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The expression of the gap junction molecule connexin-45 (Cx45; GJC1) in lymphatic endothelium and its functional relevance were not previously known. We found that Cx45 was expressed widely in the endothelium of murine lymphatics, in both valve and non-valve regions. Cell-specific deletion of Cx45, driven by a constitutive Cre line (Lyve1-Cre) or an inducible Cre line (Prox1-CreERT2), compromised the function of lymphatic valves, as assessed by physiological tests (back leak and closure) of isolated, single-valve vessel segments. The defects were comparable to those previously reported for loss of Cx43 and, like Cx43, deletion of Cx45 resulted in shortening and/or increased asymmetry of lymphatic valve leaflets, providing an explanation for the compromised valve function. In contrast to Cx43, LEC-specific deletion of Cx45 did not alter the number of valves in mesenteric or dermal lymphatic networks, or the expression patterns of the canonical valve-associated proteins PROX1, ITGA9 or CLAUDIN5. Constitutive deletion of Cx45 from LECs resulted in increased backflow of injected tracer in popliteal networks in vivo and compromised the integrity of the LEC permeability barrier in a subset of collecting vessels. These findings provide evidence for an unexpected role of Cx45 in the development and maintenance of lymphatic valves.

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Connexin45 (GJC1) is expressed in mouse lymphatic endothelium and required for lymphatic valve function

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Abstract
The expression of the gap junction molecule connexin-45 (Cx45; GJC1) in lymphatic endothelium and its functional relevance were not previously known. We found that Cx45 was expressed widely in the endothelium of murine lymphatics, in both valve and non-valve regions. Cell-specific deletion of Cx45, driven by a constitutive Cre line (Lyve1-Cre) or an inducible Cre line (Prox1-CreERT²), compromised the function of lymphatic valves, as assessed by physiological tests (back leak and closure) of isolated, single-valve vessel segments. The defects were comparable to those previously reported for loss of Cx43 and, like Cx43, deletion of Cx45 resulted in shortening and/or increased asymmetry of lymphatic valve leaflets, providing an explanation for the compromised valve function. In contrast to Cx43, LEC-specific deletion of Cx45 did not alter the number of valves in mesenteric or dermal lymphatic networks, or the expression patterns of the canonical valve-associated proteins PROX1, ITGA9 or CLAUDINS5. Constitutive deletion of Cx45 from LECs resulted in increased backflow of injected tracer in popliteal networks in vivo and compromised the integrity of the LEC permeability barrier in a subset of collecting vessels. These findings provide evidence for an unexpected role of Cx45 in the development and maintenance of lymphatic valves.

Key words: lymphedema, Cx43, Evan’s Blue Dye, valve function tests, lymphatic valve development
Introduction

A major function of the peripheral lymphatic system is to return excess fluid and protein from the interstitium back to the blood circulation. After absorption into lymphatic capillaries, lymph is transported through lymphatic vessel networks by two major mechanisms: 1) the active, spontaneous pumping of collecting lymphatic vessels and 2) the passive compression of lymphatic collectors and capillaries by adjacent tissues (e.g., skeletal muscle). Both mechanisms rely on the proper functioning of one-way lymphatic valves, interposed at short distances, to prevent the backflow of lymph.

Lymphatic valves (LVs) are thin, highly flexible, bileaflet structures formed by two layers of LECs surrounding a matrix core whose composition includes collagen (1), laminin-5 (2), integrin α9 (2), elastin (3) and fibronectin (2). LVs are typically comprised of two semicircular leaflets that insert into the vessel wall along their outer edges. The midpoints of each leaflet oppose each other across the vessel wall while the downstream free edges form an elliptical opening in an enlarged sinus area. Valves are normally biased to be open in the absence of a trans-valvular luminal pressure difference ($\Delta P$) and gate passively according to the prevailing $\Delta P$. When the leaflets close, there is a variable amount of interleaflet overlap, with an increased area of overlap presumably leading to a tighter seal. The magnitude of the $\Delta P$ required for valve closure depends strongly on vessel diameter such that low values of $\Delta P < 0.3$ cmH2O (inflow < outflow) are sufficient to close valves when vessel diameter is small, whereas larger values of $\Delta P$ are required when vessel diameter is near maximal (4). This relationship between diameter and the $\Delta P$ required for valve closure is confirmed by numerical modeling, which predicts that expansion of the vessel lumen at the base and lateral margins of the valve increases tension on the leaflets, requiring more force to close (5).

The current hypothesis for LV formation is that oscillatory shear stress initiates the activation of a gene transcription program involving Prox1, Gata2, Foxc2 and other factors (6-9). Prox1 is critical for LEC specification and Foxc2 for the initiation of valve formation (10). Selective deletion of Foxc2 from LECs in late embryonic or early post-natal stages results in reduced LV density and a significant percentage of valves with severe back leak (7). Foxc2 haplodeficient mice have normal viability, but the number of LVs is reduced by ~50% and many of the remaining valves have slight back leak (11, 12). Numerous other regulators of LEC growth and/or matrix production (13) have been implicated in lymphatic valve formation and/or maintenance, but it remains to be defined how they interact to produce functionally normal LVs. In addition to the regulators mentioned above, several LEC-LEC junctional molecules are critical for LV development and/or maintenance, including VE cadherin (14, 15) and at least three connexin (Cx) isoforms (16-18). Missense mutations in Cx43 (GJA1) and Cx47 (GJC2) are each linked to the development of lymphedema in humans (19-21) and women with mutations in GJC2 have an increased likelihood of developing lymphedema after breast cancer surgery (22).

In a previous study, images of Cx45 staining in the LMC layer of mouse and human collecting lymphatics revealed additional faint Cx45 signal in axially-oriented cells underneath the LMC layer (23), suggesting that Cx45 might also be expressed in lymphatic endothelium. The goal of the present study was to assess whether Cx45 is indeed expressed in LECs and if so, to test the consequences of Cx45 deficiency on LV development and function. Here, we identified Cx45 expression in LECs throughout the popliteal, mesenteric and dermal lymphatic networks, in both valve and non-valve regions. Expression was confirmed in popliteal lymphatics by PCR analysis and by LEC-specific expression of GFP driven by the endogenous Cx45 promoter (24, 25). In vivo tests using Lyve-1Cre;Cx45+/−/mice revealed backflow of injected dye into side branches of popliteal lymphatic collectors as well as peri-lymphatic dye leakage. Ex vivo tests of valve function in control and Cx45-deficient
popliteal lymphatic vessels indicated that Cx45 deletion in developing LVs, using a constitutive Lyve1-Cre, resulted in back leak defects and an increased percentage of valves with abnormal back leak. Deletion of Cx45 after LV development was complete, using an inducible Prox1-CreER$^{T2}$, resulted in a significant increase in the fraction of LVs with back leak. Thus, Cx45 is a previously unidentified LEC connexin isoform that contributes markedly to the development and, to a lesser extent, maintenance of functional LVs.

Results

Excision of the floxed Cx45 sequence using Prox1-CreER$^{T2}$ resulted in GFP expression (24) in popliteal lymphatics isolated from 1-to 2-month-old mice. GFP-positive LECs were prominent along the entire vessel length, in both valve and non-valve regions, as evident from live fluorescence images of cannulated, pressurized vessels from Prox1-CreER$^{T2}$;Cx45$^{f/f}$ mice. Of the three valves in the vessel segment in Fig. 1A, oriented with the normal flow direction from left to right, valves 1 and 3 appeared to have normal-shaped sinuses and normal-length leaflets, but valve 2 had a relatively indistinct sinus and somewhat short leaflets. Similar GFP fluorescence patterns in LECs were evident in lymphatic collectors from other regions of Prox1-CreER$^{T2}$;Cx45$^{f/f}$ mice, including superior cervical lymphatics, inguinal-axillary lymphatics and mesenteric lymphatics (not shown).

Popliteal valves in vessels from Prox1-GFP and Lyve1-Cre;Cx45$^{f/f}$ mice were imaged using confocal microscopy to obtain higher resolution of the leaflet structure, and examples are shown in Fig. 1B-J, each oriented with the normal flow direction from right to left. For confocal imaging, the vessels were pressurized and fixed overnight with 1% PFA prior to immunostaining with an anti-GFP antibody. After deconvolution of each image, a maximal projection of the reconstructed z-axis image stacks was generated. Views of a valve from a Prox1-GFP mouse with the leaflets open are shown in Fig. 1B-D and were obtained by setting inflow pressure (Pin) ≥ outflow pressure (Pout); views of the same valve with the leaflets closed were obtained by setting Pout > Pin (Fig. 1E-F). Views of another closed valve from a Lyve1-Cre;Cx45$^{f/f}$ mouse are shown in Fig. 1H-J, in which GFP is expressed in both LECs and macrophages and in which a small gap (at arrows in I, J) is evident at the left margin where the leaflets insert into the wall. Although the gap could be an indication of a partially defective valve, in this case it was more likely an artifact of fixation, because valve function tests on this same valve before fixation required an adverse pressure gradient (∆P; Pout-Pin) < 0.5 cmH$_2$O for closure compared to a ∆P > 5 cmH$_2$O after fixation. Rotatable movies of these confocal stacks are provided in Supplemental Movies 1 and 2.

These results show that Cx45 is expressed in LECs and suggest that mice deficient in Cx45 exhibit both morphologically normal and abnormal LVs.

PCR analysis was used to check for Cx45 mRNA in native LECs. For this analysis inguinal-axillary lymphatic vessels were used because they contained more cells than popliteal vessels. Since Cx45 is highly expressed in LMCs (23), the LEC population was purified before performing PCR. Inguinal-axillary lymphatic vessels were dissected from multiple Prox1-GFP mice and carefully cleaned to remove any attached small blood vessels or capillaries. After enzymatic digestion to obtain single cells (~5000 cells/vessel), the cell suspension was purified by FACS, sorting on the GFP signal, and then probed for known LEC and LMC markers. As expected, the preparation showed message for the LEC markers Prox1, eNOS, CD31 and Flt4, but was negative for the canonical LMC markers Myh11, Ca$_1$.2 and Cnn1, as shown in Fig. 2A (see Table 1 for a list of primer sequences, gene accession numbers and amplicons). The purified LEC lysate from similarly obtained lymphatics was probed for the major vascular connexin isoforms previously detected in whole lymphatic vessels (23). Messenger RNA was detected for Cx45, Cx43, and Cx37, but not for Cx47 or Cx40 (Fig. 2C). The absence of Cx47 expression, despite the use of two different primers, is puzzling, but in agreement with analysis of whole inguinal-axillary
lymphatic vessels in a previous study (23). It is possible that Cx47 detected in earlier studies (26, 27) may be explained if its expression is restricted to a certain developmental stage, or to lymphatic vessels from certain regions, or to valve leaflets; in the latter case, our detection method would not be sufficiently sensitive. Samples of mouse brain tissue served as positive controls for the primers used in these assays (Fig. 2, B, D).

Deletion of Cx45 was confirmed by analysis of FACS-purified LECs from inguinal-axillary lymphatic vessels of Prox1-CreERT2;Cx45f/f mice, where the expression of Cx43 and Cx37 (although faint) continued to be detected in the absence of message for Cx45 (Fig. 2E, with positive controls for primers shown in Fig. 2F). For the latter analysis Prox1-GFP rather than Cx45f/f vessels were used as the reference sample, because a GFP signal was needed to sort and purify the control LEC population. See Suppl. Fig. 2 for the gating windows used in the FACS analysis.

Subsequently, qPCR was performed on FACS-sorted LECs from Prox1-GFP and Prox1-CreERT2;Cx45f/f mice to quantitatively test if Cx45 deletion resulted in upregulation of other lymphatic Cx isoforms (Suppl. Fig. 2). As expected, Cx45 message was reduced to <10% of control in LECs from Prox1-CreERT2;Cx45f/f vessels while Cx43 and Cx37 mRNA levels were not significantly different between LECs from Prox1-GFP and Prox1-CreERT2;Cx45f/f vessels. The signals for Cx47 were too low for meaningful analysis. Thus, deletion of Cx45 did not result in significant compensatory upregulation in the mRNAs of other LEC connexins.

Next, in vivo evidence for backflow was examined in lymphatic networks of control and Cx45-deficient mice. Evan’s Blue Dye (EBD) was injected into the foot pads of anesthetized Cx45f/f or Lyve1-Cre;Cx45∆/f mice and allowed to fill the popliteal lymphatic network. The skin over the dorsal-medial surface of the thigh in each leg was opened and retracted to expose the two dye-filled, popliteal afferent lymphatic vessels. As shown in Fig. 3A, both popliteal vessels were visible on either side of the saphenous vein of a Cx45f/f mouse with no indication of EBD backflow into any side branches. Under similar conditions in a Lyve1-Cre;Cx45∆/f mouse, backflow was evident in two side branches of the lateral popliteal afferent and one side branch of the medial popliteal afferent (Fig. 3B). We also noted that a subset (5 of 14) of popliteal vessels in Lyve1-Cre;Cx45f/f mice appeared to be leaky, as evident in the lateral vessel (left side of the vein) in Fig. 3C. The number of popliteal afferents with backflow into side branches and the number of leaky popliteal vessels were quantified and plotted in Fig. 3D-E. There were significant increases in both the number of vessels with backflow and the number of leaky vessels in Lyve1-Cre;Cx45f/f mice compared to Cx45f/f mice.

The occurrence of backflow in vivo and vessel images such as those shown in Fig. 1A, J were suggestive of possible LV defects in Cx45-deficient lymphatic vessels. Therefore, LV development was characterized in Lyve1-Cre;Cx45f/f mice at embryonic and neonatal stages in the mesentery. Examination of the mesenteries of E18.5 embryos revealed no obvious reductions in the number of valves in Lyve1-Cre;Cx45f/f vessels, compared to vessels from Cx45f/f mice, based on the number of Prox1-high nuclei clusters present in mesenteric lymphatic networks in situ (Fig. 3F-G). Quantification revealed no significant differences from control vessels in the number of mesenteric valves per vessel at E18.5 (Fig. 3H). The diameters of mesenteric lymphatic vessels were similar between the two strains (Fig. 3I), indicating that there was no collecting vessel hyperplasia at E18.5. Mesenteries from P10 pups were stained for Prox1; no significant differences were observed in the Prox1 staining pattern or the number of valves per vessel length (Fig. 3L) or lymphatic vessel diameter (Fig. 3M). Cx45 deletion using Lyve1-Cre did not interfere with the expression of the canonical LEC valve proteins Prox1, Vegfr3 and Itga9, as the immunostaining patterns of these proteins were similar in mesenteric lymphatic vessels from Cx45f/f and Lyve1-Cre;Cx45f/f P10 pups (Fig. 3N-O, top panel). Likewise, Cx45 deletion did not appear to affect
the expression patterns of the tight junction molecule Claudin-5 or the adherens junction molecule VE-cadherin (Fig. 3N-O, lower four panels). We also checked for defects in the lymphovenous valves (LVVs). LVVs are the entry points for lymph into the venous system, the first valves to develop outside the heart (28) and valves in which defects are often associated with lymphatic disorders (29). There were no obvious defects in the morphology of the LVVs at E16.5 in Lyve1-Cre;Cx45<sup>Δ/Δ</sup> mice, or in the immunostaining pattern for the valve regulatory molecule Prox1 in LVVs (Suppl. Fig. 3A, B, arrows). The venous valves that develop in the vicinity of LVVs also appeared to be normal (Suppl. Fig. 3A, B, arrowheads).

Lyve1-Cre;Cx45<sup>Δ/Δ</sup> mice did not exhibit any obvious systemic pathologies, any decrease in viability, or the chylous ascites and/or chylothorax previously reported in mice with deficiencies in Cx43 and/or Cx37 (26). Collectively, these results suggest that constitutive deletion of Cx45 does not alter the number of valves in the mesenteric lymphatic network or the gross morphology or expression patterns of canonical LEC valve proteins in LVs and LVVs, although this analysis does not rule out subtle differences in valve leaflet structure or function.

The leak of EBD in some vessels, as shown in Fig. 3C, E suggests that a subtle permeability defect may not be reflected in the immunostaining patterns of Claudin-5 and VE-cadherin, or may be indicative of the requirement of Cx45 in maintaining vessel integrity during adulthood but not at neonatal stages, or may reflect differences between the roles of Cx45 in LECs of mesenteric vs. popliteal lymphatic vessels.

Tests of valve function (30) were then performed on popliteal lymphatics from Lyve1-Cre;Cx45<sup>Δ/Δ</sup> mice, in which Cx45 was deficient during development, and from Prox1-Cre<sup>ERT2</sup>;Cx45<sup>f/f</sup> mice in which LVs were allowed to develop normally followed by postnatal deletion of Cx45 at P30-60. Low pressure back leak tests were used to measure the degree of valve competency when pressures were within the presumed normal range for mice (0.5 -10 cmH<sub>2</sub>O). Closure tests were used to detect possible changes in valve stiffness, in which higher-than-normal adverse pressure gradients would be needed to close the valve. All valve function tests were conducted on single-valve popliteal lymphatic segments ex vivo, with spontaneous contractions eliminated.

For back leak tests, Pin was held constant at 0.5 cmH<sub>2</sub>O, while measuring pressure on the upstream side of the valve using a servo-null micropipette. With the servo-null pipette tip in place, Pout was raised ramp-wise from 0.5 to 10 cmH<sub>2</sub>O over the course of ~40 sec. Diameter was also measured on the upstream side of the valve near the Pin pipette (11). Normal valves closed when Pout exceeded Pin by a fraction of a cmH<sub>2</sub>O (typically <0.3 cmH<sub>2</sub>O at this level of Pin). Images of a 1-valve popliteal lymphatic segment are shown in Fig. 4 at the start (A) and end (B) of the test and illustrate how only the downstream half of the vessel distends at Pout=10 cmH<sub>2</sub>O (Fig. 4B, arrowhead) while any pressure increase in the upstream half is prevented by a competent, closed valve.

Representative recordings of low pressure back leak tests are shown in Fig. 4C-F for 4 genotypes of mice: WT (C57Bl/6), Lyve1-Cre;Cx45<sup>Δ/Δ</sup>, Prox1-Cre<sup>ERT2</sup>;Cx45<sup>f/f</sup> (haplodeficient in Cx45) and Prox1-Cre<sup>ERT2</sup>;Cx45<sup>f/f</sup>. The WT valve behaved normally (Fig. 4C), with the valve closing as Pout exceeded ~0.2 cmH<sub>2</sub>O and with no detectable change in Psn at Pout = 10 cmH<sub>2</sub>O (i.e., Psn-Pin = 0 cmH<sub>2</sub>O). In contrast, the valve in the Lyve1-Cre;Cx45<sup>Δ/Δ</sup> vessel exhibited some back leak (Psn-Pin = 1.0 cmH<sub>2</sub>O) at the maximal Pout level (Fig. 4D), but well short of the theoretical maximum back leak of ~4.75 cmH<sub>2</sub>O (halfway between Pout = 10 cmH<sub>2</sub>O and Pin = 0.5 cmH<sub>2</sub>O, with additional variability contributed by the relative resistances of the vessel segments between the pipette tips and the valve). Diameter also increased on the inflow side as Pout rose, consistent with back leak through the closed valve. A similar amount of back leak was recorded in the Prox1-Cre<sup>ERT2</sup>;Cx45<sup>f/f</sup> vessel (Fig. 4F), but only a minute amount of back leak was evident in the Prox1-Cre<sup>ERT2</sup>;Cx45<sup>f/f</sup> vessel (Fig. 4E).
The data sets for back leak included 3 groups of control vessels—WT (C57Bl/6J, the background strain for Prox1-CreERT\(^2\)), Lyve1-Cre (no floxed gene) and Cx45\(^{-/-}\) (no Cre)—as well as 3 groups of Cx45-deficient vessels: Lyve1-Cre;Cx45\(^{-/-}\), Prox1-CreERT\(^2\);Cx45\(^{-/-}\) and Prox1-CreERT\(^2\);Cx45\(^{+/+}\). To analyze the back leak data, the individual sets of Psn values during Pout ramps from 0.5 to 10 cmH\(_2\)O were averaged over 0.5 cmH\(_2\)O Pout intervals by binning the raw Psn data and computing the mean and variance for each interval (Fig. 4G-L). For statistical analysis of that data, repeated measures 2-way ANOVAs with Tukey’s post-hoc tests compared the back leak data within each group to the control value at the start of the Pout ramp. The analyses revealed significantly elevated levels of back leak for Lyve1-Cre;Cx45\(^{-/-}\) at Pout levels above 5 cmH\(_2\)O.

For statistical comparisons between the genotypes, the single value of back leak recorded at the end of the ramp (Pout = 10 cmH\(_2\)O) was used for each vessel (Fig. 4M). Of 17 Lyve1-Cre;Cx45\(^{-/-}\) and 10 Lyve1-Cre;Cx45\(^{+/+}\) vessels studied (data were combined after a Mann-Whitney U test revealed no significant differences between the two groups and hereafter referred to as Lyve1-Cre;Cx45\(^{-/-}\) mice), 17 exhibited back leak within the ‘normal’ range, 6 showed intermediate back leak and 4 were essentially incompetent (i.e., back leak >3.7 out of an estimated maximal 4.75 cmH\(_2\)O). Of 14 Prox1-Cre;Cx45\(^{+/+}\) valves, 11 exhibited no back leak and 3 exhibited intermediate back leak. 1 of 8 valves tested 6-7 wk post-induction were leaky, compared to 2 of 6 valves tested 18 wk post-induction, suggesting a possible trend for these valves to become leakier with time. Not all of the control data sets were normally distributed, so non-parametric tests were used. Kruskal-Wallis tests with Dunn’s multiple comparisons post-hoc tests compared differences in back leak between Lyve1-Cre;Cx45\(^{-/-}\), Lyve1-Cre and Cx45\(^{+/+}\) valves and between Prox1-Cre;Cx45\(^{+/+}\), Prox1-Cre;Cx45\(^{-/-}\) and Cx45\(^{+/+}\) valves. The results revealed that back leak was significantly elevated only in Lyve1Cre;Cx45\(^{-/-}\) valves, suggesting that constitutive deletion of Cx45 from LECs induced back leak defects.

As an alternate analysis of the back leak data, we calculated the fraction of leaky valves for each multi-valve segment that was initially isolated and cleaned (Fig. 4N). The numbers were more limited and the data were more variable because neither the number of valves in the segment nor the success rate of the valve tests for each vessel could be controlled. Therefore, to strengthen this analysis, the data for the three “control groups” (WT + Cx45\(^{+/+}\) + Lyve1-Cre) were combined. As stated in the Methods, Cx45\(^{+/+}\) and Lyve1-Cre mice were backcrossed to WT mice so that the three strains were congenic. We first verified, using a Kruskal-Wallis test, that there were no significant differences in back leak between the three strains. We then defined a “leaky” valve as having back leak higher than the mean + 1 SD of the pooled 3 control groups; this value equaled 0.24 cmH\(_2\)O at Pout = 10 cmH\(_2\)O. The fraction of valves in the unshortened vessel segment with back leak higher than this level were then classified as leaky. This analysis concurred with the data in Fig. 4M in showing that the combined Lyve1-Cre;Cx45\(^{-/-}\) + Lyve1-Cre;Cx45\(^{+/+}\) vessels had a significantly higher percentage of leaky valves than the combined control vessels, and a significantly higher percentage of leaky valves than Prox1-CreERT\(^2\);Cx45\(^{+/+}\) valves. Collectively, these analyses show that constitutive deletion of Cx45 from LECs consistently induced back leak defects compared to control valves or to valves with inducible deletion of Cx45 from LECs.

Next, we analyzed closure tests performed on the same valves. Recall that closure tests reflect the stiffness of the valve leaflets rather than the degree to which the leaflets seal. Representative examples of closure tests (all at a single value of Pin, 10 cmH\(_2\)O) are shown in Fig. 5. The protocol and associated measurements are illustrated by the recording for the Lyve1-Cre;Cx45\(^{-/-}\) vessel in Fig. 5B. As Pout rose, the valve remained open (with increasing backflow) until it closed at Pout ~24 cmH\(_2\)O, marked by a sharp drop in Psn, which had risen from 10 to 15.8 cmH\(_2\)O. At this moment in time the value of Pout-Pin (the \(\Delta P\) for closure) was 14 cmH\(_2\)O. The
closure test for the WT valve is indicative of a normal valve in that the valve closed when $\Delta P$ was $\sim 1$ cmH$_2$O and

Ps1 instantly returned from 11 to its baseline value of 10 cmH$_2$O (Fig. 5A). The observation that Ps1 did not fall
back to 10 cmH$_2$O in the Lyve1-Cre;Cx45$^{+/-}$ vessel when the valve closed (Fig. 5B) is further evidence that there
was significant pressure back leak through that closed valve [back leak is even more obvious in this test than the

Ps1 test at the lower value of Pin (0.5 cmH$_2$O) shown in Fig. 4D]. Closure tests for Prox1-CreER$^{272}$;Cx45$^{+/f}$ and Prox1-

CreER$^{272}$;Cx45$^{+/f}$ valves showed values of $\Delta P$ for closure that were intermediate between those of the WT valve

and the Lyve1-Cre;Cx45$^{+/-}$ valve. These tests were repeated over the presumed physiological pressure range for

mouse lymphatics (from 0.1 to 10 cmH$_2$O), allowing the generation of the curves shown in panels of Fig. 5E-H,

which characterized the complete relationship between the $\Delta P$ for closure and the baseline pressure, with each

valve described by a concave curve. The curve for the WT valve was similar to WT curves described in previous

studies (4, 31), with a maximal value of $\Delta P$ $\sim 2$ cmH$_2$O. For our standard analysis, $\Delta P$ for closure was plotted as a

function of normalized diameter, rather than the raw Pin value, resulting in sets of upward convex curves for

each valve studied, as shown in Fig. 5I-N. The red symbols/lines indicate the data for the four valves shown in

panels E-H. The closure curves for three of the Lyve1-Cre;Cx45$^{+/-}$ (combined with Lyve1-Cre;Cx45$^{+/f}$) valves were

notably distinct (Fig. 5N, red arrow) as represented by flat curves at $\Delta P = 30$ cmH$_2$O, indicating that these valves

were completely incompetent and incapable of closing at the maximum pressure gradients imposed. Three

other Lyve1-Cre;Cx45$^{+/-}$ valves became incompetent or nearly incompetent after diameter exceeded $\sim 70\%$ of

Dmax (one was the vessel shown in Fig. 5B). No valves in any of the other groups exhibited this behavior,

although one Lyve1-Cre valve had an abnormal high $\Delta P$ for closure as normalized diameter exceeded 0.8.

Because the sets of $\Delta P$ values for closure vs. D/Dmax curves were difficult to compare statistically due to

continuous x- and y-axes, the single value of $\Delta P$ at a given physiological Pin level (0.5 cmH$_2$O) was used for

comparisons between genotypes. Not all data sets were normally distributed, so non-parametric tests were

used. Kruskal-Wallis tests with Dunn’s multiple comparisons tests were used to compare differences in the $\Delta P$

for closure between Lyve1-Cre;Cx45$^{+/-}$, Lyve1-Cre and Cx45$^{+/-}$ valves and between Prox1-CreER$^{272}$;Cx45$^{+/f}$, Prox1-

CreER$^{272}$;Cx45$^{+/f}$ and Cx45$^{+/-}$ valves (Fig. 5O). Despite the incompetent Lyve1-Cre;Cx45$^{+/-}$ valves noted above, the

differences between the genotypes did not reach statistical significance. As an alternative analysis, we

calculated the fraction of popliteal vessels with an abnormal $\Delta P$ required for closure at Pin = 0.5 cmH$_2$O and

compared the Cx45-deficient valves with the combined control (WT + Cx45$^{+/-}$ + Lyve-Cre) valves. Similar to the

back leak analysis, the threshold for determining that a valve had an abnormal $\Delta P$ for closure was calculated

from the mean + 1 SD of the combined three control groups (threshold $\Delta P = 1.02$ cmH$_2$O). Significant differences

were noted in the percentage of LVs with abnormally high $\Delta P$ values for Lyve1-Cre;Cx45$^{+/-}$ compared to the

combined controls. Collectively, the results in Fig. 5O-P suggest that a subset of Lyve1-Cre;Cx45$^{+/-}$ valves were

incompetent, and that deletion of Cx45 interfered with the ability of LVs to close under an adverse pressure

gradient.

Previously, leaflets in Rasa1-deficient lymphatic and venous valves were found to be significantly reduced in

length (31), due to an inability of LV-forming LECs to export collagen IV, leading to the loss of leaflet LECs over

time and eventual valve incompetency when a threshold number of leaflet LECs was exceeded (32). A similar

analysis of Cx43-deficient valves also showed the same trend, with shorter leaflets in valves from Lyve1-

Cre;Cx43$^{+/-}$ mice compared to valves in Cx43$^{+/-}$ control mice (33). Here, we made similar measurements of leaflet

dimensions under brightfield microscopy, as defined in Fig. 6A, to test if they might reveal obvious structural

differences that could explain back leak defects in Lyve1-Cre;Cx45$^{+/-}$ valves. Ideally, the area of overlap between
the two leaflet cusps in a closed valve would be the best predictor of valve competency, defined as the absence of back leak when closed. Although such measurements might be possible in confocal reconstructions, fixation potentially altered the valve properties, as demonstrated in Fig. 1J. Under brightfield illumination, measurements of leaflet overlap were almost never possible in side-views of the valves required for our functional tests. Likewise, neither measurement of the cusp length “e”, or the actual length of the leaflet edge (the curved insertion paths of the leaflets from their common base to their tips) were measured while focusing on the lower surface of the vessel, and then the corresponding approximations of leaflet lengths in the opposite wall (b and b’) were measured while focusing on the upper surface of the vessel. Images of an actual valve are shown in Fig. 6B to illustrate the practical difficulties of such measurements.

Measurements of the number of valves per length of each cannulated vessel (before shortening to a single valve segment) did not reveal any significant differences in valve density among the 6 genotypes (Fig. 6C). This result is consistent with the comparable mesenteric LV densities observed in WT and Lyve1-Cre;Cx45\(^{-/+}\) mice in Fig. 3. However, there were significant differences in several of the leaflet dimensions between genotypes. Notably, significantly lower values of the leaflet length, the average of \((a+a’)/2\) and \((b+b’)/2\) (Fig. 6D-E), and a significantly lower degree of leaflet symmetry, the ratio of the two terms (Fig. 6F), were evident in Lyve1-Cre;Cx45\(^{-/+}\) vessels, consistent with a possible contribution of these factors to back leak. Normalizing leaflet length to diameter (d) did not alter these conclusions. The difference between a and b for the Lyve1-Cre;Cx45\(^{-/+}\) valve in Fig. 6B illustrates one example of the asymmetry observed in some Cx45-deficient valves. One other Lyve1-Cre;Cx45\(^{-/+}\) valve contained only a single leaflet, accounting for the lowest points in panels E-F of Fig. 6.

These results indicate that the Lyve1-Cre;Cx45\(^{-/+}\) mice had significantly shorter and asymmetric leaflets when compared to control littermates.

Discussion

Our results demonstrate that Cx45, a previously unidentified LEC connexin, contributes to normal LV function. Cx45 expression is uniformly distributed in LECs of collecting lymphatic networks, in and across LVs. The distribution of Cx45 in lymphatic endothelium contrasts with those of Cx43, Cx37 and Cx47 (16) as Cx43 and Cx37 are differentially expressed on the upstream and downstream sides of the LV leaflets (26), and Cx47 is expressed only in a subset of valve cells (26). Searches of published scRNA-seq databases (34-36) independently confirm Cx45 (GJC1) expression in LECs. The uniform axial distribution of Cx45 in lymphatic collectors suggests that its expression may be regulated in a different way than Cx37 and Cx43, perhaps independent of shear stress. Constitutive deletion of Cx45 from the lymphatic endothelium, using Lyve1-Cre, results in elevated back leak in 42% of popliteal LVs and an abnormally high adverse pressure gradient required for closure in 38% of popliteal LVs. Postnatal deletion of Cx45 after lymphatic valves have developed results in 21% of popliteal lymphatic valves with elevated back leak in Prox1-CreER\(^{T2}\);Cx45\(^{-/+}\) valves. The absence of significant back leak or closure defects in Prox1-CreER\(^{T2}\);Cx45\(^{-/+}\) valves suggests that a single allele of Cx45 is sufficient to protect against valve defects. The leak of EBD from some Lyve1-Cre;Cx45\(^{-/+}\) vessels in vivo suggests that Cx45 may be required to maintain a normal LEC permeability barrier in collecting lymphatic vessels. It is interesting that dye leak only occurred in a subset of vessels, and usually over the entire visible length of the vessel, rather than being punctate as in some other genotypes with a disrupted permeability barrier (37, 38); this possibly may be related to higher intraluminal pressures in the leaky vessels. Future experiments with quantitative measurements of...
albumin or solute permeability under defined conditions (39, 40) would be valuable in confirming this finding and probing the underlying mechanism.

What is the relative importance of other Cx isoforms for valve function compared to Cx45? The only other functional data are from a previous study of Lyve-1-Cre;Cx43<sup>f/f</sup> popliteal lymphatic valves by Munger et al. (33), in which constitutive Cx43 deletion resulted in some degree of back leak in 67% (4 of 6) of LVs, and complete incompetence in 17% (1 of 6) of LVs. In comparison, we find that 42% of LVs in Lyve-1-Cre;Cx45<sup>−/−</sup> popliteal vessels have some degree of elevated back leak and that 24% (4 of 17) are completely defective. These comparisons suggest somewhat comparable roles for Cx45 and Cx43 in the maintenance of mature, functioning LVs, but with Cx43 deletion also affecting the total number of LVs that develop. The combination of back leak and closure defects in Cx45-deficient LVs implies that a fraction of those valves may never close under physiological conditions and that those which do close will permit varying degrees of back flow, resulting in inefficient lymph transport.

The mechanism by which Cx45 deficiency produces back leak remains unknown. Connexins enable the flux of ions and small molecules between cells and thus are more likely to play roles in LEC-LEC communication than in determining the structural integrity of cell-cell junctions. However, Cx45-mediated signaling might regulate the trafficking or localization of other junctional proteins such as occludins or cadherins that may be required at LEC-LEC junctions to maintain valve integrity. A model of LV leaflet behavior predicts that back leak is most likely to occur at the lateral margins where the leaflets insert into the wall (5). LEC-LEC junctions are particularly pronounced in these regions (Fig. 1B, E) and reinforcement may be needed to protect the valve against the unique mechanical forces associated with closure. Analyses of Claudin5 and VE-cadherin immunostaining patterns at low magnification in vivo did not reveal any obvious changes in the localization of these junctional proteins, but perhaps higher resolution confocal imaging would. We attempted to identify possible structural abnormalities in Cx45-deficient valves by measuring leaflet dimensions under brightfield microscopy during our functional tests, reasoning that such measurements could be used by others to guide identification of defective valves in the absence of functional tests. Confocal reconstructions of LEC fluorescence after subsequent fixation of the valves provided better resolution of the degree of leaflet overlap, but fixation itself altered the valve properties (Fig. 1I-J). Image analyses of unfixed valves indicated that constitutive Cx45 deficiency was associated with shorter leaflet lengths and increased leaflet asymmetry (Fig. 6). Shorter valve leaflets have been previously shown in connection with Rasa1 deficiency (31) and Cx43 deficiency (33), and are predicted to decrease valve competency in one model of simulated valve function (41); however, valve asymmetry has not been proposed previously as a contributor to valve dysfunction, nor has this property been modeled. Shorter leaflet insertion paths on one side of the valve seemingly would decrease the amount of leaflet overlap on that side when the valve is closed, and thereby enhance the likelihood of back leak. The tendency for leaflets to be asymmetrical in Lyve-1-Cre;Cx45<sup>−/−</sup> vessels is suggestive of a disruption in the progression from stage 3 to stage 4 of valve development (42), in which the leaflet margins on one side have inserted into the wall and extended downstream, but the leaflet margins on the other side have not yet inserted into the opposite wall (43). Similarly, deletion of Cx45 from mature valves may induce reversion from stage 4 to stage 3. Whether it is possible to probe the sequence of events leading to leaflet asymmetry remains to be determined.

Although our valve tests were conducted ex vivo, the conditions used were physiologically relevant. Some studies have used backflow of injected dye in vivo as an indicator of defective lymphatic function (9, 26, 38) and our results from EBD injection protocols in Fig. 3A indicate a quantitative and significant increase in the number
of Lyve1Cre;Cx45−/− popliteal lymphatic afferents that exhibit backflow into side branches. However, these measurements depend on the volume of dye injection and the unknown pressure head generated during injection to ensure adequate lymphatic network perfusion. A brief period of backflow is known to occur during the lymphatic contraction cycle (44, 45) and backflow can occur during diastole in normal valves if a slight adverse pressure gradient (<0.3 cmH2O) is present and sufficient to drive retrograde flow but insufficient to produce closure (4). Thus, a valve might be characterized as abnormal in vivo, based on retrograde dye flux, even though it would fit our criteria for a normal valve (Figs. 4, 5) if studied ex vivo under defined pressures (i.e., with Pin and Pout known and spontaneous contractions blocked). Unlike EBD backflow protocols, the pressures used in ex vivo tests have been demonstrated to be physiologically relevant. Pressures in the mouse lymphatic system are not well documented, but recent measurements in mouse mesenteric lymphatic collectors indicate pressures of ~4 cmH2O in control mice and ~15 cmH2O in mice with partial lymphatic obstruction (46). As the distance from the heart to the lower leg is ~5 cm in an adult mouse (23, 47), lymph would need to be transported against a standing column of fluid up to ~5 cmH2O if a mouse with incompetent lymphatic valves was oriented in the vertical position (i.e., with LVs unable to disrupt the column of lymph). Our typical Pout ramp protocol to 10 cmH2O is required to detect low levels of back leak for subtle defects characteristic of Foxc2+/− and Prox1-Cre; Foxo1f/f mice (11, 12), but in Cx45-deficient vessels we detected significant back leak at Pout levels well below 10 cmH2O (Fig. 4L). Closure tests were conducted at multiple pressures/diameters below and including 10 cmH2O, and the ΔP for closure at Pin = 0.5 cmH2O was chosen as a representative pressure for statistical tests between genotypes (Fig. 5O). Importantly, valves with a ΔP for closure > 30 cmH2O, such as those shown in Fig. 5N, would be predicted to never close, even in contracting vessels.

Finally, although there are no published reports of human lymphedema associated with mutations in Cx45 (GJC1), this connexin is critically important for a number of other physiological systems, most notably for the conduction of electrical signals in the developing heart (48, 49). Loss-of-function mutations in Cx45 cause familial atrial fibrillation and conduction disease in humans (50, 51) and global deletion of Cx45 is lethal in mice (52, 53). Because of the importance of Cx45 in the developing cardiac conduction system, it is likely that most human loss-of-function mutations in Cx45 are lethal, so that defects in LVs that could potentially produce lymphedema would not be detected.

In conclusion, we have shown that Cx45, in addition to being expressed in lymphatic smooth muscle cells, is functionally expressed in LECs throughout collecting lymphatic networks in mice and is required, along with other LEC connexin isoforms, for the development and maintenance of competent LVs.

Methods

Mice. Our study examined male and female animals, and similar findings are reported for both sexes. Cx45−/− mice (25) were a gift from Klaus Willecke, University of Bonn. Prox1-CreERT2 mice (2) were a gift from Taija Mäkinen, Uppsala University. Prox1-GFP mice (54) were a gift from Young-Kwon Hong, University of Southern California. Cx45−/−, Lyve1-GFPCre, Prox1-GFP and Prox1-CreERT2 mice were backcrossed to WT mice (C57Bl/6J, strain #000664, Jackson Laboratory, Bar Harbor, ME) for at least 6 generations. Lyve1-GFPCre mice express eGFP under the Lyve1 promoter, but eGFP expression is not affected by recombination; therefore, these mice are hereafter referred to as Lyve1-Cre mice. For genotyping, genomic DNA was extracted from tail clips.
using the HotSHOT method. Genotypes were determined by PCR with 2xM-PCR OPTI Mix (Catalog # B45012, Bimake, Houston, TX) according to the provider’s instructions. Mice of both sexes were studied.

To delete Cx45 embryonically, male Lyve1-Cre+/mice were bred with female Cx45+/mice and progeny that carried a germ line deletion of one Cx45 allele are designated as Lyve1-Cre;Cx45−/mice. Alternate matings (female Lyve1-Cre+/ mice with male Cx45−/mice) produced some Lyve1-Cre;Cx45+/mice of both sexes. To delete Cx45 postnatally, we bred Prox1-CreERT2;Cx45f/f mice and induced them with five consecutive daily i.p. injections of 100 mg tamoxifen (10mg/kg; in safflower oil) beginning at P30. The deletion of Cx45 accomplished by Cre-recombinase activity led concomitantly to the expression of the reporter enhanced green fluorescent protein (25). Mice were studied 2-3 weeks later. Mice were studied at E18.5 and P10 for mesenteric valve counting and at P42-126 for valve function tests.

**Vessel isolation and cannulation.** Mice were anesthetized with ketamine/xylazine (100/10 mg/kg, i.p.) and placed in the prone position. The dissection and cannulation of popliteal and mesenteric lymphatic vessels was performed as described previously (37, 55). After cannulation, the stage containing the vessel, chamber, pipette holders and micromanipulators was moved to an inverted microscope. The backs of the micropipettes were connected to low pressure transducers and a computerized pressure controller (56), allowing independent control of inflow (Pin) and outflow (Pout) pressures. The vessel axial length was adjusted with pressures briefly set to 10 cmH2O. Custom LabVIEW programs (National Instruments; Austin, TX) acquired analog data from the pressure transducers simultaneously with vessel inner diameter, as detected from video images acquired using a Basler firewire camera (56, 57).

**Valve function tests.** Luminal pressure on the inflow side of the valve (Psn) was measured with a servo-null micropipette inserted through the wall. An initial hole was made with a pilot micropipette, which was then removed and replaced with a servo-null micropipette (tip diameter = 3-5 µm). After insertion, the servo-null micropipette was advanced to seal the hole. The calibration of the servo-null pipette was checked, and adjusted as needed, after raising Pin and Pout simultaneously between 0.5 and 10 cmH2O. Back leak tests and valve closure tests were performed as described previously (37). To ensure accurate and consistent measurements of valve back leak, 1) all three transducers (Pin, Psn, Pout) were calibrated before each experiment; 2) the same pair of cannulation pipettes was used for all experiments to maintain consistent pipette resistances; 3) the pipettes were cleaned after each experiment with boiling water and acetone and checked before each valve test to ensure that the tips were free of debris (if needed, debris was cleared by sliding 14 µm suture several times through the tip); 4) the lines were free of bubbles; 5) the Psn pipette calibration was rechecked at the end of the valve test.

After function tests were completed, some vessels were fixed in 1% PFA for 5-10 min at room temperature while pressurized, then fixed overnight in 1% PFA at 4°C followed by repeated rinsing with and storage in PBS containing 0.1% sodium azide, for subsequent immunostaining.

**Solutions and Chemicals.** Krebs buffer contained: 146.9 mM NaCl, 4.7 mM KCl, 2 mM CaCl2·2H2O, 1.2 mM MgSO4, 1.2 mM NaH2PO4·H2O, 3 mM NaHCO3, 1.5 mM Na-HEPES, and 5 mM D-glucose (pH = 7.4). A buffer of the same composition (“Krebs-BSA”) also contained 0.5% bovine serum albumin. Krebs-BSA buffer was present both luminally and abluminally during cannulation, with the abluminal solution constantly exchanged with Krebs during the experimental protocol. For Ca2+-free Krebs, 3 mM EGTA replaced CaCl2·2H2O. Purified BSA was
obtained from (US Biochemicals; Cleveland, OH), MgSO₄ and Na-HEPES from ThermoFisher Scientific (Pittsburgh, PA) and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) except as otherwise specified.

**Confocal Imaging.** Fixed lymphatic vessels were washed in PBS (x3), permeabilized with a 0.1% Triton X-100 solution in PBS for 1 hr, blocked for non-specific binding using a 5% donkey serum (Sigma, Cat. No. D9663) in PBS for 2 hours at 4°C, and then incubated at 4°C overnight with primary antibodies. The antibodies were anti-GFP [1:300] (Invitrogen #A-11122) and anti-Cd144 (VE-cadherin) [1:500] (BD Pharmingen #550548) in a PBS solution containing 5% donkey serum. Vessels were washed for 2-4 hours in PBS at 4°C, exchanging the PBS 3-4 times over the washing period. Vessels were then incubated overnight with secondary antibodies (donkey anti-rabbit and donkey anti-rat at 1:500 dilution, BD Pharmingen #A21206 and #A48272, respectively) at 4°C. The vessels were washed with PBS for 2 hours on a rocker platform replacing the PBS solution every ~30 minutes, then incubated in a DAPI solution (ThermoScientific Cat. No. EN62248 1mg/mL stock and used at 1:1000 dilution) for 15 minutes at room temperature. Finally, the vessels were transferred to a myography chamber, where they were re-cannulated and pressurized. Fluorescence image stacks were acquired on a Leica DMI8 inverted fluorescence microscope using an Andor Dragonfly 202 high-speed confocal imaging platform equipped with Borealis™ enhanced illumination (40-μm pinhole disk). Images were acquired with a Zyla PLUS 4.2 Megapixel sCMOS camera and an HCX PL APO 40x/1.10W CORR objective using excitation from 405-nm, 488-nm, and 647-nm solid state diode laser lines. Z-axis image-stacks were acquired in 0.24 µm intervals, then processed and rendered using Imaris x64 9.7.2 as 3-dimensional orthographic isometric projections.

**Immunohistochemistry.** Immunohistochemistry (IHC) was performed according to detailed protocols published recently (Geng et al. 2016). A modified iDISCO protocol (Bio-protocol 11(20): e4186) was used for whole-mount sections of the dorsal skin and mesentery. For staining with VE-cadherin antibody, a previously described whole-mount IHC protocol was used (Geng et al. 2016; Methods Mol Biol 2018;1846:85-96). Primary antibodies for immunohistochemistry included rabbit anti-PROX1 (catalog 11-002, Angiobio), rat anti–mouse CD31 (catalog 553370, BD Pharmingen), rat anti–mouse VE-cadherin (catalog 550548, BD Pharmingen), chicken anti-GFP (catalog ab13970, Abcam) and rabbit anti-mouse CLDN5 (catalog 34-1600, Thermo Fisher Scientific). Goat anti–human PROX1 (catalog AF2727), goat anti–mouse VEGFR3 (catalog AF743) and goat anti–mouse ITGA9 (catalog AF3827) were from R&D Systems. Secondary antibodies for IHC included Cy3-conjugated donkey anti-rabbit, Cy3-conjugated donkey anti-goat, Cy5-conjugated donkey anti-rat, FITC-conjugated donkey anti-chicken, Alexa 488–conjugated donkey anti-goat, and Alexa 488-conjugated donkey anti-rabbit antibodies, purchased from Jackson ImmunoResearch Laboratories (catalogs 711-165-152, 705-165-147, 712-175-150, 703-095-155, 705-545-147, and 711-547-003 respectively). Alexa 488–conjugated donkey anti-rat antibody was purchased from Invitrogen (catalog A-21208).

**Quantification of mesenteric lymphatic valves.** Mesenteric lymphatic valves were identified by high PROX1 staining of collectors from the mesenteric lymph nodes to the point where the major collecting vessels branched into the gut wall. Staining was quantified using a Nikon Eclipse 80i microscope equipped with an Andor camera and Nikon Elements Software. For E18.5 embryonic mesentery, the total number of valves were counted on two vessels per mesentery and the average number was presented for each animal. Four embryos were analyzed for each genotype. For P10 mesentery, the total numbers of valves were normalized to the vessel length. Four vessels from each mesentery were quantified and four animals were analyzed for each genotype. To determine the vessel diameter, five locations were measured along a single mesenteric vessel and the average of the five measurements was presented for each animal. For E18.5 embryonic mesentery, two vessels were measured for
each animal and four animals were analyzed for each genotype. For P10 pups, four vessels were measured for each animal and four animals were analyzed for each genotype. Measurements were made using Nikon Elements Software.

**FACS analysis.** Inguinal-axillary lymphatic vessels from Prox1-GFP mice or tamoxifen-treated Prox1-CreER\(^{T2}\);Cx45\(^{f/f}\) mice were dissected and cleaned of fat and connective tissue. Cleaned vessel segments were digested into single cells as described previously (58). LECs expressing eGFP were sorted by fluorescence-activated cell sorting (FACS) using a Beckman-Coulter MoFlo XDP instrument with excitation laser (488 nm) and emission filter (530 ± 40 nm), a 70 µm nozzle, a sheath pressure of 45 psi and sort rate of 100 events per second. Sorting was performed at the Cell and Immunobiology Core facility at the University of Missouri.

**RNA isolation, end-Point RT-PCR and quantitative, real-time PCR.** Total RNA was extracted from FACS-sorted cells and end-point PCR performed as described previously (58). All primers (listed in Table 1) were designed to amplify intron-spanning DNA regions. Real-time PCR (qPCR) was performed on cDNA prepared from each sample using 2x PrimeTime Gene Expression Master Mix (IDT, Coralville, IA) with predesigned TaqMan probes (IDT, Coralville, IA), as listed in Table 2. Real-time PCR protocols were as follows: preheating to 95°C for 3 min, 45 cycles of two-step cycling of denaturation at 95°C for 15 sec and annealing/extension steps of 30 sec at 60°C. Data collection was carried out using a Bio-Rad CFX 96 Real-Time Detection System (software version Bio-Rad CFX Manager 3.1; Bio-Rad, Hercules, CA, USA). For analyses, the results were expressed as a ratio of target gene/reference gene (β-actin).

**In vivo tests of backflow.** An anesthetized mouse was placed face down on a heating pad and each hindlimb was extended and held in place with a piece of tape. A small amount (~5-10 µL) of Evan’s Blue Dye (EBD; 1% in sterile saline) was injected into the dermis of the foot pad using a 30-gauge needle. Typically, the filling of both popliteal afferents in each leg required that half the dye volume be injected into the top of the foot and the other half into the side of the foot. The skin over the two primary popliteal afferents in the thigh region was then opened, retracted and covered with Krebs-BSA. Any loose connective tissue or fat was removed to ensure maximum visibility of the popliteal afferents. Images of the popliteal network were collected using a Leica S9i dissecting microscope (magnification 10-40x) with built-in camera and stored on an SDHC card. Successful EBD dye injection was confirmed by filling of both afferent vessels and the popliteal nodes. If needed, gentle pressure was applied to the injection site with a cotton swab to facilitate filling. Dye backflow into side branches was monitored in real time and documented with photos. In each case of “backflow” we confirmed that EBD was flowing from one of the main vessels backward into a side branch, rather than in the other direction. The procedure was then repeated for the other leg. Popliteal afferents that did not completely fill with EBD were not counted in the analysis.

**Statistics.** Microsoft Excel was used to compile the initial data and to calculate back leak pressures (Pout-Psn) and values of ΔP for closure (Pout-Pin). Igor (Wavemetrics, Lake Oswego, OR) was used for the display of representative traces. LabVIEW was used to read raw data from text files and bin that data into discrete Pout intervals for subsequent statistical analysis. Prism v9 (Graphpad, San Diego, CA) was used for summary plots and statistics. Specific statistical tests for the various data sets are described in the respective figure legends. The normality of each data set was tested prior to using analysis of variance and, if normality was not satisfied in a majority of the standard Prism normality tests, then non-parametric tests (e.g., Kruskal-Wallis or Mann-Whitney U tests) were used instead of ANOVAs. When not explicitly stated, p values <0.05 are considered statistically significant.


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FIGURE LEGENDS

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Fig. 1. Cx45 is expressed in LECs of popliteal afferent lymphatics. A) Live GFP fluorescence signal (maximum projection) in a 3-valve popliteal lymphatic vessel segment from a Prox1-CreERT2;Cx45/f mouse. The normal direction of flow is left to right. The mouse was ~2 months old when induced with tamoxifen. B-G Orthographic isometric projections from 3-dimensional reconstructions of confocal Z-stacks from a Prox1-GFP vessel with the valve in an open position B-D) or closed position E-G). The normal direction of flow is right to left. H-J Orthographic isometric projections from 3-dimensional reconstructions of confocal Z-stacks from a Lyve1-Cre;Cx45/f vessel with a valve displaying deficient seal in the presence of an adverse pressure gradient. Note the small gap between the two closed leaflets near the insertion point in I and J (indicated with arrows). Vessel segments were stained with an anti-GFP antibody (green) and DAPI (blue). Panels B and E also display staining of VE-Cadherin (red). Scale bars are 50 μm.

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Fig 2. PCR evidence for expression of Cx43, Cx45, Cx37 in LECs of popliteal afferent lymphatics and the effective deletion of Cx45 using Lyve1-Cre. A-B) PCR of FACS-purified LECs sorted from inguinal-axillary lymphatics from Prox1-GFP mice (A) show expression of LEC genes but not LMC genes, with brain homogenate (B) serving as a positive control for all primers. C) Cx45, Cx43 and Cx37 expression in purified LECs (C) and in brain homogenate (D). Two different Cx47 primers were used. E) Prox1CreERT2;Cx45/f vessels expressed Cx43 and Cx37 (faint signal) but lacked Cx45. F) brain homogenate served as a positive control for primers in E. All gels are representative of at least 3 similar experiments. Myh11 = Myosin heavy chain 11; Cav1.2 = L-type voltage-gated calcium channel; Cnn1 = Calponin1.

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Fig 3. Constitutive Cx45 deletion leads to EBD backflow and leakage but does not interfere with the development of mesenteric LVs. A-B) Images of popliteal afferent lymphatics in the intact hindlimbs of Cx45/f and Lyve1-Cre;Cx45/f mice after footpad injection of EBD. The bottoms of the images are toward the feet. A) Dye is contained in both Cx45/f vessels but B) backflow into side branches occurred at three sites (black arrows) in two Lyve1-Cre;Cx45/f vessels. LAT= lateral; MED= medial. Scale bar in B is 1 mm. C) One Lyve1-Cre;Cx45/f popliteal afferent was leaky without any backflow. D) The number of side branches (per popliteal afferent) with backflow after EBD injection. E) The number of popliteal lymphatics that leaked EBD. Means ± SEM. Significant differences between Cx45/f and Lyve1-Cre;Cx45/f vessels assessed using a Mann-Whitney U test. N=4, n=14 for Lyve1Cre;Cx45/f; N=4, n=16 for Cx45/f. F-G) Mesenteric lymphatic valves from E18.5 Cx45+/f and Lyve1-Cre;Cx45+/f embryos were morphologically normal. Mesenteric arcades from Cx45/f and Lyve1-Cre;Cx45/f/ embryos were stained using the indicated antibodies. The number of lymphatic valves per vessel (yellow arrowheads in F-G) were counted and quantified. H-I) Neither the number of valves per vessel nor the vessel diameter was affected by the deletion of Cx45. N=4 pups per genotype. Mean ± SD with each dot representing an embryo. Unpaired t tests were used for statistical analysis. J-K) The mesenteries of Cx45+/f and Lyve1-Cre;Cx45+/f P10 pups were stained for Prox1. No significant differences were observed in either the number of valves per vessel length (L) or vessel diameter (M). Mean ± SD. Unpaired t tests were used for statistical analysis. N=4 pups per genotype. N-O) The expression patterns of INTEGRIN-α9, CLAUDIN-5 and VE-CADHERIN were indistinguishable between Cx45+/f and Lyve1-Cre;Cx45+/f P10 mesenteric valves. N=4 pups per genotype.

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Fig. 4. Back leak tests. Images of a popliteal lymphatic containing a single valve (A) before and (B) after elevation of Pout to 10 cm H2O. C-F) Representative examples of back leak tests for valves from (C) WT, (D) Lyve1-Cre;Cx45/f, (E) Prox1-CreERT2;Cx45+/f and (F) Prox1-CreERT2;Cx45/f mice. Arrowheads indicate time during the
Pout ramp when each valve closed. Values of back leak (Psn-Pin) at Pout = 10 cmH₂O are stated above the traces for each of the valves. G-L) Summary of back leak measurements as a function of Pout for each of the six genotypes. The raw Psn data were binned in 0.5 cmH₂O Pout intervals before determining the mean and variance of Psn for each interval. Asterisks in L indicate significant differences (p < 0.05) for Lyve1-Cre;Cx45⁻/⁻ valves compared to their initial (control) values at Pout > 5 cmH₂O, as determined by a 2-way, repeated measures ANOVA with Tukey’s post-hoc tests. None of the other groups had significant increases in Psn. M) Comparisons of back leak at Pout = 10 cmH₂O between genotypes. Lyve1-Cre;Cx45⁻/⁻ and Lyve1-Cre;Cx45⁺/⁺ data were combined and listed as Lyve1-Cre⁻/⁻;Cx45⁻/⁻. Comparisons between Lyve1-Cre;Cx45⁻/⁻, Lyve1-Cre and Cx45⁻/⁻ valves and between Prox1-CreERT²;Cx45⁻/⁻, Prox1-CreERT²;Cx45⁺/⁺ and Cx45⁻/⁻ valves were made using Kruskal-Wallis tests with Dunn’s post-hoc tests. None of the other groups had significant increases in Psn. N) Comparisons between Lyve1-Cre;Cx45⁻/⁻, Prox1-CreERT²;Cx45⁻/⁻, Prox1-CreERT²;Cx45⁺/⁺ and the combined control groups were made using a Kruskal-Wallis test with Dunn’s post-hoc tests. Differences between Lyve1-Cre;Cx45⁻/⁻ and combined controls and between Lyve1-Cre;Cx45⁻/⁻ and Prox1-CreERT²;Cx45⁻/⁻ valves were statistically significant. Comparisons not indicated were not significant at p < 0.05. In panels G-N, data are means ± SEM. WT N=16, n=32; Lyve1-Cre;Cx45⁻/⁻ and Lyve1-Cre;Cx45⁺/⁺ combined N=9, n=24; Lyve1-Cre N=6, n=14; Cx45⁻/⁻ N=4, n=12; Prox1-CreERT²;Cx45⁻/⁻ N=4, n=14; Prox1-CreERT²;Cx45⁺/⁺ N=2, n=8.

Fig. 5. Closure tests. Representative recordings of closure tests for valves from (A) WT, (B) Lyve1-Cre;Cx45⁻/⁻, (C) Prox1-CreERT²;Cx45⁻/⁻ and (D) Prox1-CreERT²;Cx45⁺/⁺ mice at a baseline pressure of Pin = 10 cmH₂O. Arrows indicate when the valve closed during the ramp. B) Shows determination of ΔP from Pout-Pin at the moment of valve closure. E-H) Complete back leak curves vs. baseline pressures for the same valves, with the filled red circles representing the ΔP values in the top panels. I-N) Complete closure tests for the 6 genotypes of mice. The red symbols/curves are the data shown in panels E-H. Normal valves had a ΔP for closure <10 cmH₂O when vessel diameter was maximal. Three Lyve1-Cre;Cx45⁻/⁻ valves were completely incompetent at all pressures and a fourth valve became incompetent when diameter exceeded 70% of the maximal diameter. O) Comparisons of ΔP for closure valves at Pin = 0.5 cmH₂O for the six different genotypes. A Kruskal-Wallis test was used to determine significant differences for each genotype compared to the control groups. No significant differences were found. P) Alternate analysis comparing the fraction of vessels with abnormal ΔP for closure at Pin = 0.5 cmH₂O between the different genotypes. Because of the higher variability in this parameter between vessels, the control groups (WT + Lyve1-Cre + Cx45⁺/⁺) were combined and the threshold for classifying a valve as abnormal was determined from the mean + 1 SD of the combined controls. A Kruskal-Wallis test with Dunn’s post-hoc tests showed a significant difference between Lyve1-Cre;Cx45⁻/⁻ valves and the combined controls. Means ± SEM. In panel O, the values of N and n are the same as in Fig. 4. In panel P, WT N=16, n=24; Lyve1-Cre;Cx45⁻/⁻ and Lyve1-Cre;Cx45⁺/⁺ combined N=9, n=12; Lyve1-Cre N=6, n=10; Cx45⁻/⁻ N=4, n=6; Prox1-CreERT²;Cx45⁻/⁻ N=4, n=8; Prox1-CreERT²;Cx45⁺/⁺ N=2, n=5.

Fig. 6. Valve dimension measurements made under brightfield microscopy during functional tests. A) Schematic of a prototypical lymphatic valve as viewed from the top or side; b and b’ are on the opposite surface of the valve sinus and are not shown. Neither are the commissures shown, but when present they extend downstream from the intersections of a and a’ and the intersections of b and b’. B) Brightfield images of valves...
in Prox1-CreERT2;Cx45f/f and Lyve1-Cre;Cx45Δ/f vessels, with arrows pointing to the downstream ends of the leaflets. C) Intervalve distances in the 6 different genotypes of mice studied. D-F) Measurements of average leaflet length (D), shortest leaflet length (E) and leaflet symmetry (F) in the 6 genotypes of mice. Significant differences were determined using one-way ANOVAs with Dunn’s multiple comparison post-hoc tests to the respective “control” genotype. Non-significant comparisons are not marked. WT N=16; Cx45Δ/f N=4; Lyve1-Cre N=6; Prox1-CreERT2;Cx45Δ/f N=2; Prox1-CreERT2;Cx45+/f N=2; Lyve1-Cre;Cx45Δ/f N=8. The number of vessels for each group are reflected in the number of data points shown in the graphs.

Suppl. Fig. 1. FACS analysis. FACS analysis of GFP+ cells sorted from Prox1-GFP and Prox1-CreERT2;Cx45Δ/f vessels, with sorting windows circled.

Suppl. Fig. 2. qPCR analysis reveals no significant changes in Cx43 or Cx37 mRNA levels after Cx45 deletion. qPCR analysis of Cx isoform mRNA levels in GFP+ cells sorted from Prox1-GFP and Prox1-CreERT2;Cx45Δ/f vessels. Comparisons between Prox1-GFP and Prox1-CreERT2;Cx45Δ/f groups were made using Mann-Whitney U tests; * = p<0.05.

Suppl. Fig. 3. Deletion of Cx45 does not interfere with the development of lymphovenous valves (LVVs). Venous and lymphovenous valves were unaffected by the deletion of Cx45. A) E18.5 Cx45+/f and B) Lyve1-Cre;Cx45Δ/f embryos were frontally sectioned and the junctions of the jugular (JS) and subclavian veins (SCV) were analyzed using the indicated antibodies. SVC= superior vena cava. No obvious defects were observed in the lymphovenous valves (arrows) or the venous valves (arrowheads) of Lyve1-Cre;Cx45Δ/f mice. Images in each panel are representative of 4 experiments.

Suppl. Movie 1. Movie showing rotatable image stacks for the open valve in Fig. 1B-D.

Suppl. Movie 2. Movie showing rotatable image stacks for the closed valve in Fig. 1E-F.

STUDY APPROVAL
All procedures were approved by the institutional review boards at the University of Missouri, OMRF and Tulane School of Medicine and complied with the standards stated in the “Guide for the Care and Use of Laboratory Animals” (National Institutes of Health, revised 2011).

DATA AVAILABILITY
The data sets shown in all the figures are listed in an associated spreadsheet of “Supporting Data Values”. Data will be made available from the corresponding author upon request. LabVIEW code for data collection and analyses is available at https://doi.org/10.5281/zenodo.8286107 and https://doi.org/10.5281/zenodo.8286119.

AUTHOR CONTRIBUTIONS
MJD and RSS designed the experiments. MJD, XG, JAC-G, SDZ and ML performed the experiments and analyzed the results. MJD, AMS and RSS drafted the manuscript. All authors edited the manuscript and approved the final version.

ACKNOWLEDGEMENTS

We gratefully acknowledge the technical support of Shanyu Ho and the gifts of Cx45floxed mice, Prox1-CreERT2 mice and Prox1-GFP mice, as stated in Methods. This research was supported by National Institutes of Health grants R01-HL122608 and R01-HL122578 to MJD, R00-HL141143 to JAC-G and R01HL-131652, R01HL-163095 and R01HL-133216 to RSS.

CONFLICT OF INTEREST STATEMENT

The authors have declared that no conflict of interest exists.
### Table 1. Primer sequences, gene accession numbers and amplicons.

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Fig. 1. Cx45 is expressed in LECs of popliteal afferent lymphatics. A) Live GFP fluorescence signal (maximum projection) in a 3-valve popliteal lymphatic vessel segment from a Prox1-CreER<sup>T2</sup>;Cx45<sup>−/−</sup> mouse. The normal direction of flow is left to right. The mouse was ~2 months old when induced with tamoxifen. B-G) Orthographic isometric projections from 3-dimensional reconstructions of confocal Z-stacks from a Prox1-GFP vessel with the valve in an open position B-D) or closed position E-G). The normal direction of flow is right to left. H-J) Orthographic isometric projections from 3-dimensional reconstructions of confocal Z-stacks from a Lyve1-Cre;Cx45<sup>Δ−/−</sup> vessel with a valve displaying deficient seal in the presence of an adverse pressure gradient. Note the small gap between the two closed leaflets near the insertion point in I and J (indicated with arrows). Vessel segments were stained with an anti-GFP antibody (green) and DAPI (blue). Panels B and E also display staining of VE-Cadherin (red). Scale bars are 50 μm.
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Fig 2. PCR evidence for expression of Cx43, Cx45, Cx37 in LECs of popliteal afferent lymphatics and the effective deletion of Cx45 using Lyve1-Cre. A-B) PCR of FACS-purified LECs sorted from inguinal-axillary lymphatics from Prox1-GFP mice (A) show expression of LEC genes but not LMC genes, with brain homogenate (B) serving as a positive control for all primers. C) Cx45, Cx43 and Cx37 expression in purified LECs (C) and in brain homogenate (D). Two different Cx47 primers were used. E) Prox1CreER\textsuperscript{T2};Cx45\textsuperscript{fl/fl} vessels expressed Cx43 and Cx37 (faint signal) but lacked Cx45. F) brain homogenate served as a positive control for primers in E. All gels are representative of at least 3 similar experiments. Myh11 = Myosin heavy chain 11; Cav1.2 = L-type voltage-gated calcium channel; Cnn1 = Calponin1.
(A) Cx45+/f
(B) Lyve-1-Cre;Cx45+/f
(C) Cx45+/f
(D) # vessels with backflow
(E) # leaky vessels
(F) Cx45+/f
(G) Lyve-1-Cre;Cx45+/f
(H) Number of valves per vessel
(I) Vessel diameter (µm)
(J) Cx45+/f
(K) Lyve-1-Cre;Cx45+/f
(L) Number of valves per mm
(M) Vessel diameter (µm)
(N) Cx45+/f
(O) Lyve-1-Cre;Cx45+/f
Fig. 3. Constitutive Cx45 deletion leads to EBD backflow and leakage but does not interfere with the development of mesenteric LVs. A-B) Images of popliteal afferent lymphatics in the intact hindlimbs of Cx45\textsuperscript{+/f} and Lyve1-Cre;Cx45\textsuperscript{+/f} mice after footpad injection of EBD. The bottoms of the images are toward the feet. A) Dye is contained in both Cx45\textsuperscript{+/f} vessels but B) backflow into side branches occurred at three sites (black arrows) in two Lyve1-Cre;Cx45\textsuperscript{+/f} vessels. LAT= lateral; MED= medial. Scale bar in B is 1 mm. C) One Lyve1-Cre;Cx45\textsuperscript{+/f} popliteal afferent was leaky without any backflow. D) The number of side branches (per popliteal afferent) with backflow after EBD injection. E) The number of popliteal lymphatics that leaked EBD. Means ± SEM. Significant differences between Cx45\textsuperscript{+/f} and Lyve1-Cre;Cx45\textsuperscript{+/f} vessels assessed using a Mann-Whitney U test. N=4, n= 14 for Lyve1Cre;Cx45\textsuperscript{+/f}; N=4, n=16 for Cx45\textsuperscript{+/f}. F-G) Mesenteric lymphatic valves from E18.5 Cx45\textsuperscript{+/f} and Lyve1-Cre;Cx45\textsuperscript{+/f} embryos were morphologically normal. Mesenteric arcades from Cx45\textsuperscript{+/f} and Lyve1-Cre;Cx45\textsuperscript{+/f} embryos were stained using the indicated antibodies. The number of lymphatic valves per vessel (yellow arrowheads in F-G) were counted and quantified. H-I) Neither the number of valves per vessel nor the vessel diameter was affected by the deletion of Cx45. N=4 pups per genotype. Mean ± SD with each dot representing an embryo. Unpaired t tests were used for statistical analysis. J-K) The mesenteries of Cx45\textsuperscript{+/f} and Lyve1-Cre;Cx45\textsuperscript{+/f} P10 pups were stained for Prox1. No significant differences were observed in either the number of valves per vessel length (L) or vessel diameter (M). Mean ± SD. Unpaired t tests were used for statistical analysis. N=4 pups per genotype. N-O) The expression patterns of INTEGRIN-\(\alpha\)9, CLAUDIN-5 and VE-CADHERIN were indistinguishable between Cx45\textsuperscript{+/f} and Lyve1-Cre;Cx45\textsuperscript{+/f} P10 mesenteric valves. N=4 pups per genotype.
Fig. 4. Back leak tests. Images of a popliteal lymphatic containing a single valve (A) before and (B) after elevation of Pout to 10 cmH$_2$O. (C-F) Representative examples of back leak tests for valves from (C) WT, (D) Lyve1-Cre;Cx45$^{−/−}$, (E) Prox1-CreER$^{T2}$;Cx45$^{−/−}$ and (F) Prox1-CreER$^{T2}$;Cx45$^{+/−}$ mice. Arrowheads indicate time during the Pout ramp when each valve closed. Values of back leak (Ps-$P_{in}$) at Pout = 10 cmH$_2$O are stated above the traces for each of the valves. (G-L) Summary of back leak measurements as a function of Pout for each of the six genotypes. The raw Ps-$P_{in}$ data were binned in 0.5 cmH$_2$O Pout intervals before determining the mean and variance of Ps-$P_{in}$ for each interval. Asterisks in L indicate significant differences (p< 0.05) for Lyve1-Cre;Cx45$^{−/−}$ valves compared to their initial (control) values at Pout levels > 5 cmH$_2$O, as determined by a 2-way, repeated measures ANOVA with Tukey’s post-hoc tests. None of the other groups had significant increases in Ps-$P_{in}$. (M) Comparisons of back leak at Pout = 10 cmH$_2$O between genotypes. Lyve1-Cre;Cx45$^{−/−}$ and Lyve1-Cre;Cx45$^{+/−}$ data were combined and listed as Lyve1-Cre;Cx45$^{−/−}$. Comparisons between Lyve1-Cre;Cx45$^{−/−}$, Lyve1-Cre and Cx45$^{+/−}$ valves and between Prox1-CreER$^{T2}$;Cx45$^{−/−}$, Prox1-CreER$^{T2}$;Cx45$^{+/−}$ and Cx45$^{+/−}$ valves were made using Kruskal-Wallis tests with Dunn’s post-hoc tests. Back leak was significantly higher in Lyve1-Cre;Cx45$^{−/−}$ valves compared to Lyve1-Cre or Cx45$^{+/−}$ valves. (N) Fraction of leaky valves for each genotype calculated using the threshold criteria for a leaky valve as the mean + 1 SD of the pooled control groups (WT + Cx45$^{+/−}$ + Lyve1-Cre). Comparisons between Lyve1-Cre;Cx45$^{−/−}$, Prox1-CreER$^{T2}$;Cx45$^{−/−}$, Prox1-CreER$^{T2}$;Cx45$^{+/−}$ and the combined control groups were made using a Kruskal-Wallis test with Dunn’s post-hoc tests. Differences between Lyve1-Cre;Cx45$^{−/−}$ and combined controls and between Lyve1-Cre;Cx45$^{−/−}$ and Prox1-CreER$^{T2}$;Cx45$^{+/−}$ valves were statistically significant. Comparisons not indicated were not significant at p <0.05. In panels G-N, data are means ± SEM. WT N=16, n=32; Lyve1-Cre;Cx45$^{−/−}$ and Lyve1-Cre;Cx45$^{−/−}$ combined N=9, n=24; Lyve1-Cre N=6, n=14; Cx45$^{+/−}$ N=4, n=12; Prox1-CreER$^{T2}$;Cx45$^{−/−}$ N=4, n=14; Prox1-CreER$^{T2}$;Cx45$^{+/−}$ N=2, n=8.
**Fig. 5. Closure tests.** Representative recordings of closure tests for valves from (A) WT, (B) Lyve1-Cre;Cx45−/−, (C) Prox1-CreER<sup>122</sup>;Cx45<sup>−/−</sup> and (D) Prox1-CreER<sup>122</sup>;Cx45<sup>+/−</sup> mice at a baseline pressure of $P_{in} = 10 \text{ cmH}_2\text{O}$. Arrows indicate when the valve closed during the ramp. **B**) Shows determination of $\Delta P$ from $P_{out} - P_{in}$ at the moment of valve closure. **E-H**) Complete back leak curves vs. baseline pressures for the same valves, with the filled red circles representing the $\Delta P$ values in the top panels. **I-N**) Complete closure tests for the 6 genotypes of mice. The red symbols/curves are the data shown in panels E-H. Normal valves had a $\Delta P$ for closure <10 cmH$_2$O when vessel diameter was maximal. Three Lyve1-Cre;Cx45<sup>−/−</sup> valves were completely incompetent at all pressures and a fourth valve became incompetent when diameter exceeded 70% of the maximal diameter. **O**) Comparisons of $\Delta P$ for closure values at $P_{in} = 0.5 \text{ cmH}_2\text{O}$ for the six different genotypes. A Kruskal-Wallis test was used to determine significant differences for each genotype compared to the control groups. No significant differences were found. **P**) Alternate analysis comparing the fraction of vessels with abnormal $\Delta P$ for closure at $P_{in} = 0.5 \text{ cmH}_2\text{O}$ between the different genotypes. Because of the higher variability in this parameter between vessels, the control groups (WT + Lyve1-Cre + Cx45<sup>+/−</sup>) were combined and the threshold for classifying a valve as abnormal was determined from the mean + 1 SD of the combined controls. A Kruskal-Wallis test with Dunn’s post-hoc tests showed a significant difference between Lyve1-Cre;Cx45<sup>−/−</sup> valves and the combined controls. Means ± SEM. In panel O, the values of $N$ and $n$ are the same as in Fig. 4. In panel P, WT $N=16$, $n=24$; Lyve1-Cre;Cx45<sup>−/−</sup> and Lyve1-Cre;Cx45<sup>+/−</sup> combined $N=9$, $n=12$; Lyve1-Cre $N=6$, $n=10$; Cx45<sup>+/−</sup> $N=4$, $n=6$; Prox1-CreER<sup>122</sup>;Cx45<sup>+/−</sup> $N=4$, $n=8$; Prox1-CreER<sup>122</sup>;Cx45<sup>+/−</sup> $N=2$, $n=5$. 
**A**

Top view

Side view

**B**

Prox1Cre;Cx45^{f/f} Lyve1Cre;Cx45^{A/f}

**C**

Intervalle distance (mm)

**D**

Average leaflet length (μm)

**E**

Shortest leaflet length (μm)

**F**

Leaflet symmetry
Fig. 6. Valve dimension measurements made under brightfield microscopy during functional tests. A) Schematic of a prototypical lymphatic valve as viewed from the top or side; b and b’ are on the opposite surface of the valve sinus and are not shown. Neither are the commissures shown, but when present they extend downstream from the intersections of a and a’ and the intersections of b and b’. B) Brightfield images of valves in Prox1-CreERT2;Cx45f/f and Lyve1-Cre;Cx45∆f/f vessels, with arrows pointing to the downstream ends of the leaflets. C) Intervale distances in the 6 different genotypes of mice studied. D-F) Measurements of average leaflet length (D), shortest leaflet length (E) and leaflet symmetry (F) in the 6 genotypes of mice. Significant differences were determined using one-way ANOVAs with Dunn’s multiple comparison post-hoc tests to the respective “control” genotype. Non-significant comparisons are not marked. WT N=16; Cx45f/f N=4; Lyve1-Cre N=6; Prox1-CreERT2;Cx45f/f N=2; Prox1-CreERT2;Cx45∆f/f N=2; Lyve1-Cre;Cx45∆f/f N=8. The number of vessels for each group are reflected in the number of data points shown in the graphs.
Suppl. Fig. 1. FACS analysis. FACS analysis of GFP+ cells sorted from \textit{Prox1-GFP} and \textit{Prox1-CreER^{T2};Cx45''} vessels, with sorting windows circled.
Suppl. Fig 2.
Suppl. Fig. 2. qPCR analysis reveals no significant changes in Cx43 or Cx37 mRNA levels after Cx45 deletion. qPCR analysis of Cx isoform mRNA levels in GFP+ cells sorted from Prox1-GFP and Prox1-CreER\textsuperscript{T2};Cx45\textsuperscript{-/-} vessels. Comparisons between Prox1-GFP and Prox1-CreER\textsuperscript{T2};Cx45\textsuperscript{-/-} groups were made using Mann-Whitney U tests; * = p<0.05.
Suppl. Fig 3.

(A) Cx45^+/f

(B) Lyve1-Cre;Cx45^A/f
Suppl. Fig. 3. Deletion of Cx45 does not interfere with the development of lymphovenous valves (LVVs). Venous and lymphovenous valves were unaffected by the deletion of Cx45. A) E18.5 Cx45+/f and B) Lyve1-Cre;Cx45Δ/f embryos were frontally sectioned and the junctions of the jugular (JS) and subclavian veins (SCV) were analyzed using the indicated antibodies. SVC= superior vena cava. No obvious defects were observed in the lymphovenous valves (arrows) or the venous valves (arrowheads) of Lyve1-Cre;Cx45Δ/f mice. Images in each panel are representative of 4 experiments.