Lymph flow regulates collecting lymphatic vessel maturation in vivo

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Introduction
The lymphatic vascular system regulates tissue fluid homeostasis, immune cell trafficking, and the absorption of dietary fats (1). Lymphatic capillaries lack basement membranes and maintain openings between endothelial cells (ECs) to facilitate the uptake of interstitial fluid, proteins, lipids, and inflammatory cells. In contrast, larger collecting lymphatics are tight vascular structures specialized for fluid transport (2). Collecting lymphatics transport lymph back to the venous circulation in a low-pressure, low-flow system that operates without a central pump (3) and have therefore developed specific features that facilitate fluid transport. Prominent among these are intraluminal valves that prevent the backflow of lymph, and smooth muscle cells (SMCs) that cover the endothelium between valves to provide an intrinsic pumping force (4).

Lymphatic valves are composed of 2 intraluminal leaflets, each of which is formed by 2 layers of lymphatic ECs (LECs) separated by an extracellular matrix-rich core (5). The genetic and molecular program that underlies lymphatic valve formation has been recently revealed by studies of humans with primary lymphedema syndromes and mutant mice (6–8). These studies have culminated in a recently described multi-step process in which the LEC transcription factors PROX1 and FOXC2 play primary and essential roles (9). In the embryonic mouse mesentery, PROX1 and FOXC2 become upregulated in a subset of LECs at E16 (9).

Upregulation of these transcription factors precedes and controls a complex process of EC reorientation and migration as the valve leaflets develop and mature. Genetic studies in mice implicate a large number of genes in this complex second phase of valve development, including connexins 37 and 43, EfnB2, integrin α9, laminin α5, Gata2, and others (7, 10–13). The events that initiate the process of lymphatic valve formation are currently unknown, but recent in vitro studies of LECs have suggested that fluid forces may play an important role (7, 9).

ECs that line the lumen of blood and lymphatic vessels are exposed to mechanical forces generated by fluid flow. In the high-pressure, high-flow blood vasculature, hemodynamic forces generated by the beating heart are substantial and regulate EC alignment and proliferation, as well as vascular tone and remodeling (reviewed in ref. 14). The fluid shear force experienced by arterial ECs is approximately 75 times greater than that experienced by LECs in large lymphatic vessels (15), and forces in the developing lymphatic vasculature may be even lower. Recent in vitro studies suggest that fluid shear force can be sensed by LECs in tissue culture and therefore may regulate LEC gene expression and lymphatic vessel development (9, 16). Testing this hypothesis in vivo during actual lymphatic development is difficult, however, because genetic manipulations that prevent lymphatic flow also directly impair initial lymphatic vessel growth (6, 17, 18) and surgical interventions to mechanically block lymph flow in the mouse embryo are technically challenging and may also affect lymphatic growth.

To address the role of fluid shear force during lymphatic valve development and collecting vessel formation in vivo, we have taken advantage of CLEC2-deficient mice in which lymphatics develop normally but lymphatic flow is opposed by the backflow of

Fluid shear forces have established roles in blood vascular development and function, but whether such forces similarly influence the low-flow lymphatic system is unknown. It has been difficult to test the contribution of fluid forces in vivo because mechanical or genetic perturbations that alter flow often have direct effects on vessel growth. Here, we investigated the functional role of flow in lymphatic vessel development using mice deficient for the platelet-specific receptor C-type lectin–like receptor 2 (CLEC2) as blood backfills the lymphatic network and blocks lymph flow in these animals. CLEC2-deficient animals exhibited normal growth of the primary mesenteric lymphatic plexus but failed to form valves in these vessels or remodel them into a structured, hierarchical network. Smooth muscle cell coverage (SMC coverage) of CLEC2-deficient lymphatic vessels was both premature and excessive, a phenotype identical to that observed with loss of the lymphatic endothelial transcription factor FOXC2. In vitro evaluation of lymphatic endothelial cells (LECs) revealed that low, reversing shear stress is sufficient to induce expression of genes required for lymphatic valve development and identified GATA2 as an upstream transcriptional regulator of FOXC2 and the lymphatic valve genetic program. These studies reveal that lymph flow initiates and regulates many of the key steps in collecting lymphatic vessel maturation and development.
Lymph flow is impeded in developing Clec2−/− mice. CLEC2 is a platelet receptor that mediates intravascular hemostasis at the lympho-venous junction and prevents the backflow of venous blood into the lymphatic vascular system (19, 22). Although primary lymphatic vessels form normally, Clec2−/− animals exhibit severe cutaneous edema by E14 and both chylous ascites and chylothorax after birth (20, 22), phenotypes suggestive of lymphatic dysfunction due to obstruction. To determine if the presence of blood in lymphatic vessels obstructs lymph flow during lymphatic development in Clec2−/− animals, we first examined the intestine histologically between E15.5 and E18.5, the time period during which lymphatic vessels invade the gut and presumably begin to carry lymph from that organ (23). Edema was not observed in either control or CLEC2-deficient intestines at E15.5, but Clec2−/− animals exhibited significant intestinal-wall edema at E16.5 and marked edema at E18.5 compared with control littermates (Figure 1, A–F, and Supplemental Videos 1 and 2). For these studies, we used 4-week-old Clec2−/−;Pf4-Cre mice in which CLEC2 is deleted specifically from platelets because global CLEC2-deficient mice fail to develop normal lymph nodes (26, 27), and Clec2−/−;Pf4-Cre mice exhibit delayed post-natal lethality, most likely due to incomplete deletion in the megakaryocyte (22). Real-time imaging of microparticles revealed lymphatic pulsation in both Clec2−/−;Pf4-Cre and control mice. Particle trajectories in the control mice were characterized by a brief period of retrograde displacement followed by a greater antegrade motion, resulting in considerable net forward displacement during each contraction cycle (Figure 1Q and Supplemental Video 1). In contrast, Clec2−/−;Pf4-Cre mice exhibited rapid pulses of forward displacement that reversed to retrograde flow, resulting in a net reverse displacement (Figure 1Q). Despite the lack of forward lymph flow, smooth muscle contraction appeared normal, or enhanced, in Clec2−/− lymphatics (Supplemental Video 2). Together, these studies demonstrate that loss of CLEC2 does not impair primary lymphatic vessel growth but results in markedly reduced lymph flow afterwards.
Figure 1. Lack of detectable lymph flow in developing mesenteric lymphatics of Clec2/−/− mice. (A–F) Clec2/−/− mice exhibit edema in the intestine wall after E15.5. H&E staining of cross sections of embryonic intestine is shown at E15.5, E16.5, and E18.5. Scale bars: 100 μm. (G and H) LEC elongation in P1 mesenteric lymphatics was measured by staining for PROX1 (green) and VE-cadherin (red). Yellow lines indicate representative measurements of cell length and width. Scale bars: 50 μm. (I) Quantitation of intestine wall thickness. n = 2 mice per timepoint for each genotype and 2 sections per mouse with at least 3 wall thickness measurements per section. (J) Quantitation of LEC elongation using length/width ratio. n = 4 animals at E15.5 and 5 animals at P1 per genotype. Over 100 LECs were measured per mouse. All values are means ± SEM. *P < 0.05, ***P < 0.001. P value calculated by Student’s t test. (K–N) Oral lymphangiogram 2 hours after neonatal ingestion of Bodipy-FL C16. White arrows indicate lymphatic vessels in the mesentry. Images are representative of 5 mice per genotype. (O and P) In vivo measurement of lymphatic flow by bead tracking in surviving 4-week-old in Clec2/−/− vs. Clec2/−/−; Pf4-Cre mice with blood-filled lymphatic phenotype. Yellow circles indicate position of bead in each frame of video over one contraction cycle. (Q) Quantitation of bead displacement (μm) over time relative to starting point in Clec2/−/− (black asterisks) vs. Clec2/−/−; Pf4-Cre (white circles). Representative images shown from bead tracking in 3 mice per genotype.

Lymphatic valve development is blocked in CLEC2-deficient animals. The observations that CLEC2-deficient embryos undergo normal lymphatic vessel growth but fail to establish normal lymphatic flow indicate that these animals provide a means of testing the role of lymph flow on lymphatic vessel development in vivo. As recently described, a primary step in lymphatic valve formation is upregulation of PROX1 expression in LECs at the site of valve formation, an event that is evident by E16.5 in the WT mouse mesentery (9). Immunostaining of PROX1 in WT neonatal mesenteric lymphatic vessels revealed a large number of PROX1 HI LEC clusters that mark the sites of developing valves (Figure 2, A and B). PROX1 HI LEC clusters were often located at vessel branch points and found at a frequency of approximately 1 valve every 2.5 mm of mesenteric lymphatic vessel length at P1 (Figure 2G). CLEC2-deficient lymphatics exhibited very few PROX1 HI LEC clusters, even though lymphatic vessel number was not decreased (Figure 2, C and D), and PROX1 expression in nonvalvular LECs was similar to that in WT animals (Figure 2, A–D). Quantification of valve number per mm vessel length or per vessel branchpoint revealed an 80% reduction in the number of lymphatic valves in Clec2−/− animals compared with Clec2+/+ controls (Figure 2G). CLEC2 is a transmembrane receptor that is expressed primarily by platelets, and the effects of CLEC2-deficiency on valve formation in the lymphatic system are therefore most likely to reflect effects of blood backflow on lymph forward flow rather than an LEC-intrinsic defect. To rule out the possibility that CLEC2 is required cell autonomously in LECs during lymphatic valve formation, we analyzed valve formation in Clec2−/−; Pf4-Cre+ animals in which CLEC2...)
is deleted specifically in megakaryocytes and platelets but not ECs (22, 28). Clec2<sup>−/−</sup>; Pf4-Cre<sup>+</sup> animals exhibited a severe loss of lymphatic valve development that was indistinguishable from that observed in Clec2<sup>−/−</sup> animals, indicating that CLEC2 expression in platelets, and not LECs, is required for lymphatic valve formation (Figures 2H and Supplemental Figure 2). Venous valve development requires many of the same genes as lymphatic valve formation (11, 29), and CLEC2-deficient animals do not exhibit any blood vascular defects (22). To exclude an unexpected role for CLEC2 in valve formation in general, we examined the formation of valves in the femoral vein of Clec2<sup>−/−</sup> animals. Venous valve formation was preserved in Clec2<sup>−/−</sup> animals (Figure 2, E and F), suggesting that loss of lymphatic flow, and not loss of CLEC2, results in failure to form lymphatic valves in CLEC2-deficient animals.

**Figure 2. Lymphatic valve development is blocked in Clec2<sup>−/−</sup> mesenteric lymphatics.** (A–D) Whole-mount staining for PROX1 in P1 neonatal mesentery was used to identify lymphatic valves. White arrows indicate PROX1<sup>HI</sup> lymphatic valves. Scale bars: 200 μm. Representative images shown from 6 mice per genotype. (E and F) Analysis of venous valves in the femoral vein of P6 pups by visualization of PROX1-GFP, which is expressed in venous valve BECs. White arrows indicate venous valves. Representative images shown from 4 mice per genotype. (G) Quantitation of lymphatic valve number in neonatal Clec2<sup>−/−</sup> and Clec2<sup>+/+</sup> littermates. n = 6 mice per genotype. (H) Quantitation of lymphatic valve number in neonatal platelet-specific conditional Clec2<sup>fl/−</sup>; Pf4-Cre and Clec2<sup>fl/−</sup>; Pf4-Cre littermates. n = 3 mice per genotype. All values are means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, calculated by Student’s t test. (K–T) Analysis of SMC coverage of Clec2<sup>−/−</sup> and Clec2<sup>+/+</sup> littermates. (I–N) Staining of mesenteric vessels for smooth muscle actin (red) and lymphatic ECs (PROX1-GFP, green) in P4 neonates. V, vein; A, artery; L, lymphatic. White dotted line in K–L outlines lymphatic vessels. White arrows indicate lymphatic valves. Scale bars: 200 μm. Representative images shown from 3 mice per genotype. (O–R) High-magnification confocal microscopy of P4 lymphatic valve regions (smooth muscle actin, red) and lymphatic ECs (PROX1-GFP, green). V, valve. Scale bars: 100 μm. (S and T) Histology of E18.5 thoracic duct (TD) for smooth muscle actin (red) and lymphatic ECs (PROX1-GFP, green). Scale bars: 100 μm. Representative images shown from 2 mice per genotype.
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**Figure 3. Failure of mesenteric lymphatic vessel remodeling and valve initiation in Clec2–/– mice.** (A–F) Analysis of mesenteric lymphatic vessel morphology and patterning at E15.5 (A and B), E16.5 (C and D), and E18.5 (E and F) in Prox1-GFP BAC transgenic embryos. White arrows indicate sites of lymphatic valve formation. Vascular architecture and valve formation are shown diagrammed on the right. Scale bars: 200 μm. (G and H) Quantitation of valve number (G) and vessel branchpoint number (H) in E18.5 Clec2–/– and Clec2+/+ mesenteric lymphatics. n = 9 embryos per genotype. (I) Schematic of the late gestation WT mesenteric lymphatic network demonstrating vessels denoted as primary, secondary, and tertiary based on branching and distance from the intestine. (J) Quantitation of lymphatic vascular hierarchy in E18.5 Clec2–/– and Clec2+/+ embryos. Lymphatic vessel width was measured at the 3 levels of the lymphatic vascular tree indicated in I. n = 4 embryos per genotype. At least 6 vessels at each level were measured per embryo. All values are means ± SEM. *P < 0.05, ***P < 0.001, calculated by Student’s t test.

To determine if loss of lymph flow has any impact on smooth muscle recruitment to developing collecting vessels, we next examined the appearance and extent of SMC coverage in PROX1-GFP+ CLEC2-deficient and control littermates. At P4, Clec2–/– animals exhibited dilated mesenteric lymphatics with fewer lymphatic valves compared with control littermates (Figure 2, I and J). At this timepoint, SMC coverage of WT collecting vessels was very light and difficult to detect by whole-mount anti–smooth muscle actin (SMA) staining (Figure 2, K, M, O, and Q). In contrast, collecting vessels in P4 CLEC2-deficient animals were coated with a thick layer of SMCs, including regions overlying sites of immature valve formation (Figure 2, L, N, P, and R). Similarly, examination of the thoracic duct at E18.5 revealed only scattered SMCs in the WT embryo but abundant SMC coverage of the dilated CLEC2-deficient thoracic duct (Figure 2, S and T). These findings are remarkably similar to those reported in FOXC2-deficient animals (6), consistent with regulation of FOXC2 by lymph flow in vivo. More importantly, these observations suggest that flow and fluid shear forces negatively regulate SMC recruitment to collecting vessels, an observation that may explain the regional differences in SMC coverage between lymphangion (the unit of collecting vessel between valves) and valve regions of WT collecting lymphatics.

Hierarchical remodeling of collecting lymphatic vessels is impaired in CLEC2-deficient animals. LECs grow into the mesentery along preexisting blood vessels and reach the developing intestine by E15, a timepoint prior to the onset of lymphatic flow (11, 23). This nonfunctional, primary mesenteric lymphatic network is composed of highly branched vessels of similar diameter. After E16...
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were more uniform in caliber and did not change markedly in number with increased distance from the gut wall (Figure 3D). These changes were more marked at E18.5, when control embryos exhibited a fully remodeled, hierarchical vasculature of small vessels proximal to the gut leading to largercollecting lymphatics farther from the gut, whereas lymphatics in the Clec2−/− embryo mesentery were approximately the same caliber and resembled earlier timepoints (Figure 3E and F). Consistent with a failure to remodel, Clec2−/− mesenteric lymphatics maintained a higher number of branches compared with controls at E18.5 (Figure 3H). To quantify vascular hierarchy during maturation of the mesenteric collecting system, lymphatic vessel width was measured at 3 levels of the vascular tree: primary (closest to the intestine), secondary (one branch point from the intestine), and tertiary (2 branch points from the intestine) (Figure 3I). In E18.5 control embryos, lymphatic vessels were smallest most proximal to the intestine and largest in the more distal tertiary vessels. Strikingly, lymphatics in Clec2−/− embryos were of similar width at all 3 levels of the vascular tree, indicating a failure of the vasculature to remodel into a hierarchical pattern characteristic of the collecting network (Figure 3I). These findings suggest that lymph fluid shear force is required to remodel the immature lymphatic plexus into a mature, hierarchical collecting system that contains valves.

To examine the maturation of the few lymphatic valves that form in Clec2−/− animals, PROX1hi LEC clusters were stained for the presence of other lymphatic valve markers such as FOXC2, laminin α5, and CX37. PROX1hi valves in E18.5 Clec2−/− lymphatics demonstrated expression of FOXC2 and CX37, but laminin α5 staining revealed that the valve leaflets in Clec2−/− mice exhibited immature ring-shaped valve leaflets rather than the mature V-shaped structure (Supplemental Figure 3, A–N). Although most Clec2−/− animals die before weaning due to chylous ascites, we found that a small proportion of Clec2−/− animals live to adulthood if abdominal ascites is drained by abdominal paracentesis. Examination at 5 weeks of age revealed a partial recovery in the formation of lymphatic valves in the living Clec2−/− animals, although valve numbers were still much lower than in WT littermates (Supplemental Figure 4, A–I). Qualitatively, lymphatic valves in the mesenteric lymphatic network remodel to form a hierarchical set of collecting vessels in which smaller vessels near the gut wall feed into larger vessels that are more distant from the gut wall (ref. 23 and Figure 3). Since the remodeling process that creates the final lymphatic collecting vessel network takes place at the same time that valves form, we hypothesized that it might also be driven by fluid shear force and therefore disrupted in CLEC2-deficient animals. To test this hypothesis and more precisely determine the stage at which lymphatic valve development is disrupted in Clec2−/−embryos, vascular remodeling and valve formation were followed using PROX1-GFP reporter mice (31). At E15.5, mesenteric lymphatic vessel patterning and morphology were identical in Clec2−/− and control littersmates, and neither exhibited PROX1 hi LEC clusters at E18.5, when control embryos exhibited a fully remodeled, hierarchical pattern in which vessels closer to the intestine were smaller and more numerous than those farther from the intestine in WT animals (Figure 3C). In contrast, in E16.5 Clec2−/− embryos, mesenteric lymphatic vessels...
5-week-old Clec2–/– animals appeared malformed with an immature ring-like morphology and asymmetric leaflets. These findings indicate that normal lymph flow is required for lymphatic valve initiation, as well as for later valve maturation.

**Lymphatic collecting vessel remodeling requires lymph flow but not valve function.** The finding that CLEC2-deficient mice fail to both remodel their mesenteric lymphatic network and form lymphatic valves may be explained either by an independent requirement for fluid shear force in both of these processes or by a requirement for valves in vascular remodeling. To distinguish between these 2 possible mechanisms, we next studied mesenteric lymphatic remodeling in integrin α9–null mice that fail to develop mature, functional valves due to loss of LEC adhesion to the fibronectin subunit EIIIA (12). In agreement with published reports (12), Itga9–/– mice exhibited approximately 50% reduction in the overall number of PROX1HI lymphatic valves at E18.5, and the valves that formed exhibited immature ring structures that have previously been shown to lack functional leaflets (Figure 4, E–G, and ref. 12). Despite these defects in valve formation, oral lymphangiography revealed lymph transport into the mesenteric lymphatics of Itga9–/– embryos (Figure 4, E–H). These studies demonstrate that valve function is not necessary for early lymphatic flow and that flow, and not valves, is required for the lymphatic remodeling that creates the mature collecting system.

**Shear stress induces expression of genes required for lymphatic valve development.** Our studies of lymphatic valve development and collecting vessel remodeling in Clec2–/– embryos point to lymph flow and fluid shear forces as essential drivers of these late developmental processes. Recent studies have identified a number of genes that are specifically upregulated in developing lymphatic valves in vivo, including Prox1, Foxc2, Cx37, Itga9, and Gata2 (7, 9, 10, 12), as well as some — such as Lyve1 — that are downregulated in both valves (13) and mature collecting vessels (32). To directly investigate the role of fluid shear in directing these molecular events, we next tested the genetic response of human LECs to fluid shear forces designed to reproduce those in collecting lymphatic vessels using an in vitro parallel plate flow chamber and pulsatile flow system (33). Since the shear levels in the developing lymphatic network are still unknown, lymphatic flow was modeled according to actual shear stress and lymph flow values measured in adult rat mesenteric lymphatics. Dixon et al. observed reversing flow with a maximum shear stress of approx-
vessels and lymphatic valves in vivo (32), is also downregulated by lymphatic flow in vitro. Interestingly, the expression of PROX1, a master regulatory transcription factor required for LEC specification and valve development (34, 35), was not altered by lymphatic fluid shear forces in vitro (Figure 5B). Finally, the expression of KLF2, EFNB2, and NRP1 — genes that are known to be regulated by high shear stress in blood ECs (36–38) — were also upregulated in LECs exposed to lymphatic fluid shear forces (Figure 5C), although levels were lower than have been previously reported for BECs exposed to higher shear forces (36). While a role for KLF2 in lymphatic vessel development has not been reported, loss of EFNB2 or NRP1 results in lymphatic valve defects in vivo (13, 30), and SEMA3A-NRP1 has been shown to repel SMC recruitment at sites overlying lymphatic valves (30), a process that is defective in Clec2–/– mice. Western blot analysis confirmed the upregulation of GATA2, FOXC2, and CX37 proteins by lymphatic shear stress at the protein level (Figure 5D). Unexpectedly, the FOXC2 protein band detected after shear stress exhibited a higher molecular weight of approximately 3.5 dynes/cm² a minimum shear stress of approximately −1 dyne/cm², and an average shear stress of 0.64 dynes/cm² (15). To reproduce these forces in vitro, we exposed LEC for 48 hours to a pulsatile, reversing flow regimen with approximate maximum and minimum wall shear stress of 3.25 dynes/cm² and −1.45 dyne/cm², respectively. Although there is a reversal component for a portion of the cycle, the net flow is forward, with an average of 0.67 dynes/cm² (lymphatic shear stress, Figure 5A). Dextran (5%) was added to the cell culture media to increase the dynamic viscosity of the media to 2.738 cP in order to reach the desired wall shear stress magnitude while maintaining a low-flow rate similar to that seen in lymph in vivo. Gene expression was measured in LEC exposed to lymphatic fluid shear forces versus static control cells. The lymphatic fluid shear force regimen increased the expression of 4 genes — FOXC2, CX37, ITGA9, and GATA2 — that are known to be upregulated and play essential roles in valve-forming LEC in vivo (refs. 6, 7, 10, 12, and Figure 5B). Significantly, LYVE1, a gene that is downregulated in both collecting vessels and lymphatic valves in vivo (32), is also downregulated by lymphatic flow in vitro. Interestingly, the expression of PROX1, a master regulatory transcription factor required for LEC specification and valve development (34, 35), was not altered by lymphatic fluid shear forces in vitro (Figure 5B). Finally, the expression of KLF2, EFNB2, and NRP1 — genes that are known to be regulated by high shear stress in blood ECs (36–38) — were also upregulated in LECs exposed to lymphatic fluid shear forces (Figure 5C), although levels were lower than have been previously reported for BECs exposed to higher shear forces (36). While a role for KLF2 in lymphatic vessel development has not been reported, loss of EFNB2 or NRP1 results in lymphatic valve defects in vivo (13, 30), and SEMA3A-NRP1 has been shown to repel SMC recruitment at sites overlying lymphatic valves (30), a process that is defective in Clec2–/– mice. Western blot analysis confirmed the upregulation of GATA2, FOXC2, and CX37 proteins by lymphatic shear stress at the protein level (Figure 5D). Unexpectedly, the FOXC2 protein band detected after shear stress exhibited a higher molecu-
lar weight, indicating posttranslational modification (Figure 5D). FOXC2 phosphorylation at numerous serine/threonine residues has been reported in LECs, and phosphorylation of FOXC2 regulates its ability to bind chromatin (39). Thus, it is likely that fluid shear regulates FOXC2 function both by increasing its mRNA expression and by altering its phosphorylation state. These findings establish a fluid shear force regimen for exploring the role of low, reversing flow on LEC signaling, and demonstrate that lymphatic fluid shear forces are sufficient to induce a gene expression program in LECs that closely reproduces the gene expression program known to govern lymphatic valve development in vivo.

**GATA2 controls shear-induced regulation of many lymphatic valve genes.** How does fluid shear force drive the genetic program that controls collecting vessel maturation? A recent study of valve development in mice has proposed that PROX1 and FOXC2 are the key initiators of the gene expression program that underlies valve development (9). Additionally, recent studies have suggested that the transcription factor GATA2 also plays a role in lymphatic valve development using in vitro studies and mice with endothelial-specific deletion of Gata2 (7, 40). To further test this mechanism and better define the genetic pathway triggered by lymphatic fluid shear force, we utilized our in vitro shear stress assay. siRNA knockdown of FOXC2 prevented the rise in CX37 expression but did not affect the levels of ITG49 or GATA2 (Supplemental Figure 5A). PROX1 knockdown reduced the levels of FOXC2 and CX37, but did not affect the shear-mediated increase in ITG49 and only partially reduced the rise in GATA2 (Supplemental Figure 5B). In contrast to the incomplete blockage of valve gene expression with siFOXC2 or siPROX1, knockdown of GATA2 in LECs exposed to lymphatic shear blocked the rise in expression of all of the shear-dependent, valve-associated genes (i.e., FOXC2, CX37, ITG49) (Figure 6A). GATA2 knockdown had no effect on the shear-induced expression of KLF2, EFNB2, or NRPI (Figure 6B). GATA2 was upregulated in response to lymphatic fluid shear force in LEC treated with siFOXC2, placing GATA2 upstream of FOXC2 in this process (Supplemental Figure 5A). Interestingly, loss of GATA2 did not prevent the increase in FOXC2 protein size by shear (Figure 6C), indicating that another GATA2-independent pathway is required for phosphorylation of FOXC2. Together, these in vitro shear stress studies confirm the reported roles of PROX1 and FOXC2 in the valve-development program, suggest that GATA2 is the most upstream regulator of this program identified to date, and reveal that some genes (e.g., ITG49) are regulated by fluid shear and GATA2 in a manner that is independent of FOXC2 and PROX1. A third group of genes, such as KLF2, EFNB2, and NRPI, are shear-regulated in LEC but do not require GATA2, FOXC2, or PROX1, indicating that other parallel molecular pathways are activated by fluid shear force. In an effort to discover an upstream, global regulator of LEC responses to fluid shear force, several genes previously suggested to play a role in mechanosensing were knocked down, including PIEZO1 and PIEZO2 (41), KLF2 (42), KLF4 (43), and VEGFR3 (44). However, none of these genes were required for LEC response to reversing shear force in our in vitro system (Supplemental Figure 6).

To determine the effect of loss of lymphatic flow on the expression of these core transcription factors in vivo, we performed whole-mount immunostaining of mesentery from control and Clec2−/− embryos at E17.5. FOXC2, GATA2, and PROX1 were all highly upregulated in LEC nuclei at sites of developing valves in Clec2−/− embryos (Figure 6, D–G). In contrast, none of these transcription factors were upregulated in Clec2−/− embryos (Figure 6, H–K), while basal, nonvalvular LEC expression of PROX1 and FOXC2 were normal. These studies demonstrate that loss of lymphatic flow is associated with failure to upregulate GATA2, FOXC2, and PROX1, as well as loss of valve formation in vivo.

**Discussion**

Hemodynamic forces are a prominent characteristic of the high-flow blood vascular environment and have been shown to regulate many aspects of cardiovascular development and pathology, including development of the heart and outflow tract, remodeling of the yolk-sac vasculature, and formation of atherosclerotic plaques (45–47). Whether fluid shear force also plays an important role in the low-pressure, low-flow lymphatic vascular network has been harder to determine. Recent studies have reported that LECs respond to fluid shear force in cell culture and have suggested that such forces may regulate valve development (9). Testing the role of fluid shear force during lymphatic growth and development in vivo has been challenging, however, because both physical and genetic approaches to reducing lymph flow typically also affect lymphatic vessel growth. To circumvent these limitations, we have taken advantage of recently characterized CLEC2-deficient mice in which the retrograde movement of blood into the lymphatic network provides an extrinsic means of blocking lymph flow without affecting intrinsic lymphatic growth and development. Our studies reveal that lymph flow is not required for the primary formation of the lymphatic vasculature but is essential for its remodeling to form a hierarchical network of collecting vessels that contain valves and for proper SMC coverage, all canonical aspects of mature collecting vessels.

The key finding in our study is that lymph flow regulates many of the processes by which lymphatic collecting vessels are formed. Collecting lymphatics are distinguished from capillary lymphatics by their valves, the hierarchical structure by which they deliver lymph to the thoracic duct and venous system, and by the SMCs that help propel lymph through the system. Our studies indicate that flow is required to initiate valve formation, to direct the vascular remodeling that converts a primary mesenteric plexus into a hierarchical drainage system, and to control the extent of SMC coverage. The finding that valves fail to form in CLEC2-deficient animals provides important in vivo proof that flow initiates valve formation in the lymphatic system, as first suggested by in vitro LEC studies (9), and suggests that oscillatory flow may also drive valve formation in blood vessels. Blood vessel valves form exclusively in the lower-pressure, efferent venous system and are not evident until after birth in the mouse (29). Our in vitro studies and those of others (9) identify a distinct requirement for reversing fluid shear force in triggering the valve program in LECs. Reversal of flow in the venous system is largely due to negative thoracic pressure during respiration, a process that does not take place until after birth (48). Thus, it is possible that the induction of a GATA2/FOXC2/PROX1 pathway by oscillatory shear is a common mechanism for initiation of vessel valve formation. The finding that vascular remodeling to create a hierarchical collecting vessel network is lost in CLEC2-deficient mice that lack lymph flow is consistent with studies of the effect of blood flow on blood vessel development in zebrafish embryonic.
vessels (49) and in both chick (50) and mouse yolk sacs (46). In all cases, a major role of fluid flow is not to drive new vessel growth but to remodel a primary, homogeneous vascular network into a hierarchical vascular network that functions more efficiently. Perhaps most surprising is the finding that CLEC2-deficient animals exhibit excess SMC association with collecting lymphatic vessels, indicating that lymphatic flow negatively regulates SMC recruitment. Studies of SMC recruitment in the blood vascular system have focused on factors such as PDGFβ that are required to recruit SMCs to ECs (51), but these studies are relevant to high-flow vessels, such as arteries, where the role of SMCs is to provide tensile strength and regulate vessel tone and blood pressure. In contrast, SMCs in collecting lymphatics contract rhythmically and function as a pump in a manner that must be coordinated with valve function. SMCs cover the LECs between, but not over, valves so lymph is propelled forward without compromising valve function in a basic unit of the lymphangion (52). Our findings suggest that oscillatory flow simultaneously stimulates the LEC valve program and suppresses the LEC signals that recruit SMCs to create the functional lymphangion. The observation that CLEC2-deficient and FOXC2-deficient animals exhibit almost identical defects in both valve formation and SMC recruitment in lymphatic collecting vessels (6) provides genetic evidence for a model in which flow directs the formation of key components of the lymphatic collecting vessel through control of FOXC2. How flow-directed FOXC2 signaling in LECs directs SMC coverage is not yet known. However, it has recently been found that mesenteric lymphatic collecting vessels, but not arteries or veins, express the secreted factor SEMA3A and its receptor NRPI, and that loss of SEMA3A or blockade of NRPI results in a similar phenotype of excess SMC recruitment (30). Thus SEMA3A is a candidate for this regulatory mechanism.

The concept of a specific endothelial response to reversing fluid shear force that underlies valve development, vascular remodeling, and SMC recruitment during collecting vessel formation is supported by our in vitro studies of LEC responses to flow. Reversing shear forces that closely mimic those in the rat collecting lymphatic system were sufficient to drive changes in LEC gene expression that faithfully reproduce those associated with valve development in vivo. Knockdown studies identified GATA2 as a primary driver of these changes, as loss of GATA2 blocked upregulation of FOXC2, CX37, and ITGα9, while loss of FOXC2 blocked CX37 but neither ITGα9 nor GATA2. GATA2 has been demonstrated to play a necessary role in lymphatic development in the mouse (40), and is mutated in human MonoMAC syndrome in which primary lymphedema is common (7). Although reversing fluid shear force activated expression of genes known to also be upregulated by high, steady fluid shear (i.e., KLF2, EFNB2, NRPI), these were unaffected by loss of GATA2. Thus GATA2 appears to function in an endothelial response to reversing shear that resembles in vivo lymph flow conditions and is molecularly distinct from that of steady, unidirectional shear. The role of PROX1 in this process remains unclear, as this transcription factor is highly upregulated in valve-forming LEC in vivo but is not altered by reversing or laminar shear in vitro (Figure 6 and ref. 9). Future studies that further define the genetic pathway induced by reversing fluid shear in LECs are required to better understand the role of GATA2 in this process and the basis of PROX1 upregulation.

An important consideration in the interpretation of our in vivo studies is whether all of the effects of CLEC2 deficiency on lymphatic development can be attributed to changes in lymph flow. The nature of the lymphatic vascular defect associated with loss of CLEC2 or the CLEC2 signaling effectors SYK, SLP-76, or PLCγ2 has been a matter of considerable investigation and debate, but our recent studies reveal that this pathway mediates a lympho-venous hemostatic mechanism in which the LEC surface protein PODOPLANIN directly activates platelets through CLEC receptors and the SYK/SLP76/PLCγ2 intracellular signaling pathway to generate thrombi that prevent the low-pressure, low-flow lymphatic system from being filled with blood from the higher-pressure, higher-flow venous system (19, 22). Acutely, loss of this pathway results in the backflow of blood into both the developing and mature lymphatic network at sites of lympho-venous connection (19). Chronically, SLP76-deficient animals that survive to adulthood develop a complex arterio-lympho-venous vascular shunt in which blood flows in a forward manner through mesenteric lymphatics (16), but this shunt does not form until maturity and therefore does not impact the present studies of collecting-vessel development. The formation of the primary mesenteric lymphatic plexus is undisturbed in CLEC2-deficient embryos (Figure 3), as is that of lymphatic vessel growth into the intestine itself (Supplemental Figure 1). These observations are consistent with prior studies demonstrating normal cutaneous lymphatic growth despite the presence of blood (22, 53). In addition, the mesenteric lymphatic vascular changes reported here are only seen after signs of reduced lymphatic drainage, such as edema. These observations and the finding that platelet-specific loss of CLEC2 is sufficient to confer these lymphatic remodeling and valve defects are most consistent with a model in which CLEC2 signaling has no direct role in LECs but impacts lymphatic development through inhibition of forward lymph fluid flow. A final consideration in the interpretation of our findings is whether the presence of blood in the developing lymphatics of Clec2+/− mice contributes to any of the observed changes in collecting vessel or valve development. We cannot absolutely exclude this possibility, but several lines of evidence argue against it. First, venous valve formation requires many of the same factors (e.g., ITGα9, PROX1, and EFNB2) as lymphatic valve formation (5, 29), but venous valves form normally in CLEC2-deficient neonates. Thus, platelet CLEC2 function is not broadly required for vascular valve formation. Second, although blood is present in lymphatic vessels from the earliest timepoint in development (i.e., lymph sacs at E10.5) defects are only observed in the latest stages of lymphatic development that are coincident with the onset of lymphatic flow (e.g., after E15.5 in the mesentery and intestine). Third, the observation that valves gradually form in postnatal Clec2−/− animals despite the presence of blood is more consistent with a delayed response to reduced flow than the presence of a soluble factor in the blood that specifically blocks lymphatic valve formation (Supplemental Figure 4). Finally, in our in vitro studies demonstrate that reversing fluid shear force is fully sufficient to drive the molecular changes observed in valve-forming LECs in vivo (Figure 5B). It is therefore highly likely that the loss of lymphatic vascular remodeling and valve formation in CLEC2-deficient animals is due to loss of normal lymphatic flow by blood backflow rather than some unknown inhibitor that is present in blood but not lymph.
In summary, our study takes advantage of a unique mouse genetic model to test the role of flow during lymphatic vascular development in the absence of any intrinsic defects in LECs or lymphatic vessels. The findings reveal that lymph flow drives many of the processes by which an early lymphatic vessel and plexus become a mature collecting vessel and network. The earliest changes induced by lymph flow are the initiation of the valve development program and remodeling of the primitive lymphatic plexus to a hierarchical collecting network. Shortly afterward, collecting vessel lymph flow restricts the number of and sites of SMC associations, a regulatory mechanism that appears important to direct formation of a functional lymphangion capable of efficiently transporting lymph. In vitro studies suggest that many of these effects of flow are mediated by a GATA2-FOXC2 transcriptional program in LECs. While we do not yet fully understand the nature of the fluid shear forces in the developing mouse embryonic lymphatic system, these studies broaden the role of fluid shear in lymphatic vascular development. Future studies are necessary to address how other factors such as PROX1 are regulated, to understand the extent to which changes in lymph flow contribute to primary or acquired human lymphatic disorders, and to test whether fluid forces drive similar processes in the venous system.

Methods

Mouse lines. Clec2-, Clec2+, and Pf4-Cre mice have been previously described by our lab and others (19, 22, 28). Prox1-GFP (ab56696, Abcam), and Lyve-1 (R&D Systems). Embryonic or neonatal mesenteric lymphatics were whole-mount stained following fixation in 4% PFA. Whole gut plus mesentery was stained immediately upon termination of shear stress. RNA was isolated using the RNeasy Plus Mini Kit (Qiagen). cDNA was made using SuperScript III First-Strand Synthesis System (Invitrogen) following manufacturer instructions. qPCR analysis of gene expression was performed on StepOnePlus or 7900HT Real-Time PCR System (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems). Analysis of relative gene expression was carried out using the comparative CT method (ΔCT) using GAPDH as the reference housekeeping gene. Each value shown is the average of each qPCR reaction performed in triplicate.

Statistics. Quantifications and measurements of images were performed blind using ImageJ software (NIH). Quantification of gut wall edema was performed by measuring distance from just below the epithelial layer to the outer edge of the intestine on H&E-stained 6-μm sections. Quantification of LEC elongation was performed using Z-stacks of PROX1+ lymphatic vessels and measuring maximal length and maximal width of each LEC using VE-cadherin staining to mark cell borders. Only LECs located in a lymphangion were used for these measurements, taking care to exclude LECs near branchpoints or near lymphatic valves because those LECs tend to experience disturbed shear and are not elongated. Quantification of lymphatic valve number was performed by counting the number of PROX1+ clusters of LEC as a single valve. This number was divided by total lymphatic vessel length in a given image, or by total number of branchpoints in an image. Quantification of vascular hierarchy was performed by subdividing the mesenteric lymphatic vascular tree into 3 groups based on proximity to the gut wall. Primary vessels are the smallest vessels that directly emanate from the gut. Several primary vessels coalesce at a node and become secondary vessels, and most distal from the gut, several secondary vessels coalesce at a node and become a large ter-
tary vessel. Categorization of primary, secondary, and tertiary vessels was based on distance from gut, with a node/branchpoint of vessels usually serving as the boundary between groups. Average vessel width was quantified by measuring vessel diameter in 6 lymphangions per animal and 3–4 animals per group at each location. Data shown are expressed as mean ± SEM, and number of samples per condition are indicated in figure legends. Statistical significance was determined by unpaired 2-tailed Student’s t test. P values less than 0.05 were considered statistically significant.

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