Vascular Endothelial Growth Factor Receptor-2 Promotes the Development of the Lymphatic Vasculature

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Abstract

Vascular endothelial growth factor receptor 2 (VEGFR2) is highly expressed by lymphatic endothelial cells and has been shown to stimulate lymphangiogenesis in adult mice. However, the role VEGFR2 serves in the development of the lymphatic vascular system has not been defined. Here we use the Cre-lox system to show that the proper development of the lymphatic vasculature requires VEGFR2 expression by lymphatic endothelium. We show that Lyve-1-Cre;Vegfr2flox/flox mice possess significantly fewer dermal lymphatic vessels than Vegfr2flox/flox mice. Although Lyve-1-Cre;Vegfr2flox/flox mice exhibit lymphatic hypoplasia, the lymphatic network is functional and contains all of the key features of a normal lymphatic network (initial lymphatic vessels and valved collecting vessels surrounded by smooth muscle cells (SMCs)). We also show that Lyve-1Cre mice display robust Cre activity in macrophages and in blood vessels in the yolk sac, liver and lung. This activity dramatically impairs the development of blood vessels in these tissues in Lyve-1-Cre;Vegfr2flox/flox embryos, most of which die after embryonic day 14.5. Lastly, we show that inactivation of Vegfr2 in the myeloid lineage does not affect the development of the lymphatic vasculature. Therefore, the abnormal lymphatic phenotype of Lyve-1-Cre;Vegfr2flox/flox mice is due to the deletion of Vegfr2 in the lymphatic vasculature not macrophages. Together, this work demonstrates that VEGFR2 directly promotes the expansion of the lymphatic network and further defines the molecular mechanisms controlling the development of the lymphatic vascular system.

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Introduction

The lymphatic vasculature transports immune cells, absorbs dietary fats, and regulates tissue fluid homeostasis by returning fluid and macromolecules to the blood vascular system [1]. Insufficiency of the lymphatic vascular system leads to the formation of lymphedema, a condition characterized by massive swelling of affected limbs, fibrosis, and impaired immunity [1]. Despite these recent advances, the molecular mechanisms driving the expansion of the lymphatic network remain largely unknown.

There is growing evidence that VEGFR2, a receptor tyrosine kinase activated by VEGF-A, -C and -E, stimulates lymphangiogenesis. Overexpression of VEGFR2 ligands in the skin of adult mice and in tumors induces the growth of lymphatic vessels [18–21]. Furthermore, VEGF-A has been shown to promote lymphangiogenesis in the corneal micropocket assay in a VEGF-C/-D/-R3 independent manner [21]. To gain a better understanding of how VEGFR2 stimulates lymphangiogenesis, numerous in vitro experiments have been performed with LECs. These reports have shown that VEGF-A/VEGFR2 signaling promotes LEC proliferation, migration, and tube formation as well as the permeability of LEC monolayers [22–27]. We recently reported that VEGF-A stimulation of LECs leads to the phosphorylation of VEGFR2 on several tyrosine residues (Tyr 951, Tyr 1054, Tyr 1059, Tyr 1175 and Tyr 1214), and promotes protein kinase C dependent phosphorylation of ERK1/2 and PI3-K dependent phosphorylation of Akt [28]. The activation of both of these pathways was required for VEGF-A/VEGFR2-induced proliferation and migration of LECs [28]. These studies have begun to be supported by the expression pattern of molecular markers of LECs [15], lineage tracing experiments [16], and by the unique mutant phenotypes of genetically modified mice (reviewed in [17]).
shed light on the molecular pathways and cellular processes activated by VEGFR2 in LECs. However, the role VEGFR2 serves in the development of the lymphatic vasculature has not been explored, in part, because Vegfr2 knockout mice die before the lymphatic vascular system forms [29]. In the present study, we use the Cre-lox system to overcome this obstacle and conditionally inactivate Vegfr2 in LECs to characterize its function in the development of the lymphatic vascular system.

Results

Adult Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flox/flox</sup> mice display lymphatic hypoplasia

Lyve-1 is a hyaluronan receptor highly expressed by lymphatic capillaries but not by collecting lymphatic vessels or valves [30,31]. Although collecting lymphatic vessels and valves do not express Lyve-1, they are thought to arise from Lyve-1-positive LECs [30,32]. Lyve-1<sup>Cre</sup> mice were recently developed to conditionally delete floxed Vegfr2 (flanking loxP) DNA sequences in collecting lymphatic vessels and valves [34]. Lymphatic vessels in skin whole-mounts from Lyve-1<sup>Cre</sup> or mT/mG mice did not express GFP. However, Lyve-1<sup>Cre</sup>;mT/mG mice displayed strong GFP expression in macrophages as well as in lymphatic capillaries, collecting lymphatic vessels, and valves (Figure 1A, 1B). This result confirms previous reports documenting that Lyve-1-negative collecting vessels arise from Lyve-1-positive vessels [30,32] and indicates that the Lyve-1<sup>Cre</sup> mouse can be used to excise floxed DNA sequences in LECs that give rise to lymphatic capillaries, collecting lymphatic vessels and valves.

Lyve-1<sup>Cre</sup> mice were bred with Vegfr2<sup>flox/flox</sup> mice (Vegfr2<sup>lox/lox</sup>) to investigate the role Vegfr2 serves in the development of the lymphatic vasculature. Immunofluorescence staining of sections of adult ear skin with antibodies against Lyve-1 and Vegfr2 revealed that, on average, 90.6% of the Lyve-1-positive LECs lacked Vegfr2 in Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flox/flox</sup> mice (n = 3 mice; Figure 1C-H). Vegfr2 expression by dermal blood vessels was unaffected in Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flox/flox</sup> mice. Whole-mount immunofluorescence staining of ear skin for Lyve-1 showed a highly branched network of lymphatic capillaries in Vegfr2<sup>flox/flox</sup> mice (Figure 2A). In contrast, Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flox/flox</sup> mice exhibited a hypoplastic network of lymphatic capillaries (Figure 2B). Quantitative analysis showed that there were significantly fewer lymphatic branch points in Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flox/flox</sup> mice (6.042 ± 0.198, n = 6) than in Vegfr2<sup>flox/flox</sup> mice (11.29 ± 0.940, n = 6; Figure 2C). However, lymphatic vessel diameter was not significantly different between Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flox/flox</sup> mice (52.65 ± 1.303, n = 6) and Vegfr2<sup>flox/flox</sup> mice (49.05 ± 1.675, n = 6; Figure 2D).

Vegfr2 is not required for the maturation of collecting lymphatic vessels

Lymphatic capillaries and collecting lymphatic vessels are differentially covered by SMCs. Lyve-1-positive lymphatic capillaries are free of SMCs whereas Lyve-1-negative collecting vessels are covered by SMCs. Interestingly, defects in the patterning of the lymphatic vasculature have been associated with the mis-localization of SMCs on Lyve-1-positive lymphatic vessels [30,31,35]. To determine whether the hypoplastic lymphatic network in Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flox/flox</sup> mice was aberrantly covered by SMCs, we stained ear skin from adult mice for Lyve-1 and smooth muscle actin. This revealed that the Lyve-1-positive lymphatic networks in Vegfr2<sup>flox/flox</sup> and Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flox/flox</sup> mice were not...
Lymphatic function is normal in adult Lyve-1wt/Cre;Vegfr2flox/flox mice

The dramatic reduction in lymphatic vessel density led us to assess lymphatic function in Lyve-1wt/Cre;Vegfr2flox/flox mice. Evans blue dye (EBD) was injected into the hind paws of Vegfr2flox/flox and Lyve-1wt/Cre;Vegfr2flox/flox and was rapidly transported to the popliteal and iliac lymph nodes in both strains of mice (Figure 4; n = 6 of each genotype). EBD dye did not reflux into the mesenteric lymph nodes of Lyve-1wt/Cre;Vegfr2flox/flox mice, indicating that lymphatic valves functioned properly in these mice. Additionally, Lyve-1wt/Cre;Vegfr2flox/flox mice did not exhibit lymphedema, chylous ascites or chylothorax.

Lyve-1wt/Cre;Vegfr2flox/flox embryos display lymphatic hypoplasia

Because adult Lyve-1wt/Cre;Vegfr2flox/flox mice display lymphatic hypoplasia, we characterized lymphatic development in Lyve-1wt/Cre;Vegfr2flox/flox embryos. First, we measured the size of the jugular lymph sacs in Vegfr2flox/flox and Lyve-1wt/Cre;Vegfr2flox/flox embryos. At E14.5, the cross-sectional area of the jugular lymph sacs was not significantly different between Vegfr2flox/flox (380,110 ± 76,279 pixels2; n = 8) and Lyve-1wt/Cre;Vegfr2flox/flox embryos (287,350 ± 59,931 pixels2; n = 5; Figure 5A-C). This suggests that Vegfr2 is not required for the development of lymph sacs. Next, we performed whole-mount immunofluorescence staining of skin to assess the patterning of the lymphatic vasculature in E14.5 Vegfr2flox/flox and Lyve-1wt/Cre;Vegfr2flox/flox embryos. This analysis revealed that the density of lymphatic vessels was significantly lower in Lyve-1wt/Cre;Vegfr2flox/flox embryos (11.63 ± 0.5239; n = 3) than in Vegfr2flox/flox embryos (19.37 ± 0.5783; n = 3; Figure 5D-F). However, the diameter of lymphatic vessels was not significantly different between Lyve-1wt/Cre;Vegfr2flox/flox (26.00 ± 5.859 pixels; n = 3) and Vegfr2flox/flox embryos (23.75 ± 1.652 pixels; n = 4; Figure 5G). To determine whether the reduction in lymphatic vessel density was due to decreased proliferation of LECs, we stained tissues for podoplanin and phospho-histone H3. We observed that the number of proliferating LECs was lower in Lyve-1wt/Cre;Vegfr2flox/flox embryos than in Vegfr2flox/flox embryos at E14.5 and E16.5 (Figure 5H). However, this reduction was only statistically significant at E16.5. These findings indicate that Vegfr2 is required for the
proper growth and expansion of lymphatic vessels during embryonic development.

Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> embryos display reduced viability and have fewer blood vessels in the yolk sac, liver and lung than Vegfr2<sup>flx/flx</sup> embryos.

Crosses between Vegfr2<sup>flx/flx</sup> and Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> mice revealed that most Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> mice die after E14.5 (Table 1 and Figure 5I). Importantly, Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> embryos were not edematous at E14.5 or at later time points (Figure 5J-M). At E14.5, the lymphatic vessel diameter was not significantly different between Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> (26.00±5.859 pixels; n=3) and Vegfr2<sup>flx/flx</sup> embryos (19.37±0.5783; n=3). At E16.5, 26.94% of LECs in Vegfr2<sup>flx/flx</sup> embryos (n=4) were phospho-Histone H3-positive whereas 9.73% of LECs in Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> embryos (n=4) were phospho-histone H3-positive. At E16.5, 26.94% of LECs in Vegfr2<sup>flx/flx</sup> embryos (n=4) were phospho-histone H3-positive whereas 13.69% of LECs in Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> embryos (n=7) were phospho-histone H3-positive. (II) Graph showing the percent of viable Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> mice at different developmental stages. (J-M) Images of non-edematous E14.5 (J,K), E16.5 (L) and E18.5 (M) Vegfr2<sup>flx/flx</sup> and Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> embryos. ** indicates P < 0.01; *** indicates P < 0.001.

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Figure 5. Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> embryos exhibit lymphatic defects.

(A,B) Immunofluorescence staining for Lyve-1 showing jugular lymph sacs in E14.5 Vegfr2<sup>flx/flx</sup> and Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> embryos. (C) Graph showing that lymph sac area is not different between Vegfr2<sup>flx/flx</sup> (380,110±76,279 pixels<sup>2</sup>; n=8) and Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> embryos (287,350±59,931 pixels<sup>2</sup>; n=5). (D,E) Images of back skin from E14.5 Vegfr2<sup>flx/flx</sup> and Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> embryos stained with antibodies against podoplanin (green) and phospho-histone H3 (red). (F) At E14.5, there are significantly fewer lymphatic branch points in Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> embryos (11.63±0.5239; n=3) than in Vegfr2<sup>flx/flx</sup> embryos (19.37±0.5783; n=3). (G) At E14.5, lymphatic vessel diameter is not significantly different between Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> (26.00±5.859 pixels; n=3) and Vegfr2<sup>flx/flx</sup> embryos (19.37±0.5783; n=3). (H) Graph showing that there are fewer proliferating lymphatic endothelial cells in Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> embryos than in Vegfr2<sup>flx/flx</sup> embryos at E14.5 and E16.5. At E14.5, 19.45% of LECs in Vegfr2<sup>flx/flx</sup> embryos (n=4) were phospho-Histone H3-positive whereas 9.73% of LECs in Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> embryos (n=4) were phospho-histone H3-positive. At E16.5, 26.94% of LECs in Vegfr2<sup>flx/flx</sup> embryos (n=4) were phospho-histone H3-positive whereas 13.69% of LECs in Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> embryos (n=7) were phospho-histone H3-positive. (I) Graph showing the percent of viable Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> mice at different developmental stages. (J-M) Images of non-edematous embryos. ** indicates P < 0.01; *** indicates P < 0.001.

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Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> embryos display reduced viability and have fewer blood vessels in the yolk sac, liver and lung than Vegfr2<sup>flx/flx</sup> embryos.

Crosses between Vegfr2<sup>flx/flx</sup> and Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> mice revealed that most Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> mice die after E14.5 (Table 1 and Figure 5I). Importantly, Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> embryos were not edematous at E14.5 or at later time points (Figure 5J-M). However, Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> embryos tended to be smaller than their wildtype littermates. Therefore, we examined Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> embryos for a potential cardiovascular defect. Lyve-1 has recently been reported to be expressed by BECs in the yolk sac, liver and lung [36]. Indeed, crosses with mT/mG reporter mice demonstrated that Lyve-1<sup>wt/Cre</sup> mice display Cre recombinase activity in BECs in these tissues (Figure S1). To determine whether the vasculature was altered in these tissues, we performed immunohistochemistry with markers of BECs. Whole-mount immunofluorescence staining for CD31 revealed that there were significantly fewer blood vessel branch points in E12.5 Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> yolksacs (41.06±6.362, n=4) than in E12.5 Vegfr2<sup>flx/flx</sup> yolksacs (106.3±3.157, n=3) (Figure 6A-C). Immunohistochemistry for endomucin showed that there were significantly fewer blood vessels in E14.5 Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> livers (27.00±4.263, n=4) than in E14.5 Vegfr2<sup>flx/flx</sup> livers (45.88±2.850, n=4) (Figure 6D-F). Additionally, immunofluorescence staining revealed that the density of blood vessels was significantly lower in E16.5 Lyve-1<sup>wt/Cre</sup>;Vegfr2<sup>flx/flx</sup> lungs (18.67±0.962; n=5) than in E16.5 Vegfr2<sup>flx/flx</sup> lungs (28.39±1.145; n=5; Figure 6G-I). These observations reveal that the loss of Vegfr2 in BECs in the yolk sac, liver and lung severely impairs vascular development in these tissues.
Lyve-1wt/Cre;Vegfr2flox/flox embryos do not display a heart defect

Endocardium has been reported to express Lyve-1 throughout embryogenesis [36]. Therefore, we examined Lyve-1wt/Cre;Vegfr2flox/flox embryos for a heart defect. An embryo with poor cardiac function will usually display pericardial edema and a blood-engorged liver [37]. Importantly, Lyve-1wt/Cre;Vegfr2flox/flox embryos did not exhibit pericardial edema (n = 9) or blood-engorged livers (n = 4; Figure S2). Additionally, Lyve-1wt/Cre;Vegfr2flox/flox embryos did not display obvious structural defects of the heart at E14.5 (n = 4) or E16.5 (n = 5; Figure S2). Staining for endomucin did not reveal differences in the integrity of the endocardium between Vegfr2flox/flox and Lyve-1wt/Cre;Vegfr2flox/flox embryos (Figure S2). This prompted us to characterize the expression of Lyve-1 in the heart. We stained hearts from E16.5 wildtype embryos for Lyve-1 and found that it was expressed in a faint scattered pattern by the endocardium (Figure S3). Our observation is in agreement with Gordon et al., (2008), who reported a “salt and pepper” pattern of Lyve-1 expression by the endocardium [36]. This reduced expression pattern for Lyve-1 may result in low levels of Cre recombinase and explain why the endocardium is not compromised in Lyve-1wt/Cre;Vegfr2flox/flox embryos. Together, these findings suggest that Lyve-1wt/Cre;Vegfr2flox/flox embryos do not suffer from a heart defect.

Vegfr2 is not expressed by macrophages and inactivation of Vegfr2 in the myeloid lineage does not affect lymphatic development

Crossoes with mT/mG reporter mice revealed that Lyve-1Cre mice exhibit Cre recombinase activity in macrophages as well as lymphatic vessels (Figure 1A, 1B). Therefore, several experiments were performed to rule out the possibility that the lymphatic phenotype of Lyve-1wt/Cre;Vegfr2flox/flox mice was due to the inactivation of Vegfr2 in macrophages. First, we explored the expression of Vegfr2 by macrophages. Whole-mount immunofluorescence staining showed that Vegfr2 was not expressed by macrophages in the ear skin of Vegfr2wt/GFP mice (Figure S4). Next, the LysozymeCre strain was used to conditionally delete target sequences in the myeloid lineage. LysozymeCre mice were bred with mT/mG reporter mice to characterize the expression pattern of Cre recombinase and to trace the fate of cells of the myeloid lineage. All LysozymeCre;mtT/mG mice displayed strong GFP expression by macrophages in the ear skin (Figure S5). GFP did not co-localize with Vegfr3 in the ear skin of LysozymeCre;mtT/mG mice (Figure S5; Table 1. Observed number of mice following crosses between Vegfr2flox/flox and Lyve-1wt/Cre;Vegfr2flox/flox mice.

| Genotype                              | E12.5 | E14.5 | E16.5 | E18.5 | Neonates |
|---------------------------------------|-------|-------|-------|-------|----------|
| Lyve-1wt/wt;Vegfr2wt/flox             | 6     | 16    | 19    | 21    | 72       |
| Lyve-1wt/wt;Vegfr2flox/flox           | 4     | 19    | 20    | 19    | 67       |
| Lyve-1wt/Cre;Vegfr2wt/flox            | 8     | 14    | 23    | 13    | 72       |
| Lyve-1wt/Cre;Vegfr2flox/flox          | 6     | 16    | 7     | 5     | 7        |
| Total number of mice                   | 24    | 65    | 69    | 58    | 218      |

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Vegfr2 is not expressed by macrophages and inactivation of Vegfr2 in the myeloid lineage does not affect lymphatic development

Figure 6. The density of blood vessels is reduced in the yolk sac, liver and lung in Lyve-1wt/Cre;Vegfr2flox/flox embryos. (A,B) Whole-mount immunofluorescence staining of yolk sacs from E12.5 Vegfr2flox/flox and Lyve-1wt/Cre;Vegfr2flox/flox embryos for CD31. (C) There are significantly fewer blood vessel branch points in yolk sacs from Lyve-1wt/Cre;Vegfr2flox/flox embryos (41.06 ± 3.662, n = 4 embryos) than Vegfr2flox/flox embryos (106.3 ± 3.157, n = 3 embryos). (D-F) Immunohistochemical staining of E14.5 livers for endomucin showing significantly fewer blood vessels in Lyve-1wt/Cre;Vegfr2flox/flox embryos (27.00 ± 4.263, n = 4 embryos) than in Vegfr2flox/flox embryos (45.88 ± 2.850, n = 4 embryos). (G-I) Immunofluorescence staining of E16.5 lungs for endomucin (red) and DAPI (blue) showing that the density of blood vessels is lower in Lyve-1wt/Cre;Vegfr2flox/flox (18.67 ± 0.962; n = 5) embryos than in Vegfr2flox/flox embryos (28.89 ± 1.445; n = 5). * indicates P || 0.05; *** indicates P || 0.001. **** indicates P || 0.0001.
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Table 1. Observed number of mice following crosses between Vegfr2flox/flox and Lyve-1wt/Cre;Vegfr2flox/flox mice.
n = 5 mice). This finding indicates that cells genetically marked by LysM^WT do not differentiate into LECs during normal murine development and is in agreement with another report using the LysM^WT line [30]. LysM^WT mice were then crossed with Vegfr2^fl/fl mice to determine whether deleting Vegfr2 in myeloid cells affects the development of the lymphatic vasculature. Whole-mount immunofluorescence staining of ear skin for Lyve-1 revealed that the density of lymphatic vessels was not significantly different between Vegfr2^WT/WT (8.350 ± 0.278, n = 5 mice) and LysM^WT/Vegfr2^fl/fl (9.150 ± 0.5895, n = 5 mice) littermates (Figure 7A-D). Furthermore, the diameter of lymphatic vessels was not significantly different between Vegfr2^WT/WT (52.98 μm ± 1.328, n = 5 mice) and LysM^WT/Vegfr2^fl/fl (50.05 μm ± 2.031, n = 4 mice) littermates (Figure 7A-D). EBD was also effectively transported from injected hind paws to popliteal and iliac lymph nodes in all Vegfr2^WT/WT and LysM^WT/Vegfr2^fl/fl mice (data not shown). Together, these data reveal that Vegfr2 is not required in the myeloid lineage for the proper development of the lymphatic system. This demonstrates that the lymphatic phenotype of Lyve-1^WT/Vegfr2^fl/fl mice is due to the ablation of Vegfr2 in LECs, not macrophages.

Discussion

VEGFR2 is widely recognized as an essential gene driving the formation of the blood vasculature during embryogenesis. The present study demonstrates that VEGFR2 also directly promotes the development of the lymphatic vasculature. We show that the density, but not diameter, of lymphatic vessels is dramatically reduced in Lyve-1^WT/Vegfr2^fl/fl mice. Additionally, we demonstrate that lymphatic vessels in Lyve-1^WT/Vegfr2^fl/fl mice properly mature into collecting vessels. These findings indicate that VEGFR2 is required for the expansion, but not the specification or maturation, of the lymphatic vasculature.

The lymphatic vasculature of mammals can grow by undergoing sprouting lymphangiogenesis, in which new lymphatic vessels emerge from pre-existing vessels, or circumferential lymphangiogenesis, which is characterized by an increase in the diameter of lymphatic vessels [19]. Sprouting lymphangiogenesis increases the number of lymphatic branch points and complexity of the lymphatic network. Therefore, if VEGFR2 stimulates sprouting lymphangiogenesis, there should be fewer lymphatic branch points in Lyve-1^WT/Vegfr2^fl/fl mice than in Vegfr2^WT/WT mice. On the other hand, if VEGFR2 promotes circumferential lymphangiogenesis, lymphatic vessels should have a smaller diameter in Lyve-1^WT/Vegfr2^fl/fl mice than in Vegfr2^WT/WT mice. Importantly, we found that the number of lymphatic branch points was greatly reduced in Lyve-1^WT/Vegfr2^fl/fl mice and that the width of lymphatic vessels was not significantly different between wildtype and mutant mice. In contrast to a previous report [19], our results suggest that VEGFR2 signaling promotes sprouting lymphangiogenesis rather than circumferential lymphangiogenesis. The earlier model proposing that VEGFR2 stimulates circumferential rather than sprouting lymphangiogenesis was based on experiments showing that adenoviral and transgenic overexpression of VEGF-E (VEGFR2-specific ligand) preferentially induces the enlargement of lymphatic vessels instead of the formation of new lymphatic vessels [19]. The discrepancy between our findings and this previous report could be due to the fact that overexpression of VEGF-E increases blood vessel permeability [19], an effect which may contribute to the enlargement of lymphatic vessels. Alternatively, the effect of VEGF-E on lymphatics may be unique to this factor, as adenoviral expression of VEGF-A^iso induces lymphatic vessel sprouting in vitro, albeit to a lesser extent than VEGF-C [19]. Furthermore, VEGF-A has also been shown to induce sprouting lymphangiogenesis in mouse corneas and in vitro by LEC spheroids [21,39]. Together, these observations suggest that the function of VEGFR2 in lymphatic vessels is similar to its function in blood vessels, where it also stimulates the budding of new vessels from pre-existing vessels [40].

Although it is well established that lymphatic vessels can grow by sprouting, the underlying mechanism of sprouting lymphangiogenesis is not well defined. In contrast, the process of sprouting hemangiogenesis is well characterized. Growing blood vessels are led by specialized tip cells which migrate and extend numerous filopodia to probe the microenvironment for directional cues [40]. These cells are followed by stalk cells that proliferate in response to growth factors and thereby promote vessel extension [40]. VEGF-A/VEGFR2 signaling plays a critical role in regulating tip cell activity (migration/filopodia extension) and stalk cell proliferation [41]. VEGFR2 may also control sprouting lymphangiogenesis in a similar fashion. In vivo, VEGFR2 is expressed by growing lymphatic vessels and their filopodia [42]. Whether the LECs extending filopodia are true tip cells has yet to be determined. Nevertheless, VEGFR2 at the end of a growing lymphatic vessel could be involved in sensing directional signals and migration. Additionally, VEGFR2 could promote vessel extension by stimulating the proliferation of LECs. We found that LEC proliferation is reduced in Lyve-1^WT/Vegfr2^fl/fl embryos. Furthermore, we and others have previously shown that VEGF-A/VEGFR2 signaling promotes LEC migration and proliferation in vitro [28]. Therefore, the loss of VEGFR2 in LECs in Lyve-1^WT/Vegfr2^fl/fl mice may impair lymphangiogenesis by affecting the migration and proliferation of LECs.

Figure 7. Deleting Vegfr2 in the myeloid lineage does not affect the development of the lymphatic vascular system. (A,B) Whole-mount immunofluorescence staining of ear skin from Vegfr2^WT/WT and LysM^WT/Vegfr2^fl/fl mice for Lyve-1. (C) Quantitative analysis showing that the number of lymphatic branch points in the ear skin of Vegfr2^WT/WT (8.850 ± 0.278, n = 5 mice) and LysM^WT/Vegfr2^fl/fl mice (9.150 ± 0.5895, n = 5 mice) are not significantly different from one another. (D) Furthermore, lymphatic vessel diameter is not significantly different between Vegfr2^WT/WT (52.98 μm ± 1.328, n = 5 mice) and LysM^WT/Vegfr2^fl/fl mice (50.05 μm ± 2.031, n = 4 mice).

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During development specific lymphatic vessels acquire a collecting vessel phenotype, a process involving the recruitment of mural cells and formation of intraluminal valves. Recent studies of genetically modified mice have identified several genes that participate in collecting vessel maturation, such as Ang2, Ephrinb2, and Nfatc1 [30–32,43,44]. Importantly, Vegfr2 signaling induces the expression of Ang2, nuclear translocation of Nfatc1, and is regulated by Ephrinb2-mediated internalization and trafficking [24,32,45]. These observations, as well as the high expression of Vegfr2 by collecting vessels and valves [19] and data not shown), led us to test the hypothesis that Vegfr2 participates in the maturation of lymphatics into collecting vessels. To our surprise we found that Lyve-1wt/Cre;Vegfr2flox/flox mice develop normal collecting lymphatic vessels. Mural cells were properly associated with lymphatic vessels and intraluminal valves were present in Lyve-1wt/Cre;Vegfr2flox/flox mice. The lack of an abnormal collecting vessel phenotype may be due to compensation by Vegfr3, which is also expressed by collecting vessels and valves [19]. Future work with Vegfr3 and Vegfr2 mutant mice will help elucidate the role these receptors serve in the remodeling of the lymphatic system.

During the course of our study we discovered that most Lyve-1wt/Cre;Vegfr2flox/flox mice die during embryonic development. Previously described mutant embryos that display fatal lymphatic defects die from edema [15,46]. However, Lyve-1wt/Cre;Vegfr2flox/flox embryos were not edematous at any of the time points analyzed. This suggests that Lyve-1wt/Cre;Vegfr2flox/flox embryos do not die from a lymphatic defect. Therefore, the Lyve-1wt allele must induce the loss of Vegfr2 in a different cell type that is required for survival. In agreement with a previous study that documented Lyve-1 expression in blood vessels of the yolk sac, liver and lung [36], we found that Lyve-1wt/Cre mice expressed Cre recombinase in blood vessels of the yolk sac, liver and lung. We also found that the density of blood vessels was dramatically reduced in these tissues in Lyve-1wt/Cre;Vegfr2flox/flox embryos. Proper development of the yolk sac vasculature is required for mice to survive to birth [37]. Therefore, Lyve-1wt/Cre;Vegfr2flox/flox embryos may die of a yolk sac vascular defect. Additionally, vascular defects in the liver and lung may also contribute the lethal phenotype of Lyve-1wt/Cre;Vegfr2flox/flox mice. Select Lyve-1wt/Cre;Vegfr2flox/flox mice may survive because they display low Cre recombinase activity in BECs in the yolk sac, liver and lung. Alternatively, differences in genetic background may contribute to the survival of a subset of Lyve-1wt/Cre;Vegfr2flox/flox mice.

In conclusion, we show that Vegfr2 directly promotes the expansion of the lymphatic vessel network. This newly identified function of Vegfr2 further defines the molecular pathways controlling the development of the lymphatic vasculature and sheds light on the mechanisms by which therapeutic agents targeting Vegfr2 inhibit lymphangiogenesis.

Materials and Methods

Ethics Statement

Experiments performed with mice were carried out in accordance with an animal protocol approved by the IACUC of the University of Texas Southwestern Medical Center (APN 0974-07-03-1).

Mice and genotyping

Vegfr2<sup>glo</sup> (Balb/c genetic background) [47], mT/mG (mixed genetic background) [34], Lyve-1<sup>Cre</sup> (129 and C57Bl/6 mixed genetic background) [33], LysM<sup>F<sub>os</sub></sup> (C57Bl/6 genetic background) [48] and Vegfr2<sup>d<sub>Cre</sub></sup> (CD1 genetic background) [49] mice have been described previously. Vegfr2<sup>glo</sup> mice were genotyped with the following primers: 5'-TGG-AGA-GCA-AGG-GGC-TGC-TAG-C-C' and 5'-CTT-TCC-ACG-CTCTT-GCC-TAG-CTA-GT-G-3' to yield a 322 bp wildtype band and a 439 bp mutant band. mT/mG mice were genotyped with the following primers: 5'-TGG-TGC-TGC-CTC-TGC-CTCT-3', 5'-TCA-ATG-GGG-GGG-GTG-CTGT-T-3' to yield a 330 bp wildtype band and a 250 bp mutant band. Lyve-1<sup>Cre</sup> mice were genotyped with the following primers: 5'-TGC-AC-TGCT-AGG-CTC-TCT-3', 5'-TGA-CCA-ACA-GAA-GGG-TTA-CTG-3', and 5'-GAG-GAT-GGG-GAC-TGA-AAC-TG-3' to yield a 425 bp wildtype band and a 210 bp mutant band. LysM<sup>F<sub>os</sub></sup> mice were genotyped with the following primers: 5'-CCC-AGA-AAT-GCC-AGA-TTA-CG-3', 5'-TTA-CAG-TCG-GCC-AGG-CTG-AC-3', and 5'-CTT-GGG-CTG-CCCA-GAA-TTTT-CTC-3' to yield a 700 bp wildtype band and a 350 bp mutant band.

Antibodies

The following primary antibodies were used for immunohistochemistry or immunofluorescence staining of mouse tissues: rabbit anti-Lyve-1 (abcam, cat no. ab14917), goat anti-Lyve-1 (R&D Systems, cat no. AF215), rat anti-endomucin (Santa Cruz, cat no. sc-65495), rat anti-CD31 (BD Biosciences, cat no. 553900), Cy3-conjugated mouse anti-smooth muscle actin (Sigma, cat no. C6198), hamster anti-podoplanin (abcam, cat no. ab11936), rat anti-Vegfr3 (eBioscience, 14-5988-81), rabbit anti-phospho-histone H3 (Millipore, cat no. 06-570) and rabbit anti-Vegfr2 (T014, purified in our laboratory [50]). All secondary antibodies were from Jackson ImmunoResearch.

Whole-mount immunofluorescence staining

Tissues were fixed overnight at 4°C in 1% PFA, washed with PBS (6 x 15 minutes), permeabilized for 1 hour with PBS + 0.3% Triton-X 100, then blocked with overnight at 4°C in PBS + 0.3% Triton-X 100 + 20% Aquablock (East Coast Biologics, cat no. PP02-P0691). Tissues were then incubated overnight at 4°C with primary antibodies diluted in PBS + 0.3% Triton-X 100. Following this step, tissues were washed with PBS + 0.3% Triton-X 100 (3 X 40 minutes), incubated overnight at 4°C with the appropriate secondary antibodies, then washed again with PBS + 0.3% Triton-X 100 (3 X 40 minutes). Coverslips were mounted with ProLong Gold plus DAPI (Invitrogen, P36931). Slides were analyzed using a Nikon Eclipse E600 microscope and images captured using NIS-Elements imaging software.

Quantitative analysis of lymphatic branch points and diameter

For adult mice, the number of branches was counted in 4 images from each mouse at 10X magnification. For embryos, the number of branches was counted in 1-6 images from each mouse at 10X magnification. The same images were used to assess lymphatic vessel diameter. A grid with lines spaced 75 μm from one another was placed over each 10X image. Vessel diameter was measured with the NIS-Elements imaging software at locations where two perpendicular grid lines intersected a lymphatic vessel.

Immunofluorescence and immunohistochemical staining of tissue sections

Embryos were fixed overnight at 4°C in 4% PFA, washed with 50% EtOH, processed, and then sectioned at 5 μm for staining. Slides were deparaffinized with xylene and rehydrated through a descending EtOH series. Antigen retrieval was performed with
0.01 M citric acid (pH = 6.0) in a pressure cooker. Slides were then washed with PBS and blocked for 1 hour with TBST + 20% Aquablock. Primary antibodies diluted in TBST + 5% BSA were then added and allowed to incubate overnight at 4°C. Slides were then washed with TBST then secondary antibodies diluted in TBST + 5% BSA were added and allowed to incubate for 1 hour at room temperature. Slides were then washed again with TBST and coverslips were mounted with ProLong Gold plus DAPI. Immunohistochemistry was performed using a similar protocol except endogenous peroxidase activity was blocked by incubating slides with hydrogen peroxide diluted in MeOH and signal was detected via the DAB chromogen system (Dako, cat no. K3468).

Evans blue dye lymphangiography

The popliteal and iliac regions were examined for lymph transport following the injection of Evans blue dye (1% w/v) into the hind paws of mice anesthetized with isoflurane and kept warm with a heating pad.

Assessment of Vegfr2 expression by lymphatic vessels

Frozen sections of adult ear skin were stained with antibodies against Lyve-1 and Vegfr2. Four images at 20X magnification were taken of each section of ear skin. The number of Vegfr2-positive and Vegfr2-negative LECs in each image was manually counted. The number of Vegfr2-positive LECs was divided by the total number of LECs and then multiplied by 100 to determine the percent of Vegfr2-positive LECs.

Assessment of LEC proliferation

Tissue sections of E14.5 and E16.5 embryos were stained with antibodies against podoplanin and phospho-histone H3. Six images at 40X magnification were taken of each tissue section. The number of phospho-histone H3-positive and phospho-histone H3-negative LECs in each image was manually counted. The number of phospho-histone H3-positive LECs was divided by the total number of LECs and then multiplied by 100 to determine the percent of LECs proliferating.

Statistical analysis

Data were analyzed using GraphPad Prism statistical analysis software (Version 5.0). All results are expressed as mean ± SEM. Unpaired student’s T-tests were performed to test means for significance. Data were considered significant at P<0.05.

Supporting Information

Figure S1 Lyve-1Cre is expressed by blood endothelial cells in the yolk sac, liver and lung. Representative images of Lyve-1Cre/+;mT/mG tissues showing GFP expression by blood endothelial cells in the yolk sac, liver, and lungs. (TIF)

Figure S2 Lyve-1Cre/+;Vegfr2flox/flox embryos do not display a cardiac defect. (A,B) H & E stained sections of E14.5 Vegfr2flox/flox and Lyve-1Cre/+;Vegfr2flox/flox embryos. (C,D) Endomucin immunolabeled sections of E16.5 Vegfr2flox/flox and Lyve-1Cre/+;Vegfr2flox/flox embryos. Hearts appear normal and pericardial edema is not present in E14.5 or E16.5 Lyve-1Cre/+;Vegfr2flox/flox embryos. (E,F) H & E stained sections of livers from E14.5 Vegfr2flox/flox and Lyve-1Cre/+;Vegfr2flox/flox embryos. (TIF)

Figure S3 Lyve-1 is not strongly expressed by endocardium in wildtype embryos. Endomucin is strongly expressed by blood endothelial cells in the lung and by endocardium (arrow). In contrast, Lyve-1 is strongly expressed by blood endothelial cells in the lung but not by endocardium (arrow). Lyve-1 was present in a faint “salt and pepper” pattern in the heart. (TIF)

Figure S4 Macrophages do not express Vegfr2. (A-C) Whole-mount immunofluorescence staining showing a GFP (Vegfr2-negative)-Lyve-1-positive macrophage in the ear skin of a Vegfr2+/-;GFP mouse. (TIF)

Figure S5 LysMCre is not expressed by lymphatic endothelial cells. (A,B) GFP is not expressed in ear skin from mT/mG mice. (C) GFP expression by macrophages is shown in a whole-mount preparation of ear skin from an adult LysM+/-;mT/mG mouse. (D) GFP (green) does not co-localize with VEGFR3 (red) in the ear skin of a LysM+/-;mT/mG mouse. (TIF)

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Author Contributions

Conceived and designed the experiments: MTD RAB. Performed the experiments: MTD KW SMM. Analyzed the data: MTD RAB. Contributed reagents/materials/analysis tools: OC. Wrote the paper: MTD RAB.
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