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Establishment and maintenance of blood-lymph separation

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Abstract

Hippocratic Corpus, a collection of Greek medical literature, described the functional anatomy of the lymphatic system in the fifth century B.C. Subsequent studies in cadavers and surgical patients firmly established that lymphatic vessels drain extravasated interstitial fluid, also known as lymph, into the venous system at the bilateral lymphovenous junctions. Recent advances revealed that lymphovenous valves and platelet-mediated hemostasis at the lymphovenous junctions maintain life-long separation of the blood and lymphatic vascular systems. Here, we review murine models that exhibit failure of blood-lymph separation to highlight novel mechanisms and molecular targets for the modulation of lymphatic disorders. Specifically, we focus on the transcription factors, cofactors, and signaling pathways that regulate lymphovenous valve development and platelet-mediated lymphovenous hemostasis, which cooperate to maintain blood-lymph separation.

The cardiovascular system, composed of a central pump (heart) and a network of conduits (blood vessels), is the first functional organ system to develop during mammalian embryogenesis. The heart and blood vessels collaborate to send oxygen- and nutrient-rich blood throughout the embryo to support and sustain its growth and development. Initial assembly of the vascular system is termed vasculogenesis [1], a complex process whereby endothelial cells, which will eventually line all vessels in the body, differentiate from the mesoderm into a primitive plexus of blood vessels [2]. Subsequent angiogenesis, remodeling, expansion, and maturation forms a hierarchical network of vessels of varying calibers and composition mediating efficient nutrient delivery and waste removal throughout the body [3, 4]. The blood vascular system is further differentiated molecularly and functionally into two serial systems - a pre-capillary high-pressure arterial system and a post-capillary low-pressure venous system [5–7].

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The lymphatic vasculature, a complementary low-pressure system of channels, develops in a process called lymphangiogenesis [8]. Historically, descriptions of the lymphatic system can be traced back to the ancient Greeks; however, it was not until the 17th century that a more systematic study of the lymphatics took place [9]. Gasparo Aselli, an Italian anatomist, through the dissection of abdomens of dogs in unfed and fed state, described the presence of ‘veiue albae aut lacteae’ in the intestine or ‘vein like vessels that lack pulsation and transport a milky fluid’ within them [9]. He also described the presence of valves within these vessels and inferred they might help prevent fluid backflow. Subsequently, scholars of the 18th and 19th centuries detailed the anatomy of the lymphatic system and examined the primary significance of the lymphatics in health and in disease [9].

During the early 20th century, two groups put forth theories addressing the developmental origin of the lymphatic system [10, 11]. Using ink injection into embryos, Florence Sabin proposed that the lymphatic system originated by budding from the centrally located cardinal vein and then grows peripherally to all other tissues [10]. Huntington and McClure, deriving observations from wax reconstruction of cat embryos at different developmental stages, postulated that lymphatics develop from peripheral tissues and then make connections with the central venous system [11]. Almost a century later, the use of modern molecular and genetic tools largely support Sabin’s theory [12]. However, more recent evidence reveals that several organ specific lymphatic vessels draw some lymphatic endothelial cells from local sources including those of the heart, the intestine and the skin [13–15]. Integrating these more-recent theories, current models suggest that central cardinal vein derived lymphatics cooperate with local sources to form mature lymphatic vessel endothelium [16, 17]. Defective development of the blood and/or lymphatic vascular system can vary in severity with some cases resulting in embryonic death while others present as congenital vascular anomalies manifesting after birth [18, 19].

The lymphatic system performs multiple tasks, including draining excess protein-rich interstitial fluid back into the systemic circulation, trafficking immune cells from tissues to lymph nodes for antigen presentation and immune surveillance, and transferring lipid-rich chyle from the intestine to the blood [20, 21]. To facilitate the draining of interstitial fluid, blind ended capillaries interspersed throughout the tissue interstitium collect excess fluid and traffic it through increasingly larger vessels towards the bilateral lymphovenous junction (LVJ). The right LVJ interfaces with the right lymphatic duct at the right subclavian and jugular vein junction [22] while the left LVJ joins with the thoracic duct at the junction created by the left subclavian and left jugular veins [23]. The lymphatic endothelium lining the lymphatic vessels is contiguous with the central venous system at the LVJ [24], yet the lymphatic vessels remain clear of blood. These findings suggest systems in place to prevent the backflow of blood into the lymphatic system and raise a critical question: how is the separation of blood - lymphatic system established and maintained?

Lymphovenous Junction in health and disease

Detailed studies on human embryos in the early 20th century, revealed the structural and developmental anatomy of the human LVJ, including the identification of a valve guarding the junction [10]. Subsequent studies in cadavers and surgical patients identified the

common features and variations in terminal branches of thoracic duct and its interface with the central venous system [25–28]. The thoracic duct returns lymph fluid from the lower limbs, abdomen, pelvis, left thorax, head, and neck into venous circulation. The right lymphatic duct drains lymph from the right thorax, right upper limb, and right cervical regions [22]. Approximately 87.5% humans have the thoracic duct with a single opening into the venous system, while the rest have 2 or more openings [23]. In almost half of humans, the thoracic duct opens either into the left internal jugular vein or the venous angle at the left internal jugular vein and left subclavian veins. The thoracic duct drains lymph exclusively into the left LVJ in nearly 95% of humans [23]. This level of variability isn't unexpected as developmental processes during embryogenesis lead to striking variability in venous patterning. Further investigation of the complex anatomical variations of the thoracic duct would help to facilitate clinical procedures such as cannulation of the terminal thoracic duct across the lymphovenous junction.

The lymphovenous valve (LVV) is composed of bicuspid leaflets that open obliquely into the central venous system, positioning one of the cusps as anatomically superior to the other [29]. At the LVJ, the thoracic duct is narrow with a thin endothelial layer and a sub-endothelial layer populated by smooth muscle fibers, elastic fibers, and collagenous fibers. The internal elastic lamina is a thin network-like structure surrounded by two or three layers of longitudinally arranged smooth muscle. The medial layer is composed of few connective tissue or elastic fibers. Scanning electron microscopy demonstrated thinner smooth muscle layer and fine connective tissue fibers near the LVJ. HRP-based immunostaining identified strong expression of extracellular matrix proteins, such as Collagen I, Collagen III, and Laminin within the trunk of human thoracic duct [29]. However, high-resolution microscopy combined with immunofluorescent-staining characterizing the expression of these extracellular matrix proteins within the thoracic duct and the venous angle (Pirogoff's angle) is lacking.

Recent advances in imaging, such as high-resolution ultrasonography, enable visualizing the terminal thoracic duct and motion of the LVVs [30]. In humans, measurement of thoracic duct pressure is an important marker of LVV function. During inspiration, central venous pressure drops and the venous walls relax. This generates a pressure differential across the LVV, forcing it open and driving anterograde drainage of lymph from the thoracic duct into the venous system. In contrast, expiration increases central venous pressure, abolishing this pressure gradient forcing the LVV leaflets closed [31, 32]. Pathologic increases in central venous pressure, a common feature of conditions such as chronic renal failure and congestive heart failure, reverse the pressure gradient across the LVV, causing a breakdown of blood-lymph separation [31]. Notably, not all venous reflux into the thoracic duct is pathologic - physiological venous reflux into the thoracic duct has been observed in approximately 20% patients by CT scans [30, 33]. Despite our longstanding knowledge of the lymphatic system's existence and function, it wasn't until recently that the molecular and cellular basis of LVJ formation have begun to be unraveled.

Lymphovenous valve development

The murine system is a great model for studying cardiovascular developmental processes such as LVV development. With the discovery of molecular markers specific to the lymphatic system, such as a transcription factor Prospero Homeobox 1 (Prox1), more detailed anatomical studies of the LVJ using the murine model system became possible (Figure 1A-D) [34–36]. For instance, Prox1 immunostaining analysis revealed pairs of symmetric LVVs located bilaterally in murine embryos. One LVV is situated dorso-medially relative to the other at the confluence of the jugular lymph sac, internal jugular veins, and external jugular veins. The other LVV is located at the junction of the lymph sac, superior vena cava, external jugular vein, and subclavian vein [34]. The valve leaflets are made of two layers of endothelial cells: 1) An outer layer of Lympho-Venous Valve Endothelial Cells (LVV-ECs) oriented towards the lumen of the superior vena cava in continuity with the endothelial lining of the jugular or subclavian veins; and 2) An inner layer of Lymphatic Endothelial Cells (LECs) oriented towards the lumen of the lymph sac (Figure 1B-D) [34, 35]. LVV-ECs are characterized by high expression of: Prox1; GATA binding protein 2 (Gata2); Forkhead Box C2 (Foxc2); integrin subunit alpha 9 (Itga9); and Integrin subunit alpha 5 (Itga5), low expression of Fms related tyrosine kinase 4 (Flt4, also known as Vegfr3), and no expression of Podoplanin (Pdpn) or Lymphatic vessel endothelial hyaluronan receptor 1 (Lyve1) (Figure 1B) [35]. In contrast, LECs express reduced levels of Prox1, Gata2, and Foxc2 but express typical lymphatic markers such as Flt4, Lyve1, and Pdpn (Figure 1B) [35].

Murine LVV development is orchestrated in a step-wise manner beginning at embryonic day 12 after fertilization (E12) with specification of LVV-ECs from venous endothelial cells (Figure 1B). In the next 12-hours, LVV-ECs undergo delamination, elongation, and perpendicular alignment to form an immature valve-like structures (Figure 1C). This structure continues developing until birth in a process characterized by endothelial cell elongation, extracellular matrix deposition, and recruitment of a variety mural cells to the valve leaflets (Figure 1D) [35].

Molecular regulation of blood-lymph separation

Murine genetic models are a valuable resource for understanding the molecular basis of blood-lymph separation (Table 1). Several mouse models with targeted deficiencies of specific proteins, including Prox1, Foxc2, Gata2, Itga5, Histone deacetylase 3 (Hdac3), Gap junction protein alpha 4 (Gja4, also known as Connexin 37), Cytochrome P450 family 26 subfamily B member 1 (Cyp26b1), EPH receptor B4 (EphB4), and Catenin Beta 1 (Ctnnb1), display defective LVV formation and/or failure of blood-lymph separation at various stages of development (Table 1, Figure 2) [34, 35, 37–42]. Mice with reduced expression of Prox1 (*Prox1^{+/−}*) demonstrate defective specification of LVV-ECs and lack of LVVs [34]. Instead, the jugular lymph sacs in *Prox1^{+/−}* mice abnormally connect to the central veins due to increased fluid pressure within the lymph sac. Furthermore, a small fraction of the embryos displayed peripheral blood-filled lymphatic vessels due to reflux of blood from the high-pressure venous vessels to low-pressure lymphatic vessels. This body of work demonstrates

that *Prox1* functions both as a marker of LVVs and a critical regulator of their formation and maintenance [34].

Mutations within human *FOXC2*, a member of the forkhead family of transcription factors, are frequently associated with lymphedema-distichiasis syndrome, characterized clinically by swelling of the limbs (lymphedema) and the growth of extra eyelashes (distichiasis) [43]. *Foxc2*-knockout mice recapitulate this phenotype, demonstrating a causative role for *Foxc2* in human lymphedema-distichiasis[44]. *Foxc2* heterozygous mutant mice have a reduced number of LVVs while homozygous mutants lack LVVs altogether [35]. The presence of embryonic edema negatively correlates with the number of LVVs, reinforcing their important role in normal lymphatic function. Mechanistically, loss of *Foxc2* leads to defective specification of LVV-ECs and reduced *Gja4* expression [35, 45]. Interestingly, *Foxc2*-heterozygous and *Gja4*-null embryos show blood-filled dermal lymphatic vessels, suggesting that *Foxc2* and *Gja4* are components of a common molecular pathway regulating blood-lymph separation [46]. Furthermore, loss of *Gja4* and *Gja1* (also known as Connexin 43) expression in murine embryos causes blood to fill the thoracic duct in addition to dermal, mesenteric, and intercostal lymphatic vasculature [47]. These embryos lacked an aberrant connection between blood vessels and lymphatic vasculature, suggesting defective LVV formation [47]. Indeed, a recent study revealed defective LVV leaflet formation in *Gja4*-null mice [35]. Although *Gja4* is dispensable for LVV-EC specification, it is required for the coordinated invagination of LVV-ECs. This further establishes requirement of Connexins in collective cell behavior, such as migration, during embryogenesis [48–51].

GATA2, a zinc-finger transcription factor, is frequently mutated in patients presenting with primary lymphedema [52, 53]. Analysis of mice genetically deficient for endothelial *Gata2* show anomalous LVV leaflets and defective blood-lymph separation. Interestingly, *Prox1* expression is reduced in the *Gata2*-null LVV-ECs, possibly resulting in loss of LVV-EC identity in the developing LVV leaflets [35, 41]. Mechanistically, *Gata2* interacts with regulatory elements upstream of *Prox1* and *Foxc2* loci to enhance *Prox1* and *Foxc2* expression in LVV-ECs [41].

Recently, we identified a novel and unexpected LEC-specific function of *Hdac3*, a member of an evolutionarily conserved group of histone-modifying enzymes, during LVV formation [40]. Mice lacking *Hdac3* in LECs exhibit anomalous LVV morphogenesis, failure of blood-lymph separation, and postnatal lethality, demonstrating that *Hdac3* functions in a lymphatic endothelial cell-autonomous manner during LVV development [40]. Consistent with this, platelet or macrophage specific *Hdac3* knockout mice demonstrate normal blood-lymph separation. Mechanistically, *Hdac3* promotes flow-responsive *Gata2* expression in developing LECs. In response to flow, transcription factors *Tal1*, *Ets1/2* and *Gata2* recruit *Hdac3* at a deeply conserved intragenic *Gata2* enhancer (divergence ~350 million years ago). *Hdac3* recruits p300, a histone acetyl transferase, which activates the intragenic *Gata2* enhancer, promoting *Gata2* transcription [40]. Reinforcing the importance of this enhancer, patients with monoallelic missense mutation (c.1017+572C>T) or intragenic microdeletion (c.1017+512del28) within this enhancer element, have reduced GATA2 expression and lymphatic dysfunction [54–58]. Further cementing this interaction, mice lacking this *Gata2*

intragenic enhancer exhibit reduced Gata2 expression and phenocopy the endothelial knockout of Gata2 [41, 57, 59, 60].

In addition to specific transcription factors and cofactors, recent studies have identified numerous signaling pathways that regulate blood-lymph separation including: retinoic acid (RA), Wnt, MAPK, Notch, Polycystin, and Integrin pathways [37, 38, 61–65]. Mice lacking *Cyp26b1*, a cytochrome P450 enzyme that catalyzes the breakdown of RA, display enlarged blood filled lymph sacs and abnormal LVV leaflets with increased number of Prox1⁺ cells [37]. Mechanistically, loss of *Cyp26b1* results in robust activation of RA signaling within the developing lymphatic system, which drives over-specification and proliferation of LEC progenitor cells [37]. Recent work from the Srinivasan lab demonstrates that activity of the Wnt- β -catenin signaling pathway is essential for LVV development. Mice with LEC-specific deletion of *Ctnnb1*, encoding beta-catenin, lack LVV valves and LVV-EC differentiation, thus failure of blood-lymph separation [38]. Similarly, tissue-specific deletion of Wntless (Wls) in mice also cause failure of LVV valve formation, suggesting that complementary secretion of Wnt ligands from smooth muscle cells and LECs are important for LVV development [66].

Rho GTPases, a family of small signaling G proteins and a subfamily of Ras signaling, regulate endothelial cell migration and vascular development [67–69]. Emerging evidence shows that Rac1, a Rho GTPase, regulates migration of committed LECs during lymph sac specification from the cardinal vein [70]. Embryos lacking endothelial *Rac1* exhibit blood-filled lymphatic vessels and embryonic lethality. Rac1-deficient LECs show reduced expression of Vegfr3, suggesting that Vegfc mediated persistent LEC migration is likely required for establishment of blood-lymph separation [70]. Additionally, a recent study from the Oliver lab demonstrates that GTPase signaling is a key regulator for blood-lymph separation [71]. Embryos lacking Ras interacting protein 1 (*Rasip1*), a vascular-specific regulator of GTPase signaling, exhibit blood-filled lymphatic vessels [71]. Similarly, LEC-specific ablation of *Ras-related protein 1* (*Rap1*) causes blood-filled lymphatics and defective LVV formation in embryonic mice, suggesting that Rap1 functions downstream of Gs-coupled adrenomedullin signaling pathway to regulated LVV development [72]. Sprouty related EVH1 domain containing 1 and 2 (*Spred1/2*) function downstream of GTPases and negatively regulate Vegfc/Vegfr3-dependent RAS-Mitogen activated protein kinase (MAPK) signaling pathway. Deletion of *Spred1* and *Spred2* genes in mice leads to erythrocyte-filled lymphatic vessels, suggesting that Vegfc-Vegfr3-mediated hyperactivation of RAS-MAPK signaling could lead to defective blood-lymph separation [61]. Interestingly, RAF1^{S259A} mediated constitutive activation of RAS-MAPK signaling in mice does not cause failure of blood-lymph separation [62]. Hence, Vegfc-Vegfr3 may activate additional pathways to regulate separation of blood and lymphatic systems.

Notch signaling pathway genes, whose expression is noted around the lymphovenous junction, are critical for LEC specification and blood-lymph separation [63, 64]. Mice lacking *Notch1* or expressing constitutively active *Notch1* in LECs exhibit failure of blood-lymph separation via distinct mechanisms. Loss of Notch1 signaling in mice promotes specification of Prox1⁺ LECs, leading to enlarged lymph sacs and defective LVV formation and failure to partition the lymphatic and vascular spaces [63, 64]. In contrast, mice with

gain-of Notch1 signaling exhibit reduced specification of Prox1⁺ LECs, thus failure of blood-lymph separation [63, 64]. Components of the calcium-based polycystin signaling pathway, Polycystin1 (*PKD1*) and *PKD2*, are frequently mutated in patients with autosomal dominant polycystic kidney disease [73, 74]. Less frequent defects seen in these patients, such as intracranial aneurysms, allude to broader functions of PKD1/2. Indeed, mice lacking both *Pkd1* and *Pkd2* exhibit blood-filled lymphatic vessels and jugular lymph sac, indicating that polycystin signaling is required for blood-lymph separation [65, 75]. Mechanistically, Pkd1 and Pkd2 regulates filopodia formation and directed migration of LECs to establish complex lymphatic capillary network [65, 75].

Hydrops fetalis is a life-threatening disorder characterized by fluid accumulation in the fetus. As a major regulator of tissue fluid balance, a dysfunctional lymphatic system is implicated in nearly 15% of non-immune-related cases of hydrops fetalis [76]. A recent study of two families with a history of non-immune hydrops fetalis (NIHF) found mutations in the EPH receptor B4 (*EPHB4*), a component of the receptor tyrosine kinase (RTK) signaling pathway [42]. Mice with targeted deletion of *EphB4* within LECs demonstrate defective LVV development, shortened valve leaflets, abnormal clustering of Prox1⁺ cells at the LV Junction, and blood-filled dermal lymphatic vessels [42]. Very recently the Caron laboratory defined a case of NIHF associated with mutation in calcitonin receptor-like receptor (*CALCRL*), a component of G protein-coupled receptor signaling pathway [77]. Murine embryos lacking LEC *Calcrl* or nonendothelial receptor activity modifying protein 2 (*Ramp2*) exhibit severe NIHF, placental dysfunction, and lethality similar to human NIHF [77]. However, LVV formation and blood-lymph separation appear normal in *Calcrl*-null mice. Other mutations identified in cases of non-immune related hydrops fetalis include *CCBE1*, *FAT4* and *PEIZO1*, however their role in LVV development is unexplored [78–81].

Components of Integrin signaling pathways are critical regulators of lymphatic development [8, 82, 83] and multiple Integrin receptors have been implicated in the regulation of blood-lymph separation [82, 83]. Mice lacking *Itga5*, a major fibronectin receptor, exhibit defects both in the separation of blood-lymphatic vasculature and formation of the LVVs [39]. These defects appear to be due to defective assembly of fibronectin in developing LVVs. In contrast, *Itga9* knockout mice exhibit proper separation of blood-lymphatic vasculature and normal-appearing LVVs [84]. However, these mice have anomalous large platelet thrombi both at the LVVs and throughout the thoracic duct, suggesting that LV hemostasis could be sufficient to maintain blood-lymphatic separation [84]. The coxsackie- and adenovirus receptor (CAR), a cell-adhesion molecule, is expressed in developing lymphatic vasculature [85]. Embryos lacking CAR gene, *Cxadr*, exhibit erythrocyte-filled lymphatic vessels, suggesting a failure of blood-lymph separation [86]. Despite this, the function of CAR-mediated signaling in LVV development is unknown.

Platelet mediated Inter-vascular Hemostasis

Platelet mediated hemostasis at the LVJ prevents backflow of venous blood into the lymphatic circulation, and is required to both establish and maintain blood-lymph separation [84]. In contrast to the platelet mediated hemostasis that occurs after endothelial cell injury in blood vessels, platelet hemostasis at the LVJ involves the interaction of platelets with

uninjured lymphatic endothelial cells at the LVJ. This interaction activates platelets, resulting in a platelet plug and thrombus formation specifically at the LVJ [84]. Pioneering studies from the Kahn lab defined the role of hematopoietic cells in blood-lymph separation using mice lacking immune receptor activated platelet signaling proteins lymphocyte cytosolic protein 2 (*Lcp2*, also known as *Slp76*) or spleen associated tyrosine kinase (*Syk*) [87]. These mice exhibited blood-filled lymphatic vessels, chylothorax, and chylous ascites, demonstrating compromise of the lymphatic system [84, 87, 88]. Irradiated wild-type mice transplanted with *Lcp2*-deficient hematopoietic cells recapitulated the phenotype of *Lcp2* knockout mice, supporting the notion that *Lcp2* and *Syk* are required specifically within hematopoietic cells for blood-lymph separation [87, 89] and confirming a role for hematopoietic cells in LVJ integrity.

Analyses of how hematopoietic cells regulate the separation of blood and lymphatic vascular systems identified a platelet cell surface receptor C-type lectin domain family 1 member b (*Clec1b*, also known as *Clec2*), which mediates sequential activation of *Lcp2* and *Syk*, therefore controlling platelet activation [88, 90–93]. Subsequent studies characterized *Pdpn*, an O-glycosylated protein expressed on the surface of LECs, as a ligand for *Clec1b* [88, 94, 95]. The interaction between *Pdpn* and *Clec1b* robustly induces tyrosine phosphorylation of the hemi-immunoreceptor tyrosine-based activation motif (ITAM) YxxL. Activation of this motif leads to platelet aggregation via activation of *Syk*, *Lcp2*, linker for activation of T-cells (LAT), and phospholipase C Gamma 1 (*Plcg1*) [88, 90, 91, 96–101]. Disruption of platelet aggregation with an anti-*Pdpn* antibody or acetyl salicylic acid administration in mice results in blood-filled lymphatics, demonstrating functional significance of platelet-mediated hemostasis in blood-lymph separation [84, 95]. Consistent with this, genetic experiments in mice revealed that megakaryocyte or platelet *Clec1b* and *Lcp2* are critical for maintenance of blood-lymph separation [84, 88, 93, 95, 102, 103]. *Clec1b* activation in platelets also promotes *Syk* activation via PI3K/Tec-Bruton's tyrosine kinase (*Btk*) [104]. *Tec* and *Btk* double knockout embryos exhibit blood-filled lymphatics suggesting that this pathway is required for blood-lymph separation [104]. *Clec1b* function in other hematopoietic lineages, including neutrophils and macrophages, appears to be dispensable for LVJ integrity [88, 103].

Mice lacking platelets (*Meis1*-null) or definitive hematopoiesis (*Runx1*-null) have blood-filled lymphatic vessels, reinforcing the association between platelets, hematopoietic cells, and blood-lymph separation [12, 102]. A recent study from the Mäkinen laboratory identified that platelets also maintain venous integrity during embryonic vascular morphogenesis [105]. Specifically, embryos lacking *Clec1b* have impaired blood vessel integrity, shown by the presence of extravascular erythrocytes and platelets [105]. In turn, lymphatic vessels clear the extravasated erythrocytes from tissues resulting in a blood-filled lymphatic phenotype [105].

Conditional deficiency of O-glycan synthase (*C1Galt1*), the enzyme required for o-glycosylation of *Pdpn* in hematopoietic and endothelial cells, resulted in intestinal blood-lymph mixing and reduced expression of *Pdpn* [106]. Interestingly, a bone marrow transplant with *C1Galt1*-deficient hematopoietic cells in mice did not induce breakdown of blood-lymph separation, revealing that *Pdpn* functions in non-hematopoietic cells [106].

Interestingly, *Pdpm*-null mice exhibit partially penetrant blood-lymph separation phenotype at postnatal stages, suggesting additional ligands of Clec1b in LECs [95]. In contrast, a recent study demonstrates that *Pdpm* may maintain blood-lymph separation throughout postnatal life [107]. At the developing LVJ, platelet-Clec1b and LEC-*Pdpm* interact to promote platelet aggregation around E13.5 [95]. Consistent with this, *Pdpm*-null mice lack platelet aggregation at LVJ. Similarly, administration of an anti-Clec1b antibody in mice causes a rapid breakdown of blood-lymph partitioning, seen by the emergence of blood-filled intestinal lymphatics [84]. Retrograde flow of blood into the lymphatic vasculature via the thoracic duct, ultimately ending with blood-filled intestinal lymphatics, was observed in *Clec1b*-deficient mice. This demonstrates that interaction between platelets and endothelial cells likely occurs proximal to the intestine. Mice lacking lymph nodes, such as *Lta*- or *Rorc*-knockout mice, do not exhibit blood filled lymphatics, revealing that the site of platelet-LEC interaction is not in the lymph node [84]. Syk inhibitor treated mice have normal blood-lymph separation, demonstrating that even low levels of platelet signaling is sufficient to maintain blood-lymph separation [84]. Overall, these studies highlight the critical in vivo role of *Pdpm*-Clec1b interaction and the importance of platelet aggregation in effecting blood lymph separation.

These results raise an interesting question: How does a thrombus at the LVJ prevent backflow of venous blood, yet allow unimpeded anterograde lymph flow? Recent work from the Griffin laboratory demonstrates that *Chd4*, a component of the NuRD chromatin repressive complex, is required for establishment of blood-lymph separation in mice [108]. LEC-specific ablation of *Chd4* in mice increases expression of Plasminogen activator Urokinase (*Plau*), an anticoagulant enzyme, leading to dissolution of the thrombus at the LVJ. Genetic deletion of *Plau* rescued both thrombus formation and blood-lymph separation defect in *Chd4*-null embryos. Despite rescuing the embryonic phenotype, *Chd4*-null/*Plau*-null pups were not recovered at birth, suggesting *Plau*-independent clot dissolution later in development [108]. Currently three models have been proposed to explain how the LVJ could both maintain forward lymph drainage and prevent blood reflux [109]. First, the dynamic lymphovenous clot model proposes that thrombi at the LVJ block retrograde blood flow while increasing pressure within the jugular lymph sac or thoracic duct. Increased lymphatic pressure crosses a threshold, dislodges the thrombus, and allows transient anterograde lymph flow [109]. The second, the pressure mediated dormant valve model, suggests that some LVJs allow anterograde lymph flow while others prevent retrograde venous blood flow [109]. Lastly, the lymph fluid lysis model claims that forward lymph flow occurs when clot lysis factors accumulate in the lymph in close proximity to the LECs. The local increase in these factors facilitates the dissolution of the clot and transient anterograde lymph flow. In contrast, venous blood contact with the LECs abrogates this local accumulation of lytic factors, promoting the clot formation and re-establishing blood/lymph segregation [109].

Both LVV development and thrombus formation are required for blood-lymph separation, however, they are independent processes. For example, *Prox1*-heterozygous mice lacking LVVs demonstrate extensive thrombus formation in the thoracic duct. Despite a normal appearing LVJ thrombus, some *Prox1*-heterozygous embryos have blood-filled dermal lymphatics, revealing that the LVVs are required for segregation of blood and lymphatic

systems [84]. Similarly, *Clec1b*-deficient mice have normal LVVs despite the absence of a thrombus at the LVJ, demonstrating that platelet-LEC interactions and thrombus formation aren't required for LVV development [84]. Although they have normal appearing LVVs, *Clec1b*-deficient mice present with a blood-filled lymphatic network. The influx of blood into the lymphatic vascular space in *Clec1b*-null embryos disrupts anterograde lymphatic flow, a potent stimulator of lymphatic valve development. Indeed, *Clec1b*-deficient embryos exhibit reduced number of lymphatic valves, indicating that proper blood-lymph partitioning is required for lymphatic valve development. Overall, these data support a model where disruption of lymphovenous hemostasis reduces lymph flow impairs lymphatic valve development, and leads to lymphedema. However, this model raises questions regarding antiplatelet therapy approach to treat thrombotic diseases. Aspirin or adenosine 5'-diphosphate receptor antagonists could inhibit lymphovenous hemostasis in pathological conditions with elevated venous pressure leading to lymphatic insufficiency, thus lymphedema. Future studies in disease settings will reveal the effect of antiplatelet therapies on lymphovenous hemostasis, thus separation of blood and lymphatic vasculature.

Conclusions and Perspective –

Although the lymphatic vasculature has been described as early as fifth to the third century B.C., scientific progress over the past 20 years has begun to define the cellular and molecular pathways involved in separation of blood and lymphatic systems. Using the murine model system, multiple transcription factors, cofactors, receptors, ligands, and signaling components that are necessary for the establishment and/or maintenance of blood-lymph separation have been identified. These factors regulate all aspects of LVV-EC development, the formation of LVVs, the establishment of platelet-mediated hemostasis, and the establishment of distinct blood and lymphatic vascular spaces. There remains a clear need to better understand the mechanisms that regulate LVV structure, function, and lymph flow across LVJ in patients with altered vascular dynamics, such as in cardiomyopathies, chronic renal failure, lymphedema, and congestive heart failure. Emerging high-resolution ultrasound and minimally invasive cannulation techniques may help elucidate the disease-specific alterations of lymph flow at the LVJ and launch investigation into the LVV and LVJ dynamics in vascular pathophysiology. Generation of adult animal models mimicking these pathophysiological alterations will be critical for developing better diagnostics and effective treatment.

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Figure 1:

(A-D) Cartoon model of developing murine lymphovenous valve (A) in a stage-specific (B-D) manner. LVV-EC differentiate at E12.0 (B) and undergo delamination (B), aggregation (C), and maturation (D) to form functional LVVs at LVJ. Lymph sac (LS, Green), External jugular vein (EJV, Blue), Internal jugular vein (IJV, Blue), Superior vena cava (SVC, Blue), Lymphovenous valve endothelial cells (LVV-EC, Yellow), and Lymphatic endothelial cells (LECs, Red),

Figure 2:
(A-B) Cartoon model of blood-lymph separation defect due to anomalous LVVs and/or lymphovenous hemostasis.

Table 1:

Mouse models of blood-filled lymphatics

Gene	Gene Function	Genotype	Explanation of blood-filled lymphatics	References
<i>Prox1</i>	Transcription Factor	<i>Prox1</i> ^{+/-}	Defective specification of LVV-ECs and lack of LVVs	[34]
<i>Foxc2</i>	Transcription Factor	<i>Foxc2</i> ^{+/-} <i>Foxc2</i> ^{-/-}	Reduced number or complete absence of LVVs, defective specification of LVV-ECs	[35]
<i>Gata2</i>	Transcription Factor	<i>Gata2</i> ^{F/F} ; <i>Prox1-Cre</i> ^{ERT2} <i>Gata2</i> ^{F/-} ; <i>Cdh5-Cre</i> ^{ERT2}	Anomalous LVV leaflets	[35, 41]
<i>Hdac3</i>	Transcription cofactor, Deacetylase enzyme	<i>Hdac3</i> ^{F/F} ; <i>Tek-Cre</i> <i>Hdac3</i> ^{F/F} ; <i>Cdh5-Cre</i> <i>Hdac3</i> ^{F/F} ; <i>Lyve1-Cre</i>	Shortened and anomalous LVV leaflets; Defective perpendicular alignment of LVV-ECs	[40]
<i>Cyp26b1</i>	Monooxygenase enzyme, Retinoic Acid Signaling	<i>Cyp26b1</i> ^{-/-}	Abnormal LVV leaflets composed of increased number of Prox1 ⁺ cells	[37]
<i>Ctnnb1</i>	Wnt Signaling; Component of adherens junction	<i>Ctnnb1</i> ^{F/F} ; <i>Lyve1-Cre</i>	Absence of LVV valves and LVV-EC differentiation	[38]
<i>Wls</i>	Wnt Signaling Ligand	<i>Wls</i> ^{F/F} ; <i>Lyve1-Cre</i> ; <i>Tagln-Cre</i>	Absence of LVVs	[66]
<i>Lrp5</i> and <i>Lrp6</i>	Wnt Signaling Receptor	<i>Lrp5</i> ^{F/F} ; <i>Lrp6</i> ^{F/F} ; <i>Lyve1-Cre</i>	Absence of LVVs	[66]
<i>Rac1</i>	Small GTPase, Hydrolase enzyme, RAS signaling	<i>Rac1</i> ^{F/F} ; <i>Tie1-Cre</i>	Defective LEC migration	[70]
<i>Rasip1</i>	Regulator of small GTPase, RAS signaling	<i>Rasip1</i> ^{F/F} ; <i>Prox1-Cre</i> ^{ERT2}	Enhanced LEC proliferation	[71]
<i>Spred1</i> and <i>Spred2</i>	Inhibitor of MAP kinase, RAS signaling	<i>Spred1</i> ^{-/-} ; <i>Spred2</i> ^{-/-} <i>Spred1</i> ^{-/-} ; <i>Spred2</i> ^{+/-}	Enhanced LEC proliferation	[61]
<i>Rap1a</i> and <i>Rap1b</i>	Regulator of small GTPase, RAS signaling	<i>Rap1a</i> ^{F/F} ; <i>Rap1b</i> ^{F/F} ; <i>Lyve1-Cre</i> <i>Rap1a</i> ^{F/F} ; <i>Rap1b</i> ^{F/F} ; <i>Prox1-Cre</i> ^{ERT2}	Defective LVV formation	[72]
<i>Notch1</i>	Receptor, Notch signaling	<i>Notch1</i> ^{F/F} ; <i>Prox1-Cre</i> ^{ERT2}	Defective LVV formation; Increased LEC differentiation	[63, 64]
<i>Notch1 intracellular domain</i>	Receptor, Notch signaling	<i>NIIC</i> ; <i>Prox1-Cre</i> ^{ERT2}	Decreased LEC differentiation	[63]
<i>Pkd1</i>	Membrane Protein, Calcium Signaling	<i>Pkd1</i> ^{-/-}	Defective migration of LEC progenitor cells; Defective polarity, elongation, and adherens junction of LECs	[65, 75]
<i>Pkd2</i>	Membrane Protein, Calcium Signaling	<i>Pkd2</i> ^{-/-}	Defective migration of LEC progenitor cells	[65]
<i>Ephb4</i>	Receptor tyrosine kinase, RTK signaling	<i>Ephb4</i> ^{F/F} ; <i>Prox1-Cre</i> ^{ERT2}	Shortened and anomalous LVV leaflets	[42]
<i>Itga5</i>	Receptor, Integrin signaling	<i>Itga5</i> ^{F/F} ; <i>Pdgfrb-Cre</i>	Failure of LVV formation	[110]
<i>Itgb3</i>	Receptor, Integrin signaling	<i>Itgb3</i> ^{-/-}	Impaired lymphovenous hemostasis	[84]
<i>Cxadr</i>	Cell-adhesion	<i>Cxadr</i> ^{F/F} ; <i>CMV-Cre</i> ^{ERT2}	Defective cell-cell junctions in LECs	[86]
<i>Gja4</i>	Cell-adhesion	<i>Gja4</i> ^{-/-}	Defective LVV-EC invagination	[35]
<i>Pdpr</i>	Cell-adhesion	<i>Pdpr</i> ^{-/-} <i>Pdpr</i> ^{F/F} ; <i>Tek-Cre</i> <i>Pdpr</i> ^{F/F} ; <i>Prox1-Cre</i> ^{ERT2}	Failure of LEC-platelet interaction; Failure of platelet aggregation	[88, 95, 107, 111]

Gene	Gene Function	Genotype	Explanation of blood-filled lymphatics	References
<i>Lcp2</i>	TCR-activated tyrosine protein kinase	<i>Lcp2^{-/-}</i> <i>Lcp2^{F/-}; Vav-Cre</i>	Failure of platelet aggregation	[87, 88, 112]
<i>Syk</i>	Non-receptor tyrosine kinase	<i>Syk^{-/-}</i> <i>Syk^{F/F}; Vav-iCre</i>	Failure of platelet aggregation	[87, 103, 113–115]
<i>Clec1b</i>	Calcium-dependent lectin-like receptor	<i>Clec1b^{-/-}</i> <i>Clec1b^{F/F}; PF4-Cre</i>	Failure of platelet aggregation Loss of venous integrity	[84, 88, 93, 103, 105, 116]
<i>Tec</i> and <i>Btk</i>	Non-receptor tyrosine kinase	<i>Tec^{-/-}; Btk^{-/-}</i>	Failure of platelet activation / aggregation	[104]
Meis1	Transcription Factor	<i>Meis1^{-/-}</i>	Complete lack of platelets; Failure of platelet aggregation	[102]
<i>C1Galt1</i>	Glycosyltransferase	<i>C1Galt1^{F/F}; Tek-Cre</i> <i>C1Galt1^{F/F}; Mx1-Cre</i>	Mosaic expression of both blood and LEC markers	[106]
<i>Chd4</i>	Transcription Cofactor	<i>Chd4^{F/F}; Cdh5-Cre</i> <i>Chd4^{F/F}; Lyve1-Cre</i>	Lack of fibrin-rich thrombi at LVVs	[108]
<i>Fermt3</i>	Cell-adhesion; Integrin signaling	<i>Fermt3^{-/-}</i>	Failure of platelet aggregation	[95]
<i>Plcg2</i>	Transmembrane signaling enzyme	<i>Plcg2^{-/-}</i>	Mosaic expression of both blood and LEC markers	[96]
<i>Angptl4</i>	Secreted Factor, Angiogenesis	<i>Angptl4^{-/-}</i>	Loss of Prox1 expression	[117]