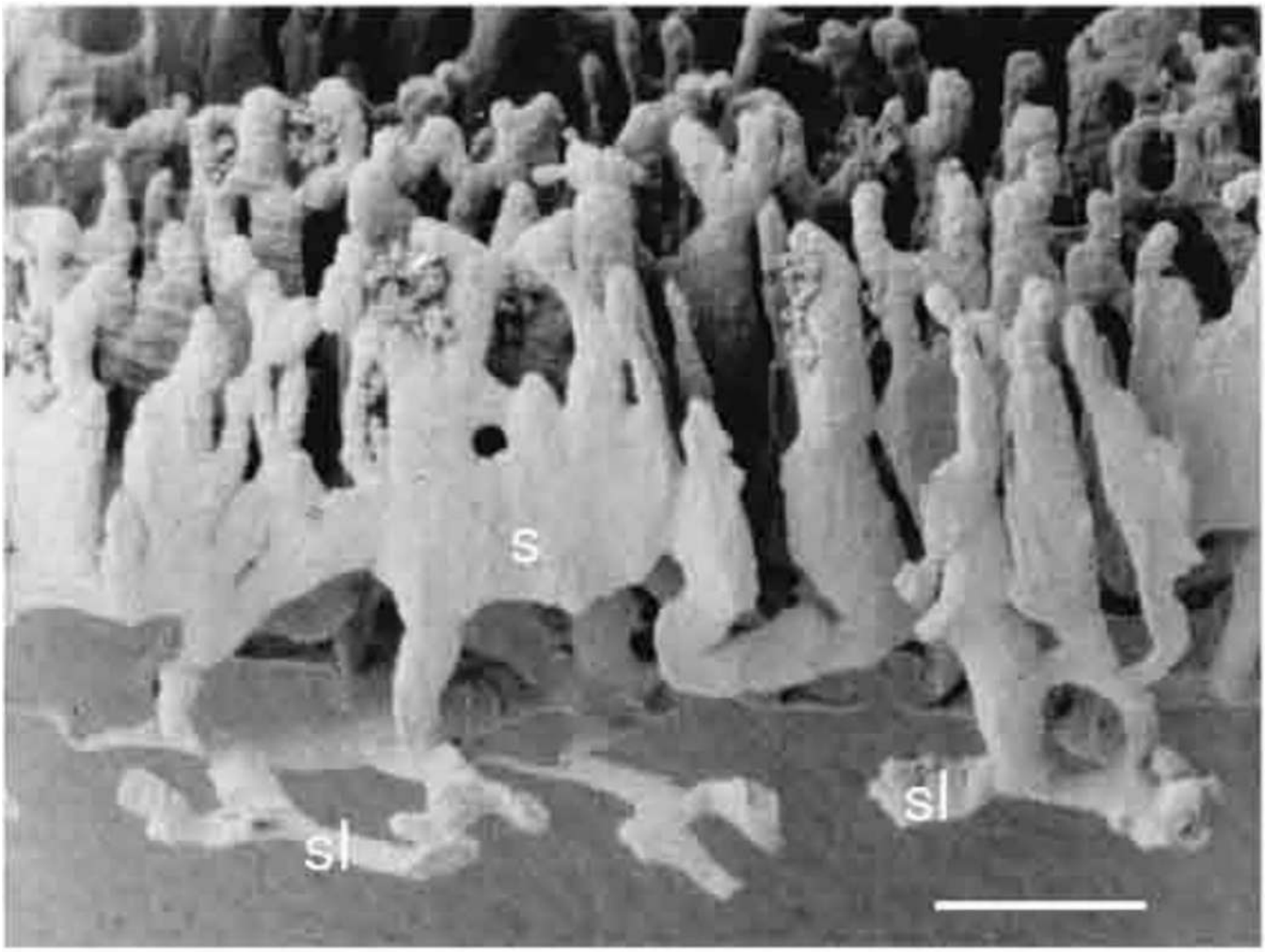

Fig. 9.

Lymph lacteals of the rat small intestine. The image is of a corrosion cast of the rat upper small intestine viewed by scanning electron microscopy. Blind-ended lymph lacteals coalesce at the bottom, and this sinus (s) then connects to the submucosal lymphatic plexus (sl). Scale bar= 200 μm. The image is from reference (795) with permission.

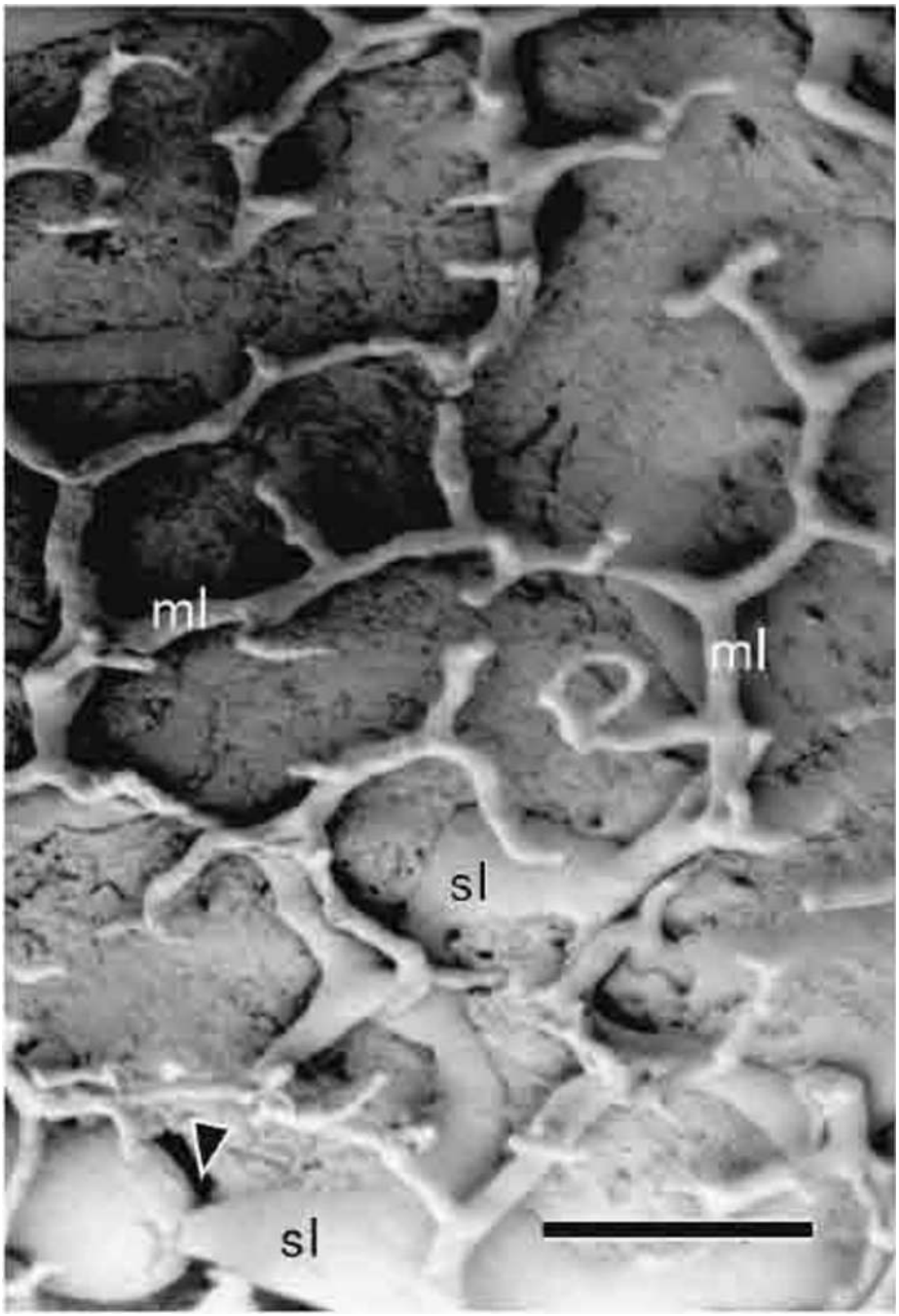

Fig. 10.

Scanning electron micrograph of a lymphatic corrosion cast of rat cecum. The mucosal lymphatic (ml) capillaries form a network with many blind-ended vessels. These networks then drain in to thicker submucosal lymphatics (sl). The arrowhead indicates a constriction point indicative of a secondary valve. Scale bar = 500 μm. The image is from reference (795) with permission.

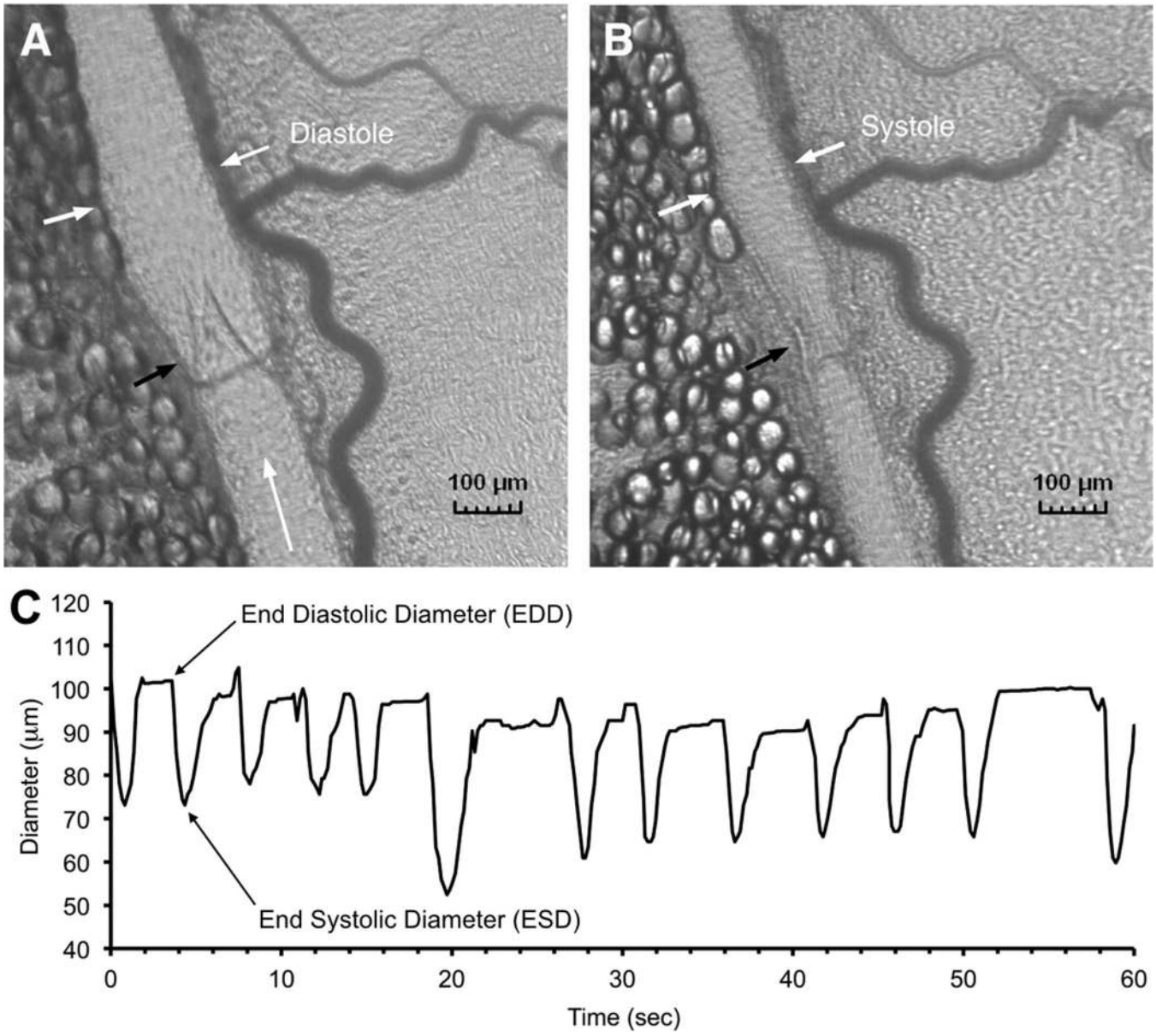

Fig. 11.

Collecting lymphatics of the rat mesentery contract intrinsically. Panels *A* and *B* show a typical mesenteric collecting lymphatic vessel of an anesthetized rat, observed by intravital microscopy during its phases of diastole (*A*) and systole (*B*). The thick white arrows denote the vessel walls, with the lumen in between. The black arrow indicates the site of a secondary valve separating two lymphangions, which prevents backflow of lymph. Panel *C* shows a trace of diameter versus time acquired from a mesenteric lymphatic vessel using intravital microscopic video recording. The trace shows cyclic changes in diameter. The points at which the end diastolic diameter (EDD) and end systolic diameter (ESD) for a single contraction cycle are shown. These images and data are from reference (128) with permission.

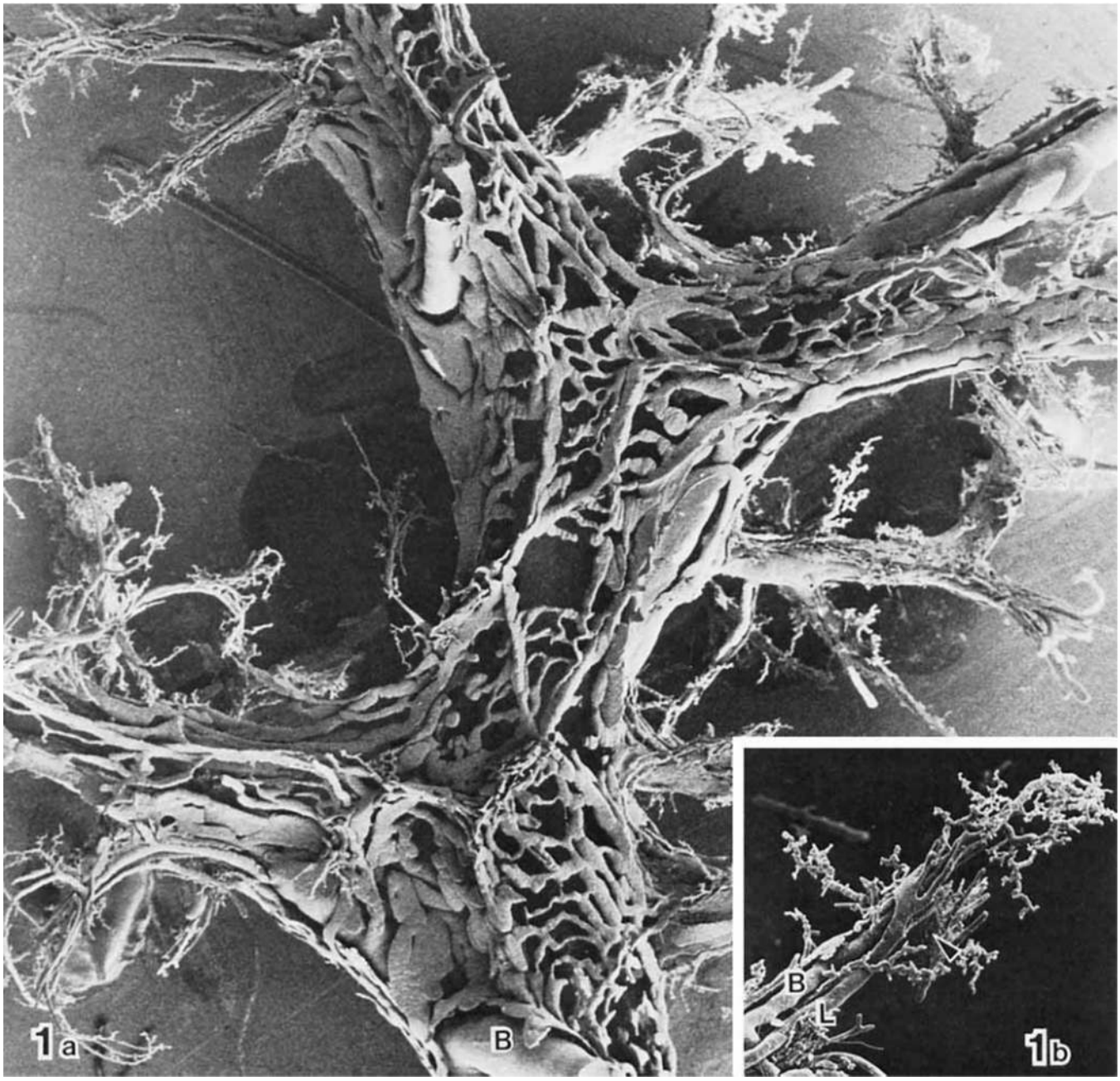

Fig. 12.

Lymphatic networks surrounding portal tracts in the rabbit liver. Corrosion casts were prepared after injection of resin into the bile ducts, which then leaked out and entered the lymphatic networks. Scanning electron microscopy was used to view the corrosion casts. The large panel (1a) shows a low power (30×) image of the rich lymphatic networks around the bile duct (B). The high power image (80×) in panel 1b shows where resin leaked from the bile duct (B) into the initial lymphatics (L). These images are from reference (1164) and reproduced with permission.

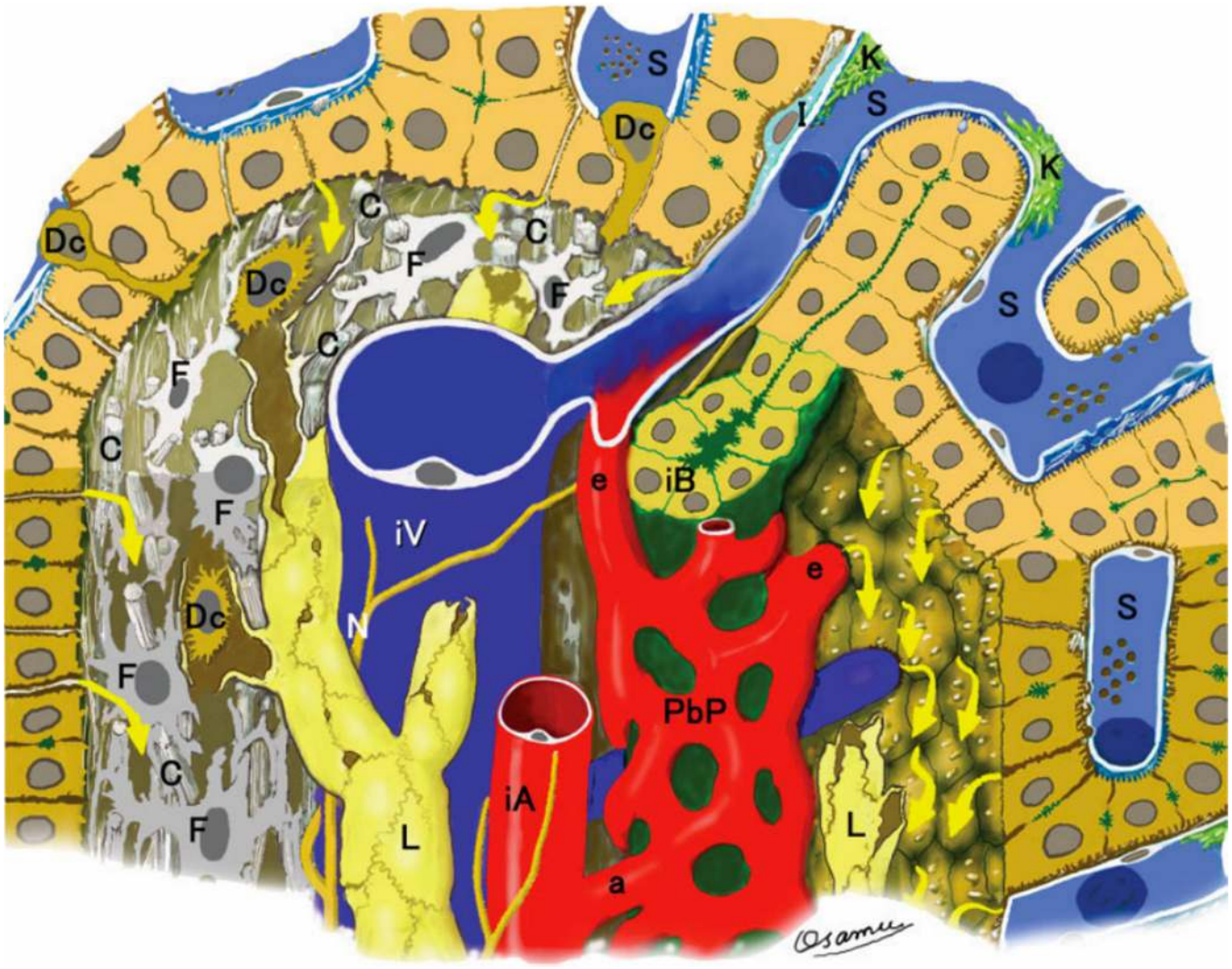

Fig. 13.

Diagram of fluid flow and cell migration pathways from the liver sinusoids to the portal lymphatics. Fluid draining from the liver sinusoids (S), indicated by the arrows, presumably passes through the space of Disse, channels of the limiting space, and through the portal tract interstitial space to reach the portal lymphatic vessels (L). Other cells and structures shown include dendritic cells (Dc), collagen fibers (C), interlobular artery (iA), interlobular vein (iV), interlobular bile duct (iB), fibroblasts (F), Ito (stellate) cell (I), Kuppfer cell (K), nerve (N), peribillary capillary plexus (PbP), afferent vessel of PbP (a), and efferent vessel of PbP (e). Reproduced from reference (794) with permission.

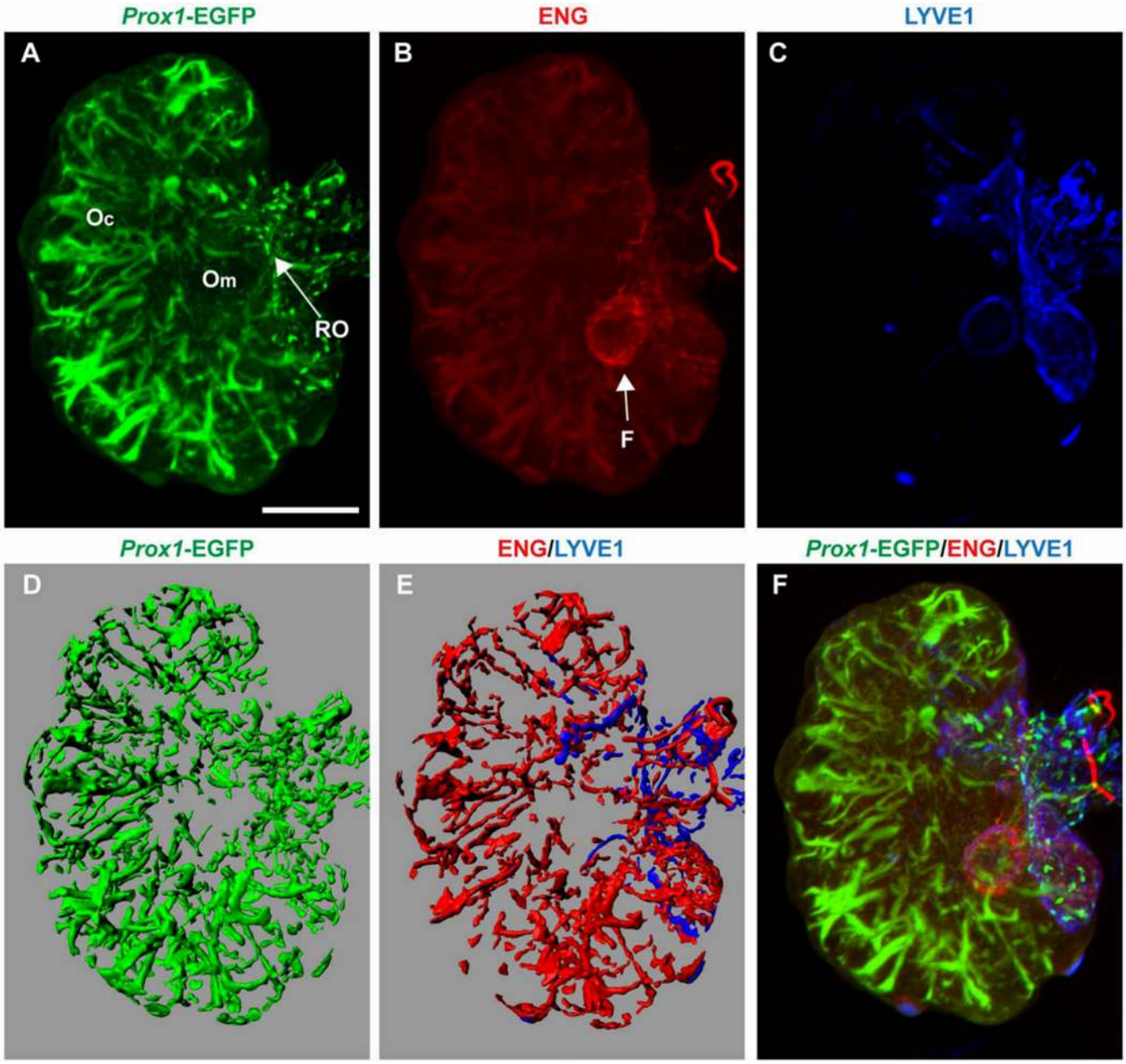

Fig. 14.

Lymphatic networks of the mouse ovary visualized in *Prox1*-EGFP reporter mice. *A.* Lymphatic networks (green) arise from the rete ovarii (RO), indicated by the arrow, and extend into the ovarian medulla (Om) and ovarian cortex (Oc). *B.* Blood vessels were labeled with an anti-endoglin (ENG) antibody immunofluorescence labeling (red). The arrow indicates the follicle(F). *C.* Lyve1 was also labeled (blue) and was localized mainly to lymphatics at the ovarian rete and extraovarian rete. Panel *D* shows a 3-dimensional representations of *Prox1*-EGFP-positive lymphatic vessels, while in panel *E* a similar 3-dimensional model shows Lyve1-positive lymphatics (blue) overlaid with endoglin-positive vessels. Panel *F* shows a composite image with *Prox1*-EGFP (green), endoglin (red), and

Lyve1 (blue) labels, showing some overlap and also distinct patterns of the blood and lymphatic vessel networks. Scale bar = 1 mm. The images are reproduced from reference (1031) with permission.

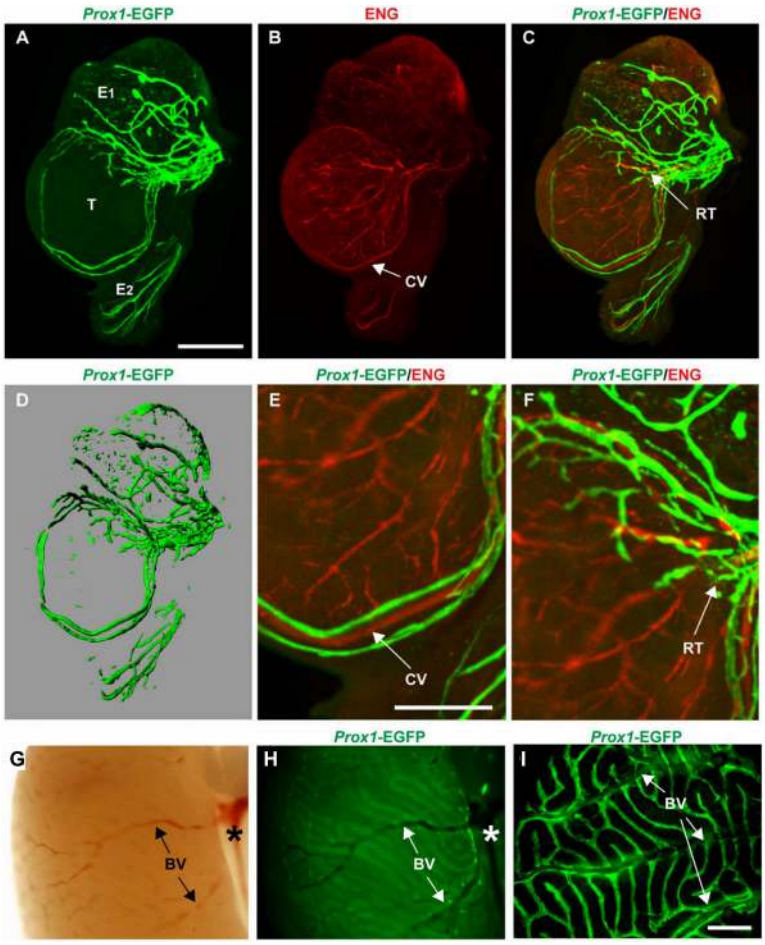

Fig. 15.

Lymphatics in the testes of *Prox1*-EGFP reporter mice. *A*. During late gestation (E17.5), EGFP-positive lymphatics sprout from the spermatic cord across the surface of the testis (T). Lymphatics are also found on the head (E1) and tail (E2) of the epididymis. The scale bar = 500 μm and applies to panels *A*-*C*. *B*. Blood vessels were also visualized in the same specimen using an anti-endoglin antibody (ENG). *C*. The *Prox1*-EGFP-positive lymphatics (green) and ENG-labeled blood vessels (red) did not occupy the same space. Some yellow areas show overlap of the two fluorescence signals from different planes. *D*. A three-dimensional representation of the *Prox1*-EGFP-positive lymphatic network from panel *A*. Panel *E* shows a magnified region from panel *C* showing the lymphatic vessels (green) running parallel to the coelomic vessel (CV). Panel *F* shows a magnified region of the rete testes. The scale bar for panels *E and F* = 250 μm. *G*. Brightfield whole mount view of the adult mouse testis surface, showing blood vessels (BV) and the spermatic cord (asterisk). *H*. EGFP signal can be observed from the same surface view, however there is much background due to *Prox1*-EGFP expression within spermatids located in the testis. *I*. A confocal image better shows the *Prox1*-EGFP-positive lymphatic network located within the tunica albuginea of the adult testis. Panel *I* scale bar = 600 μm. The images are reproduced from reference (1031) with permission.

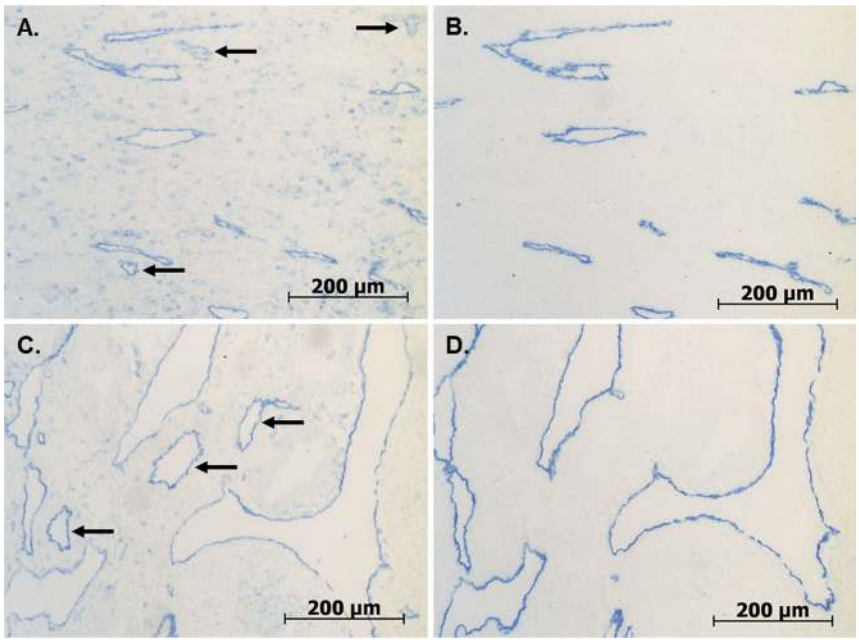

Fig. 16.

Endometrial lymphatics and blood vessels in women dilate in response to progestin treatment. Hysterectomy samples were obtained from women who either received no treatment, or intrauterine progestin therapy for heavy menstrual bleeding. Panels *A* and *B* are endometrial sections from controls, and *C* and *D* are from those treated with LNG-IUS. Sections were immunolabeled to identify either CD31 *(A* and *C*) or D2–40 (podoplanin, *B* and *D*). Reproduced from reference (277) with permission.

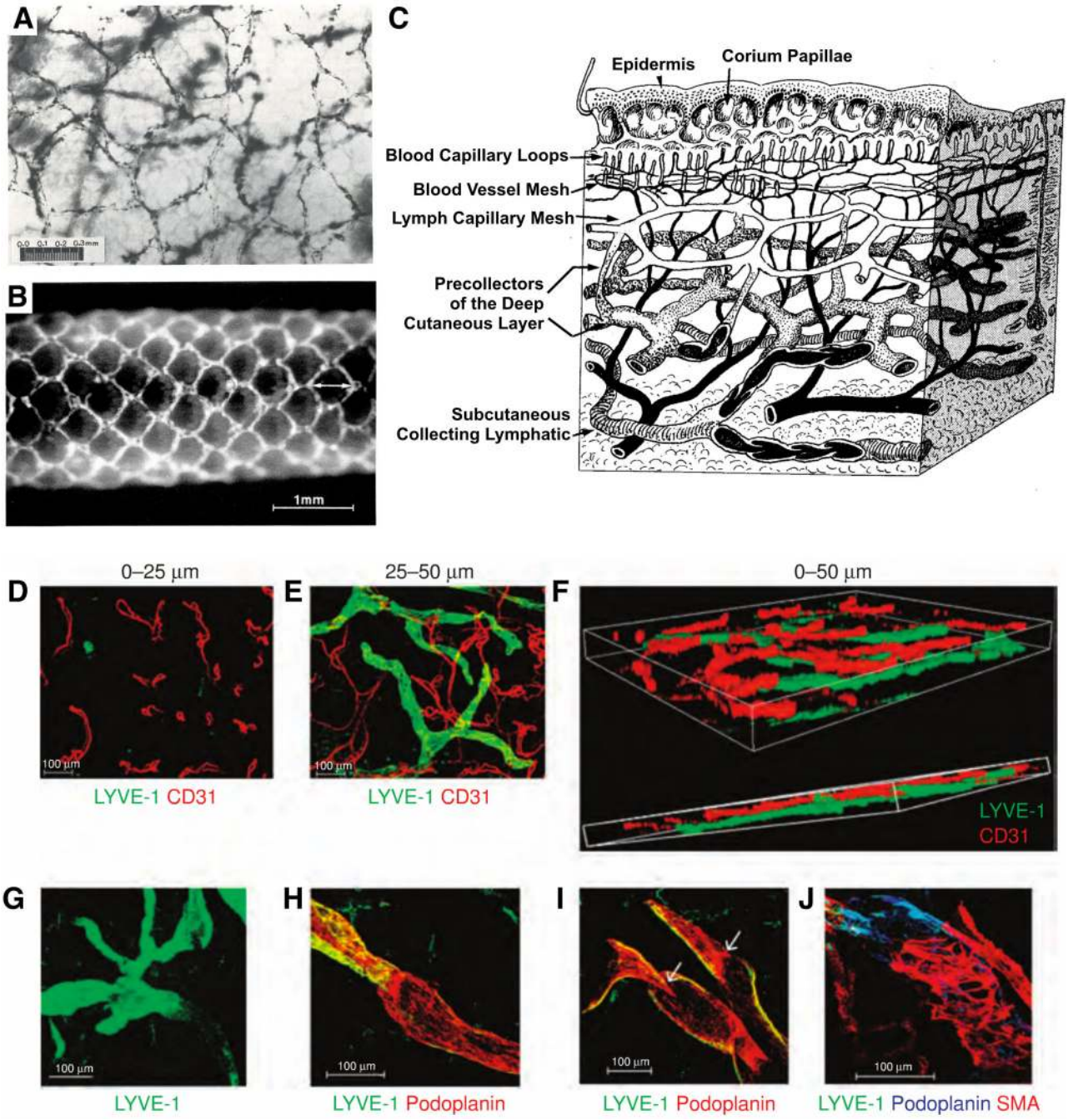

Fig. 17.

Lymphatic vessel networks of the skin. *A.* This image shows a translucent preparation of dorsal skin from the human foot in which the initial lymphatic network was labeled with India ink absorbed into the network after a subcutaneous injection. *B.* A view of the mouse tail with a fluorescent microscope, after intradermal injection at the distal tail with FITC-dextran-2000kDa reveals the polygonal lymphatic capillary network. *C.* A block diagram of the cutaneous lymphatic and blood vessel networks in human skin. *D-J.* Confocal microscopic images of human skin. *D.* Only PECAM-1-positive capillary networks are

visible 0–25 μm below the dermoepipermal junction. *E.* At 25–50 μm both capillary networks and initial lymphatics identified by LYVE1 labeling are visible. *F.* Three-dimensional reconstruction of these networks. *G.* Decrease in LYVE1-labeling in a lymphatic network at the interface where collecting lymphatics appear in the skin. *H* and *I.* *P*odoplanin-positive endothelial cells within lymphatics remain, despite the decrease in LYVE1 labeling. *J.* The appearance of smooth muscle actin-positive smooth muscle cells at the collecting lymphatic interface. The images are from references (562, 597, 1122) and reproduced here with permission.

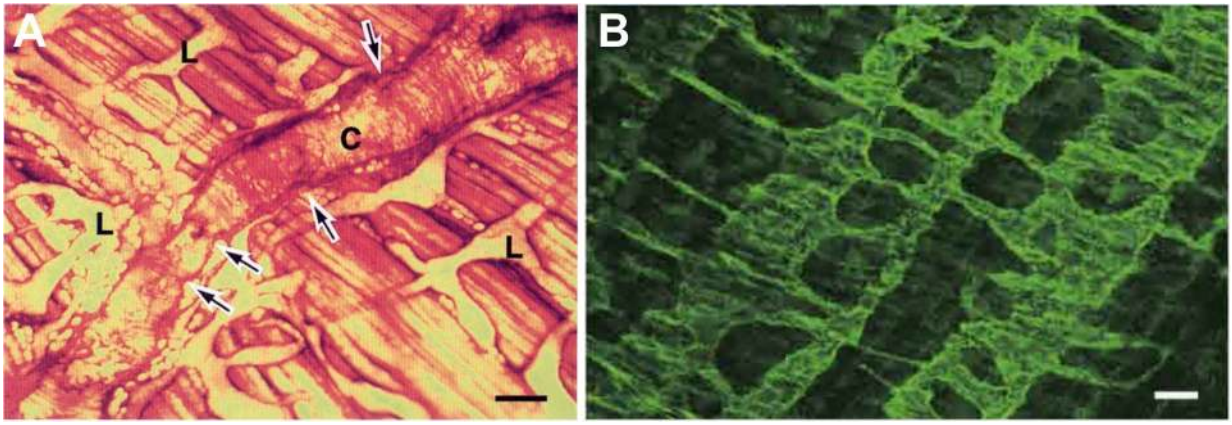

Fig. 18.

Lymphatic networks of the diaphragm. *A*. Subpleural lymphatics in the diaphragm from a 23-week old rat, stained with 5'Nase and viewed by light microscopy. Scale bar = 200 μm. L= lymphatic capillaries; C = collecting lymphatic vessel. The arrows indicate circular smooth muscle on the collecting lymphatic. *B*. Lyve1 immunolabeling of lymphatic lacunae, a lattice-like network featuring irregular and wide shapes, of the peritoneal side of the rat diaphragm. Scale bar = 50 μm. The images are reproduced from references (795, 973) with permission.

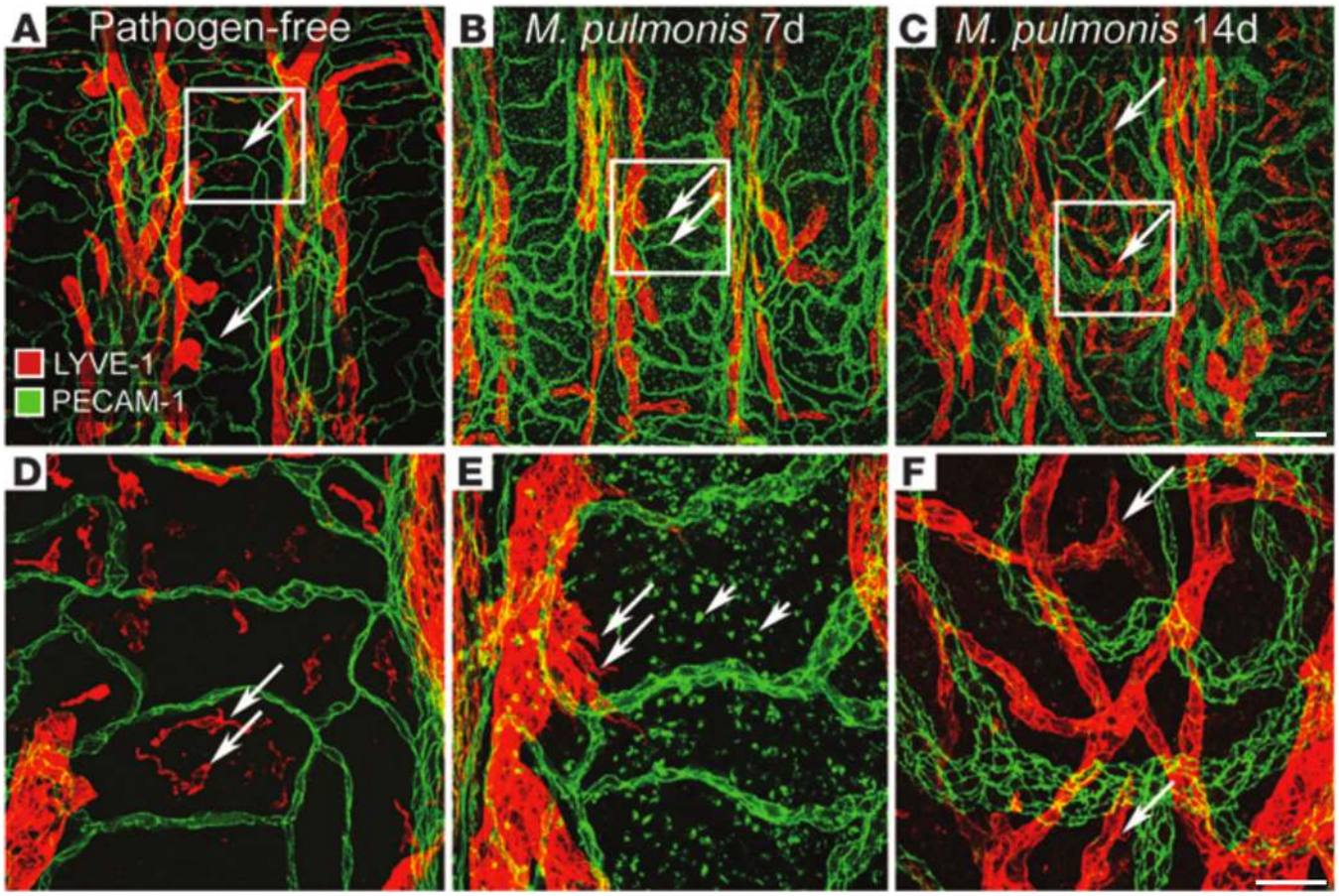

Fig. 19.

Mouse tracheal microvascular and lymphatic vessel networks, and changes in response to *M. pulmonis* infection. *A*. Blood vessel (green) and lymphatic (red) networks in a flat whole mount of the trachea from a pathogen-free C57BL/6 mouse. Capillaries (arrows) cross the cartilage, but lymphatics do not cross. *B*. After 7 days of *M. pulmonis* infection, capillaries (arrows) crossing the cartilage are widened. *C*. After 14 days of *M. pulmonis* infection, the blood vessels appear larger, and abundant lymphatic sprouts (arrows) are apparent. Panels *D-F* show enlargements of the boxed regions in panels *A-C*. In panel *D*, Lyve1-positive lymphatic sprouts are absent, but there is Lyve1 expression on some leukocytes (arrows). *E*. An influx of leukocytes, many labeling for PECAM-1 (short arrows) accompanies the vessel changes. The large arrows indicate lymphatic sprouts. *F*. Enlarged blood vessels and abundant lymphatic sprouts (arrows) are present. The scale bar for the upper panels = 200 μm, and for the lower panels = 50 μm. Reproduced from reference (64) with permission.

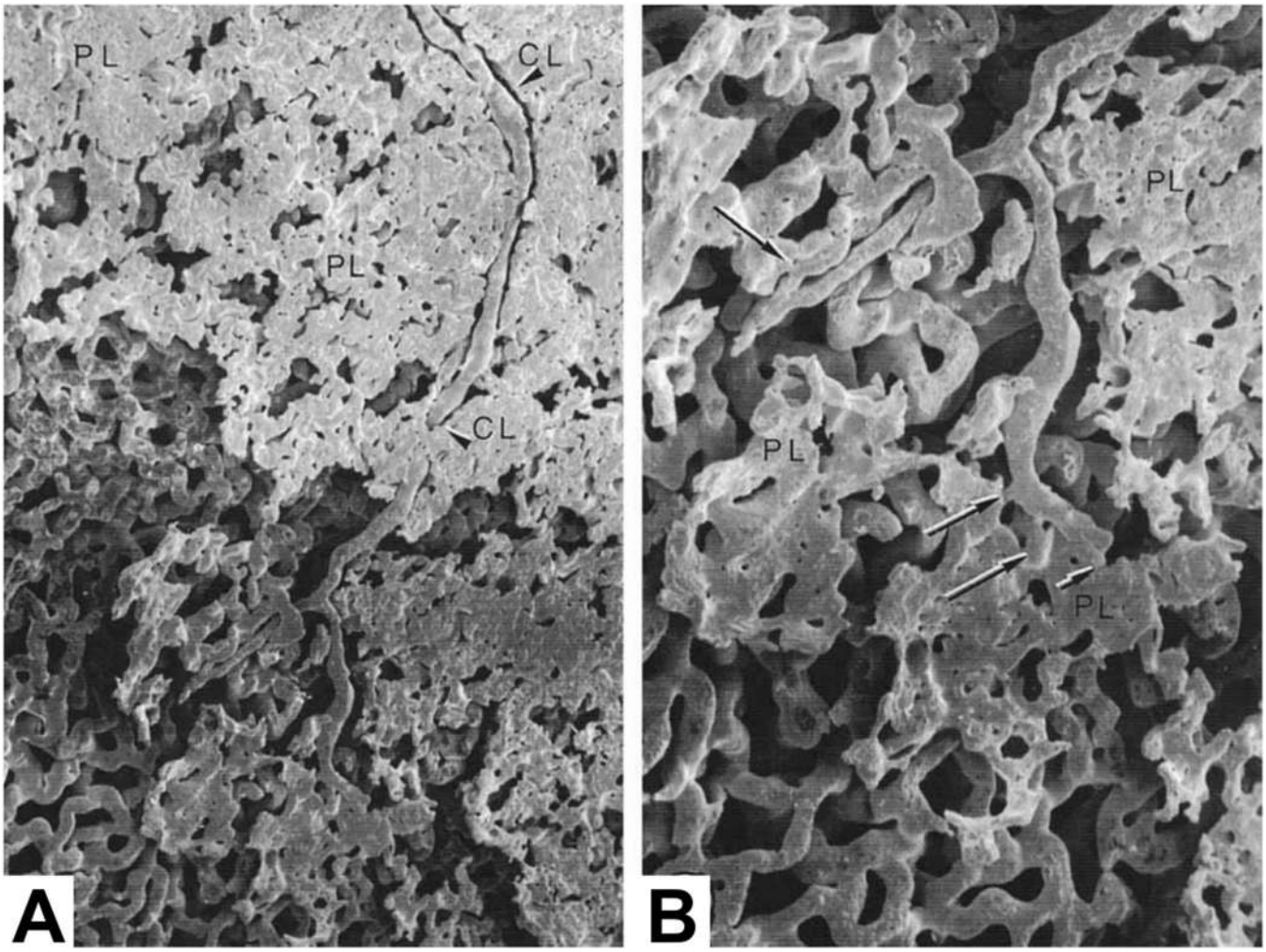

Fig. 20.

Scanning electron micrographs of corrosion casts of lymphatics in the rat lung visceral pleura. *A*. In the first image (320× magnification) he initial lymphatics are flat and ribbon-like (termed prelymphatics in the original paper; PL). These connect to conduit lymphatics (CL, denoted by arrowheads). *B*. In the second image (640 × magnification), the arrows denote the connections between the flat, ribbon-like lymphatics and conduit lymphatic vessel. Reproduced from reference (17) with permission.

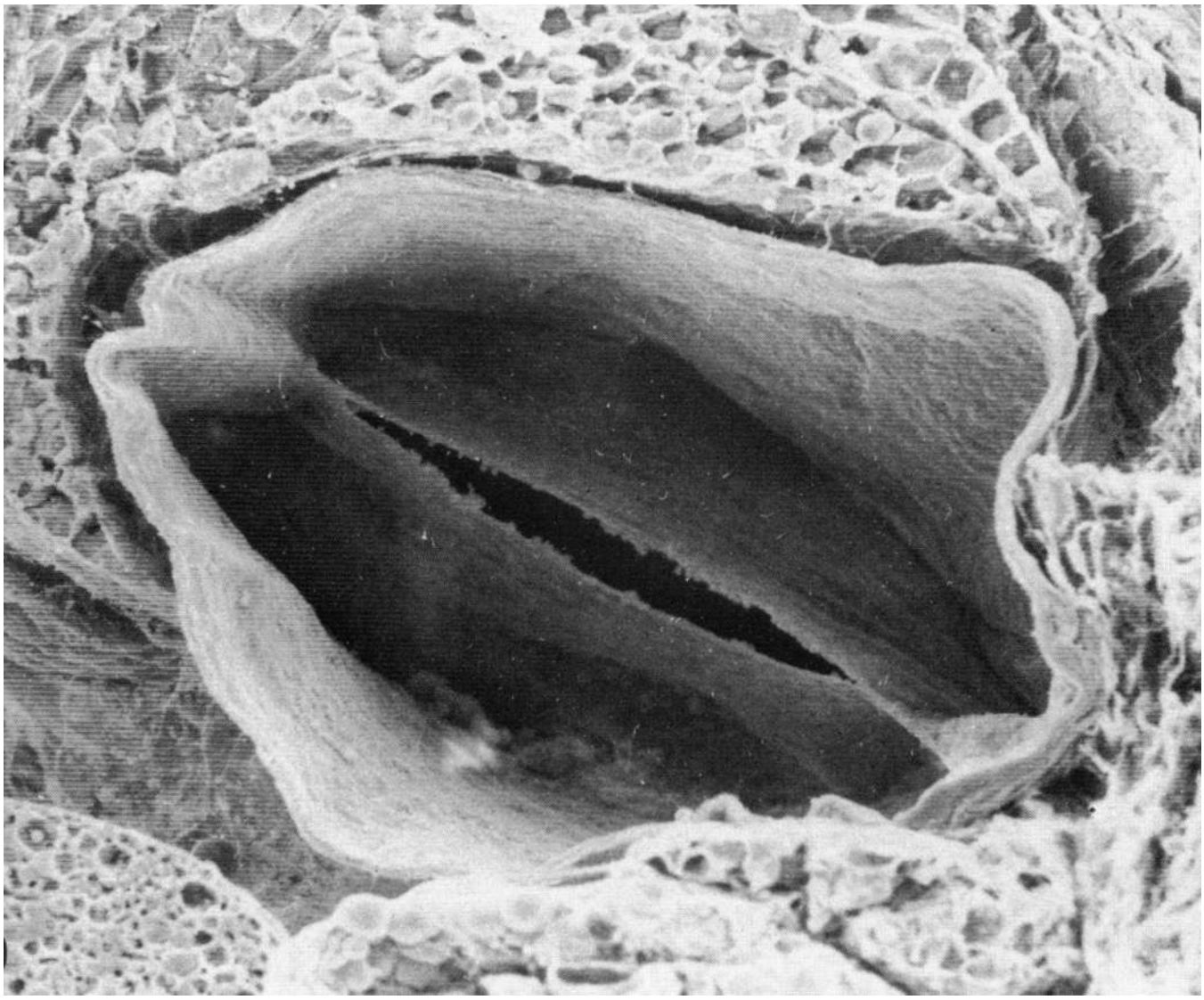

Fig. 21.

A cross-sectional view of a secondary valve in a pulmonary lymphatic vessel. The pair of leaflets originates along the circumference of the inner lymphatic wall, projecting to the lumen of the vessel. Reproduced from reference (583) with permission.

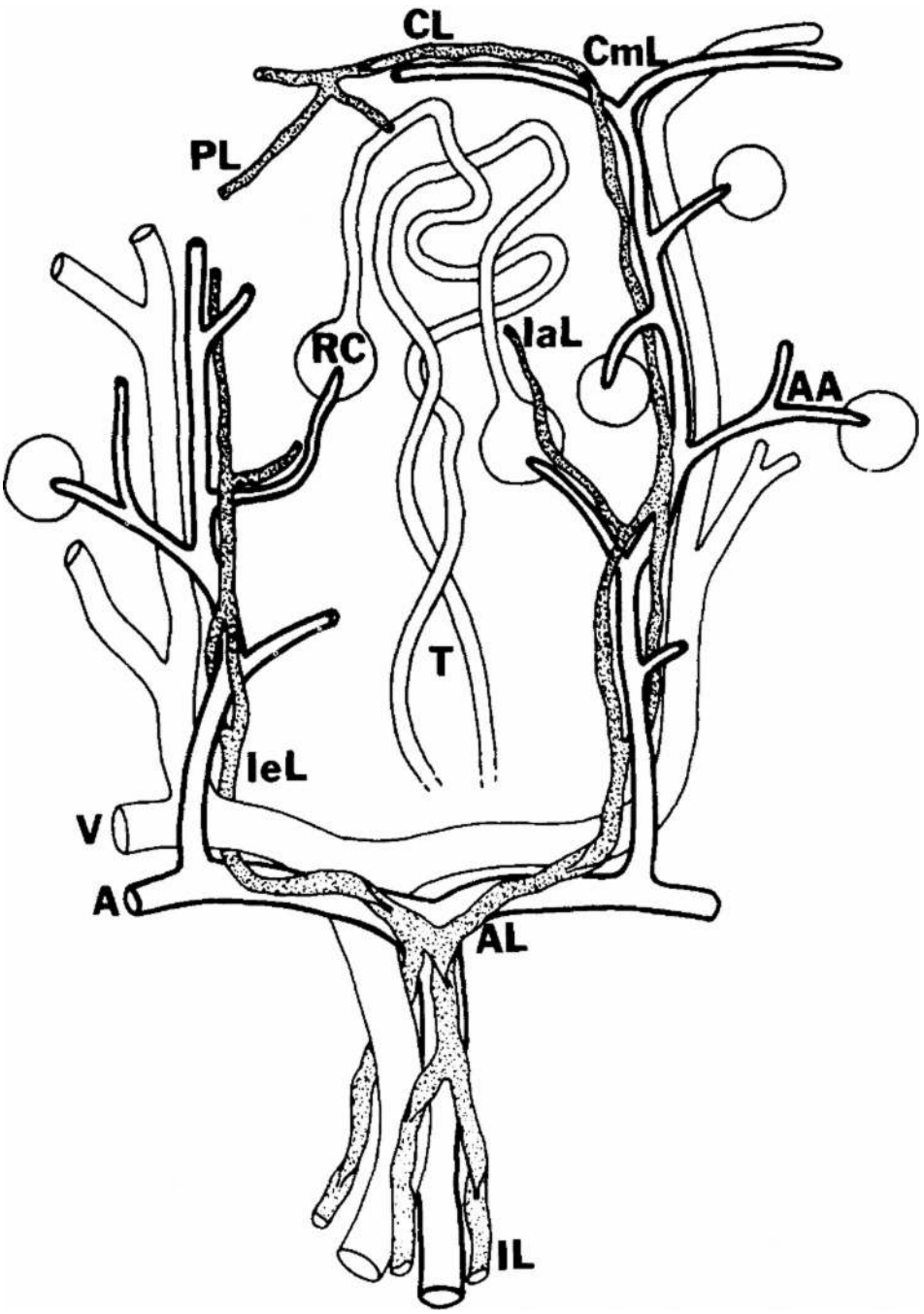

Fig. 22.

A schematic diagram of lymphatics in the kidney, in relation to the local microvasculature and nephrons. Intralobular lymphatic capillaries (IaL) originate near renal tubules (T), renal corpuscles (RC), or afferent arterioles (AA). These lymphatics feed into interlobular lymphatics (IeL). The capsular lymphatics (CL) receive lymph from the perforating lymphatics (PL) in the superficial cortex, and from communicating lymphatics (CmL) that follow arteries (A) or veins (V) that occasionally pierce the renal capsule. The interlobular and communicating lymphatics both drain into arcuate lymphatics (AL) with valves. The

arcuate lymphatics coalesce into interlobar lymphatics (IL), which drain into the hilar lymphatics, which are contractile collecting lymphatics. Reproduced from reference (777) with permission.

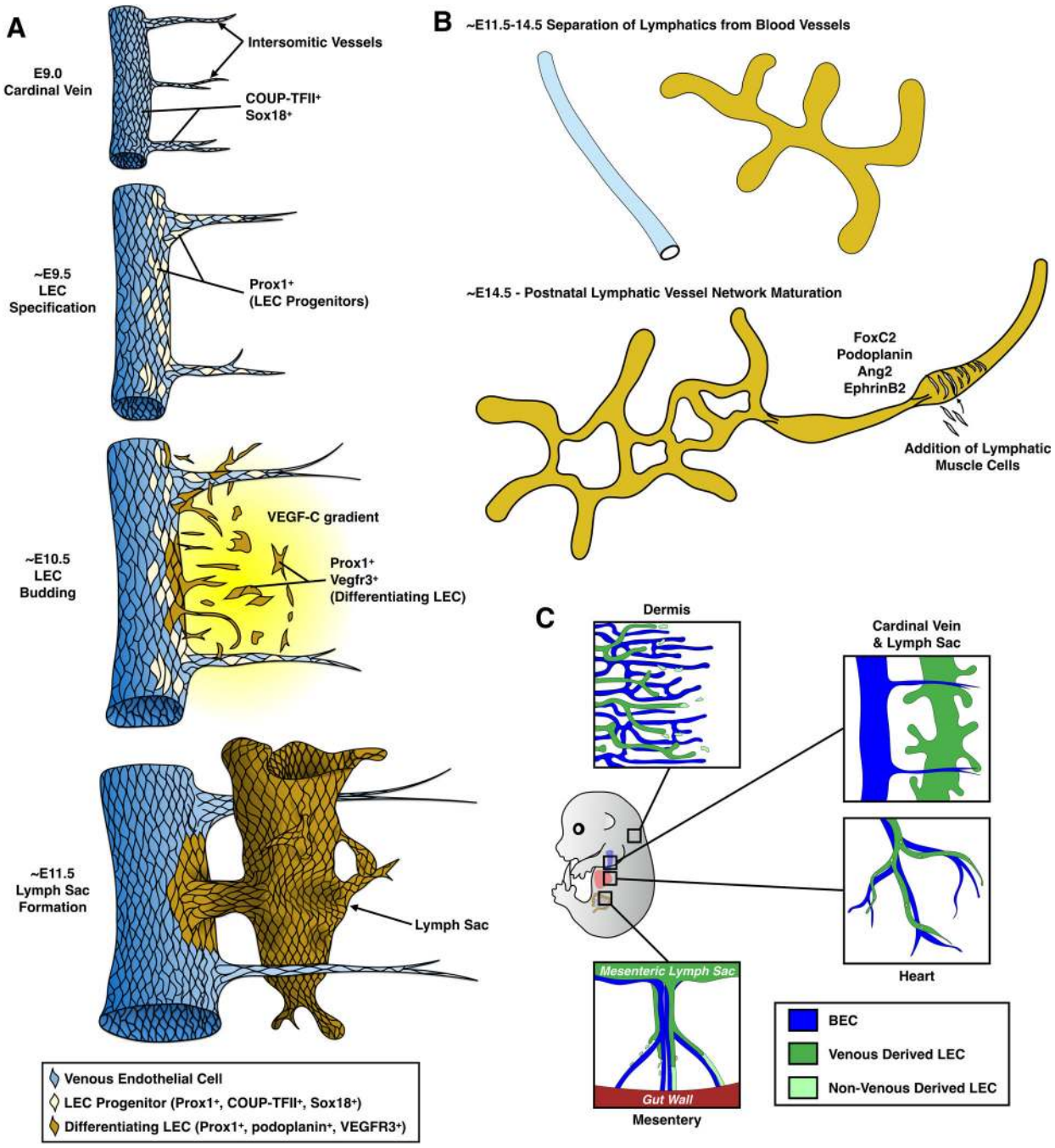

Fig. 23.

Schematic diagrams of early development of the mammalian lymphatic vasculature and different origins of the organ-specific lymphatic vessels. *A*. Sagittal view of the key steps during the formation of the first lymphatic structure-lymph sac along the cardinal vein (CV) from E9.0 to E11.5 in the mouse embryo. Around E9.5, the transcription factors CoupTFII and Sox18 induce *Prox1* expression in a subpopulation of venous ECs in the CV and intersomitic vessels (ISV). The appearance of the Prox1-expressing LEC progenitors indicates that LEC specification has commenced. Most of those progenitors start to leave the

CV and ISV and migrate in the surrounding mesenchyme in response to VEGFC gradient. The differentiating LECs maintain the expression of Vegfr3 mediated by Prox1. As LEC migrate and proliferate in an interconnected manner, they assemble together to form lymph sacs around E11.5. B. Both the venous derived LEC (green) and non-venous derived LEC (light green) contribute to the formation of the lymphatic vasculature in the dermis, the heart, and the mesentery while the lymph sacs are formed by only the venous derived LEC. *C.* As LEC proliferate and sprout out of the lymph sacs, they start to remodel into the collecting lymphatic vessels and the lymphatic capillaries around E14.5. In the course of the maturation of the lymphatic vessel network, the collecting lymphatic vessels form intraluminal valves to prevent lymph backflow and recruit smooth muscle cells to the outside of the vessels to facilitate pumping during lymph transport. The lymphatic capillaries develop button-like junctions to serve as the primary valves for fluid and cell entry into the lymphatic vessels.

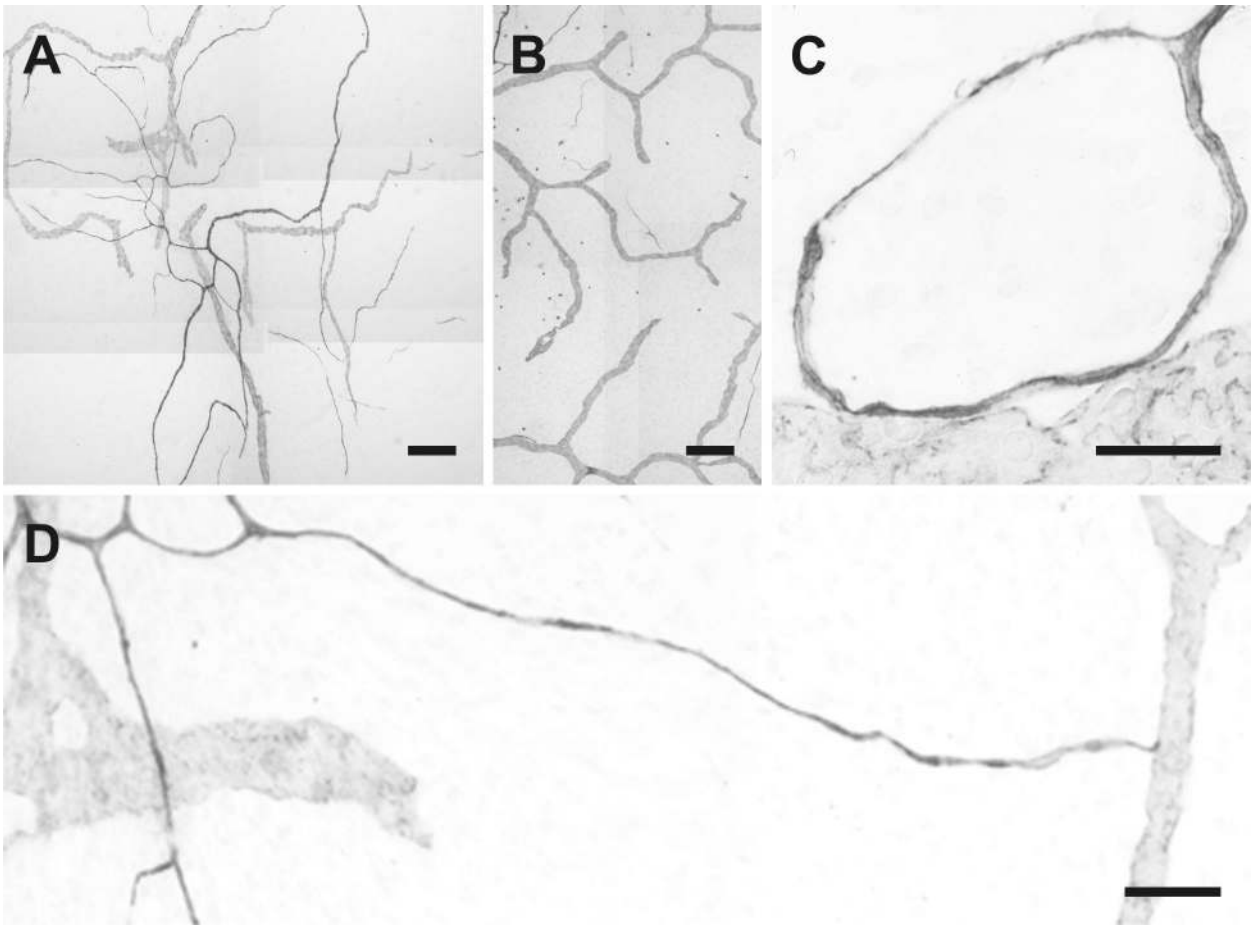

Fig. 24.

Representative images of initial lymphatic network patterns in adult rat mesenteric tissues. PECAM labeling identifies both lymphatic and blood endothelial cells across the hierarchy of intact networks. Lymphatic vessels are distinguishable based on a lighter labeling intensity and increased diameter. A) Image of a microvascular network containing both lymphatic and blood vessels. B) An image of a microvascular network region containing only lymphatic vessels. Note even in the lymphatic only region, disconnected endothelial segments characteristic of blood capillary sprouts can be observed. C) Example of apparent blood and lymphatic capillary patterning coordination. D) Example of an apparent blood-to-lymphatic capillary connection. Scale bars: A, B = 500 μm, C = 100 μm, D = 200 μm.

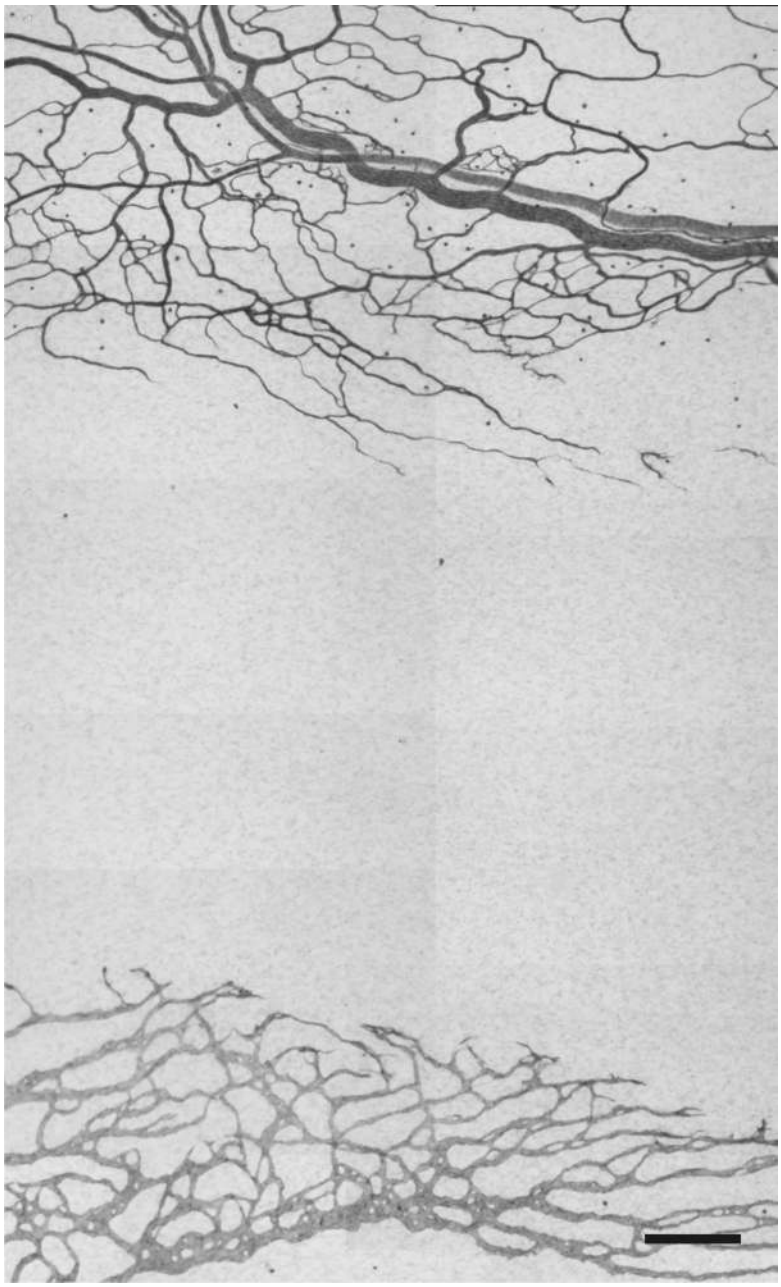

Fig. 25.

Representative image of simultaneous lymphatic and blood microvascular network growth in the same adult rat mesenteric tissue. The angiogenic blood microvascular network (top) and the lymphangiogenic lymphatic network (bottom) appear to be growing toward the same avascular interstitial space. This example motivates the emerging area of lymphatic biology research focused on the common cellular and molecular dynamics involved in the coordination of growth between the two systems. Scale bar = 500 μm.

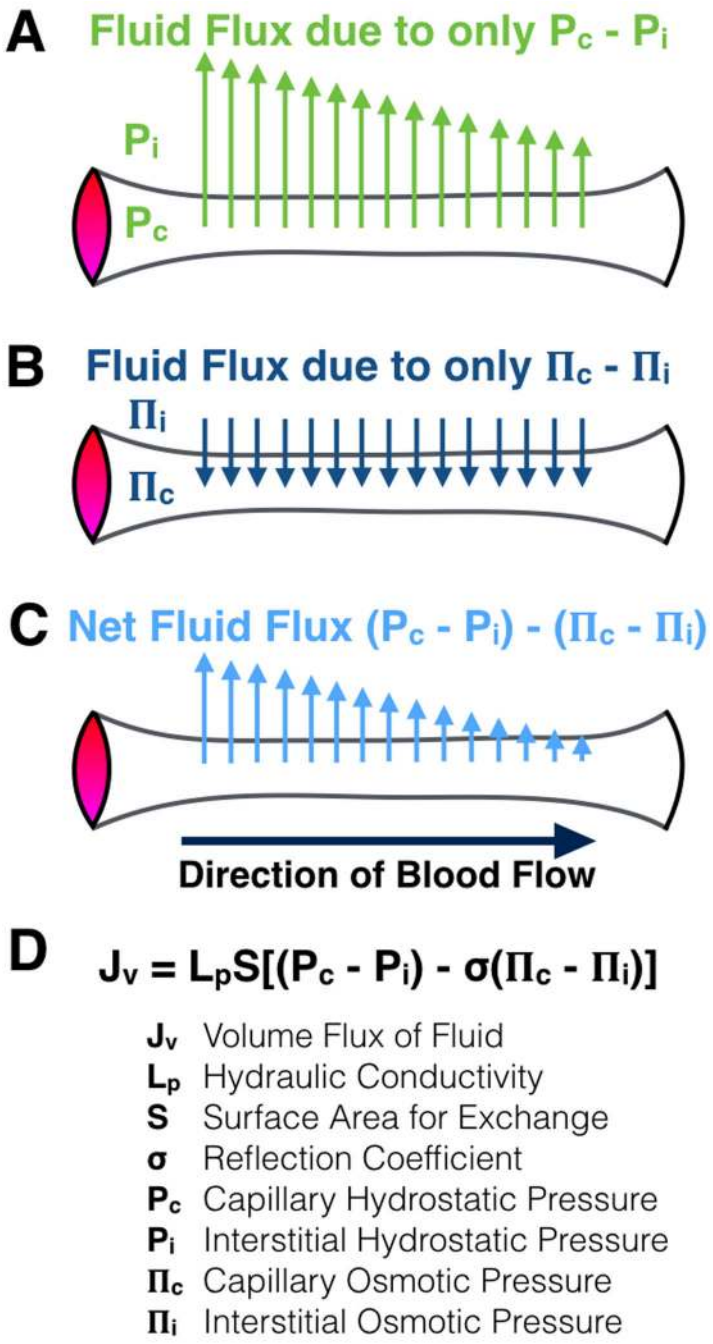

Fig. 26.

The Starling Forces and microvascular leakage. The Starling forces include the hydrostatic and osmotic pressures that drive fluid flow across the microvascular wall. *A*. The net hydrostatic pressure gradient, determined as the capillary hydrostatic pressure (P_c) minus the surrounding tissue's interstitial hydrostatic pressure (P_i) favors fluid flux out of the capillary. Note that P_c, which is determined by the upstream arterial and downstream venous hydrostatic pressures, decreases along the length of the capillary when moving away from the arterial side (left) and getting closer to the venous side (right), while p_i. is equivalent

along the entire length of the capillary. *B*. The capillary osmotic pressure ($\prod_c$) is determined primarily by plasma proteins, while the interstitial osmotic pressure ($\prod_i$) is determined by the protein content in the interstitial space. The osmotic (or oncotic) pressure gradient, determined by the difference between $\prod_c$ and $\prod_i$, favors fluid entry into the capillary, and is generally constant along the length of the capillary. *C*. The resulting net fluid flux when considering both the hydrostatic and osmotic pressure gradients favors extravasation, although the flux decreases along the length of capillary. *D*. The current equation utilized to describe these forces also includes additional factors, including the hydraulic conductivity of water (L_p), the surface area for exchange (S), and the reflection coefficient for plasma proteins (σ). These factors take into account changes that may occur due to increased blood flow and capillary recruitment (which affects S), and changes in microvascular wall integrity (which affects L_p and σ).

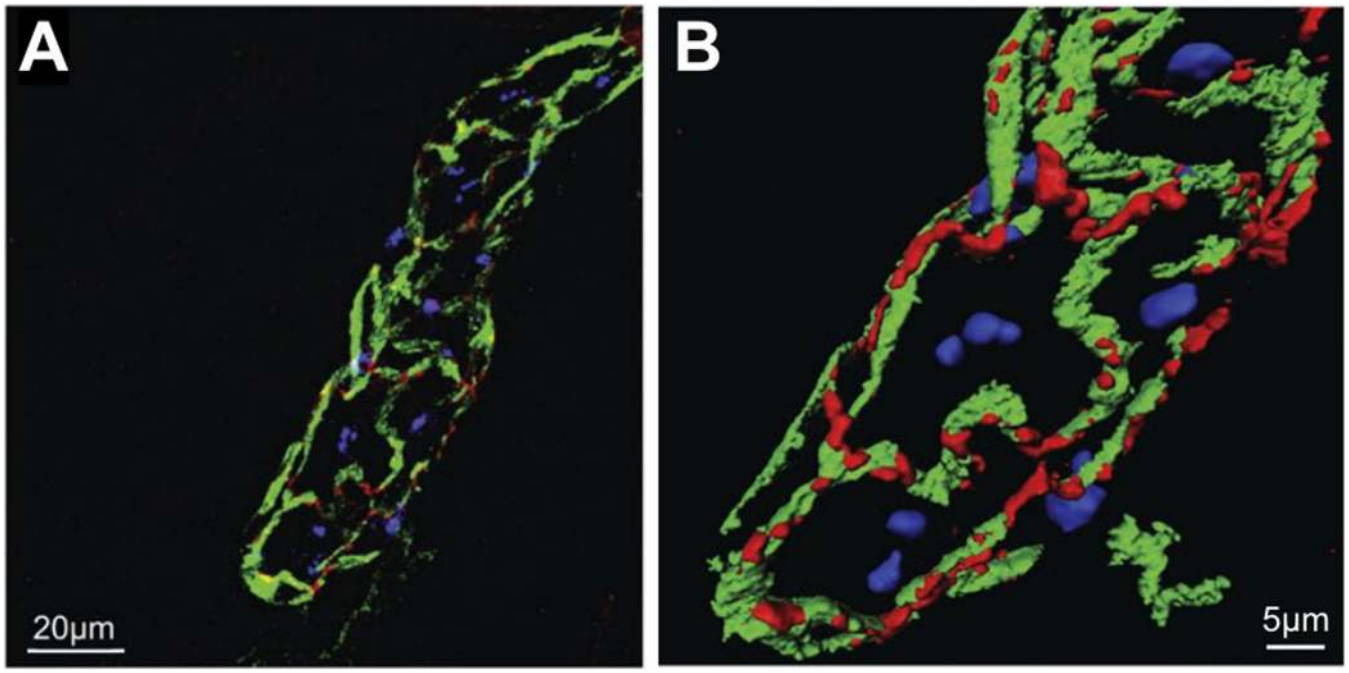

Fig. 27.

CCL21 depositions located in specific regions of initial lymphatic endothelial cells. *A*. Confocal image rendering of triple labeling for Lyve1, CCL-21, and VE-cadherin of a mouse ear initial lymphatic show the distinct sites of CCL21 deposition. Panel *B* shows a closer view. Reproduced from reference (1050) with permission.

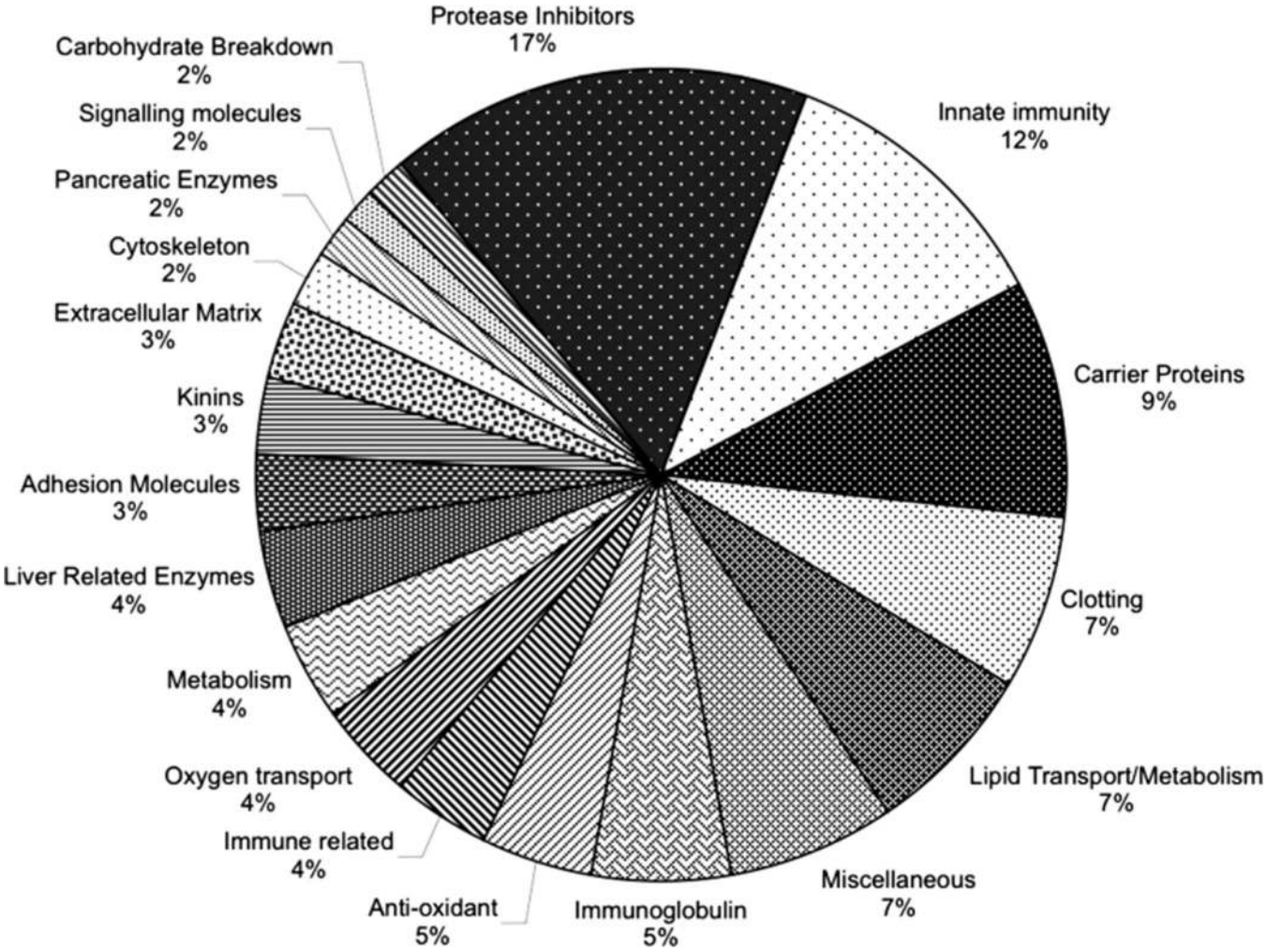

Fig. 28.

The pie chart shows the protein composition of rat mesenteric lymph described in the proteomic study by Mittal et al (705). The percentages are based on the number of identified, non-redundant proteins, classified into their functional groups. Reproduced from reference (705) with permission.

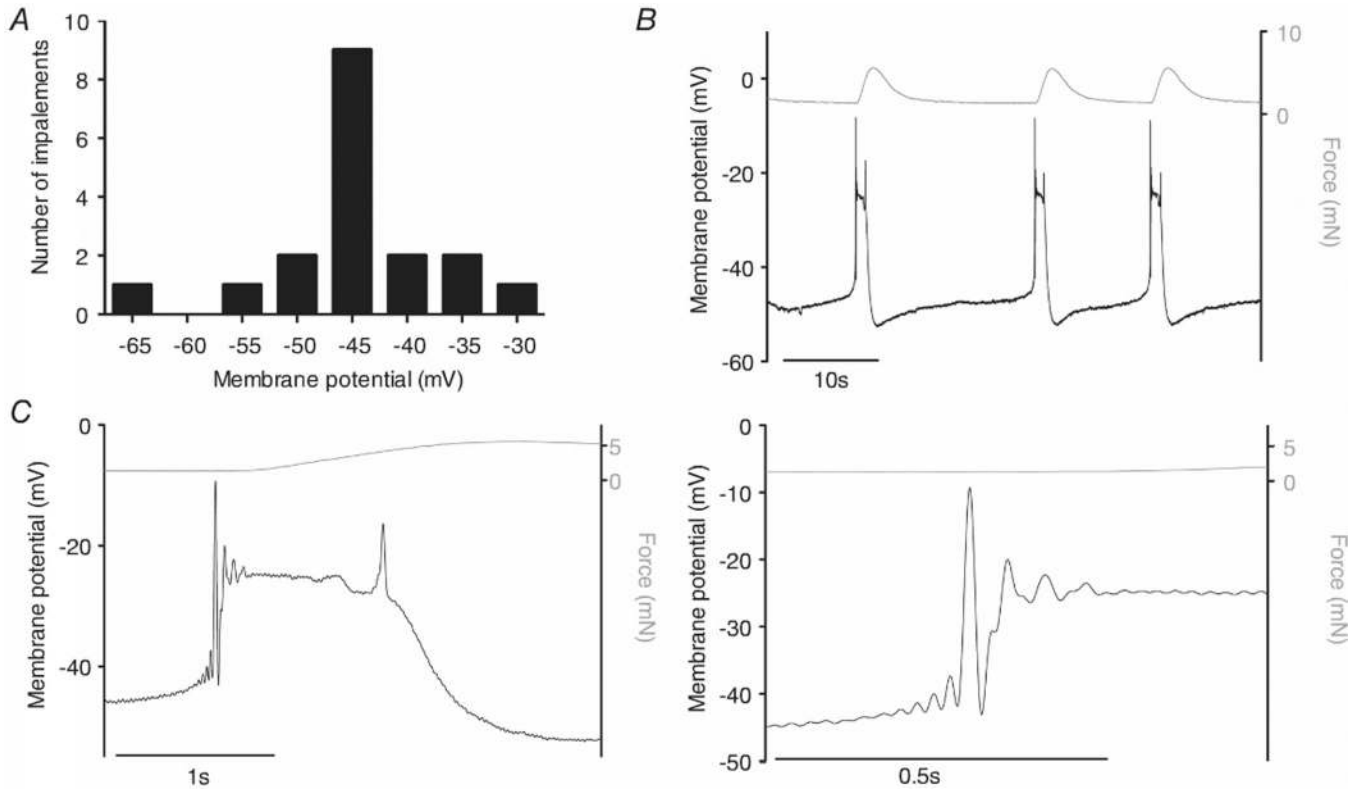

Fig. 29.

Action potentials measured from human mesenteric lymphatic vessels. *A*. The frequency distribution shows measurements of resting potential from 18 lymphatic smooth muscle impalements from 10 different vessels. *B*. The lower trace shows changes in membrane potential over time, including action potentials. The upper trace shows contractions of the lymphatic vessel over the same time frame. These occur immediately after the start of each action potential. *C*. The two traces show greater detail of the changes in membrane potential and force. Note the transient hyperpolarizations that occur just prior to the upstroke of the action potential. This figure is reproduced from reference (1062) with permission.

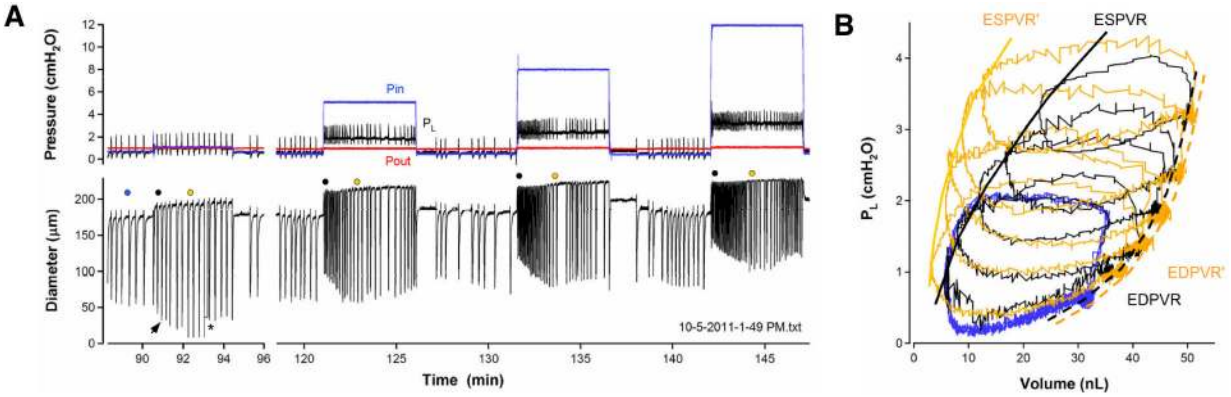

Fig. 30.

Impact of increasing preload at constant afterload on contractions of isolated rat mesenteric lymphatic vessels. *A*. Time course of lymphatic pressure (P_L, black trace) and diameter when the inflow pressure (P_{in}, blue trace) is raised to various levels while the outflow pressure (P_{out}, red trace) is held constant. After each step increase in Pin, the frequency of the phasic contractions initially increases and gradually becomes slower. The amplitude of the phasic contractions initially decreases but then becomes larger (arrow). In panel *B*, pressure-volume (P-V) loops were plotted from the same data. The blue trace represents three consecutive contraction cycles prior to the upward steps in P_{in}, at the time point indicated by the blue dot in panel *A*. The black traces show single contraction cycles at the times indicated by the black dots shown in panel *A*, which correspond to the time points immediately after the upward steps in P_{in}. The gold traces show single contraction cycles corresponding to the times indicated by the gold dots in panel *A*, each about 1 min after the upward pressure step. Evaluation of the end-systolic P-V relationship (ESPVR) between the black and gold traces shows a leftward shift, while the end-systolic P-V relationship (EDPVR) was unchanged, indicating an increase in lymphatic contractility. This figure is modified from reference (961) with permission.

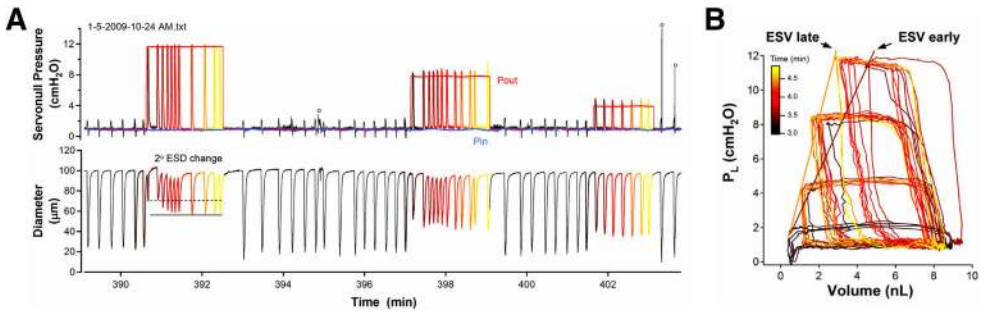

Fig. 31.

Impact of increasing afterload at constant preload on contractions of isolated rat mesenteric lymphatic vessels. *A*. Time course of lymphatic pressure (P_L, black trace) and diameter when the outflow pressure (P_{out}, red trace) is raised to various levels while the inflow pressure (P_{in}, blue trace) is held constant. The open circles denote spike artifacts in the P_L recording due to table vibration of the tip touching the vessel wall. The dotted horizontal line indicates the level of the ESD for the initial phasic contraction after the step increase in P_{out}. The solid horizontal line indicates the ESD for the 8^{th} - 12^{th} phasic contractions.. *B*. P-V plots were drawn for the each of the pressure steps in panel *A*. The P-V loops corresponding to each pressure step are plotted. The color-coding corresponds to the two phasic contractions prior to the upward pressure (dark brown) proceeding to the last phasic contraction prior to the downward pressure step (yellow). Linear fits to the ESPVR are shown for the first P-V loop after the pressure step (ESV early) and the last P-V loop (ESV late). The shift in ESPVR indicates an increase in lymphatic contractility. This figure is modified from reference (249) with permission.

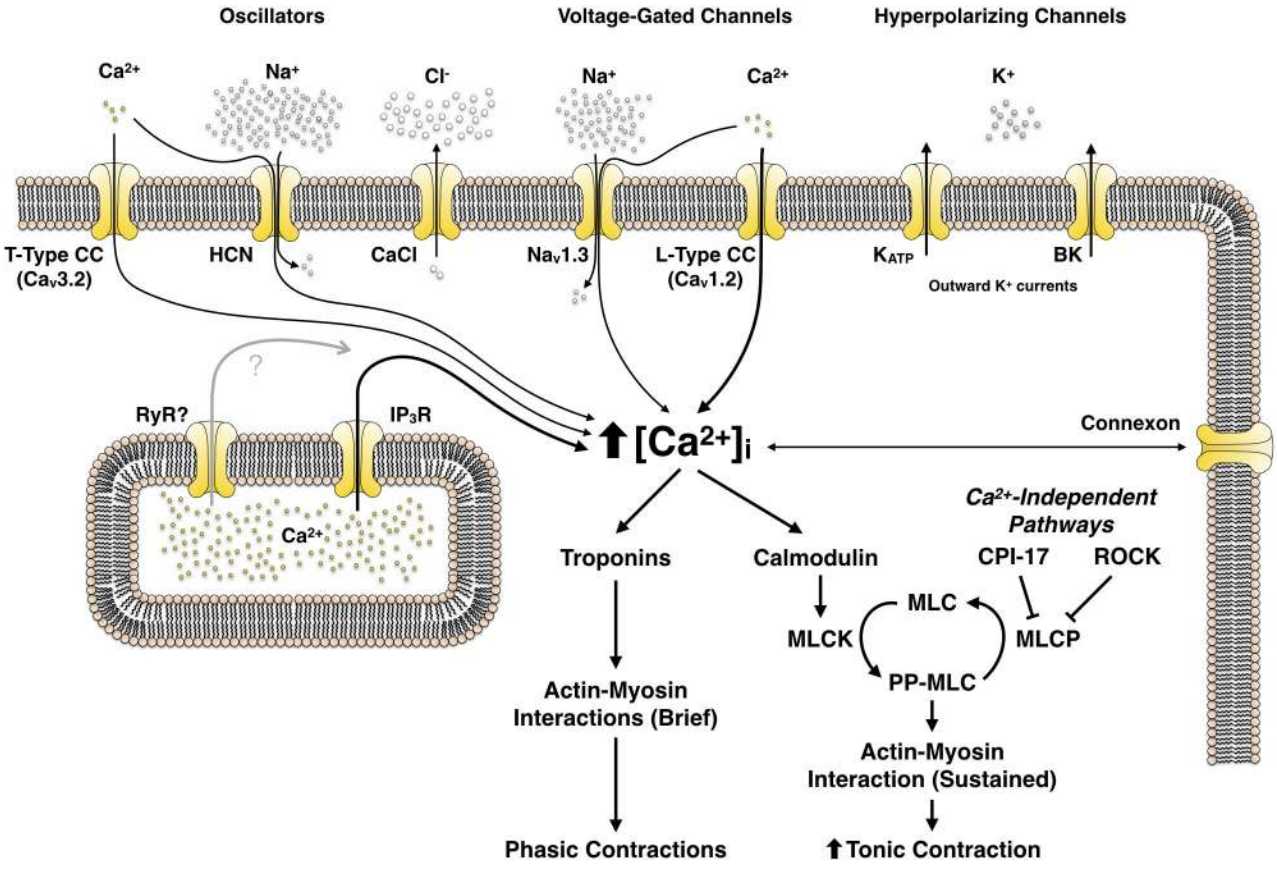

Fig. 32.

Signaling Cascades leading to contraction in lymphatics. Several ion channels affect membrane potential and allow for transient increases in intracellular free Ca^{2+} ($[Ca^{2+}]_i$), which subsequently can activate signaling pathways that cause phasic and tonic contractions. Plasma membrane channels that contribute to oscillations in membrane potential ("Oscillators") include T-type Ca^{2+} channels (Cav3.2), HCN channels, and CaCl channels. Voltage-gated channels that allow for rapid influx of Na^+ and Ca^{2+} to generate action potentials include Nav1.3 and Cav1.2. Hyperpolarizing channels include K_{ATP} and BK channels. Release of Ca^{2+} from the sarcoplasmic reticulum involves IP_3-receptor channels and possibly ryanodine-receptor channels. Elevations in $[Ca^{2+}]_i$ allow for activation of troponins and binding of Ca^{2+} to calmodulin. Troponins facilitate brief actin-myosin interactions that cause phasic contractions. Ca^{2+}-calmodulin activates myosin light chain kinase (MLCK), causing specific phosphorylation of myosin light chains (MLC) allowing for sustained interaction of myosin with actin and generation of tone. This pathway may be modulated by Ca^{2+}-independent signaling by CPI-17 or ROCK, which inhibit the MLC phosphatase, which in turns allows for sustained phosphorylation of MLC.

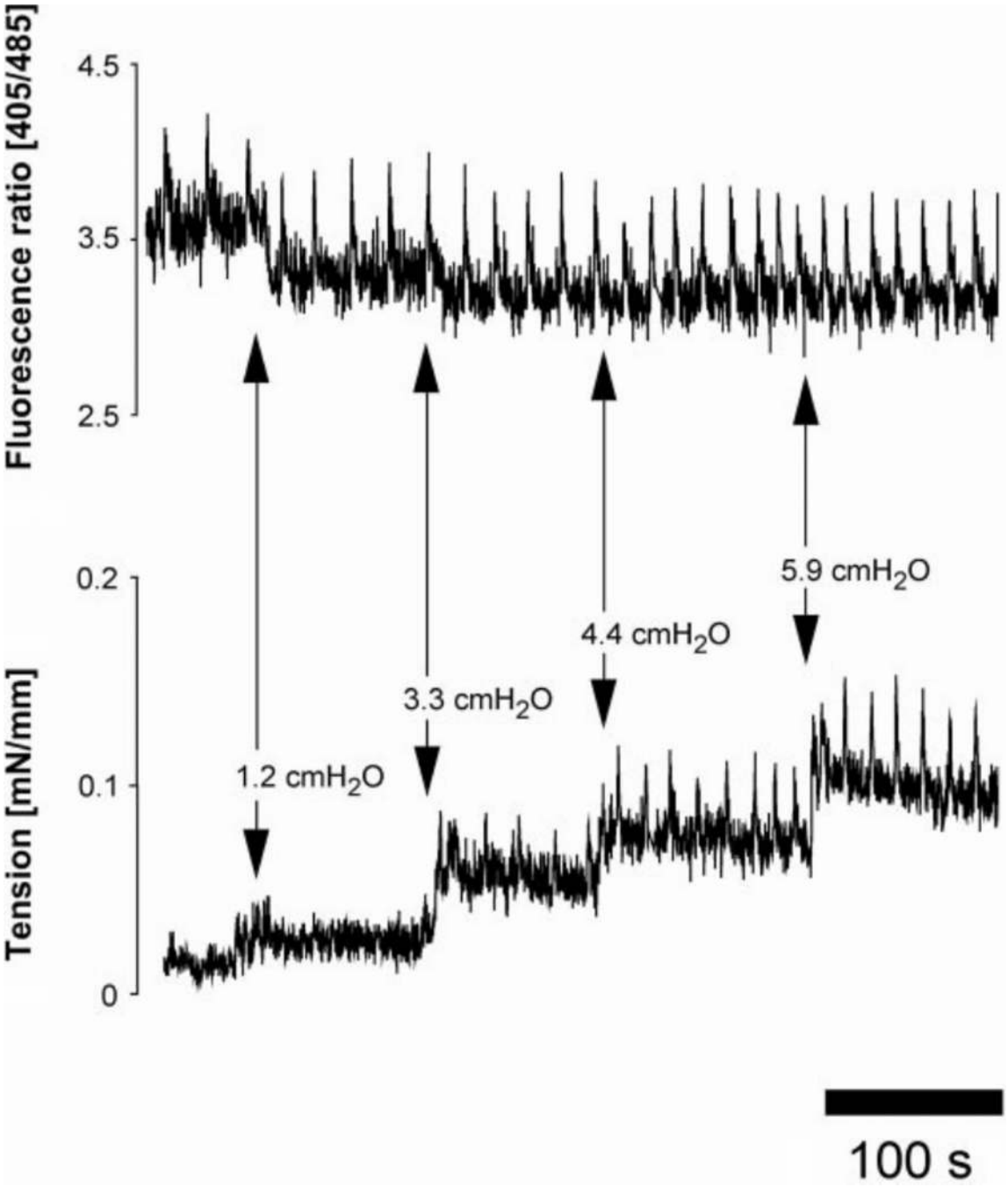

Fig. 33.

Changes in Ca^{2+}-dependent fluorescence tension of an isolated rat thoracic duct in response to stretch. The arrows depict the time points at which the vessel was stretched. Reproduced from reference (981) with permission.

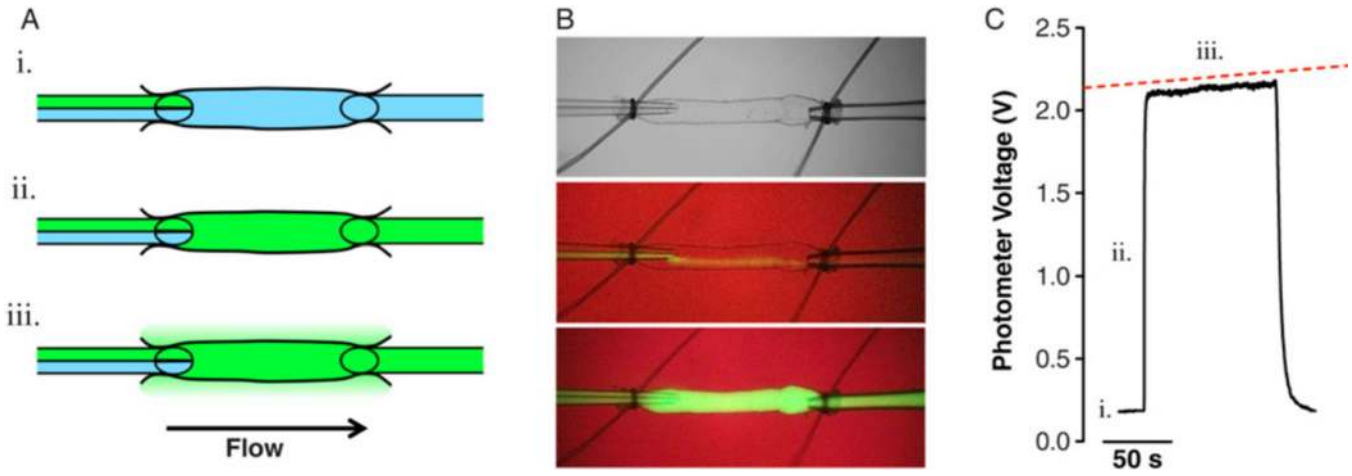

Fig. 34.

Determination of solute permeability of isolated, cannulated collecting lymphatic vessels. *A*. Mouse mesenteric lymphatic vessels were isolated and cannulated onto glass micropipettes. The inflow pipette (left) is a "theta" pipette with a septum that allows for perfusion with solutions from two different reservoirs, indicated by the blue and green colors (i). Flow is controlled by raising inflow pressure in either the blue (no tracer) or green (fluorescent tracer) reservoirs. When tracer is infused, it initially is only observed in the lumen of the vessel (ii), but over a short amount of time some of the tracer leaks across the vessel wall into the surrounding bath (iii). Panel B shows a brightfield image of a cannulated lymphatic (top), a fluorescent image during perfusion with physiological salt solution with no tracer (middle), and during perfusion with solution containing fluorescent albumin (bottom). The red background in the fluorescent images is due to an infrared filter that allows for measurement of diameter throughout recording. *C*. A trace of the fluorescence intensity of the vessel and surrounding area is recorded with a photometer. Initially, baseline fluorescence intensity is recorded, corresponding to image (i) in panel *A*. When the fluorescent albumin is infused, corresponding to image (ii) in panel *A*, there is a step increase in the intensity, followed by a gradual increase (iii) due to the flux of the tracer across the vessel wall. After washout, the fluorescence intensity returns to baseline. The initial rise (ii in Panel *C*) and the slope (iii in panel *C*) represent the initial concentration difference across the vessel wall and the solute flux of the tracer, which along with the area of the vessel wall can be utilized to solve for the solute permeability coefficient using Fick's First Law of Diffusion. Reproduced from reference (958) with permission.

Table 1:

Parameters used to describe collecting lymphatic contractions.

Term	Definition	Abbreviation	Units
Contraction Frequency	Number of phasic contractions per unit of time	CF	Min^{-1}
End Diastolic Diameter	Lymphatic diameter at the end of diastole	EDD	μm
End Systolic Diameter	Lymphatic diameter at the end of systole	ESD	μm
Contraction Amplitude	EDD-ESD	AMP	μm
Maximal Passive Diameter	Diameter obtained at a given pressure in Ca^{2+}-free bath	MaxD	μm
Tone	100% × (MaxD - EDD)/MaxD	Tone	%
Ejection Fraction	(EDD - ESD)/EDD	EF	None
Fractional Pump Flow	EF × CF (an index of active lymph flow)	FPF	min^{-1}