Altered lymphatics in an ovine model of congenital heart disease with increased pulmonary blood flow

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Datar SA, Johnson EG, Oishi PE, Johengen M, Tang E, Aramburo A, Barton J, Kuo H-C, Bennett S, Xoinis K, Reel B, Kalkan G, Sajti E, Osorio O, Raff GW, Matthay MA, Fineman JR. Altered lymphatics in an ovine model of congenital heart disease with increased pulmonary blood flow. Am J Physiol Lung Cell Mol Physiol 302: L530–L540, 2012. First published December 29, 2011; doi:10.1152/ajplung.00324.2011.—Abnormalities of the lymphatic circulation are well recognized in patients with congenital heart defects. However, it is not known how the associated abnormal blood flow patterns, such as increased pulmonary blood flow (PBF), might affect pulmonary lymphatic function and structure. Using well-established ovine models of acute and chronic increases in PBF, we cannulated the efferent lymphatic duct of the caudal mediastinal node and collected and analyzed lymph effluent from the lungs of lambs with acutely increased PBF (n = 6), chronically increased PBF (n = 6), and age-matched normal lambs (n = 8). When normalized to PBF, we found that lymph flow was unchanged following acute increases in PBF but decreased following chronic increases in PBF. The lymph: plasma protein ratio decreased with both acute and chronic increases in PBF. Lymph bioavailable nitric oxide increased following acute increases in PBF but decreased following chronic increases in PBF. In addition, we found perturbations in the transit kinetics of contrast material through the pleural lymphatics of lambs with chronic increases in PBF. Finally, there were structural changes in the pulmonary lymphatic system in lambs with chronic increases in PBF: lymphatics from these lambs were larger and more dilated, and there were alterations in the expression of vascular endothelial growth factor-C, lymphatic vessel endothelial hyaluronan receptor-1, and Angiopoietin-2, proteins known to be important for lymphatic growth, development, and remodeling. Taken together these data suggest that chronic increases in PBF lead to both functional and structural aberrations of lung lymphatics. These findings have important therapeutic implications that warrant further study.

lymph flow; lymphatic imaging; lymphatic endothelial dysfunction

Congenital and acquired abnormalities of the lymphatic circulation are well recognized in patients with congenital heart disease (14, 18, 29, 43, 49, 56, 67). For example, lymphangiectasia has been observed in infants and children with obstructive left-sided lesions, such as hypoplastic left heart syndrome with restrictive atrial septum (29, 56) or total anomalous pulmonary venous return (3). In addition, disruption of normal lymphatic development has been described in children with congenital heart defects associated with Turner and Noonan Syndromes (2, 15, 20). Perioperatively, these patients often have significant morbidity and mortality that can be attributed to these abnormalities, such as increased lung water, impairment of normal respiratory function, and an increased metabolic burden on an already compromised cardiovascular system. In addition, acquired lymphatic abnormalities may participate in the pathobiology of protein losing enteropathy and choledochoax, additional common causes of morbidity for children who have undergone surgery for cardiac defects (48). Despite these observations, investigations of lymphatic aberrations in the setting of congenital heart disease are sparse.

Congenital heart defects are commonly associated with increased pulmonary blood flow (PBF) and pressure. Elegant studies using sheep have previously demonstrated that acute increases in PBF or pressure led to increased capillary filtration of protein-poor fluid and augmented clearance of lymphatic fluid (12, 19, 42, 44, 50). However, infants born with congenital cardiac defects often live with increased PBF for months or years before their defects are surgically corrected, and it remains unknown what effect this chronically increased PBF has on the function and structure of the pulmonary lymphatic system. Furthermore, infants with congenital heart disease are often exposed to acute increases in PBF secondary to surgical placement of an aorto-pulmonary graft. The lymphatic response to this type of surgically induced acute increase in PBF and pressure has not been previously investigated.

We have previously developed a clinically relevant ovine model of a congenital cardiac defect with chronically increased PBF (55). In this model, a large vascular graft (8-mm shunt) is placed between the aorta and pulmonary artery of late gestation fetal lambs. Following spontaneous delivery, these lambs have increased PBF and pulmonary arterial pressure and demonstrate hemodynamic and morphologic features that recapitulate the human disease (Figure 1). In addition we have previously developed a model of acute increases in PBF by the postnatal placement of a similar aorto-pulmonary graft in juvenile lambs. With these models, we have elucidated important alterations in pulmonary vascular endothelial function that result from abnormal PBF, including disruption in endothelium-dependent nitric oxide (NO) signaling (5–8, 21, 30, 41, 51, 52, 54).

Interestingly, the endothelial cell is believed to play a prominent role in lymphangiogenesis. Sabin proposed over 100 years ago that lymphatic-specific endothelial cells originated from venous origin and spread outward to become a completely independent vascular compartment. Indeed, today this...
is the widely accepted model of lymphatic development (22, 34, 39, 53, 64). A subset of venous endothelial cells that express lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), a CD44 homolog, become competent for specification for a lymphatic cell lineage. Prox1, a homeobox transcription factor, then initiates a lymphatic endothelium-specific program of gene transcription while inhibiting gene expression for arterial and venous endothelial cell fates. Prox1-positive endothelial cells respond to vascular endothelial growth factor-C (VEGF-C) and its receptor, VEGFR-3, to promote vessel sprouting, growth, and extension. Further remodeling and maturation of this complementary vascular system rely on transcriptional regulation (22) and additional growth factors and signaling molecules such as Angiopoietin-2 (Ang-2) (64). Disruption in any of these signaling pathways leads to severe disturbances in, and often loss of all, lymphatic architecture and function (1, 23, 34, 38), and several of these molecules have been implicated in congenital human lymphatic disorders (1, 58). Moreover, endothelial NO signaling has also been shown to play an important role in promoting lymphatic flow in vitro (11, 24, 25) and in vivo (32).

We hypothesized that chronic increases in PBF lead to lymphatic aberrations, including endothelial dysfunction, resulting in decreased lymphatic flow. To test this hypothesis, we used our acute and chronic models of increased PBF (51, 55) and directly cannulated the efferent duct of the caudal mediastinal lymph node that represents lymphatic flow from the right lower lobe of the lung (50, 60). In this way, we quantified lung lymph flow under conditions of acutely and chronically increased PBF and measured NOx levels in lymph effluent to assess lymphatic endothelial function. We also quantified transit kinetics of contrast material through lung lymphatics by performing computed tomography (CT) lymphography. Finally, we assessed for evidence of alterations to pulmonary lymphatic architecture in chronic shunt lambs using CT lymphography, immunohistochemistry, and by measuring changes in protein expression of genes (VEGF-C, LYVE-1, and Ang-2) known to be important for the growth and development of the lymphatic vasculature (22, 34, 64).

**MATERIALS AND METHODS**

**Chronic model of increased PBF.** As previously described in detail (55), an 8.0-mm Gore-tex vascular graft, ~2 mm length (W.L. Gore and Associates, Milpitas, CA) was anastomosed between the ascending aorta and main pulmonary artery in a late-gestation fetus (137–141 days gestation, term = 145 days) from six mixed-breed Western pregnant ewes. Multiple gestation pregnancies provided the majority of normal age-matched control lambs.

Four weeks after spontaneous delivery, normal (n = 8) and shunt lambs (n = 6) were anesthetized, mechanically ventilated, and instrumented to continuously measure hemodynamics and pulmonary blood flow (55).

Through a right thoracotomy in the tenth intercostal space, the tail of the caudal mediastinal lymph node (CMN) was ligated at T10 (not shown), and the head of the CMN has been injected with Evan’s Blue Dye. The efferent duct of the CMN (CMNELD) is cannulated and lymph is collected. B: photograph of 4-wk lamb preparation showing surgical exposure of the right posteriolateral chest wall. The head of the CMN has been injected with Evan’s Blue Dye. Note the dye tracking along the CMNELD. C: rostrally, the lymph duct has been ligated with a 6-O silk tie, resulting in proximal dilatation (arrowhead) and allowing for lymph cannulation. Note as well the proximity of the thoracic duct.

Fig. 1. A: schematic of lung lymph collection. An ultrasonic flow probe measures pulmonary blood flow (PBF) through the left pulmonary artery. The tail of the caudal mediastinal lymph node (CMN) has been ligated at T10 (not shown), and the head of the CMN has been injected with Evan’s Blue Dye. The efferent lymph duct of the CMN (CMNELD) is cannulated and lymph is collected. B: photograph of 4-wk lamb preparation showing surgical exposure of the right posteriolateral chest wall. The head of the CMN has been injected with Evan’s Blue Dye. Note the dye tracking along the CMNELD. C: rostrally, the lymph duct has been ligated with a 6-O silk tie, resulting in proximal dilatation (arrowhead) and allowing for lymph cannulation. Note as well the proximity of the thoracic duct.
previously coated with heparin (tridodecylmethylammonium heparinate; Polysciences, Warrington, PA) was then inserted into the lymphatic vessel and advanced toward the head of the node. When lymph was freely flowing through the catheter, a proximal suture was tied to secure the catheter in the vessel. To minimize the known effects of variation in outflow pressure on lymph flow (17), the collection cannula was kept level with the efferent lymphatic vessel in all preparations. Similarly to avoid sudden changes in central venous pressure and its effects on lymph flow, we did not administer intravenous volume boluses immediately before or during lymph collection. Because positive end-expiratory pressure and positive pressure ventilation are known to affect (decrease) lymph flow (45), identical anesthetic protocol and similar ventilator settings were utilized on all study animals.

Animals’ vital signs, including core temperature, were monitored throughout the study, and they were given intravenous fluids and prophylactic antibiotics per protocol (55). After a 60-min recovery period, baseline values were obtained, and the hemodynamic variables and lymphatic drainage were monitored continuously for the duration of the study period. Baseline blood and lung biopsies were obtained. Lung tissue was excised, rinsed in ice-cold PBS, and apportioned for tissue histochemistry; Polysciences, Warrington, PA) was then inserted into the lymphatic vessel and advanced toward the head of the node. When lymph was freely flowing through the catheter, a proximal suture was tied to secure the catheter in the vessel. To minimize the known effects of variation in outflow pressure on lymph flow (17), the collection cannula was kept level with the efferent lymphatic vessel in all preparations. Similarly to avoid sudden changes in central venous pressure and its effects on lymph flow, we did not administer intravenous volume boluses immediately before or during lymph collection. Because positive end-expiratory pressure and positive pressure ventilation are known to affect (decrease) lymph flow (45), identical anesthetic protocol and similar ventilator settings were utilized on all study animals.

**Hemodynamic measurements.** Pulmonary and systemic arterial and right and left atrial pressures (LAP) were measured using Sorenson Neonatal Transducers (Abbott Critical Care Systems, N. Chicago, IL). Mean pressures were obtained by electrical integration. Heart rate was measured by a cardio-tachometer triggered from the phasic systemic arterial pressure pulse wave. Left PBF was measured with an ultrasonic flow meter (Transonic Systems, Ithaca, NY). All hemodynamic variables were measured continuously utilizing the Ponemah Physiologic Platform (Version 4.1) and Acquisition Interface (Model ACG-16; Data Sciences International, St. Paul, MN) and recorded with a Dell Inspiron 5160 computer (Dell, Round Rock, TX). Blood gases, pH, and arterial saturation were measured on a Radiometer ABL5 pH/blood gas analyzer (Radiometer, Copenhagen, Denmark). Hemoglobin concentration and oxyhemoglobin saturation were measured by a cooximeter (Model 682; Instrumentation Laboratory, Lexington, MA). Pulmonary vascular resistance was calculated using standard formulas. Qp:Qs was determined using the Fick principle. A modified pulse-waveform analysis was performed on the pulmonary artery pressure tracing to estimate pulmonary capillary pressure (Pcap) as previously described (13). Five pulse waveforms were sampled at 1,000 Hz during the steady-state expiratory phase and recorded the average value. Body temperature was monitored continuously with a rectal temperature probe.

**Thoracic roentgenograms.** A Sedecal high-frequency generator with 1000-mA Varian X-ray tube was used to obtain ventral-dorsal and lateral thoracic radiographs on spontaneously breathing 3–4-wk-old normal and chronic shunt lambs. Images were captured on Cannon CXD-50G 14 × 17 inch digital plates, transferred to a picture archive and communication system server, and evaluated using eFilm 2.1.2 (Merge Healthcare, Chicago, IL).

**CT lymphography.** Three weeks after spontaneous delivery, normal or chronic shunt lambs were sedated using ketamine (10 mg/kg IV) and diazepam (0.25 mg/kg IV) and were intubated with a 6.0-mm OD cuffed endotracheal tube and ventilated with a Hallowell 2000. Appropriate anesthesia was maintained using isoflurane (1.5–2.5%) with 100% oxygen. The lamb was monitored using a Criticare POET II 602, and blood pressure was followed using cuff manometry. The animals were placed in ventral recumbency on the CT couch, and the right chest wall overlying the ventral aspect of the right cranial lung lobe was shaved and cleaned. Following local infiltration of the injection area with 1–2 ml 2% lidocaine, a 27-gauge needle was inserted into the subpleural space, under ultrasonographic guidance using a wideband 5–8-MHz transducer (ATL HLD 5000; Phillips Medical Systems, Bothell, WA), and 2 ml of Visipaque (iodixanol), a nonionic iodinated contrast material (GE Healthcare, Princeton, NJ), was injected into the subpleural space and pulmonary parenchyma (36). Thoracic examinations were acquired using a helical CT scanner (GE Lightspeed 16; General Electric, Milwaukee, WI) immediately following and at 30, 60, and 75 min after injection. Examinations were performed using a forced breath-hold technique with airway pressure held at 10 cm water and scan times limited to 30 s. All examinations consisted of contiguous, transverse 0.6-mm collimated images, depending on the size of the lamb, using a moderately edge-enhancing reconstruction algorithm and 120-kVp/150-mAs technique. Field of view for all studies was determined by the smallest area to include the entire thorax. 3-D rendering was performed using GE Advantage Workstation 4.4 (GE Healthcare, Milwaukee, WI). The distribution of lymph was measured using a recently developed clinical method of lymphography by CT (36) and adapted to the pulmonary circulation. The radiographic intensity of the injected contrast material was measured at an initial time period time from a fixed two-dimensional CT slice using OsiriX (v.3.9.2.1) open-source imaging software. The same slice was remeasured at 30-min intervals for up to 120-min duration. The rate of dispersal of contrast material was calculated by determining the slope [Hounsfield units (H)/min]. Following the final scan, anesthesia was discontinued, and the animals were exsanguinated. They were recovered under direct observation until they were ambulatory and freely accessing food and water.

**Lymph/plasma protein ratio.** We used an ATAGO handheld SUR-NE refractometer (Bellevue, WA) to measure the protein concentration (g/dl) in lymph (L) and plasma (P) samples taken at concurrent time points. The L/P ratio was compared between normal and chronic shunt lambs and between post- and presamples in the acute study.
Measurement of NO. To quantify bioavailable NO, the concentration of NO and its metabolites were determined in lymph from chronic shunt, control, and acute-study lambs. In solution, NO reacts with molecular oxygen to form nitrite and with oxyhemoglobin and superoxide anion to form nitrate. Nitrite and nitrate are reduced using vanadium (III) and hydrochloric acid at 90°C. NO is purged from solution resulting in a peak of NO for subsequent detection by chemiluminescence (NOA 280; Sievers Instruments, Boulder, CO), as previously described (9, 47, 69). The sensitivity is $1 \times 10^{-12}$ mol, with a concentration range of $1 \times 10^{-9}$ to $1 \times 10^{-3}$ molar of nitrate.

Immunohistochemistry. Lung tissue harvested during the study were fixed in 4% PFA for 24 h at 4°C and then rinsed in cold PBS and transferred to a 30% sucrose/PBS solution. After 24 h at 4°C, these samples were embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetek USA, Torrance, CA), cryosectioned at 7 µm, collected onto Superfrost Plus slides (VWR Scientific, West Chester, PA), allowed to air-dry at room temperature, and stored at −80°C until needed. Multilabeling immunofluorescence was performed on serial 7-µm lung sections: tissue sections were allowed to come to room temperature, washed briefly in TBS to remove residual optimal cutting temperature compound, treated with 20 µg/ml proteinase K (Invitrogen, Life Technologies, Carlsbad, CA) in deionized water for 5 min, washed in TBS + Tween (0.03%) (TBSTw) 3 × 5 min, and blocked with Dako Antibody Diluent (Dako, Carpinteria, CA). Slides were placed in primary antibody diluent in 10% blocking serum overnight at 4°C. Antibody dilutions were as follows: 1:100 goat-lyVE1 (AF2089; R&D Systems, Minneapolis, MN), 1:100 rabbit-lyVE-1 (ab14917; Abcam, Cambridge, MA); 1:500 goat-cCL21 (AF366; R&D Systems, 1:200 mouse-αMysin (M7786; Sigma-Aldrich, St. Louis, MO). Slides were then washed 3 × 5 min in TBSTw and then placed in the appropriate secondary antibodies, Biotium (Biotium, Hayward, CA) or Alexa Fluor (Invitrogen, Life Technologies), in blocking serum for 60 min. Slides were washed in TBS for 4 × 5 min. The second-to-last wash contained DAPI (Invitrogen, Life Technologies) at 1:1,000 in TBS. Slides were mounted in Aquamount (Polysciences) and coverslipped.

Images were taken with a Hamamatsu c10600 ORCA-R2 Digital Camera on a Zeiss Axio Imager Z2 with Apotome System using ×5, 10, or ×20 DIC objectives, the X-cite 120 Mercury/Halide System, and analyzed using AxioVision 4.8.1 software (Carl Zeiss Microimaging, Thornwood, NY). A series of 20 images at 0.5–1.0-µm interval focal planes were collected into a z-stack to determine actual colocalization. Images were subsequently processed using Adobe Photoshop CS5 software (Adobe, San Jose, CA).

Preparation of protein extracts and Western blot analysis. Preparation of lung protein extracts and Western blot analysis was performed as previously described (6, 46, 68). Protein extract (25–50 µg) was used for each sample. Primary antibodies and their dilution are as follows: 1:300 rabbit-VEGF-C (ab95456; Abcam); 1:100 goat-lyVE-1 (AF2089; R&D Systems), 1:100 rabbit-lyVE-1 (ab14917; Abcam), or 1:80 rabbit-αAng-2 (AB10611; Millipore, Billerica, MA). The appropriate secondary antibodies were used, and the protein bands were visualized with ECL Western blotting reagents using high-performance chemiluminescence film (GE Healthcare, Little Chalfont, Buckinghamshire, UK) or using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Images were scanned using an Epson Perfection 4490 Photo Scanner with Epson Scan v. 3.01A (Seiko, Mahwah, NJ). All captured and analyzed images were analyzed with Adobe Photoshop CS 4 or 5 and were determined to be in the dynamic range of the system. To normalize for protein loading, blots were reprobed with the housekeeping protein, β-actin. The methodology and exposure times used were those that have previously demonstrated to be within the linear range able to detect changes in lung protein expression.

Statistical analysis. The means ± SD were calculated for hemodynamic variables, NOx levels, and Western blot analyses. Changes in variables within each animal before and after an intervention (i.e., unclipping of acute shunt) were compared by the paired t-test. Values between groups (i.e., control and shunt lambs) were compared by unpaired t-test. A P value of <0.05 was considered statistically significant. Analysis of covariance (ANCOVA) was used to evaluate if Pcap between groups demonstrated a similar dependence on LAP in the form of a regression equation: Pcap = slope × LAP + grand intercept + group intercept.

A statistically significant slope indicates dependence between Pcap and LAP, whereas the group intercept, if significantly different than zero, identifies a the contribution to Pcap beyond LAP of a group. Analysis was done using Prism 4.0c (GraphPad Software, La Jolla, CA).

RESULTS

For these studies, we used two distinct ovine models of increased PBF, an acute model and a chronic model. Comparisons in the acute model were made between preshunt and postshunt within the same animal. Comparisons in the chronic model were made between shunt lambs with age-matched, normal lambs.

Consistent with our previous studies, shunt lambs were tachypneic and failed to thrive (data not shown and Ref. 55). As seen in Fig. 2, both chest radiograph (Fig. 2, A and B) and chest CT (Fig. 2, C and D) demonstrate interstitial edema in the lungs of shunt lambs (Fig. 2, B and D) compared with normal age-matched lambs (Fig. 2, A and C).

**Hemodynamics.** Hemodynamic data for the acute and chronic models of increased PBF are presented in Table 1. Both models resulted in a significant increase in PBF (Table 1, Fig. 3A). In the acute model, PBF increased twofold postshunt opening, and in the chronic model, PBF was 4.2-fold higher in shunt-lambs compared with normal lambs at 4 wk of age.

**Lymph flow.** In the acute model, lymph flow was 3.5 ± 2.3 µl/min−1 kg−1 preshunt opening and increased to 7 ± 3.1 µl/min−1 kg−1 postshunt opening (n = 6, *P < 0.03) (Fig. 3B).

In the chronic model, lymph flow in shunt lambs was 9.5 ± 3.4 µl/min−1 kg−1 and was higher than normal lambs, 5.5 ± 2.2 µl/min−1 kg−1 (n = 6 shunt, 8 control, **P < 0.03). When normalized for PBF, the difference in averaged lymph flow postshunt opening to preshunt opening did not significantly change (P = 0.4) in the acute model; however, in the chronic model, the difference in the ratio of averaged lymph flow normalized to PBF between shunt and normal lambs was 0.07 ± 0.02, P = 0.003 (Fig. 3C). These data indicate that, under conditions of chronic increases in PBF, relative lymph flow is attenuated.

**Pcap.** To look at the association of PBF with Pcap, we evaluated Pcap from pulmonary arterial pressure waveforms (13) in each of our study groups. We found that, in the acute model, the calculated Pcap was higher after shunt opening than prior (14 ± 1.2 mmHg vs. 7.1 ± 1.2 mmHg, P < 0.009); similarly in the chronic model Pcap in normal control lambs was 7.2 ± 0.9 mmHg and was significantly elevated in chronic shunt lambs (15.6 ± 1.0 mmHg, P < 0.0001). Moreover, the slope of Pcap − LAP ANCOVA regression relationship was not statistically significant from zero (0.42 ± 0.43, P > 0.05), suggesting that Pcap was functionally independent of LAP.

**Lymph:plasma protein ratio.** To further assess the effects of increased PBF and Pcap on lung fluid and protein balance, we measured the lymph to plasma (L/P) protein ratio. In the acute model, the L/P protein ratio was 0.57 ± 0.15 preshunt opening and decreased to 0.34 ± 0.12 postshunt opening (n = 6, P = 0.0004, Fig. 4A). In the chronic model, the L/P protein ratio...
was 0.27 ± 0.16 in shunt lambs and was lower than normal lambs, 0.56 ± 0.10 (n = 6 shunt, 7 control, P = 0.002). The similar decrease in the L/P protein ratio with increased PBF in each model is consistent with the results of our Pcap calculations. This indicates that capillary intravascular hydrostatic pressure remains the main driving force for accumulation of interstitial fluid and suggests that capillary endothelial barrier function remains intact under both conditions; therefore, pulmonary interstitial fluid (and lymphatic effluent) remains protein poor as PBF increases.

**NOx levels in lymph effluent.** NOx levels (a measure of bioavailable NO) were determined in lymphatic effluent collected in both the acute and chronic models. In the acute model, NOx levels increased from 6.0 ± 3.2 µM preshunt opening to 6.5 ± 3.2 µM postshunt opening (n = 6; P = 0.03). However, in the chronic model, NOx levels were 36% lower in shunt

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**Table 1. Hemodynamic changes in the acute and chronic models of increased PBF**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ACUTE, pre</th>
<th>ACUTE, post</th>
<th>NORMAL</th>
<th>SHUNT</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPAP, mmHg</td>
<td>17 ± 7</td>
<td>21 ± 4</td>
<td>16 ± 2</td>
<td>26 ± 4†</td>
</tr>
<tr>
<td>Diastolic PA, mmHg</td>
<td>13 ± 2</td>
<td>15 ± 4</td>
<td>12 ± 2</td>
<td>21 ± 4†</td>
</tr>
<tr>
<td>Systolic PA, mmHg</td>
<td>22 ± 2</td>
<td>29 ± 8</td>
<td>23 ± 4</td>
<td>31 ± 5†</td>
</tr>
<tr>
<td>LA, mmHg</td>
<td>5 ± 1</td>
<td>8 ± 5</td>
<td>4 ± 4</td>
<td>9 ± 4*</td>
</tr>
<tr>
<td>RA, mmHg</td>
<td>5 ± 1</td>
<td>5 ± 1</td>
<td>3 ± 2</td>
<td>5 ± 3</td>
</tr>
<tr>
<td>MSAP, mmHg</td>
<td>63 ± 10</td>
<td>57 ± 14</td>
<td>75 ± 7</td>
<td>54 ± 11†</td>
</tr>
<tr>
<td>HR, beat/min</td>
<td>122 ± 20</td>
<td>156 ± 18</td>
<td>162 ± 19</td>
<td>152 ± 17†</td>
</tr>
<tr>
<td>Qp, ml·min⁻¹·kg⁻¹</td>
<td>53 ± 20</td>
<td>112 ± 37*</td>
<td>43 ± 10</td>
<td>181 ± 65†</td>
</tr>
<tr>
<td>Qp:Qs</td>
<td>1.0 ± 0.1</td>
<td>2.1 ± 0.3*</td>
<td>n/a</td>
<td>2.8 ± 0.3‡</td>
</tr>
<tr>
<td>LPVR, mmHg·ml⁻¹·min⁻¹·kg⁻¹</td>
<td>0.22 ± 0.05</td>
<td>0.12 ± 0.07</td>
<td>0.29 ± 0.12</td>
<td>0.10 ± 0.03‡</td>
</tr>
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</table>

Values are ± SD. The two columns on the left show changes in hemodynamic parameters before and after acute increases in pulmonary blood flow (PBF); the two columns on the right compare hemodynamic parameters between normal and shunt juvenile lambs. *P < 0.05 postacute vs. preacute, and †P < 0.05 shunt vs. normal chronic; ‡chronic shunt compared to an assumed ratio of pulmonary to systemic blood flow (Qp:Qs) of 1 for normal animals. MPAP, mean pulmonary arterial pressure; LA, left atrial pressure; RA, right atrial pressure; MSAP, mean systemic arterial pressure; HR, heart rate; Qp, left pulmonary arterial blood flow; LPVR, left pulmonary vascular resistance.
lambs. Washout analysis indicated that transit time of contrast increase in the overall lymphatic bed in the lungs of shunt higher volume of intralymphatic dye, or it may indicate an to lymphatic dilatation and increased conspicuity because of the shunt lymphatics; this increased density may be secondary lymphatics were dilated, pruned at the periphery, and blunted lymphatic bed. Compared with normal lung lymphatics, shunt Hounsfield units specific for iodinated contrast uptake in the subpleural space and pulmonary parenchyma of anesthetized and mechanically ventilated shunt or normal lambs. CT was increased PBF, we injected contrast dye (Visipaque) into the function of pulmonary lymphatics under chronic conditions of

Fluorescent immunohistochemistry. To better visualize and characterize the structural changes in pulmonary lymphatics at the microscopic level, we used lymph-specific markers on isolated vessels and peripheral lung tissue. Figure 6A demonstrates staining of an isolated CMN efferent lymphatic vessel from a normal lamb, confirming that chemokine ligand 21 (CCL21) specifically stains lymphatic endothelium but not isolated pulmonary artery (Fig. 6B) or isolated pulmonary vein (Fig. 6C). In Fig. 6D, double staining with LYVE-1 (488 nm, green) and CCL21 (694 nm, red) demonstrates specificity for lymphatics in peripheral lung that is also stained with DAPI (blue) to highlight the nuclei. There was overlapping expression of the two lymphatic markers (yellow) to a pulmonary (L) and a peribronchial lymphatic (PB). Consistent with the CT reconstruction and the washout analysis, pulmonary lymphatics in shunt lambs (Fig. 6, F and H) appear qualitatively larger but no different in number than in normal lambs (Fig. 6, E and G). Similarly in the acute study, we did not appreciate any qualitative difference in the size or number of lymphatics after opening the shunt (data not shown).

Western analysis. There was no significant difference in Ang-2, LYVE-1, and VEGF-C protein expression in peripheral lung homogenate prepared from biopsies taken before and after shunt opening in the acute model of increased PBF (Fig. 7A). However, there was a significant decrease of all three proteins in peripheral lung samples from shunt (Fig. 7B, n = 6, *P < 0.03 vs. acute pre). In the chronic model, the difference in the ratio of averaged lymph flow normalized to PBF between shunt and normal lambs was 0.07 ± 0.02 (n = 8 normal, 6 shunt lambs); **P = 0.003.

CT lymphography. To further evaluate the distribution and function of pulmonary lymphatics under chronic conditions of increased PBF, we injected contrast dye (Visipaque) into the subpleural space and pulmonary parenchyma of anesthetized and mechanically ventilated shunt or normal lambs. CT was performed at 75 min postinjection, (Fig. 5). We gated for Hounsfield units specific for iodinated contrast uptake in the lymphatic bed. Compared with normal lung lymphatics, shunt lymphatics were dilated, pruned at the periphery, and blunted (n = 3 control, 5 shunt). There was more absolute contrast in the shunt lymphatics; this increased density may be secondary to lymphatic dilatation and increased conspicuity because of higher volume of intralymphatic dye, or it may indicate an increase in the overall lymphatic bed in the lungs of shunt lambs. Washout analysis indicated that transit time of contrast material through the pulmonary lymphatics of shunt lambs was delayed nearly fivefold; the rate of decay of contrast intensity in controls (n = 3) was 19 ± 8.4 H/min and 3.9 ± 3.8 H/min in shunts (n = 5), P < 0.04. Thus effective lung lymph clearance under conditions of chronically increased PBF was impaired.

Fig. 3. A: PBF in ml·min⁻¹·kg⁻¹. B: lymph flow in μl·min⁻¹·kg⁻¹. In the acute model, PBF and lymph flow increased after the shunt was opened, n = 6; *P < 0.03 vs. acute pre. In the chronic model, PBF and lymph flow were greater in shunt lambs compared with normal lambs, n = 8 control, 6 shunt lambs; **P < 0.03 vs. normal chronic. C: when normalized for PBF, lymph flow is decreased following chronic increases in PBF. In the acute model, the difference in the ratio of averaged lymph flow normalized to PBF postshunt opening to preshunt opening was not significant (n = 6, P = 0.4); however, in the chronic model, the difference in the ratio of averaged lymph flow normalized to PBF between shunt and normal lambs was 0.07 ± 0.02 (n = 8 normal, 6 shunt lambs); **P = 0.003.

Fig. 4. A: Lymph/plasma (L/P) protein ratio in acute shunt studies, either with the shunt closed (preshunt), 0.57 ± 0.15 or open (postshunt), 0.34 ± 0.12, n = 6; *P < 0.003 vs. acute pre, L/P protein ratio from 4-wk normal lambs, 0.56 ± 0.1, and shunt lambs, 0.27 ± 0.16. n = 7 controls, 6 shunts; **P < 0.003 vs. normal chronic. **B: NOx levels were measured in lymph collected from 4-wk normal, 12.6 ± 3.1 μM and shunt lambs, 8.1 ± 1.3 μM, SD shown, n = 8 controls, 6 shunts; **P = 0.005 vs. normal chronic.
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DISCUSSION

The present study demonstrates alterations in the function and structure of lung lymphatics under conditions of chronically increased PBF. Our previous characterizations of the chronic shunt model demonstrated that it recapitulates the clinical symptomatology of tachypnea and failure to grow (33, 55). We now add to this characterization radiographic evidence of interstitial pulmonary edema and functional, anatomic, and molecular alterations in pulmonary lymphatics. Functional impairments include a decrease in protein-poor lymph flow normalized for PBF, associated with decreased bioavailable NO, and delayed lymph transit time through the lungs; anatomic alterations include lymphatic capillary dilatation; and molecular alterations include decreased expression of VEGF-C, LYVE-1, and Ang-2. Taken together, this study describes novel aberrations in the lymphatic circulation, with intact capillary endothelial integrity, under conditions of chronically increased PBF.

The effects of acute increases in PBF and/or capillary hydrostatic pressure on lung fluid dynamics and lymph flow have been studied previously in sheep models that included exercise, pneumonectomy, asymmetric pulmonary artery ligation, left atrial occlusion, and placement of central shunt (4, 12, 19, 26, 42, 50, 60). The results in this study demonstrate similar findings utilizing a surgically induced acute increase in PBF; this model produces increases in flow and pressure, recapitulating the changes associated with many surgical procedures for congenital heart disease. Consistent with these previous investigations, increases in lymph flow in this model were proportional to the increase in PBF (and capillary pressure). Of note, the present study was designed so that lambs with the acute and chronic models of increased PBF were studied at the same postgestational age with identically sized aorto-pulmonary grafts in place. Therefore, it is striking and novel that, in contrast to the well-described proportional increase in lymph flow with acutely increased PBF, chronic shunt lambs demonstrated a decrease in lymph flow when normalized for PBF.

We have previously described a twofold increase in pulmonary arterioles in the chronic shunt model (55). Therefore it was important to investigate whether the decrease in lymph flow in the setting of chronically increased PBF might be attributable to an increase in pulmonary vascular capacitance. This increased capacitance might decrease capillary hydrostatic pressure, transcapillary fluid filtration, and measured lung lymph flow. We therefore estimated capillary hydrostatic pressure by calculating Pcap in our different study groups. This analysis suggested that, as PBF increased, so did Pcap. To test this further, we measured the lymph-to-plasma-protein ratio in the acute and chronic models. In both models, the lymph-to-plasma-protein ratio decreased, indicating that increased intravascular hydrostatic forces promoted filtration of water and solutes across the pulmonary capillary endothelium into the interstitial compartment, in accordance with Starling principles. These data demonstrate that, despite any increase in the capacitance of the pulmonary vascular bed of shunt lambs, the increase in PBF led to an increase in capillary hydrostatic pressure. We also performed a regression analysis to determine whether there was a dependence of Pcap on LAP because LAP was higher in both acute and chronic shunt animals. This analysis indicates that, in either shunt model, the elevation in Pcap was independent of LAP. Therefore we conclude that pulmonary capillary hydrostatic pressure in acute and chronic shunt lambs was elevated due to increased PBF. Additionally, because the lymph-to-plasma-protein ratio declined to a similar extent in the acute and chronic models, chronic exposure to increased PBF did not result in measurable losses in pulmonary capillary barrier function, despite previously described pulmonary vascular endothelial dysfunction (52).

Ex vivo and in vivo studies demonstrate that NO is an important modulator of lymphatic pump activity and lymph flow. For example, in isolated and pressurized canine thoracic duct, flow has been shown to be an important stimulus for NO production by lymphatic endothelial cells (66). In rat mesenteric lymphatics, increased lymphatic contractions were associated with generation of NO (11). In addition, studies in the...
mouse tail have demonstrated decreased lymph flow velocity with either pharmacological or genetic inhibition of NO synthase (NOS), which is thought to be due to the effect of NO on the vascular tone of the collecting lymphatics (32). Taken together, the prevailing view is that, in response to an increase in interstitial fluid, lymphatic endothelial cells produce NO, which inhibits the active lymph pump but enhances lymph flow by decreasing resistance (31). To determine potential alterations in lymph NO signaling pathways in this study, we measured NOx concentrations in lymphatic effluent. In the acute model, the concentration of NOx in lymph increased by 7% after shunt opening. However, in the chronic model, the concentration of NOx in lymph was 36% lower in shunt lambs compared with normal controls. These data, although limited, suggest that NO may mediate pulmonary lymph flow similar to other vascular compartments. Interestingly, we have previously demonstrated increased lung tissue and plasma NOx concentrations in our chronic shunt model compared with normal controls, indicating that the decreased lymph effluent NOx concentrations in the present study reflect changes specific to the pulmonary lymphatic compartment (52). Further studies are warranted to determine the precise effects of lymphatic NO on pulmonary lymph flow.

In addition to lymphatic pump activity and lymph flow, NO signaling has been implicated in lymphangiogenesis. For example, exposure to an NO donor induces lymphatic endothelial
cell proliferation in a dose-dependent manner (37). In addition, VEGF-C, which can promote functional lymphangiogenesis in mice (57, 65) or rabbits (61) and help to resolve lymphedema, can promote lymphangiogenesis through activation of endothelial NOS (40). Interestingly, we observed a decrease in the expression of LYVE-1, VEGF-C, and Ang-2 protein in peripheral lung homogenate in the chronic, but not the acute, shunt model. LYVE-1, whose exact function still remains unclear, is expressed early during lymphatic endothelial cell specification (58, 64). The growth factor VEGF-C is essential for lymphatic sprouting and development (38, 64), and it has been shown to stimulate regeneration of functional lymphatics after injury or surgery (35, 65). Ang-2 is important for stabilization of post-natal lymphatics (23, 64); a lack of Ang-2 leads to defects in normal lymphatic remodeling and maturation and ineffective fluid transport (16). It is intriguing to consider that chronic increases in PBF caused by a fetal cardiac defect might disrupt normal post-natal lymphatic development in the lung; further investigations into these developmental pathways are warranted. Importantly, we did not observe any qualitative change in the number of lymphatic vessels in the lungs of chronic shunt animals by immunohistochemistry. However, we did identify enlarged lymphatic vessels, which is similar to what has been described in patients with breast cancer following axillary surgery (59) and in a mouse model of acquired lymphatic insufficiency (62) in which drainage of interstitial fluid was inadequate.

Other investigators have proposed that changes to capillary hydrostatic pressure and altered interstitial flow can lead to dynamic stress and pressure gradients that might promote morphogenic signaling that could induce lymphangiogenesis and promote lymphatic remodeling (10, 27, 28). However, in our chronic model of increased PBF, despite the chronic increase in capillary hydrostatic pressure, we observed impaired lymphatic flow, decreased bioavailable NO, and decreased expression of markers important lymphangiogenesis and remodeling: VEGF-C, LYVE-1, and Ang-2. Furthermore, washout analysis indicated that, in chronic shunt lambs, the transit time of contrast material through the lymphatics was markedly longer; thus pulmonary lymphatics in shunt lambs did not clear interstitial fluid as efficiently as normal juvenile lambs. Taken together, the evidence supports the conclusion that, with chronically increased PBF, there is impairment of extant lung lymphatics and disruption of new lymphatic growth and remodeling, which exacerbates overall pulmonary lymphatic function.

Important limitations to the present study are noteworthy. First, protein analysis of whole lung homogenate provides, at best, a crude survey of signaling molecules important for and specific to lymphatic development. Further studies are needed to isolate these aberrations to the lymphatics and to specific cell types. In addition, a detailed evaluation of lung mechanics and its association with these lymphatic abnormalities was not performed but also warrants further studies.

Both congenital and acquired lymphatic abnormalities are well recognized in patients with congenital heart disease (14, 18, 29, 43, 49, 56, 67) and remain a source of major morbidity and mortality in this patient population. Despite these observations, investigations of lymphatic aberrations in the setting of congenital heart disease are sparse. Utilizing a clinically relevant large animal model of a congenital cardiac defect with increased blood flow, the present study demonstrates developmental aberrations in both function and structure of the lymphatic circulation in the setting of chronically increased PBF and pressure. We also demonstrate a decrease in lymphatic NO production, which may play a role in the perturbation of
lymphatic function, as well as its postnatal development (31). A better understanding of these underlying mechanisms may lead to improved treatment strategies to help alleviate symptomatology and improve outcomes for these patients.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: S.A.D., P.E.O., and S.H.B. analyzed data; S.A.D. and E.T. edited and interpreted results of experiments; S.A.D. and E.G.J. prepared figures; S.A.D., E.G.J., M.A.M., and J.R.F. wrote the manuscript.
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