Pulmonary Lymphangiectasia Resulting From Vascular Endothelial Growth Factor-C Overexpression During a Critical Period

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Rationale: Lymphatic vessels in the respiratory tract normally mature into a functional network during the neonatal period, but under some pathological conditions they can grow as enlarged, dilated sacs that result in the potentially lethal condition of pulmonary lymphangiectasia.

Objective: We sought to determine whether overexpression of the lymphangiogenic growth factor (vascular endothelial growth factor-C [VEGF-C]) can promote lymphatic growth and maturation in the respiratory tract. Unexpectedly, perinatal overexpression of VEGF-C in the respiratory epithelium led to a condition resembling human pulmonary lymphangiectasia, a life-threatening disorder of the newborn characterized by respiratory distress and the presence of widely dilated lymphatics.

Methods and Results: Administration of doxycycline to Clara cell secretory protein-reverse tetracycline-controlled transactivator/tetracycline operator-VEGF-C double-transgenic mice during a critical period from embryonic day 15.5 to postnatal day 14 was accompanied by respiratory distress, chylothorax, pulmonary lymphangiectasia, and high mortality. Enlarged sac-like lymphatics were abundant near major airways, pulmonary vessels, and visceral pleura. Side-by-side comparison revealed morphological features similar to pulmonary lymphangiectasia in humans. The condition was milder in mice given doxycycline after age postnatal day 14 and did not develop after postnatal day 35. Mechanistic studies revealed that VEGF receptor (VEGFR)-3 alone drove lymphatic growth in adult mice, but both VEGFR-2 and VEGFR-3 were required for the development of lymphangiectasia in neonates. VEGFR-2/VEGFR-3 heterodimers were more abundant in the dilated lymphatics, consistent with the involvement of both receptors. Despite the dependence of lymphangiectasia on VEGFR-2 and VEGFR-3, the condition was not reversed by blocking both receptors together or by withdrawing VEGF-C.

Conclusions: The findings indicate that VEGF-C overexpression can induce pulmonary lymphangiectasia during a critical period in perinatal development.

Key Words: chylothorax ▪ lung ▪ lymphatic vessels ▪ lymphangiogenesis ▪ lymphangiomatosis, pulmonary ▪ pulmonary edema ▪ VEGFR-2 ▪ VEGFR-3

Lung lymphatics serve as routes for transport of extracellular fluid, antigens, and immune cells to lymph nodes, but this can change in conditions where lymphatics regress, overgrow, or otherwise become dysfunctional. Congenital pulmonary lymphangiectasia is a life-threatening developmental disorder where newborn infants have respiratory distress, cyanosis, pleural effusion or chylothorax, and widely dilated lymphatics in the lung. From the initial description, >150 years ago by Rudolf Virchow and many subsequent reports, pulmonary lymphangiectasia is distinguished by the presence of large lymphatic sacs, cysts, or networks around major bronchi and pulmonary blood vessels, within interlobular septa, and beneath the visceral pleura. For many years, most babies with the condition were stillborn or died soon after birth. Improvements in neonatal intensive care have led to better outcomes in some cases, but many still succumb. Pulmonary lymphangiectasia can occur in patients with congenital heart disease, can accompany chromosomal abnormalities, and can be associated with other genetic disorders. However, few patients have defects in VEGF-C signaling. Therefore, mutations in other genes that regulate lymphatic growth and maturation are likely to be important in the pathogenesis of pulmonary lymphangiectasia.
disorders, such as Noonan syndrome and Down syndrome, or can have a late onset in older children.8,10,13 Lymphangiectasia can also occur in extrapulmonary sites, including the intestine, pancreas, heart, kidneys, or in multiple organs.8,14-16

The cause of pulmonary lymphangiectasia is unknown, and no disease-specific therapies or animal models have been developed. Among the factors that could contribute to the condition, defective signaling of the lymphangiogenic factor (vascular endothelial growth factor [VEGF]-C) through its receptor VEGFR-3 is a likely candidate. The activation of VEGFR-3 signaling by VEGF-C is essential for normal development of the lymphatic vascular system17 and can promote growth of lymphatics in the adult.18-20 After proteolytic processing to the mature protein, VEGF-C can also activate VEGFR-2,21 which drives lymphangiogenesis under some conditions.22,23 VEGF-D, the other known ligand for VEGFR-3, seems to be dispensable because lymphatic development proceeds normally in its absence24 but can substitute when VEGF-C is not present.25

Lymphangiogenesis is a feature of sustained inflammation of the airways and lung20,26 and occurs in multiple other lung conditions.1 Despite the abundance of lymphatics accompanying inflammation, leaky blood vessels lead to airway mucosal edema, perhaps because the new lymphatics are immature, abnormal, or dysfunctional and unable to handle the fluid load.20,27,28 Lymphatic dysfunction in these pathological conditions contrasts with lymphatic growth promoted by engineered overexpression of VEGF-C, which can increase lymph flow and can reduce inflammatory responses in skin and joints.29,30

We sought to determine whether switching on VEGF-C to drive lymphatic growth in the respiratory tract before the onset of inflammation could ameliorate subsequent inflammatory responses. To our surprise, the activation of VEGF-C overexpression in neonatal mice—but not in adults—led to a condition resembling pulmonary lymphangiectasia. This growth of lymphangiectatic vessels differed from reported effects of VEGF-C overexpression in skin and joints.19,29,30 It also differed from reported effects of VEGF-A overexpression in the airway and lungs, where the new blood vessels grow as tubular sprouts and not as sheets, sacs, or cystic structures.31,32

This unanticipated finding provided the opportunity to obtain a better understanding of how lymphangiectasia develops in the respiratory tract. We reasoned that the finding of lymphatics growing as irregular sheets or bags instead of simple tubes after overexpression of VEGF-C in the respiratory tract could provide insights relevant to human pulmonary lymphangiectasia. Therefore, we examined the development, mechanism, and reversibility of lymphangiectasia in the airways and lung of Clara cell secretory protein (CCSP)-reverse tetracycline-controlled transactivator (rtTA)/tetracycline operator (tetO)-VEGF-C double-transgenic mice in which VEGF-C expression was driven by the promoter of CCSP under doxycycline regulation. We learned that pulmonary lymphangiectasia developed in these mice only when VEGF-C overexpression occurred during a critical period from embryonic day 15.5 (E15.5) to postnatal day 14 (P14). The development of lymphangiectasia required VEGF-C signaling through both VEGFR-2 and VEGFR-3 and involved the formation of VEGFR-2/VEGFR-3 heterodimers, unlike lymphangiogenesis in the adult that required only VEGFR-3. Once lymphangiectasia developed, the abnormal lymphatics resisted regression, even after inhibition of VEGFR-2/VEGFR-3 signaling by function-blocking antibodies or withdrawal of VEGF-C.

Methods

Mice

We generated CCSP-rtTA; tetO-VEGF-C double-transgenic mice (designated CCSP-VEGF-C mice) by breeding mice of the CCSP-rtTA driver line (The Jackson Laboratory; strain #006222, line 1)31 with mice of the tetO-mVEGF-C responder line.19 In CCSP-rtTA driver mice, doxycycline induces rapid, reversible expression of full-length mouse VEGF-C in Clara cells and alveolar type II cells of the respiratory epithelium.32 In tetO-VEGF-C responder mice, lymphangiogenesis is driven by VEGF-C overexpressed in the target cells, as previously shown in skin.32 Some transgenic mice were crossed with Prox1-green fluorescent protein mice33 to make triple-transgenic mice with green fluorescent lymphatics. Double-transgenic mice without doxycycline, which had no apparent phenotype, were used as controls. All mice were pathogen free and were housed under barrier conditions. Before being subject to experimental procedures, mice were anesthetized by intramuscular injection of ketamine (87 mg/kg) and xylazine (13 mg/kg). Each experimental group had 8 to 10 mice. All experiments were approved by the Institutional Animal Care and Use Committee of the University of California at San Francisco.

Human Specimens

Human lung specimens obtained at autopsy (case A) or by wedge biopsy (cases B and C) were fixed in formalin, embedded in paraffin, sectioned at a thickness of 5 μm, and stained with hematoxylin and eosin (H&E) or by immunohistochemistry (Methods in Online Data Supplement). The Institutional Review Board of Boston Children’s Hospital approved the study and waived the need for individual patient consent because it was restricted to a retrospective review of slides and reports and met the other institutional standards including next-of-kin consent for autopsy, parent/guardian consent for biopsy, patient confidentiality protections, and other requirements.

Doxycycline Administration

To activate VEGF-C expression, CCSP-VEGF-C mice received doxycycline (Sigma-Aldrich) at a concentration of 0.001 to 5 mg/mL in drinking water containing 5% sucrose.36 CCSP-VEGF-C littermates on normal water were used as controls, unless described otherwise. In some experiments, mice received doxycycline by intraperitoneal injection at a dose of 100 μg/g every other day (Methods in Online Data Supplement). In reversal studies, VEGF-C expression was turned off by removing doxycycline from drinking water.

Inhibition of VEGFR-2 and VEGFR-3 Signaling

Function-blocking, rat monoclonal antibodies were administered to inhibit signaling of VEGFR-2 (DC101) and VEGFR-3 (mF4-31C1; ImClone/Elit Lilly, New York, NY). Antibodies were injected intraperitoneally at an initial dose of 100 μg/g and then 40 μg/g every
other day. Control mice received sterile 0.9% NaCl, which was previously found to be indistinguishable from control rat IgG in this setting. In prevention studies, mice were given the inhibitory antibodies and doxycycline concurrently for 7 days. In reversal studies, doxycycline was given for 7 days, and then the antibodies were given for the next 7 days without doxycycline.

Preparation of Mouse Tissues

Tissues were fixed by vascular perfusion of fixative (1% paraformaldehyde in PBS; pH 7.4) through the pulmonary artery via the right ventricle, at a pressure of 20 to 40 mmHg until the lungs turned white, and then perfusion via the left ventricle for 2 minutes at 120 to 140 mmHg. Trachea, lungs, diaphragm, mesentery, and skin from the ventral midline of the abdomen were prepared as whole mounts for immunohistochemical staining (Methods in Online Data Supplement). Some tissues were embedded in compound optimal cutting temperature (Sakura Finetek) and cryosectioned or embedded in paraffin, sectioned, and stained with H&E.

In Situ Proximity Ligation Assay

Cryostat sections of trachea 10 μm in thickness were mounted on slides, stained for lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) immunoactivity (rabbit polyclonal; AngioBio), and incubated with secondary antibody antirabbit Alexa 488 (Jackson ImmunoResearch). Slides were then subjected to in situ proximity ligation assay (PLA) using Duolink kits (Olink Bioscience, Uppsala, Sweden) according to the manufacturer’s instructions. Sections were incubated overnight with primary antibodies to VEGFR-2 (rabbit antinouse, clone 55B11; Cell Signaling) and VEGFR-3 (goat polyclonal, #AF743; R&D) followed by incubation with secondary antibody-conjugated PLA probes (anti-rabbit PLUS, #LNK-92002 and anti-goat MINUS, #LNK-92006; Axxora, San Diego, NY) for 1 hour at 37°C. After the ligation and rolling circle amplification steps, the single-strand rolling circle amplification products were hybridized by 554-nm fluorophore-conjugated complementary oligonucleotides (Duolink In Situ Detection Reagents Orange). VEGFR-2/VEGFR-3 heterodimers visible as bright red fluorescent dots were imaged by confocal microscopy with a Cy3 filter. The number of PLA dots per lymphatic endothelial cell, defined as 4',6-diamidino-2-phenylindole–stained nucleus colocalized with LYVE-1, was counted in 5 sections per mouse (n=3 per group). As controls, each primary antibody was omitted, one at a time, to confirm the low or absent background signal from PLA probes.

Supplemental Methods

Methods for mouse genotyping, dosing doxycycline by intraperitoneal injection, thoracic duct imaging by the lipophilic fluorophore DiI, measuring wet-to-dry lung weight ratios, and performing immunohistochemistry, morphometric measurements of lymphatics, cell counting by fluorescence-activated cell sorting, real-time reverse transcriptase-polymerase chain reaction (RT-PCR), and Western blots are described in Methods in Online Data Supplement.

Statistical Analysis

Data are presented as mean±SEM with 8 to 10 mice per group unless otherwise indicated. Differences were assessed by ANOVA followed by Dunn–Bonferroni test for multiple comparisons. P values <0.05 were considered statistically significant.

Results

Tracheal Lymphangiectasia

VEGF-C expression was activated in the respiratory tract of double-transgenic CCSP-rtTA/rtOE-VEGF-C mice at ages ranging from late gestation to adult by administration of doxycycline. In these mice, doxycycline activates (Tet-On) VEGF-C expression in respiratory epithelial cells under control of the CCSP promoter (Methods in Online Data Supplement). VEGF-C expression was intended to expand the network of lymphatics in the airways and lung. Doxycycline in drinking water was given to pregnant mice with embryos from E15.5 to E18.5, to maternal mice with pups from age P0 to P21, or to CCSP-VEGF-C mice from age P21 to P70. Embryos younger than E15.5 were not studied because strong expression of VEGFR-3 in vascular endothelial cells can result in VEGF-C–mediated changes in blood vessels and in lymphatics. In the absence of doxycycline, double-transgenic mice had no apparent abnormalities at any of these ages.

All pups that received doxycycline in utero beginning on E15.5 (n=26) were found dead soon after birth. All had pleural effusion at autopsy. When doxycycline was started on E16.5 (n=33), 70% of newborn pups were found dead, and the remaining 30% had respiratory distress, cyanosis (Figure 1A), pleural effusion (Figure 1B), and died within a few hours. In contrast to the distinctive segmented lymphatic network of the normal trachea (Figure 1C), lymphatics, marked by LYVE-1 staining, were merged into sheets that formed the walls of irregularly shaped, flattened sacs throughout much of the mucosa (Figure 1D). When doxycycline was started at P0 (N=40), 20% of pups had chylothorax at P14 (Figure 1E) but most survived.

The distribution of lymphatics examined in tracheal cross sections from CCSP-VEGF-C mice was strikingly different in pups on water from those on doxycycline from P0 to P14 (Figure 1F and 1G). In the controls, lymphatics were scattered around the tracheal circumference, mainly between cartilage rings (Figure 1F and 1H), but in mice on doxycycline, sheets of lymphatic endothelial cells were located beneath the epithelium of almost the entire trachea (Figure 1G and 1I). To determine whether the sheets of lymphatic endothelial cells had a lumen, we evaluated cell polarity by determining the distribution of the luminal surface marker podocalyxin. The results clearly showed that podocalyxin was restricted to the surface of the lymphatic endothelium that bordered a lumen, unlike LYVE-1 which was on both surfaces (Figure 1J and 1K). Lymphatic endothelial cells in mice on doxycycline had the same polarity as the controls (Figure 1J and 1K). Together, these findings indicate that tracheal manifestations of lymphangiectasia in CCSP-VEGF-C mice were dominated by flattened sacs that had a thin lumen and were located beneath the epithelium of the entire trachea.

Pulmonary Lymphangiectasia: CCSP-VEGF-C Mice Versus Humans

Histological sections of lungs from CCSP-VEGF-C mice on doxycycline from P0 to P14 were compared with lung sections from 3 children with pulmonary lymphangiectasia. Case A was a 1-month-old girl who died from respiratory failure during an apparent pulmonary hypertensive crisis because of pulmonary vein stenosis. Examination of H&E-stained sections revealed widely dilated lymphatics around bronchi and large vessels and beneath the pleura (Figure 2A). Lymphatics were also enlarged in interlobular septa. The identity of the abnormal lymphatics was confirmed by D2-40/podoplanin immunoreactivity of the endothelial lining (Figure 2B and 2C). Enlarged lymphatics had a similar distribution in the lungs of an 11-year-old girl with pulmonary hypertension accompanied by later onset pulmonary lymphangiectasia (case B; Online Figure IA–IC) and a 6-month-old boy with pulmonary lymphangiectasia associated
with Down syndrome (case C; Online Figure ID–IF). The widely dilated appearance of lung lymphatics was similar in both cases, despite the difference in onset.

In H&E-stained sections of normal mouse lung, major bronchi and pulmonary blood vessels were surrounded by thin sleeves of loose connective tissue (Figure 2D). By comparison, major bronchi and vessels in lungs of CCSP-VEGF-C mice on doxycycline from P0 to P14 were surrounded by prominent wide spaces (Figure 2E and 2F). Lymphatics stained for VEGFR-3 and Prox1 immunoreactivity were sparse around bronchi and major vessels in lungs of normal mice (Figure 2G) but were widespread in these regions of mice on doxycycline from P0 to P14 (Figure 2H). Few lymphatics were visible in regions around alveoli in either group. Unlike human lungs, mouse lungs do not have lobules or interlobular septa, and CCSP-VEGF-C mice did not have enlarged lymphatics in interlobular septa.

Although few or no lymphatics were found in the visceral pleura of normal mice (Figure 2I and 2K), large flattened sacs lined by lymphatic endothelial cells were a conspicuous feature of the lung–visceral pleura interface of CCSP-VEGF-C mice on doxycycline (Figure 2J and 2L). To determine whether pleural mesothelial cells were actually part of the wall of these subpleural sacs, we compared the distributions of mesothelial cells identified by mesothelin immunoreactivity and lymphatic endothelial cells marked by LYVE-1 (green) and blood vessels (platelet endothelial cell adhesion molecule-1 [PECAM-1], red) in normal trachea of control newborn mouse (CCSP-VEGF-C on water). D. Widespread lymphangiectasia in trachea of CCSP-VEGF-C mouse on doxycycline from E16.5 to P0. E. Chylothorax (asterisk) in CCSP-VEGF-C mouse on doxycycline from P0 to P14. F–I. Tracheal cross sections stained for epithelial Clara cells (CCSP, red), lymphatics (LYVE-1 green), and blood vessels (PECAM-1, blue) in CCSP-VEGF-C mice on water (F and H, green) and in CCSP-VEGF-C mice on doxycycline (G and I) from P0 to P14. F. Lymphatics in control mouse are scattered around the trachea. G. Lymphatics in CCSP-VEGF-C mouse on doxycycline surround most of the trachea beneath the epithelium. Boxes in F and G marked regions in H and I show the differences in lymphatics (LYVE-1, green, arrows) and similarities of blood vessels (PECAM-1, blue, arrowheads) in the 2 conditions. J and K. Podocalyxin (red) is restricted to the luminal surface and LYVE-1 (green) is on both surfaces of lymphatic endothelium in CCSP-VEGF-C mice on water (J) or doxycycline (K) from P0 to P14, demonstrating that lymphatic sacs in lymphangiectasia have a lumen (K). Scale bar, 100 μm (C and D), 150 μm (F and G), 50 μm (H and I), and 10 μm (J and K).

**Figure 1. Lymphangiectasia in trachea of neonatal Clara cell secretory protein (CCSP)-vascular endothelial growth factor (VEGF)-C mice.**

A. Normal skin color of control newborn mouse and cyanotic skin of newborn CCSP-VEGF-C mouse on doxycycline from E16.5 to P0. B. Pleural effusion (asterisk) in newborn CCSP-VEGF-C mouse on doxycycline from E16.5 to P0. C. Segmented pattern of lymphatics (lymphatic vessel endothelial hyaluronan receptor 1 [LYVE-1], green) and blood vessels (platelet endothelial cell adhesion molecule-1 [PECAM-1], red) in normal trachea of control newborn mouse (CCSP-VEGF-C on water). D. Widespread lymphangiectasia in trachea of CCSP-VEGF-C mouse on doxycycline from E16.5 to P0. E. Chylothorax (asterisk) in CCSP-VEGF-C mouse on doxycycline from P0 to P14. F–I. Tracheal cross sections stained for epithelial Clara cells (CCSP, red), lymphatics (LYVE-1 green), and blood vessels (PECAM-1, blue) in CCSP-VEGF-C mice on water (F and H, green) and in CCSP-VEGF-C mice on doxycycline (G and I) from P0 to P14. F. Lymphatics in control mouse are scattered around the trachea. G. Lymphatics in CCSP-VEGF-C mouse on doxycycline surround most of the trachea beneath the epithelium. Boxes in F and G marked regions in H and I show the differences in lymphatics (LYVE-1, green, arrows) and similarities of blood vessels (PECAM-1, blue, arrowheads) in the 2 conditions. J and K. Podocalyxin (red) is restricted to the luminal surface and LYVE-1 (green) is on both surfaces of lymphatic endothelium in CCSP-VEGF-C mice on water (J) or doxycycline (K) from P0 to P14, demonstrating that lymphatic sacs in lymphangiectasia have a lumen (K). Scale bar, 100 μm (C and D), 150 μm (F and G), 50 μm (H and I), and 10 μm (J and K).

**Fluid Accumulation in Lungs With Lymphangiectasia**

Mice with severe pulmonary lymphangiectasia had edematous lungs and pleural effusions. Wild-type and single-transgenic
mice treated with doxycycline for 5 weeks had essentially the same average values of wet weight, dry weight, and wet/dry ratio (Figure 2M). By comparison, all of these values were significantly greater in CCSP-VEGF-C mice on doxycycline (Figure 2M). The 10% increase in wet/dry ratio reflected the significant accumulation of fluid in lungs of mice that survived for 5 weeks with pulmonary lymphangiectasia. The 25% increase in lung dry weight, without evidence of inflammatory cell influx in H&E-stained sections (data not shown), was consistent with solute accumulation and lung remodeling in the presence of lymphangiectasia.

The presence of lung edema indicated a disturbance of the balance between fluid leakage and clearance. In addition to the structural abnormalities of lymphangiectatic vessels, the lymphatic endothelial cells lacked discontinuous, button-like intercellular junctions normally found in initial lymphatics (Figure 3A and 3C). These junctions are thought to be necessary for efficient cell and fluid entry.27,28 Instead, the cells were...
joined by continuous junctions (Figure 3B and 3D), similar to those present in normal collecting lymphatics and in initial lymphatics at sites of inflammation.27,28

Abnormalities in Thoracic Duct and Other Organs
The presence of pleural effusion at birth in CCSP-VEGF-C embryos on doxycycline from E16.5 was a sign of lymphatic dysfunction in utero. We asked whether defects in the thoracic duct contributed to pleural effusion or chylothorax. The thoracic duct of normal newborn Prox1-green fluorescent protein mice on doxycycline from E16.5 was filled with chyle after nursing (Figure 3E, left), but the thoracic duct of newborn CCSP-VEGF-C mice lacked chyle and was almost invisible (Figure 3E, right). To learn why chyle was not present, the structure of the thoracic duct was examined in situ in Prox1-green fluorescent protein mice. The thoracic duct in normal mice had a tubular shape with valves at regular intervals (Figure 3F, left), but the thoracic duct of Prox1-green fluorescent protein/CCSP-VEGF-C triple-transgenic mice on doxycycline had aneurismal bulges and irregularly spaced valves (Figure 3F, right). To test the barrier function of thoracic duct wall, the lipophilic fluorophore DiI was administered in milk given orally by pipette. The thoracic duct of normal neonates was sharply defined by red fluorescence in pleural fluid.

To determine whether VEGF-C overexpression in respiratory epithelial cells had effects outside the thorax, we surveyed the lymphatics of other organs. Lymphatics on the pleural surface of the diaphragm had a tubular shape in controls but were irregular sheets and sacs in CCSP-VEGF-C mice on doxycycline (Online Figure IIIA). However, no evidence of lymphangiectasia was found outside the chest. Lymphatics in the skin and mesentery of mice on doxycycline were similar to those in controls (Online Figure IIIB and IIIC).

Critical Period of Lymphatic Sensitivity to VEGF-C
VEGF-C overexpression in late gestation or during the neonatal period had more severe consequences than that in older mice. Lymphangiectasia was found only after VEGF-C overexpression occurred in young mice. To define the age range during which VEGF-C overexpression resulted in pulmonary lymphangiectasia, we compared the effects of beginning doxycycline at increasing ages ranging from E15.5 to P0. The presence of pleural effusion at birth in CCSP-VEGF-C mice on doxycycline started at E16.5, 30% of the pups survived beyond P0, and lymphangiectasia was widespread in the lungs at P1. Lymphatics marked by VEGFR-3 immunoreactivity occupied 52% of lung sectional area instead of the normal 3% (Figure 4A and 4B). Lungs of mice on doxycycline from P0 to P7 had extensive lymphangiectasia, including subpleural lymphatics (VEGFR-3 staining, 21%; Figure 4C), but less than when started at E16.5. Mice on doxycycline for 7 days beginning at ages ranging from P14 to

![Figure 3. Abnormal lymphatic junctions and barrier function in lymphangiectasia. A and B, Adherens junctions (vascular endothelial [VE]-cadherin, black) between endothelial cells of tracheal lymphatics are shown in inverted grayscale images. Discontinuous, button-like junctions (arrows) in lymphatic of normal mouse at P14 (A) are contrasted with continuous zipper-like junctions (arrows) in lymphatic of Clara cell secretory protein (CCSP)-vascular endothelial growth factor (VEGF)-C mouse on doxycycline from P0 to P14 (B). C and D, Adherens junctions (VE-cadherin, red) between endothelial cells of lung lymphatics (Prox1, green) when compared with button-like junctions (arrows) in CCSP-VEGF-C mouse on water (C) with zipper-like junctions (arrows) in mouse on doxycycline from P0 to P14 (D). E–G, Image pairs comparing thoracic duct in normal Prox1-GFP mouse at P0 and Prox1-GFP/CCSP-VEGF-C mouse on doxycycline from E16.5 to P0. E, Thoracic duct normally filled with chyle (left) is compared with almost invisible thoracic duct in mouse on doxycycline (dashed lines, right). F, Thoracic duct with strong Prox1-GFP fluorescence in normal valves (left) is compared with thoracic duct with herniations (arrows) and abnormal valves in mouse on doxycycline (right). G, Normal thoracic duct filled with Dil-labeled chyle from milk (left) is compared with Dil extravasated from thoracic duct in mouse on doxycycline. Scale bar, 20 μm (A and D), 900 μm (E), and 250 μm (F and G).]
P35 developed mild-to-moderate pulmonary lymphangiectasia, but chylothorax did not occur and survival was not compromised (data not shown).

After age P35, doxycycline administration was accompanied by lymphatic sprouting with little or no lymphangiectasia. Lungs had few or no subpleural lymphatics. Adult mice on doxycycline from P70 to P77 had double the normal abundance of lung lymphatics (VEGFR-3 staining, 6%; Figure 4D), but the lymphatics were more normal in structure, and pleural effusion or chylothorax did not develop.

Changes in the lymphatic vasculature of the trachea provided additional insights into the age-related differences in sensitivity to VEGF-C overexpression. Tracheal lymphatics in normal neonates at P7 resembled the simple, segmental pattern of the adult (Figure 4E). Most lymphatics were located between cartilage rings; regions over cartilage rings and the trachealis muscle had few or none. When doxycycline was given from P0 to P7, what seemed to be sheets of lymphatic endothelial cells were throughout the tracheal mucosa (Figure 4F). The sheets were actually flattened bags with a narrow lumen located beneath the epithelium (Figure 1G, II, and IK). Subepithelial mucosal lymphatics were located over cartilages, between cartilages, and over the trachealis muscle (Figure 4F).

In adult CCSP-VEGF-C mice on doxycycline from P70 to P77, lymphatic sprouts grew in the mucosa overlying cartilage rings (Figure 4G and 4H) but not over the trachealis muscle. Lymphatic growth was greater caudally than rostrally, which is consistent with previous reports of the distribution of Clara cells in the tracheobronchial epithelium of mice.41 Platelet endothelial cell adhesion molecule 1 immunoreactivity of lymphatic endothelial cells resulted in stronger staining in the tracheal mucosa of neonatal and adult mice on doxycycline, but changes in tracheal blood vessels were not evident (Figure 4I–4L).

At baseline, the overall abundance of tracheal lymphatics was similar at ages P7 and P77 (area density, 33% versus 34%; Figure 4M). The value increased to 96% in neonates but only to 55% in adults on doxycycline for 7 days (Figure 4M). Lymphatics over cartilage rings, which reflected sprouting, were similarly sparse at baseline at P7 and P77 (area density, 6% versus 9%), but the value was 100% in neonates on doxycycline, where the entire region over cartilage rings was covered...
Figure 5. Vascular endothelial growth factor (VEGF)-C comparison in neonatal and adult Clara cell secretory protein (CCSP)-VEGF-C mice. A and B, VEGF-C (A) and reverse tetracycline-controlled transactivator (rtTA; B) mRNA expression in trachea of CCSP-VEGF-C mice at P7 or P77 after doxycycline for 7 days and in corresponding controls (water). †P<0.05 vs neonates on water; †P<0.05 vs neonates on water or doxycycline. C, Intensity of VEGF-C immunofluorescence, measured along dashed line with ImageJ, in tracheal epithelium imaged by confocal microscopy with the same settings. D–G, VEGF-C immunoreactivity (red) in epithelium of tracheal whole mounts of neonatal and adult mice shown alone (D and E, upper) and with CCSP (green) immunoreactivity. White boxes in D–G demarcate regions enlarged below. H, Dose–response relationship between VEGF-C mRNA expression in trachea and doxycycline concentration in drinking water. Tracheal VEGF-C mRNA was about the same (dashed line) in neonates on doxycycline at 10⁻¹ mg/mL and in adults on doxycycline at 5 mg/mL. *P<0.05 vs water (0 on x axis; 0.005 on y axis). I, Lymphangiectasia (lymphatic vessel endothelial hyaluronan receptor 1 [LYVE-1]) occurred in neonates but not in adults even when doxycycline concentration was adjusted to match VEGF-C expression at the 2 ages. J, Similarly, lymphangiectasia was present in neonates but not in adults when both were given doxycycline at a low concentration (10⁻² mg/mL). K, Western blot showing stronger bands for intermediate forms of VEGF-C (32–35 kDa) in neonates than that in the corresponding positive control. Top and middle are from different exposures of the same blot. Loading control is β-actin (bottom). Scale bar, 70 μm (C), 125 μm (D–G top; I–J left), 25 μm (D–G bottom), and 175 μm (I–J right).
Mechanism of Pulmonary Lymphangiectasia in Neonates
To explore possible mechanisms underlying the exaggerated response in neonatal CCSP-VEGF-C mice that led to lymphangiectasia, we compared the expression of VEGF-C and CCSP and the regulation of VEGF receptor signaling using changes in the trachea as readouts.

VEGF-C and rtTA mRNA
We first asked whether VEGF-C expression was greater in neonates than that in adults. Measurements by quantitative RT-PCR (qRT-PCR) of VEGF-C mRNA in the trachea revealed values in neonates on doxycycline from P0 to P7 averaging 55× those of controls without doxycycline, but the values for adults on doxycycline from age P70 to P77 were only twice the control (Figure 5A). The much larger increase in neonates resulted from smaller baseline values and larger postdoxycycline values. We next asked whether this large difference between neonates and adults was simply explained by a difference in CCSP promoter activity and thus resulted from a peculiarity of the transgene construct rather than relevant underlying biology. This proved not to be the case, because rtTA expression was greater in adults than that in neonates, both at baseline and after doxycycline (Figure 5B). Because the differences in rtTA expression were in the opposite direction to VEGF-C expression, they seemed unlikely to explain the development of lymphangiectasia in neonates. This led us to determine whether the amount or distribution of the cellular source of VEGF-C differed in neonates and adults.

Amount and Distribution of VEGF-C Immunoreactivity
Consistent with differences in expression assessed by qRT-PCR, VEGF-C immunoreactive epithelial cells had stronger staining and were more abundant in mice on doxycycline than those on water (Figure 5C) and in neonates than in adults (Figure 5D, E). VEGF-C colocalized with CCSP-immunoreactive cytoplasmic granules in Clara cells (Figure 5D and 5E, lower). Despite this difference in amount, the distribution of VEGF-C–positive cells was about the same in neonates and in adults. At both ages, VEGF-C cells were more numerous between tracheal cartilage rings than over the rings, consistent with the known distribution of CCSP-expressing Clara cells.26 VEGF-C immunoreactivity was similarly faint or absent in the tracheal epithelium of neonates and adults in the absence of doxycycline (Figure 5F and 5G). Similar results were obtained with 2 different VEGF-C antibodies (Methods in the Online Data Supplement).

Doxycycline-VEGF-C Expression Dose–Response
To test whether the difference in VEGF-C expression was indeed responsible for lymphangiectasia developing in neonates but not in adults, we matched the VEGF-C expression at the 2 ages by taking advantage of doxycycline regulation of VEGF-C transgene expression through the Tet-on system.22 The goal was to learn whether lymphangiectasia still developed in neonates when VEGF-C production was reduced to the adult level by lowering doxycycline intake.

We found that expression of VEGF-C mRNA in neonates increased in a log-linear manner with doxycycline concentration over the range of 0 to 5 mg/mL (Figure 5H). Baseline VEGF-C expression was essentially the same as in wild-type mice, indicating minimal leakage of transgene expression without doxycycline (Figure 5H). VEGF-C expression and lymphangiectasia increased progressively at doxycycline concentrations >0.01 mg/mL. The dose–response relationship enabled us to identify the doxycycline concentration (0.1 mg/mL) that produced in neonates the same low level of VEGF-C mRNA expression as found in adults. Importantly, at that concentration, lymphangiectasia developed in neonates but not in adults, even though the VEGF-C expression was matched (Figure 5I). The number and distribution of VEGF-C–positive epithelial cells were similar at both ages under this condition (Online Figure IV). Neonates developed moderate lymphangiectasia at an even lower doxycycline concentration (0.01 mg/mL), but adults had only lymphatic sprouting (Figure 5J).

Chylothorax developed only when the pups received the highest concentration of doxycycline (5 mg/mL). Mice receiving doxycycline at 0.1 mg/mL from E16.5 had 35% mortality by P0 when compared with 70% for those receiving 5 mg/mL, but all died by P4 (n=14). Pups on doxycycline at 1 mg/mL from P0 to P14 did not develop chylothorax (n=30).

Matching Doxycycline Intake in Neonates and Adults
Because neonates received doxycycline via the mother by drinking milk and adults received it by drinking water, we asked whether this difference could explain the greater expression of VEGF-C in neonates. To address the issue, we estimated the dose of doxycycline received by neonates via milk (Methods in the Online Data Supplement) and gave this dose (100 μg/g) to newborns and adults by intraperitoneal injection to circumvent the oral route altogether. Neonates—but not adults—developed lymphangiectasia, and the severity resembled that found with oral doxycycline at a concentration of 5 mg/mL (data not shown).

Proteolytic Cleavage Forms of VEGF-C
As another possible factor contributing to the age dependency of lymphangiectasia in CCSP-VEGF-C mice, we asked whether proteolytic processing of VEGF-C differed in neonates and adults. Proteolytic processing of VEGF-C from the native to mature form increases receptor-binding affinity,21 which could contribute to lymphangiogenic potency. In Western blots, the band for VEGF-C protein was similarly weak in tracheas of neonates and adults under baseline conditions (Figure 5K). When compared with the baseline value, after doxycycline for 7 days, intermediate forms of VEGF-C were 10× as abundant in neonates and only twice as abundant in adults. Yet, the mature form of VEGF-C was still faint in neonates but was readily visible in adults. Native full-length VEGF-C (58 kDa) was not detected in tracheas of mice on doxycycline at either age, but the high background from the probing antibody at the expected molecular weight limited detection of weak signals.
Together, these experiments examine multiple factors that could contribute to the age dependency of lymphangiectasia in CCSP-VEGF-C mice. Although neonates had higher expression of VEGF-C than that of adults, this difference did not explain the development of lymphangiectasia because neonates still developed lymphangiectasia when VEGF-C
expression matched that in the adult. Age-related differences in CCSP promoter activity, Clara cell distribution, or efficiency of doxycycline delivery were also excluded. Age-related differences in proteolytic processing or receptor signaling could, however, contribute.

**VEGFR-2 and VEGFR-3 Signaling in Pulmonary Lymphangiectasia**

Comparison of the distribution of VEGFR-2 and VEGFR-3, the receptors for VEGF-C revealed immunoreactivity for both receptors on lymphatics in the tracheas of neonatal and adult CCSP-VEGF-C mice under baseline conditions. VEGFR-3 immunoreactivity was mainly on lymphatics. Staining for VEGFR-2 was stronger on blood vessels than that of lymphatics (Figure 6A and 6C). The intensity of staining for the receptors did not change noticeably after doxycycline for 7 days, but both were much more widespread because of the expansion of lymphatic endothelial cells, which was much greater in neonates than in adults (Figure 6B and 6D).

Measurements of VEGFR-2 and VEGFR-3 mRNA by qRT-PCR revealed higher expression in the trachea of mice on doxycycline for 7 days and greater expression in neonates than that in adults (Figure 6E). VEGFR-3 expression was increased much more than VEGFR-2 although the apparent change in VEGFR-2 was confounded by high baseline expression of VEGFR-2 in blood vessels. The increase in VEGFR-3 was much larger in neonates (7.4-fold) than that in adults (2-fold). VEGFR-2 and VEGFR-3 protein assessed by Western blot and qRTPCR analysis. Cells with CD45 or Iba1 immunoreactivity were found in control or doxycycline-treated tracheas and lungs of neonates by immunohistochemical staining and qRT-PCR analysis. Cells with CD45 or Iba1 immunoreactivity were sparse in the tracheal mucosa of control mice and in CCSP-VEGF-C mice on doxycycline (Online Figure VA and VB). Similarly, few cells with S100A9, B220, or CD3e immunoreactivity were found in control or doxycycline-treated tracheas (Online Figure VC–VE). Double staining for VEGFR-3 and each of the 5 leukocyte markers showed no apparent association under any of the conditions (Online Figure VA–VE). The mRNA levels measured for these 5 markers revealed no significant differences in the trachea and lungs between controls and doxycycline-treated mice (Online Figure VF).

We next asked whether the increase in VEGFR-2 and VEGFR-3 expression could be explained by the greater number of lymphatic endothelial cells or whether greater receptor expression per cell made a significant contribution. We estimated the number of lymphatic endothelial cells in the trachea in 3 ways. First, the number of lymphatic endothelial cells in the tracheas was estimated by fluorescence-activated cell sorting using lymphatic endothelial cell markers after tissue digestion (Methods I Online Data Supplement). An average of only 35 lymphatic endothelial cells was obtained per trachea from neonates (Online Figure VI). Even in the trachea of adults, the number was small (120 cells per trachea), yet as a control for the methods, the number obtained from adult ears was much greater (1920 cells per mouse). Because of the poor yield from tracheas in the fluorescence-activated cell sorting approach, the low values were considered unreliable. Second, Prox1-positive nuclei were counted, and the number was scaled to the overall area of LYVE-1 immunoreactivity (Methods in the Online Data Supplement). On the basis of the method, we found that the number of lymphatic endothelial cells increased 6.5-fold in mice on doxycycline from P0 to P7, from 13787 cells to 89057 cells. Third, comparison of Prox1 mRNA expression in the trachea of P7 mice on doxycycline to corresponding baseline controls gave a 7-fold increase. According to the second and third approaches, roughly 90% of the increase in VEGFR-3 could be attributed to lymphatic endothelial cell proliferation in the trachea of neonates on doxycycline.

The relative contribution of VEGFR-2 and VEGFR-3 signaling to the development of lymphangiectasia was tested by treating newborn mice from P0 to P7 on doxycycline concurrently with function blocking antibodies to VEGFR-2 (DC101), VEGFR-3 (mF4–31C1), or both (Figure 6G). Inhibition of VEGFR-2 alone had little effect on lymphangiectasia and reduced tracheal lymphatics by only 14% when compared with age-matched controls (Figure 6H), but did result in regression of some tracheal blood vessels (data not shown), as reported previously.45 Inhibition of VEGFR-3 alone reduced lymphatics by 40%. However, when VEGFR-2 and VEGFR-3 were blocked together, the amount of lymphangiectasia was reduced 90%, which was much larger than the sum of effects of blocking the individual receptors (54% reduction), consistent with synergistic actions of the receptors (Figure 6H).

Lymphatic sprouts over cartilage rings were measured as another readout of lymphangiogenesis in neonates on doxycycline. Lymphatic sprouts were reduced 5% by blocking VEGFR-2, were reduced 21% by blocking VEGFR-3, but were reduced 91% by blocking both receptors together (Figure 6I). The much larger effect of VEGFR-2 and VEGFR-3 blockade together, in comparison with the sum of effects of blocking individual receptors (26% reduction), was additional evidence of synergistic actions of the receptors.

The link of receptor synergy to lymphangiectasia was further explored by performing similar experiments on adult mice treated with blocking antibodies during doxycycline administration from P70 to P77 (Figure 6J and 6K). These experiments showed that lymphatic sprouts over cartilage rings were reduced 33% by blocking VEGFR-2 but were reduced 94% by blocking VEGFR-3. Inhibition of both receptors together resulted in 99% reduction in lymphatic sprouts, indicating that concurrent inhibition of VEGFR-2 added little to the already large effect of VEGFR-3 blockade. The minimal increment of blocking VEGFR-2 concurrently fits with the dominance of VEGFR-3 signaling in adult lymphangiogenesis and contrasts with the synergistic actions of the 2 receptors in neonates.

**VEGFR-2/VEGFR-3 Heterodimers in Pulmonary Lymphangiectasia**

To explore the apparent synergistic actions of VEGFR-2 and VEGFR-3 in mediating the development of pulmonary
lymphangiectasia, we asked whether the exaggerated signaling in neonates involved the formation of receptor heterodimers. Using in situ PLA to detect VEGFR-2/VEGFR-3 heterodimers, we compared PLA dots (heterodimer signals) in lymphatic endothelial cells in the trachea of neonates and adults on water or doxycycline for 7 days. PLA dots were associated with lymphatic endothelial cells in all groups but were most abundant in neonates on doxycycline (Figure 7A–7D).

Limited reversibility of lymphangiectasia. A and B, Lymphatics stained for lymphatic vessel endothelial hyaluronan receptor 1 (LYVE)-1 immunoreactivity in tracheal whole mounts from Clara cell secretory protein (CCSP)-vascular endothelial growth factor (VEGF)-C mice at baseline (A) or after doxycycline from P0 to P7 and then no doxycycline from P7 to P14 (B). Lymphangiectasia is still widespread after 1 week off doxycycline. C, Expression of VEGF-C mRNA in trachea of CCSP-VEGF-C mice (n=5 per group) at baseline (green) or after doxycycline from P0 to P7 and then no doxycycline for 0 to 4 weeks (red). D and E, Extent of lymphangiectasia in trachea shown by LYVE-1 staining in trachea of P7 mice (n=5 per group) at baseline (green) or after doxycycline from P0 to P7 and then no doxycycline for 0 to 4 weeks (red). PLA dots per lymphatic endothelial cell nucleus. *P<0.05 vs water; †P<0.05 vs adults on doxycycline.

Figure 7. Vascular endothelial growth factor receptor (VEGFR)-2/VEGFR-3 heterodimers revealed in lymphatic endothelial cells by proximity ligation assay (PLA). A–D, Confocal micrographs comparing the number of PLA red dots (VEGFR-2/VEGFR-3 heterodimers) in tracheal lymphatics (lymphatic vessel endothelial hyaluronan receptor 1 [LYVE-1], green) of neonatal (A and B) and adult (C and D) CCSP-VEGF-C mice on water or doxycycline for 7 days. Nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI). Inverted grayscale versions of the same images emphasize the larger number of PLA dots in the neonate (B, bottom) than that in the adult (D, bottom). E, PLA dots marking lymphatic endothelial cells in trachea of neonatal CCSP-VEGF-C mouse on doxycycline for 7 days, shown alone (left), with LYVE-1 (middle), and with LYVE-1 and DAPI-stained nuclei (right). Scale bar, 20 μm.

Figure 8. Limited reversibility of lymphangiectasia. A and B, Lymphatics stained for lymphatic vessel endothelial hyaluronan receptor 1 (LYVE)-1 immunoreactivity in tracheal whole mounts from Clara cell secretory protein (CCSP)-vascular endothelial growth factor (VEGF)-C mice at baseline (A) or after doxycycline from P0 to P7 and then no doxycycline from P7 to P14 (B). Lymphangiectasia is still widespread after 1 week off doxycycline. C, Expression of VEGF-C mRNA in trachea of CCSP-VEGF-C mice (n=5 per group) at baseline (green) or after doxycycline from P0 to P7 and then no doxycycline for 0 to 4 weeks (red). D and E, Extent of lymphangiectasia in trachea shown by LYVE-1 staining in trachea of P7 mice (n=5 per group) at baseline (green) or after doxycycline from P0 to P7 and then no doxycycline for 0 to 4 weeks (red). PLA dots per lymphatic endothelial cell nucleus. *P<0.05 vs water; †P<0.05 vs adults on doxycycline.

Figure 7A–7D).
Confirmation that PLA dots were associated with lymphatics was obtained using LYVE-1 to mark lymphatic endothelial cells and 4′,6-diamidino-2-phenylindole to mark nuclei (Figure 7E). Measurements revealed that PLA dots per lymphatic endothelial cell nucleus were twice as abundant in neonates under baseline conditions and 4× as abundant in neonates on doxycycline when compared with corresponding values in adults (Figure 7F).

Reversibility of Pulmonary Lymphangiectasia
Because of the reversibility of doxycycline-induced activation of the CCSP-rtTA driver,34 we asked whether lymphangiectasia resolved after withdrawal of doxycycline. Newborn CCSP-VEGF-C mice were given doxycycline from P0 to P7 and then water from P7 to P14. Lymphangiectasia was still widespread at P14 (Figure 8A and 8B). To determine the rate of decrease in VEGF-C expression after doxycycline withdrawal, VEGF-C mRNA was measured in tracheas after doxycycline from P0 to P7 and then water for 1 to 4 weeks. VEGF-C expression decreased 20% at 1 week, 55% at 2 weeks, 70% at 3 weeks, and 100% at 4 weeks (Figure 8C). This relatively slow decline could reflect the slow clearance of doxycycline.45 Because of the slow decrease in VEGF-C after removal of doxycycline, we tested the reversibility of lymphangiectasia by function-blocking antibodies to VEGFR-2 and VEGFR-3 receptors while off doxycycline from P7 to P14. Lymphangiectasia was minimally reversed by this approach (Figure 8D and 8E). The reduction was 1% after inhibition of VEGFR-2, 2% after inhibition of VEGFR-3, and 10% after inhibition of both receptors (Figure 8E).

To test reversibility for a longer period, lymphangiectasia was measured in mice on doxycycline from P0 to P7 and then on water for 19 months. Lymphangiectasia was still strikingly widespread in the trachea (Figure 8F). Measurements showed that the amount of lymphangiectasia was reduced only 24% (Figure 8E), and the overall appearance of the sheets of lymphatic endothelial cells was still highly abnormal and resembled that found after inhibition of VEGFR-2 and VEGFR-3 together for a week (Figure 8D).

Discussion
This study of CCSP-VEGF-C double-transgenic mice revealed unexpectedly that a condition resembling pulmonary lymphangiectasia developed when VEGF-C was overexpressed in the respiratory epithelium of neonates. Affected mice had respiratory distress, cyanosis, pleural effusion, chylothorax, and high mortality. Like pulmonary lymphangiectasia in humans, the condition in CCSP-VEGF-C mice was characterized by abundant large sac-like lymphatics around major bronchi and pulmonary blood vessels and beneath the pleura. Lymphangiectasia was most severe when VEGF-C was overexpressed between the ages of E15.5 and P14 and was minimal or absent in mice older than P35. Mechanistic studies revealed that lymphangiectasia resulted from exaggerated responses of lymphatics of neonates to VEGF-C. Signaling of both VEGFR-3 and VEGFR-2 was required for development of lymphangiectasia in neonates, unlike lymphangiogenesis in the adult where VEGF-R3 signaling was sufficient. The involvement of both receptors in lymphangiectasia was supported by the presence of heterodimers of VEGFR-2 and VEGFR-3 in lymphatics in neonates. Once formed, lymphangiectasia resisted regression after withdrawal of VEGF-C or inhibition of its receptors.

Comparison of Lymphangiectasia in CCSP-VEGF-C Mice and Humans
CCSP-VEGF-C mice were created to determine whether the magnitude or duration of inflammatory responses in the airways could be reduced by expanding the lymphatic network to increase fluid and cell clearance. Instead, overexpression of VEGF-C in respiratory epithelial cells unexpectedly led to the growth of widely dilated lymphatics in the form of irregular sacs around major bronchi and pulmonary vessels and beneath the visceral pleura. In so doing, the model serendipitously offered the opportunity to examine the development of pulmonary lymphangiectasia. The changes in CCSP-VEGF-C mice were similar to the pathology found in pulmonary lymphangiectasia in humans, as reflected by 3 cases shown herein and many other published cases.4–6 This similarity raises the possibility of mechanistic parallels that could help in understanding how lymphangiectasia develops in humans, albeit VEGF-C overexpression in the respiratory epithelium is unlikely to contribute to the cause in humans as it does in CCSP-VEGF-C mice.

Chylothorax is among other abnormalities found in both CCSP-VEGF-C mice and in pulmonary lymphangiectasia in humans. Chylothorax in CCSP-VEGF-C mice was a manifestation of early onset and severe lymphatic dysfunction and could have resulted from thoracic duct defects because of high levels of VEGF-C in the pleural fluid, but this is yet to be demonstrated. If this is true, VEGF-C could serve as a biomarker relevant to survival and prognosis. Fluid accumulation in the lungs also occurs in both settings. In CCSP-VEGF-C mice, this was reflected by increased total lung water and wet/dry weight ratio and was accompanied by edema around major bronchi and pulmonary vessels.

Lymphatic malformations in the lung are typically in the form of a generalized lymphatic anomaly, also known as diffuse lymphangiomatosus, which overlaps clinically and histologically with pulmonary lymphangiectasia.16 Lymphatic malformations, previously termed lymphangiomas, are a source of serious morbidity in children. They most commonly involve soft tissues in the neck, axillae, groin/perineum, chest wall, tongue, and proximal limbs and occasionally affect viscer.a.15 They are generally nonfamilial and idiopathic, but an understanding of their causes is gradually unfolding because genetic underpinnings are identified in a subset of patients.13

As the search for the underlying cause of lymphangiectasia continues, recent studies of transgenic mouse embryos have implicated gain-of-function mutations in the RAS signaling pathway, including RAF1, during development of lymphatic endothelial cells.47 Furthermore, mice lacking phosphatidylinositol 3-kinase, regulatory subunit 1, develop intestinal lymphangiectasia and chyloous ascites at birth, but no changes were reported in lungs.48 It is expected that insights into the pathobiology of pulmonary lymphangiectasia will not only lead to insights into the treatment of patients with this disease, including severe forms that are increasingly survivable in the modern era of neonatal intensive care,5,49 but also
improve the understanding of the physiology and treatment of other lymphatic anomalies.

**Lymphangiectasia and Lung Inflammation**

Although bacterial pneumonia is well known to complicate chronic lung disease of all sorts, including pulmonary lymphangiectasia,^{50,51} inflammation is not a part of the classic description or classification scheme for developmental lymphatic disorders of the human thorax^{52} and is not required for diagnosis. Intraluminal lymphocytes as a component of lymph fluid can be found in lymphangiectasia,^{53} and malformed intrapulmonary lymphatic channels^{5} can be focally lined by lymphoid aggregates, but neither is considered inflammation. Similarly, macrophages can be more abundant in alveolar spaces next to lymphatic malformations^{54} but generally do not infiltrate the tissue as in sites of inflammation.

Because some inflammatory cells express VEGF-C or support VEGFR-2 and VEGFR-3 signaling, which could contribute to the development of lymphangiectasia, we determined whether inflammatory cells were recruited to the trachea or lungs of CCSP-VEGF-C mice. No such influx was found by histological examination, immunohistochemical staining, or lungs of CCSP-VEGF-C mice. No such influx was found by histological examination, immunohistochemical staining, or qRT-PCR. These findings indicate little or no role of inflammatory cells in promotion of lymphangiectasia in this model and show that VEGF-C overexpression can promote lymphangiectasia in the absence of inflammatory cell recruitment.

**Critical Period of Lymphatic Hypersensitivity to VEGF-C**

The response of lymphatics to doxycycline regulated VEGF-C overexpression was strikingly different in neonates and adults. Lymphangiectasia developed in neonatal CCSP-VEGF-C mice for a 5000-fold range of doxycycline concentration (10^{-3} to 5 mg/mL) but did not occur in adults at even the highest concentration. Similarly, lymphangiectasia developed in neonates but not in adults even when the number and distribution of VEGF-C–producing cells were matched by customizing the doxycycline administration. These findings suggest that lymphatics in neonates are more prone to abnormal growth in response to VEGF-C.

Lymphatics in the lungs of neonatal CCSP-VEGF-C mice were abundant in lung parenchyma and beneath the visceral pleura, where they were normally sparse or absent. The vessels from which the subpleural lymphatics grew in these mice was not determined, but lymphatics in the adjacent parenchyma were a likely possibility. Lymphatics in fibrotic lung disease have been suggested to form from CD11b-positive alveolar macrophages.^{55} Yet, lymphangiogenesis in adult lungs tends to occur preferentially in regions around major bronchi and blood vessels, similar to where they grow near lung tumors that overexpress VEGF-C.^{56}

It is unclear whether the type of response found in the airways and lung of CCSP-VEGF-C mice can occur elsewhere. Lymphangiectasia was not reported in studies of VEGF-C overexpression in skin of K14-rTA/tetO-VEGF-C mice,^{19} but the timing of doxycycline and methods of analysis differed from the present study. Testing the effects of VEGF-C driven by other tissue-specific promoters should expand the understanding of age-dependent differences in sensitivity of lymphatic endothelial cells to VEGF-C.

**Greater VEGF-C Expression in Neonatal CCSP-VEGF-C Mice**

An important step in assessing the basis of greater VEGF-C production in neonatal CCSP-VEGF-C mice was to ask whether the response to doxycycline of the CCSP-rTA driver was age dependent. Fortunately, CCSP-rTA driver mice have been used to control gene expression in the lung of multiple transgenic models, and multiple driver lines have been developed.^{53,54,57} Neonates in one of the CCSP-rTA driver lines were found to have greater expression than that in adults,^{58} but our measurements of rTA mRNA expression in the trachea of CCSP-rTA mice showed lower values in neonates. Consistent with this finding, no significant difference was found between neonatal and adult CCSP-rTA–driven expression of luciferase or interleukin-1β in mice derived from the same driver mice used in present study.^{33,34} These findings indicate that an age-dependent difference in CCSP-rTA expression was not essential for greater VEGF-C expression in neonates.

Although low concentrations of doxycycline led to the development of pulmonary lymphangiectasia in neonatal CCSP-VEGF-C mice, larger amounts were necessary for the development of chylothorax and changes accompanied by high mortality. Future studies are needed to elucidate factors that underlie these differences.

**Incomplete VEGF-C Processing in Neonatal Mice With Pulmonary Lymphangiectasia**

Native full-length 58-kDa VEGF-C undergoes proteolytic processing to generate the mature form, which has greater binding affinity for VEGFR-2 and VEGFR-3.^{21} The extent of proteolytic processing of VEGF-C is determined by multiple factors, and the amount of the shorter forms influences activity.^{59} We found that most of the VEGF-C in neonatal CCSP-VEGF-C mice was not the fully processed mature 20 kDa form, suggesting a limiting step in VEGF-C protein cleavage during this critical period of development. The tracheas of neonates had much more partially processed VEGF-C than that of adults. Although the difference was striking, the contribution to the abnormal response of lymphatics remains to be determined. Proteolytic processing is also required for activation of VEGF-D,^{46} and partially processed VEGF-D can induce VEGFR-2/VEGFR-3 heterodimerization.^{60} It is unknown whether partially processed VEGF-C has this property.

The binding affinity of VEGF-A isoforms for heparan sulfate proteoglycans influences angiogenic activity.^{62} Less is known of this property of VEGF-C, which does not have a conventional heparin-binding domain in the primary protein sequence. However, interaction with heparin can influence the pattern of VEGF-C–induced lymphangiogenesis.^{61} Fully processed VEGF-C can bind heparan sulfate,^{64} but the extent of binding of longer forms of VEGF-C has not been determined. Further work is needed to understand the links between proteolytic processing of VEGF-C, binding to extracellular matrix, and development of lymphangiectasia.

**Involvement of VEGFR-2, VEGFR-3, and Heterodimers in Lymphangiectasia**

On the basis of these effects of selective blocking antibodies, VEGFR-3 signaling was sufficient to drive lymphangiogenesis...
in adult CCSP-VEGF-C mice, and VEGFR-2 signaling had a minor role. A similar mechanism has been found in other models of lymphangiogenesis. Unexpectedly, VEGFR-2 was more important in neonatal mice because both VEGFR-2 and VEGFR-3 had to be blocked to prevent the development of lymphangiectasia. Inhibition of both receptors had a much larger effect on lymphangiectasia than the sum of the effects of blocking each receptor separately. The more-than-additive effect of blocking VEGFR-2 and VEGFR-3 together suggests that VEGF-C is not just simply saturating VEGFR-3 and then activating VEGFR-2. This raised the question of involvement of VEGFR-2/VEGFR-3 heterodimers in the synergistic actions of the receptors. The finding by PLA of more abundant VEGFR-2/VEGFR-3 heterodimers in lymphatics of neonates than adults is consistent with this involvement.

VEGF-C can induce the formation of VEGFR-2/VEGFR-3 heterodimers in lymphatic endothelial cells. Importantly, VEGFR-3 is phosphorylated at fewer tyrosine residues in VEGFR-2/VEGFR-3 heterodimers: tyrosines Tyr-1337 and Tyr-1363 are not phosphorylated in heterodimers because they are in VEGFR-3 homodimers. This difference could have profound effects on signal transduction. Further experiments are needed to determine the extent heterodimers contribute to the hypersensitivity of lymphatic endothelial cells to VEGF-C during the critical period.

The function-blocking antibodies used in the present studies were generated against the ligand-binding domains of VEGFR-2 and VEGFR-3, respectively. Human versions of such antibodies are currently in clinical trials as cancer therapeutics. The activation of VEGF receptors by ligand binding involves receptor dimerization. A new class of blocking antibody against human VEGFR-3 had been made to inhibit the formation of VEGFR-3 homodimers and VEGFR-2/VEGFR-3 heterodimers. A mouse version of the antidimerization antibody could help elucidate the contribution of VEGFR-2/VEGFR-3 heterodimers to pulmonary lymphangiectasia.

**Limited Reversibility of Pulmonary Lymphangiectasia**

The persistence of lymphangiectasia after inhibition of VEGFR-2 and VEGFR-3 or after long-term withdrawal of the doxycycline indicated a previously unappreciated stability of these abnormal lymphatics. VEGFR-2/VEGFR-3 signaling was evidently not required for maintenance of lymphangiectasia in this model. Persistence of newly formed lymphatics in mouse airways has been reported after resolution of airway inflammation or withdrawal of interleukin-1β in doxycycline-regulated CCSP-interleukin-1β transgenic mice. Similarly, new lymphatics in skin persist for many months after withdrawal of VEGF-C overexpression in transgenic mice. The limited regression of lymphangiectasia is in sharp contrast to the rapid regression of blood vessels in CCSP-rtTA/tetO-VEGF-A transgenic mice after withdrawal of doxycycline.

Until a strategy is found to promote regression of lymphangiectatic vessels, another approach would be to improve lymphatic function by advancing maturation or normalization of the new lymphatics. Normalization of tumor blood vessels can have beneficial effects on drug delivery. Less is known about the normalization of lymphatics, but the principle has been demonstrated in tumors where lymphatic function was improved by inhibition of TGF-β, and lymphatic maturation is promoted by treatment with glucocorticoids.

In summary, the present study revealed unexpectedly that doxycycline-regulated overexpression of VEGF-C in the respiratory epithelium of CCSP-VEGF-C mice during a critical period of perinatal development led to a condition resembling pulmonary lymphangiectasia in human infants. Newborn pups developed respiratory distress, cyanosis, and chylothorax, and sac-like lymphatics formed in the lung around major bronchi and pulmonary vessels and beneath the visceral pleura. The formation of lymphangiectasia required VEGF-C signaling through both VEGFR-2 and VEGFR-3 and involved the formation of receptor heterodimers. Lymphangiectasia was prevented by blocking VEGFR-2 and VEGFR-3 together but was little reversed by receptor blockade or withdrawal of VEGF-C. Studies of abnormal lymphatic growth in CCSP-VEGF-C mice should help to elucidate the mechanisms of formation and persistence of pulmonary lymphangiectasia in humans and to identify new diagnostic and therapeutic approaches.

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**Disclosures**

Bronislaw Pytowski is an employee of ImClone Systems, Eli Lilly and Company. The other authors report no conflicts.

**References**


Sideway comparison showed similarities of lymphatic abnormality.

In mice, VEGF-C overexpression in the lung resulted in pulmonary lymphangiectasia, which lung lymphatics are widely dilated and respiratory function is severely impaired. The condition is of unknown cause and without specific treatment. In a double-transgenic mouse in which VEGF-C was overexpressed during a critical period of perinatal development, which lung lymphatics are widely dilated and respiratory function is severely impaired. The condition is of unknown cause and without specific treatment.

**What is Known?**

- Pulmonary lymphangiectasia is a life-threatening condition often evident soon after birth where respiratory function is severely impaired because of the presence of widely dilated lymphatics in the lung.
- Pulmonary lymphangiectasia is of unknown cause, and specific treatments are lacking.
- Vascular endothelial growth factor (VEGF)-C promotes the proliferation of lymphatic endothelial cells and the growth of new lymphatics.

**What New Information Does This Article Contribute?**

- In mice, VEGF-C overexpression in the lung resulted in pulmonary lymphangiectasia only during a critical period of perinatal development.
- Side-by-side comparison showed similarities of lymphatic abnormality in the mouse model and patients with pulmonary lymphangiectasia.
- The abnormal lymphatics in mice with pulmonary lymphangiectasia resisted regression after the VEGF-C stimulus was withdrawn.

**Novelty and Significance**

Pulmonary lymphangiectasia is a potentially lethal condition in which lung lymphatics are widely dilated and respiratory function is impaired. The condition is of unknown cause and without specific treatment. In a double-transgenic mouse in which VEGF-C could be switched on in the respiratory epithelium, we found an unexpected condition resembling pulmonary lymphangiectasia in human infants. Lymphangiectasia occurred only when VEGF-C was overexpressed during a critical period of perinatal development. The synergistic action of exaggerated VEGF-C–mediated activation of VEGFR-2 and VEGFR-3 was required for the development of pulmonary lymphangiectasia. Abnormal lymphatic vessels persisted after the VEGF-C stimulus was withdrawn or receptor signaling was blocked. Insights from a better understanding of the cause and pathophysiology of pulmonary lymphangiectasia should lead to improved diagnostic and therapeutic approaches for this serious condition.
Pulmonary Lymphangiectasia Resulting From Vascular Endothelial Growth Factor-C Overexpression During a Critical Period
Li-Chin Yao, Chiara Testini, Denis Tvorogov, Andrey Anisimov, Sara O. Vargas, Peter Baluk, Bronislaw Pytowski, Lena Claesson-Welsh, Kari Alitalo and Donald M. McDonald

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Supplemental Figure I. Pulmonary lymphangiectasia in two children. A-F, Sections of wedge biopsy of lung from two cases of pulmonary lymphangiectasia stained for H&E (A, D) and D2-40/podoplanin (B) or LYVE-1 (E) to mark lymphatics. Dilated lymphatics are marked by asterisks and dashed red lines; other landmarks are marked by solid blue lines (C, F). A-C, Case B: lung of 11-year-old girl with pulmonary hypertension accompanied by later onset pulmonary lymphangiectasia. D-F, Case C: lung of 6-month-old boy with pulmonary lymphangiectasia and Down syndrome. Alveolar macrophages are abundant (D). Br, bronchus; PA, pulmonary artery. Scale bar: 150 μm.
Supplemental Figure II. Pulmonary lymphangiectasia in CCSP-VEGF mice. A-B, H&E stained sections of lung from CCSP-VEGF-C mice on water (A, control) or doxycycline (B, lymphangiectasia) from P0 to P98. Dashed red line marks region with pulmonary lymphangiectasia. Br: bronchus; PA: pulmonary artery; PV: pulmonary vein. Asterisks mark lymphatic lumen. C-D, Higher magnification view of region around the pulmonary artery in A and B to compare normal lung (C) and lung with lymphangiectasia from mouse on doxycycline (D). E-F, Pleura of normal lung (E) closely attached to underlying lung compared to subpleural lymphangiectasia (F) where the lumen forms a conspicuous space (asterisks) between the pleura and lung in mouse on doxycycline from P0 to P98. Arrows mark visceral pleura. Scale bar: 200 μm (A-B); 70 μm (C-D); 100 μm (E-F).
Supplemental Figure III. Lymphatic abnormalities restricted to the thorax. A, Low magnification images of pleural surface of diaphragm compare lymphatics (LYVE-1, green) in control mouse (single transgenic tetO-VEGF-C, upper row, left) and mouse with pulmonary lymphangiectasia (double transgenic CCSP-VEGF-C mouse on doxycycline from E16.5 to P0, upper row, right). White boxes in upper row show regions enlarged in the row beneath, where higher magnification images of diaphragm compare normal lymphatics in control mouse (lower row, left) with lymphangiectasia in CCSP-VEGF-C mouse (lower row, right). B, C, Similarity of lymphatics (LYVE-1, green) in skin (B) and mesentery (C) of control mouse (left panel) and CCSP-VEGF-C mouse on doxycycline from E16.5 to P0 (right panel). Scale bar: 250 μm (A, upper row), 100 μm (A, lower row); 300 μm (B); 200 μm (C).
Supplemental Figure IV. Matched number and distribution of VEGF-C cells in tracheal epithelium of neonatal and adult CCSP-VEGF-C mice. A-D, VEGF-C (red) and CCSP (green) immunoreactivities of epithelial cells in tracheal whole mounts of CCSP-VEGF-C mice as neonate (A, C) or adult (B, D). By using different doxycycline concentrations in drinking water in neonates and adults, VEGF-C positive cells were made roughly equal in abundance at both ages. VEGF-C positive cells are more numerous between tracheal cartilage rings than over the rings at both ages. Scale bar: 60 μm.
Supplemental Figure V. Lack of inflammatory/immune cell influx in neonatal CCSP-VEGF-C mice with pulmonary lymphangiectasia. A-E, Tracheal cross-sections from CCSP-VEGF-C mice on water (left) or doxycycline from P0 to P7 (right) showing staining for VEGFR-3 (red) in combination with a marker of pan-leukocytes (CD45, green, arrows), macrophages (Iba1, green, arrows), neutrophils (S100A9, green, arrow), T cells (CD3e, green, arrows), or B cells (B220, green, arrows). Green staining in the tracheal epithelium (arrowheads) in E is similarly present in water and doxycycline-treated mice and does not reflect B cells. Scale bar: 50 μm. F, qRT-PCR measurements comparing expression of inflammatory/immune cell markers in trachea and lung of CCSP-VEGF-C mice on water or doxycycline from P0 to P7. mRNA values for doxycycline are scaled to the corresponding value for water = 1. N = 5 mice per group.
Supplemental Figure VI. FACS counts of blood vessel endothelial cells (BECs) and lymphatic endothelial cells (LECs) from the tracheas of neonatal and adult mice. A-B, FACS analysis of total leukocytes (CD45+, CD31-, podoplanin-), LECs (CD45-, CD31+, podoplanin+), and BECs (CD45-, CD31+, podoplanin-) isolated from pools of tracheas from 6 neonatal mice (A) and from 4 adult mice (B) as described in Supplemental Methods. An average of 35 LECs per trachea was obtained from neonates (A) and 120 LECs per trachea from adults (B). FACS analysis of cells dissociated from ears (C) of two of the adult mice gave an average of 1920 LECs per pair of mouse ears, in agreement with published results.6
Supplemental Methods

**Mouse genotyping.** Mice were genotyped by PCR analysis of genomic tail DNA using primers specific for the CCSP-rtTA transgene or the tetO-VEGF-C transgene:

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**Doxycycline given by intraperitoneal injection.** The dose of doxycycline injected ip into neonatal mice was calculated to match the oral dose received from milk. The corresponding amount scaled for body weight was injected ip into adult mice. Controls received phosphate-buffered saline (PBS) by ip injection. Daily doxycycline intake of pups was estimated from the amount of milk consumed per gram of body weight and the water content of milk produced per day by the mother. Because mice drink less water when it contains doxycycline, due to the bitter taste, the calculated dose (1 mg/g) was reduced to one-tenth (100 µg/g) to achieve approximately the same drug level as consumed orally.

**Thoracic duct imaging by lipophilic fluorophore Dil.** To image the thoracic duct and detect chyle in pleural fluid, Dil fluorophore (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) at a concentration of 40 mg/ml and then diluted with half-and-half milk to a final concentration of 10 mg/ml in 25% DMSO. Pups were fed 0.3 mg Dil from a small pipette tip and kept on a heating pad for 2 hours before anesthesia, euthanasia, and dissection. Pleural fluid was removed and fluorescence tested, and the thoracic duct was photographed in situ with a fluorescence dissecting microscope (Nikon, DXM1200).

**Lung wet-to-dry weight ratio.** Lungs were removed without vascular perfusion, lightly blotted, weighed immediately to obtain the wet weight, and then dried in an oven at 65°C for 3 days until no further change in weight occurred to obtain the dry weight. The ratio of wet to dry weight was used to quantify lung water content (N = 10 per group).

**Immunohistochemistry.** Human tissues were stained with antibodies to D2-40/podoplanin (Signet Laboratories, Dedham, MA) or LYVE-1 (Reliatech, Braunshweig, Germany) and counterstained with hematoxylin. Mouse tissues were incubated with one or more primary antibodies: VEGF-C (R&D, goat polyclonal #AF752; Santa Cruz, goat polyclonal #SC7132), VEGFR-2 (rabbit polyclonal #T014, a kind gift of Dr. Rolf Brekken, University of Texas Southwestern, TX), VEGFR-3 (R&D, goat polyclonal #AF743), CCSP (rabbit polyclonal, generated in Barry Stripp’s laboratory [Reynolds, 2007 #378], Duke University, and kindly provided by Jason Rock, UCSF), VE-cadherin (eBioscience, rat anti-mouse, clone BV13), LYVE-1 (AngioBio, rabbit polyclonal #11-034 or R&D, rat polyclonal #MAB2125), PECAM-1 (Thermo Scientific, hamster anti-mouse, clone 2H8), Prox1 (AngioBio, rabbit polyclonal #AF2727), podocalyxin (R&D, goat polyclonal #AF1556), mesothelin (Santa Cruz, goat
polyclonal #SC27702), CD45 (BD Biosciences, rat polyclonal #550566), Iba1 (Wako, rabbit polyclonal #019-19741), S100A9 (Abcam, rat polyclonal #Ab105472), CD3e (BD Biosciences, hamster anti-mouse, clone 500A2) and B220 (eBioscience, rat anti-mouse, clone RA3-6B2). Secondary antibodies were labeled with Alexa Fluor 488, 594 or 647 (Jackson ImmunoResearch). Specimens were imaged with a Zeiss LSM-510 confocal microscope using Zeiss AIM 4.0 software or a Zeiss Axiohot fluorescence microscope equipped with a 3-CCD low light RGB (red, green, blue) video camera (SciMeasure, Decatur, GA).

**Measurement of lymphatics.** Lymphatics stained for LYVE-1 immunoreactivity in flattened tracheal whole mounts from mice at age P7 or P77 (N = 8 per group) were recorded in a montage of digital images (5x objective, 1x Optovar). The fractional area of LYVE-1 immunoreactivity was measured using ImageJ software (http://rsb.info.nih.gov/ij) over the first 10 consecutive cartilage/intercartilage rings beginning at the rostral end and in the corresponding region over the trachealis muscle. The area density was calculated as the percentage of total pixels with fluorescence intensity above a threshold value of 80 to 90 on a scale of 0 to 255. Lymphatic sprouts were measured as the fractional area of LYVE-1 immunoreactivity over cartilage rings (area density) by stereological point counting (N = 8 per group). A rectangular box containing a lattice of 126 regularly spaced points was superimposed on the projected image of each area. Ten regions, each having an area of 0.034 mm² for neonates and 0.134 mm² for adults, were measured per trachea. The same approach was used to measure the area density of blood vessels over cartilage rings in neonates and adults. In selected lungs (Figures 4A-D) the area density of lymphatics was measured in a montage of images by using ImageJ and expressed as the proportion of total pixels that had a VEGFR-3 fluorescence intensity above the threshold value of 100 on a scale of 0 to 255. VEGFR-3 was used for this assessment because of its greater selectivity than LYVE-1 for lung lymphatics, as judged by colocalization with Prox1.

**Measurement of lymphatic endothelial cell number.** Three approaches for estimating the size of the lymphatic endothelial cell population were used to determine whether endothelial cell proliferation could account for the 7-fold increase in VEGFR-3 expression determined by qRT-PCR in the trachea of neonatal CCSP-VEGF-C mice on doxycycline for 7 days.

1. We counted lymphatic endothelial cells by flow cytometry after dissociating cells of tracheas. Tracheas from mice at age P7 were cut into small pieces and digested in RPMI medium (Gibco) containing 4 mg/ml collagenase IV (Worthington) and 10 µg/ml DNase I (Roche) at 37°C for 1 hr. Tissue suspensions were passed through a 50 µm-cell strainer (BD Biosciences), washed, resuspended in PBS containing 2% fetal bovine serum, and stained for 20 min on ice with one or more of the following antibodies: anti-mouse CD45 conjugated to R-phycocerythrin (PE)/Cy7 (BioLegend, clone 30-F11), anti-mouse CD31 conjugated to PE (BioLegend, clone 390), anti-mouse podoplanin conjugated to allophycocyanin (APC) (BioLegend, clone 8.1.1). Lymphatic endothelial cells (LECs) were identified by FACS as CD45-, CD31+, and podoplanin+ cells. Blood vessel endothelial cells (BECs) were identified by FACS as CD45-, CD31+, and podoplanin- cells. FACS was performed using an LSRFortessa cell analyzer with FACSDiva software (BD Biosciences). Data were analyzed with FlowJo software (Tree Star).

2. We estimated the number of lymphatic endothelial cells in flattened tracheal whole mounts from control and CCSP-VEGF-C mice at age P7 (N = 4 per group) by (i) counting Prox1-positive nuclei per mm² of LYVE-1-stained lymphatics; (ii) calculating the area (mm²) of lymphatics per trachea from measurements of the total tracheal surface area (mm²) and the fractional area (%) of Prox1/LYVE-1 staining; and (iii) calculating the number of lymphatic endothelial cells per trachea as the product of (i) and (ii).

3. We estimated the size of the lymphatic endothelial cell population by using qRT-PCR to
measure Prox1 mRNA expression in the trachea of P7 mice on doxycycline and of corresponding baseline controls.

**Real-time qRT-PCR.** The trachea and lungs of CCSP-VEGF-C mice was excised after blood was removed by vascular perfusion with PBS. RNA was extracted and cDNA synthesis was performed as described previously [Baluk, 2009 #21]. Samples of 5 ng of cDNA were subjected to qRT-PCR using SYBR qRT-PCR SuperMix Universal (Invitrogen) and measured in duplicate with a Bio-Rad MyiQ detection system using the following cycling protocol: at 50°C for 2 min, and then 95°C for 10 min followed by 40 cycles at 95°C for 15 sec and then at 60°C for 60 sec. Gene expression values were normalized to β-actin, and results were presented as fold differences in mice on doxycycline compared to water controls. Primers for CD3e and B220 were proprietary (SABiosciences). The other primers (Integrated DNA Technologies) were:

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**Western blots.** Tracheas were homogenized in cold PBS buffer containing 1% Triton X-100, 1 mM sodium orthovanadate, 2 mM phenylmethylsulphonyl fluoride (PMSF), 2 µg/ml leupeptin and 0.07 U/ml aprotinin. Equal amounts of total lysates (25 µg) were separated in 15% polyacrylamide gels for VEGF-C and in 3-8% gels for VEGFR-2 and VEGFR-3 under reducing conditions. Proteins were transferred to polyvinylidene difluoride (PVDF; Invitrogen) membranes and then were blocked with 5% BSA. Blots were probed with an antibody to VEGF-C (Millipore, rabbit polyclonal; R&D, goat polyclonal), VEGFR-3 (R&D, goat polyclonal), or VEGFR-2 (Cell Signaling, rabbit-anti-mouse, Clone 55B11) followed by HRP-conjugated secondary antibodies (Dako, goat anti-rabbit or donkey anti-goat). In parallel, total cell lysates from each group were detected with anti-β-actin antibody (Cell Signaling, rabbit polyclonal) as a loading control. Signals were visualized by chemiluminescence (ECL Prime, Amersham) and LAS-4000 image system (Fujifilm Life Science). Densitometric analysis was performed using Multi Gauge V3.0 (Fujifilm Life Science).

**Supplemental References**


