Online supplemental material

Pulmonary lymphangiectasia resulting from VEGF-C overexpression during a critical period

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:

Gene	Forward	Reverse
CCSP-rtTA transgene	5'-CCTCGATGGTAGACCCGTAA-3'	5'-CGCAATGGAGCAAAAGTACA-3'
tetO-VEGF-C transgene	5'-CCAAACCGGGCCCCTCTGCTAAC-3'	5'- ACTGTCCCCTGTCCTGGTATTGAG-3'

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 [1, 2]. 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 [3].

Thoracic duct imaging by lipophilic fluorophore DiI. To image the thoracic duct and detect chyle in pleural fluid, DiI 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 DiI 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^{o}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) [4].

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 [5]. 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 Axiophot 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 mm^2 for neonates and 0.134 mm^2 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^{o}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-phycoerythrin (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 [6, 7]. 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 mm^2 of LYVE-1-stained lymphatics; (ii) calculating the area (mm^2) of lymphatics per trachea from measurements of the total tracheal surface area (mm^2) and the fractional area (%) of Prox1/LVYE-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 ^{o}C for 2 min, and then 95 ^{o}C for 10 min followed by 40 cycles at 95 ^{o}C for 15 sec and then at 60 ^{o}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:

Gene	Forward	Reverse
β-actin	5'-GGCTGTATTCCCCTCCATCG-3'	5'-CCAGTTGGTAACAATGCCATGT-3'
CD45	5'-TAACTGTGTCCGTCCAGATGG-3'	5'-TCCTCAGCACTATTGGTAGGC-3'
Iba1	5'-GCTGGAGGGGATCAACAAGC-3'	5'-TCTTCAGCTCTAGGTGGGTCT-3'
Prox1	5'-AGAAGGGTTGACATTGGAGTGA-3'	5'-TGCGTGTTGCACCACAGAATA-3'
rtTA	5'-GCCCAGAAGCTTGGTGTAGAG-3'	5'-TGGTGCCTATCTAACATCTCAATG-3'
S100A9	5'-GGAGCGCAGCATAACCACCATC-3'	5'-GCCATCAGCATCATACACTCCTCA-3'
VEGF-C	5'-GAAATGTGCCTGTGAATGTACAG-3'	5'-GACAGTCCTGGATCACAATGC-3'
VEGFR-2	5'-GGGTCGATTTCAAACCTCAATGT-3'	5'-AGAGTAAAGCCTATCTCGCTGT-3'
VEGFR-3	5'-GCAGGAGGAGGAAGAGGAGC-3'	5'-TGCATGCTGGGTGGACTATCA-3'

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

1. Jara-Almonte M, White JM. Milk production in laboratory mice. *J Dairy Sci*. 1972;55:1502-1505
2. Rath EA, Thenen SW. Use of tritiated water for measurement of 24-hour milk intake in suckling lean and genetically obese (ob/ob) mice. *J Nutr*. 1979;109:840-847
3. Perl AK, Tichelaar JW, Whitsett JA. Conditional gene expression in the respiratory epithelium of the mouse. *Transgenic Res*. 2002;11:21-29

4. Kaner RJ, Ladetto JV, Singh R, Fukuda N, Matthay MA, Crystal RG. Lung overexpression of the vascular endothelial growth factor gene induces pulmonary edema. *Am J Respir Cell Mol Biol*. 2000;22:657-664
5. Florez-Vargas A, Vargas SO, Debelenko LV, Perez-Atayde AR, Archibald T, Kozakewich HP, Zurakowski D. Comparative analysis of d2-40 and lyve-1 immunostaining in lymphatic malformations. *Lymphology*. 2008;41:103-110
6. Halin C, Tobler NE, Vigl B, Brown LF, Detmar M. Vegf-a produced by chronically inflamed tissue induces lymphangiogenesis in draining lymph nodes. *Blood*. 2007;110:3158-3167
7. Lapinski PE, Kwon S, Lubeck BA, Wilkinson JE, Srinivasan RS, Sevick-Muraca E, King PD. Rasa1 maintains the lymphatic vasculature in a quiescent functional state in mice. *J Clin Invest*. 2012;122:733-747

Supplemental Figure Legends

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. Br, bronchus; PA, pulmonary artery. **D-F,** Case C: lung of 6-month-old boy with pulmonary lymphangiectasia and Down syndrome. Alveolar macrophages are abundant (**D**).

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**).

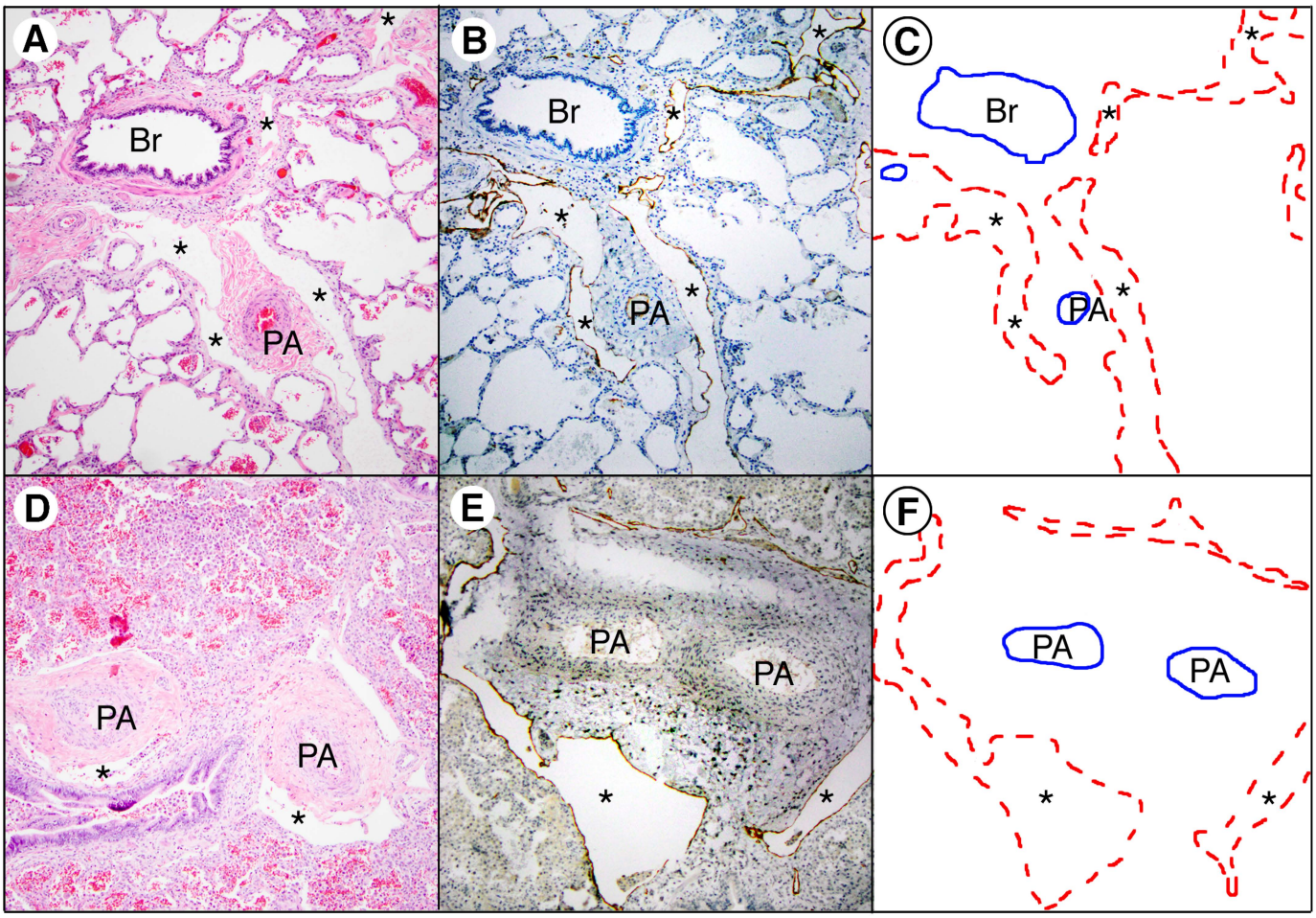
Supplemental Figure III. Lymphatic abnormalities restricted to the thorax. A, Low magnification images of pleural surface of diaphragm compare lymphatics (LYVE-1, green) in control mouse (single transgenic tetO-VEGF-C, upper row, left) and mouse with pulmonary lymphangiectasia (double transgenic CCSP-VEGF-C 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

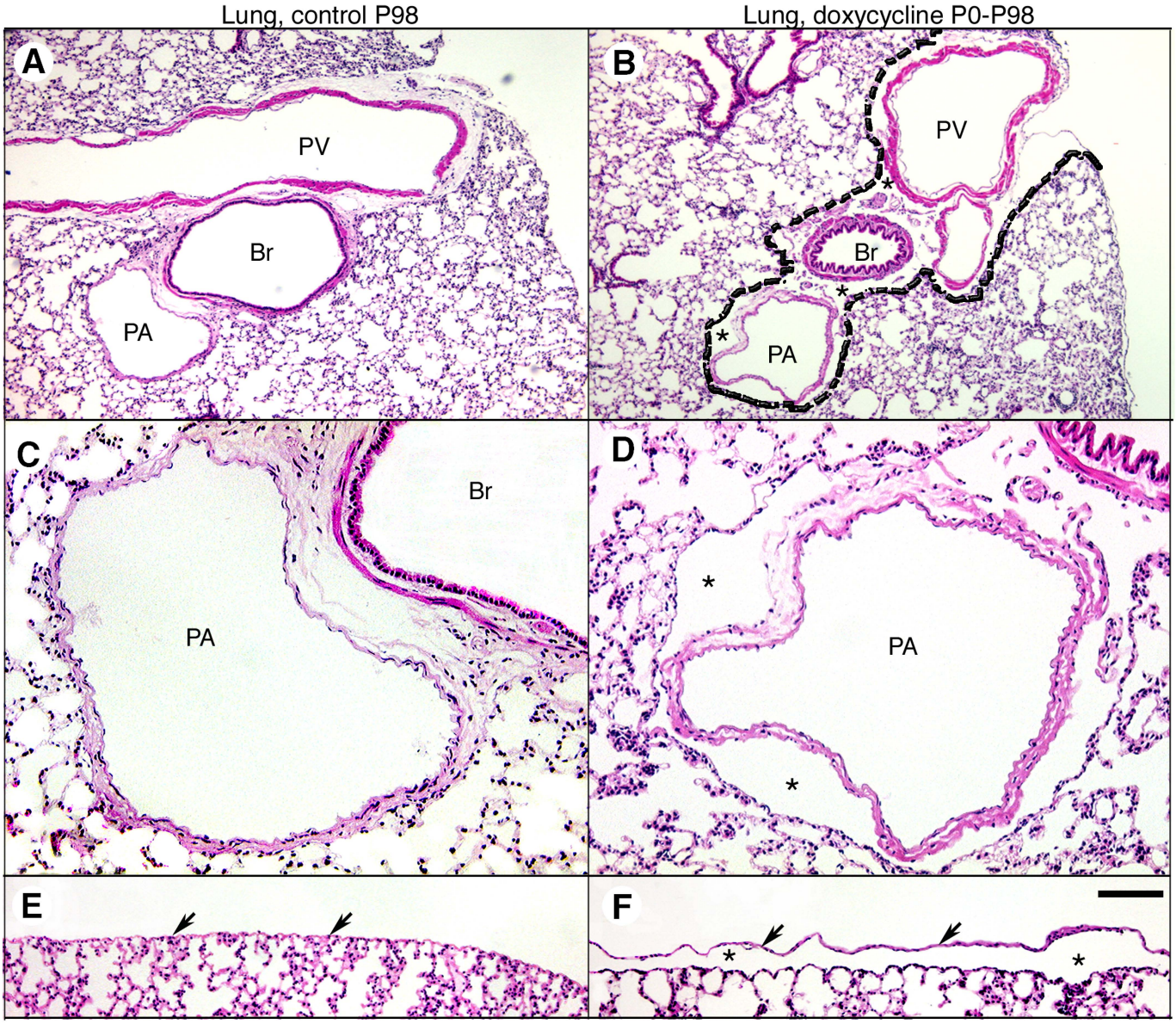
ages. VEGF-C positive cells are more numerous between tracheal cartilage rings than over the rings at both ages. Scale bar: 60 μm.

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-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-P7. mRNA values for doxycycline are scaled to the corresponding value for water = 1. *N* = 5 mice per group.

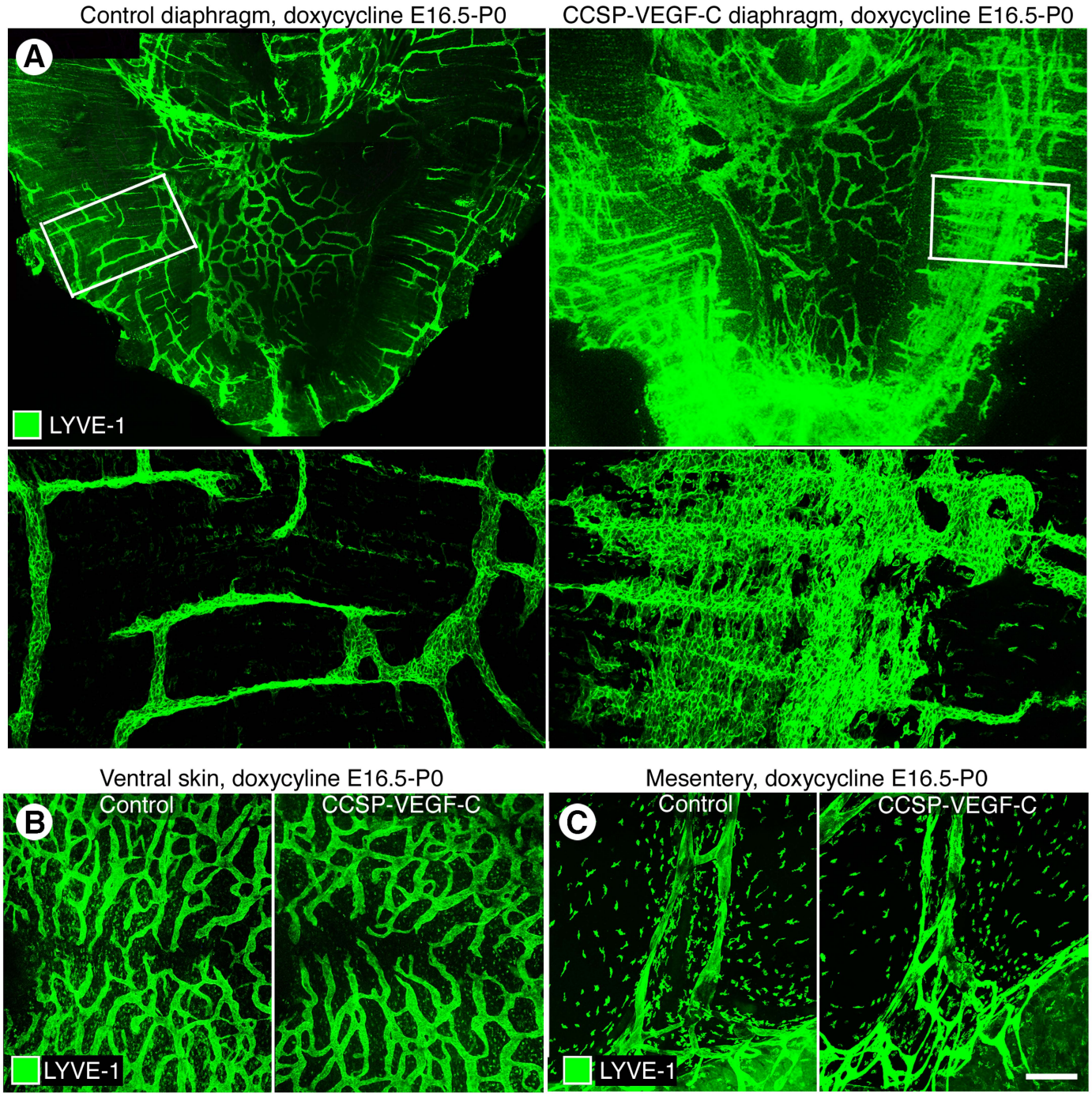
Supplemental Figure VI. FACS counts of blood vessel endothelial cells (BECs) and lymphatic endothelial cells (LECs) from the tracheas of neonatal and adult mice. A-B, FACS analysis of total leukocytes (CD45+, CD31-, podoplanin-), LECs (CD45-, CD31+, podoplanin+), and BECs (CD45-, CD31+, podoplanin-) 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].



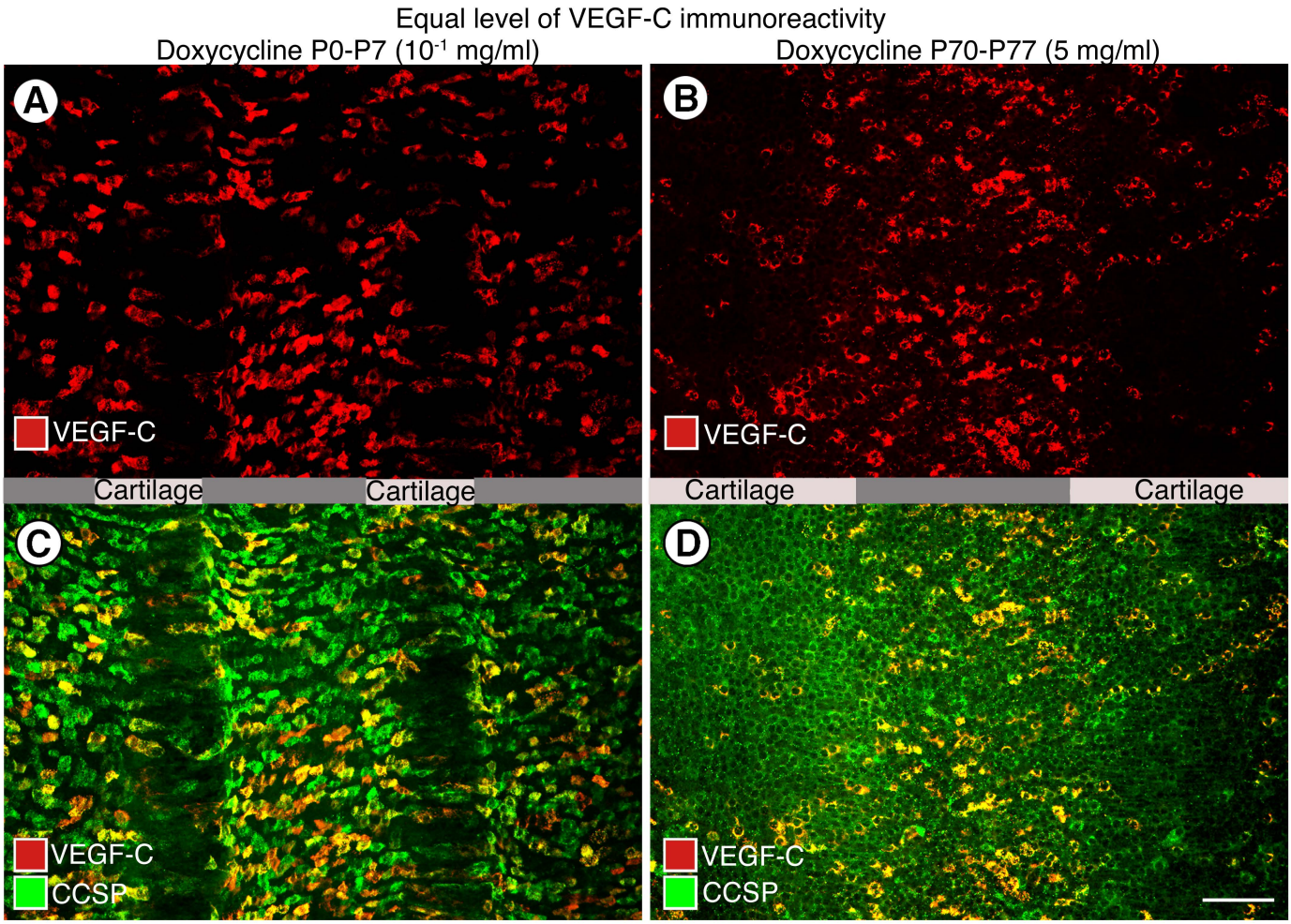
Supplemental Figure I



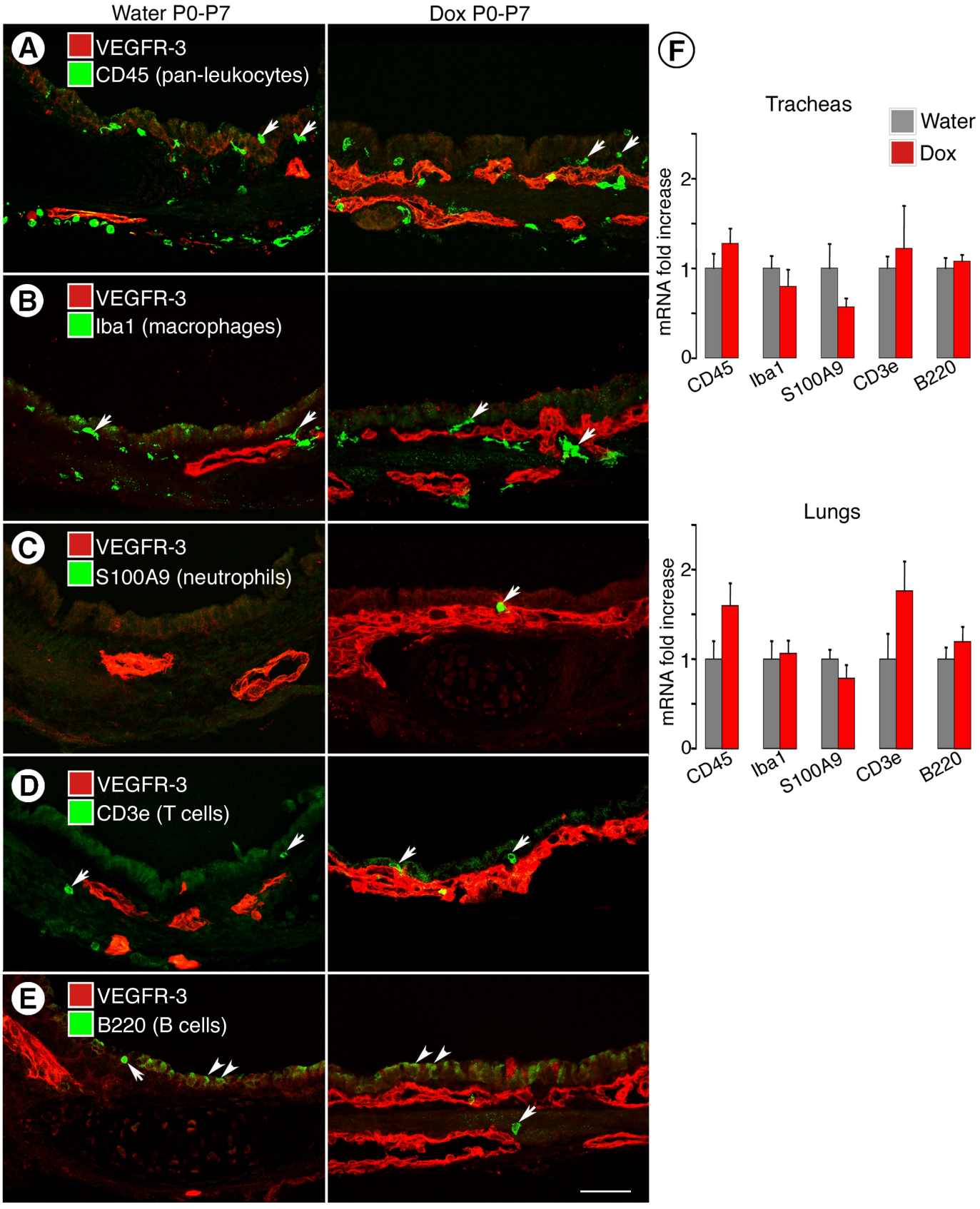
Supplemental Figure II



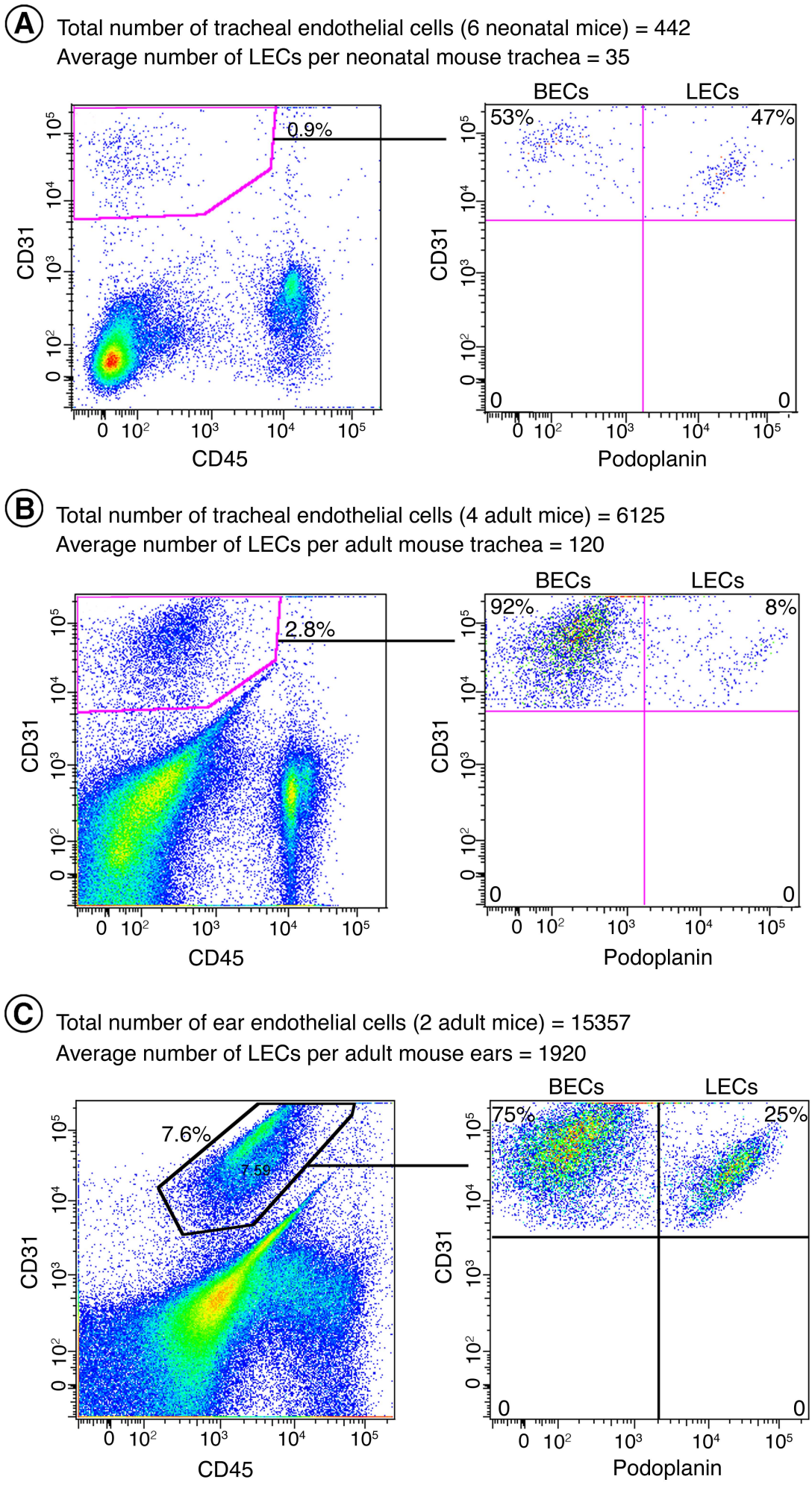
Supplemental Figure III



Supplemental Figure IV



Supplemental Figure V



Supplemental Figure VI

The NIHMS has received the file 'CCSP-VEGF-C SupplFigs+legends 012414.lcy.submit.pdf' as supplementary data. The file will not appear in this PDF Receipt, but it will be linked to the web version of your manuscript.