Published in final edited form as:

Angiogenesis. 2017 February ; 20(1): 73–84. doi:10.1007/s10456-016-9529-2.

Lymphangiogenesis in rat asthma model

Aigul Moldobaeva, John Jenkins, Qiong Zhong, and Elizabeth M. Wagner

Department of Medicine, Johns Hopkins University, Baltimore, MD

Abstract

Although bronchial angiogenesis has been well documented in allergic asthma, lymphangiogenesis has not been widely studied. Therefore, we evaluated changes in lung lymphatics in a rat model of allergen-induced asthma using house dust mite (Der p 1; 100 µg/challenge). Additionally, properties of isolated lung lymphatic endothelial cells (CD45⁻, CD141⁺, LYVE-1⁺, Prox-1⁺) were studied in vitro. Three weeks after the onset of intranasal allergen exposure (twice weekly), an increase in the number of lung lymphatic vessels was measured (34% increase) by lung morphometry. New lymphatic structures were seen predominantly in the peribronchial and periarterial interstitial space but also surrounding large airways. Isolated lymphatic endothelial cells from sensitized lungs showed enhanced proliferation (% Ki67⁺), chemotaxis, and tube formation (number and length) compared to lymphatic endothelial cells isolated from naïve rat lungs. This hyper-proliferative lymphangiogenic phenotype was preserved through multiple cell passages (2-8). Lymphatic endothelial cells isolated from naïve and HDM sensitized rats produced similar in vitro levels of VEGF-C, VEGF-D, and VEGFR3 protein, each recognized as critical lymphangiogenic factors. Inhibition with anti-VEGFR (axitinib, 0.1µM) blocked proliferation and chemotaxis. Results suggest that in vivo sensitization causes fundamental changes to lymphatic endothelium, which are retained in vitro, and may relate to VEGFR downstream signaling.

Keywords

Angiogenesis; lymphatic vessels; lung; house dust mite; allergen

INTRODUCTION

Airway biopsies as well as lung imaging demonstrate increased bronchial vascularity in the airway wall of allergic asthmatics (1-3) and allergically sensitized animal models (4, 5). Angiogenic vessels have long been known to have defective barrier function, such that fluid leakage in asthmatics occurs more readily than in normal blood vessels (2, 6). Airway edema results in further wall thickening, and this edema is known to increase airway narrowing and

Address all correspondence to: Elizabeth M. Wagner, Ph.D., Johns Hopkins Asthma and Allergy Center, Division of Pulmonary and Critical Care Medicine, 5501 Hopkins Bayview Circle, Baltimore, Maryland 21224, Telephone: 410-550-2506, FAX: 410-550-2612, wagnerem@jhmi.edu.

Author Contributions: Conception and design: AM, EMW

Experimental work: AM, JJ, QZ

Analysis and interpretation: AM, EMW

Manuscript: AM, EMW

responsiveness (7, 8). Unless there is a way to efficiently remove extravascular fluid, the likelihood of amplifying any acute airway spasm and limiting breathing, remains high. Although lymphatic vessels are the major conduit for extravascular fluid clearance in most organs, little is known about lung lymphatics in asthma. In other organs, tissue inflammation has been shown to elicit stimulus-specific increases in cytokines in lymphatic endothelial cells and proliferation of the lymphatic vasculature and draining lymph nodes (9). This lymphatic proliferative expansion suggests an increased capacity to remove edema fluid. Yet, the mere presence of airway edema in allergic asthma implies there is impaired fluid clearance. Contrary to expectations, Ebina showed that in cases of fatal asthma, there was an overall decrease in lymphatic area compared to that in normal subjects (10). Nor was there a change in lymphatic density in the upper airways of subjects with persistent rhinitis compared to normal subjects (11). Murine models have shown disparate results, with tracheal lymphatic vessels proliferating after ovalbumin sensitization (12), however no change was seen in the morphology of lung lymphatics after house dust mite sensitization (13). Shin and colleagues demonstrated that the Th2 cytokines IL-4 and IL-13, critical in the development of allergic asthma, served as negative regulators of lymphangiogenesis (14). Thus, the available data regarding allergen-induced inflammation shows disparities in results regarding lymphangiogenesis where increased fluid and inflammatory cell clearance appear to be an important physiologic process.

Due to the paucity of information concerning this important vascular network in asthma, we sought to determine its proliferative capacity in a relevant animal model. In the present work, we studied the appearance of lung lymphatics after house dust mite sensitization in a rat model where angiogenesis and airway hyperreactivity have been documented (15). We used a rat model since the rat bronchial vascular anatomy is similar to other larger mammals (16-18). Additionally, house dust mite causes hyperreactive airways disease in human subjects and therefore, the results should be more directly translatable to asthma pathology (19). Our results confirm lymphangiogenesis and provide a model to further query the structure and function of this network in removing airway edema fluid.

METHODS

Rat sensitization

All protocols were approved by the Johns Hopkins University Animal Care and Use Committee (Protocol # RA13M479). Male Brown Norway rats (BN, Charles River, 100 g) were given house dust mite allergen (HDM, Der p 1; 100 µg/challenge, Greer Laboratories) by intranasal aspiration twice/week for 3 weeks duration as done previously, and were not housed under barrier conditions (15). Rats were studied one day after the last intranasal challenge.

Histology

After rats were euthanized, lungs were immediately inflated with Z-fix (Anatech) to a pressure of 12 cmH₂O for 24 hr, then submerged in Z-fix for at least an additional 24 hr. Subsequently, the left lung was cut along its long axis into three sections (medial, intermediate, and lateral) and was embedded in paraffin. From each block, serial sections

were cut and stained with anti-LYVE-1 (see **Table 1** for antibody information) and counterstained with methyl green (0.1%; Sigma) and the chromagen liquid permanent red (Dako # K0640), which forms a permanent red reaction product at the site of the target antigen. Serial sections stained with hematoxylin and eosin (H&E) were obtained for all fixed sections. To acquire frozen sections, lungs were infused through the trachea with OCT (optimal cutting temperature) solution and cryosections (5 μ m) were produced for histological analysis. Sections were stained with anti-LYVE-1 for lymphatics, rhodamine *Griffonia simplicifolia* lectin for blood vessels and mounted with Prolong Gold antifade reagent with DAPI (all cell nuclei). Images were captured using OLYMPUS IX51 (Olympus) microscope. Additional images were captured using a confocal microscope (Zeiss LSM520).

Isolation of rat lung lymphatic endothelial cells (LEC)

Lungs from Brown Norway rats were dissected, minced and digested in collagenase (1 mg/ml; Sigma, 37°C for 15-20 min). The cellular digest was filtered through sterile mesh, centrifuged (440g for 10 min) and cells were labeled with biotinylated *Lycopersicon esculentum* lectin (labeling all endothelial cells; (20). Magnetic nanoparticles (EasySep Biotin selection kit; StemCell Technologies) were used for purification of cells according to the manufacturer's instructions and cells were cultured (0.2% gelatin-coated dishes in DMEM with 20% fetal bovine serum, antibiotics-antimycotics, 15 μ g/ml ECGS, 0.1 mM MEM nonessential amino acids, 2mM glutamine, 5 units/ml heparin). A second immune-purification step was performed after cells reached confluence using biotinylated anti-LYVE-1 and EasySep Biotin selection kit. The estimated yield from the right lung of a rat, after the second passage, was about 5×10^5 cells on average. Validation of these cells as lymphatic endothelial cells was performed using flow cytometry (FACS) at multiple passages while staining for LIVE (Live/Dead fluorescent staining kit; Invitrogen), CD141 (thrombomodulin; endothelial cell surface marker (21), CD45 (pan leukocyte marker), Prox-1 (lymphatic endothelial cell intracellular marker). As shown in a representative example (Figure 1), greater than 90% of live cells were CD141⁺, CD45⁻, Prox-1⁺. All in vitro experiments were carried out using lymphatic endothelial cells between passages 2-8 that were >90% positive for these markers by FACS.

Imaging of Lymphatic Endothelial Cells In Vitro

Lymphatic endothelial cells on 8-well slides ibiTreat (Ibidi GmbH) were fixed in 4% paraformaldehyde for 10 min at room temperature. The cells were then washed with PBS, permeabilized with 0.25% Triton X-100 in PBS for 5 min, and blocked with 5% BSA in PBS for 30 min. Cells were incubated with anti-LYVE-1 and anti-Prox-1 for 1 h at room temperature. After incubation with secondary fluorescent antibodies, slides were mounted with Prolong Gold antifade reagent with DAPI (all cell nuclei). Images were captured using an OLYMPUS IX51 microscope.

Proliferation assay

Lymphatic endothelial cells from naïve and HDM treated rats were plated on 6 well plates (50,000 cells/well) in 2% and 20% serum. 24hrs later, cells were briefly trypsinized and

labeled with anti-thrombomodulin and anti-LYVE-1 antibodies. Cells were fixed and permeabilized with fixation/permeabilization concentrate (eBioscience). To label proliferating cells, permeabilized cells were stained with anti-Ki67. Flow cytometry was performed with a FACS Aria (Becton Dickinson), and analyzed using FlowJo software (Tree Star). Lymphatic endothelial cell proliferation after VEGF receptor blockade was studied in some experiments treating cells with axitinib (0.1-10 μ M; Selleckchem) after plating. Axitinib is a tyrosine kinase inhibitor selective for vascular endothelial growth factor (VEGF) receptors -1, -2 and -3. (22)

Chemotaxis

Polycarbonate filters (5 μ m pore size, Corning Costar) were coated with 0.2% gelatin. LEC (3×10^4 cells) were added to the upper chamber of transwells (Corning) and incubated (37°C for 2 h; DMEM with 2% serum). Non-migrated cells were removed from the upper side of the membrane before the migrated cells of the lower chamber (bottom side of membrane) were fixed and stained (Diff-Quik stain set; Dade Behring). Membranes were mounted on slides and the migrated cells were counted under a microscope (20x objective) in five fields across each membrane and averaged (23). The effects of axitinib (0.1 μ M) on chemotaxis were evaluated by treating cells in the upper chamber immediately after plating.

Tube formation assay

The tube formation assay was carried out on angiogenesis slides (ibidi, GmbH), precoated with 10 μ l of Growth Factor Reduced Matrigel Matrix (BD Bioscience). LEC were seeded on Matrigel in triplicate (5×10^3 cells/well, incubated for 16h in DMEM with 2% serum). Images were taken of 3 fields in each well and the lengths (μ m) of the tubes of interconnecting cells were measured using Image J software.

Protein Expression

Because VEGF-C and VEGF-D have been shown to be critical to the process of lymphangiogenesis (24, 25), these proteins were evaluated in naïve and HDM cultured cells by ELISA (R&D). To measure their predominant receptor VEGFR3, tissue lysates were fractionated by SDS-PAGE and detected by Western blotting using anti-VEGFR3 antibody. Immunoblots were quantified by densitometry using Un-Scan-It Gel software (Silk Scientific). GAPDH was detected on immunoblots as a loading control for protein quantification.

Statistics

All data are presented as the mean $\pm$ the standard error. ANOVA was used to compare multiple groups followed by post-hoc analysis with Fisher's LSD Comparison test. Two sample comparisons (naive vs HDM) were made with unpaired t-tests. A p value ≤ 0.05 was accepted as significant.

RESULTS

Changes to lung structure after three weeks of HDM allergen challenge were evaluated by routine histology and compared to control lungs. **Figure 2** shows representative examples of

standard histologic sections (H&E) of the bronchovascular interstitial space from control (PBS) and HDM exposed rats. In the HDM exposed rats, the mass of dark staining inflammatory cells marks bronchus-associated lymphoid tissue (26, 34). Small bronchial arteries (red arrows) are prominent in this region of the airway wall. Enlarged lymphatic vessels (green arrows) are also seen in this histologic section from a rat exposed to HDM compared to a section from a control rat. Additional characterization of airway structures was obtained in frozen sections using anti-LYVE-1 to delineate lymphatic vessels. **Figure 3A** shows sections from lungs of HDM rats compared to PBS treated control rats (green; anti-LYVE-1, red: *Griffonia simplicifolia* lectin, blue: DAPI labels all cell nuclei). White arrows point to prominent lymphatic vessels. In the control rats, few lymphatics are only seen in the interstitial space between airway and pulmonary artery (PA). However, in the HDM sensitized rats, there seem to be additional new lymphatics positioned all around the airway wall, some appearing collapsed in these frozen sections. Additional images (**Figure 3B**) show increased anti-LYVE-1 label predominantly in the regions between airway and pulmonary artery with sparse to no staining in the lung periphery.

To obtain multiple lung sections for quantitative determination of lymphatic vessel numbers, lungs stained with anti-LYVE-1 antibody (**Figure 4A**) were evaluated. Arrows indicate prominent lymphatic vessels (red staining) in low power images (left panels) in both naïve and HDM exposed rats. Visual inspection suggests increased prominence of LYVE-1⁺ vessels in HDM lungs with morphometric results presented in **Figure 4B**. A significant 34% increase (*p=0.036) in the number of LYVE-1⁺ lymphatic vessels was enumerated in HDM treated rats compared to naïve rats (n=3 rats/group, 3 slices/rat). In each lung slice, the entire left lung was evaluated with most LYVE-1⁺ structures associated with airways, pulmonary arteries, and to a lesser extent, pulmonary veins. A few parenchymal structures were also counted. Airway blood vessel numbers were confirmed to be increased in these sections as previously reported (15). Average number of bronchial blood vessels increased significantly by 28% (naïve: 7.4±0.8, HDM: 9.5±0.8; p=0.03). Furthermore, the relationship between airway size and blood vessel number was significantly increased after HDM compared to naïve (p=0.002) as previously noted (15). Thus both lymphatic endothelial vessels and bronchial blood vessels increased after HDM exposure.

To evaluate lymphatic vessels for growth potential, we extracted lung lymphatic endothelial cells from naïve rats and from rats after 3 weeks of HDM exposure. Growth potential was determined by measuring proliferation, chemotaxis, and tube formation in vitro (cells from 3-4 rats/group). **Figure 5** shows these results. Lymphatic endothelial cells isolated from HDM rats and studied after 24 hrs in 2% serum as well as 20% serum, demonstrated significantly enhanced proliferation (% Ki67⁺) compared to lymphatic endothelial cells isolated from naïve control rats (**Figure 5A**; *p=0.014; naïve vs HDM). No additional stimulant was added however, there were detectable levels in serum of VEGF-C (2% serum: 110 pg/ml; 20% serum: 152 pg/ml) and VEGF-D (2% serum: 106 pg/ml; 20% serum: 270 pg/ml). Since the higher serum conditions had such a marked effect on both naïve and HDM cells, subsequent lymphangiogenesis studies were conducted only in 2% serum to examine basal properties. Chemotaxis of HDM lymphatic endothelial cells was markedly greater than cells from naïve rats (**Figure 5B**; unpaired t-test; *p=0.003). **Figure 5C** shows examples of

tube formation in vitro cultures of naïve and HDM lymphatic endothelial cells. Many more tubes with branching morphology are observed in the HDM cells compared to naïve cultures. Both the sum of all tube lengths/high power field and the number of tubes/well were significantly greater in HDM cells than naïve cells (**Figure 5D**; both are *p 0.05).

To explore one potential mechanism for the differences in overall growth potential of lymphatic endothelial cells of naïve rats compared to HDM exposed rats, protein levels of critical growth components VEGF-C, VEGF-D, and VEGFR3 protein levels were measured (cells from n=3 rats/group). **Figure 6A** shows the growth factors in both the supernatant and cell fraction normalized for total cell protein. There was no difference between the naïve and HDM cells with regard to these proteins. Interestingly, secreted VEGF-D was quite abundant in the measured supernatant. Supernatant values were corrected for background serum level. Additionally, there were no differences observed between the naïve and HDM cells in their expression of VEGFR3. (**Figure 6B**). Thus the factors typically demonstrated to be most critical for lymphatic growth did not differ between naïve and HDM cells. An additional experiment evaluating functional involvement of VEGFR pathways in proliferation and chemotaxis was performed. Using the VEGFR1,2,3 inhibitor axitinib, a dose-response assessment on lymphatic endothelial cell proliferation was determined (cells from n=3 rats/group). In **Figure 7A** the increased proliferative response of HDM lymphatic endothelial cells compared to naïve cells after vehicle treatment was confirmed and similar to that shown in Figure 5A. Axitinib had a significant anti-proliferative effect on both lymphatic endothelial cell types demonstrating the dominance of the VEGF receptors for proliferation (*p<0.05 vs naïve vehicle). Axitinib (0.1µM) blocked the HDM-induced increase in chemotaxis as well as reduced basal chemotaxis in naïve lymphatic endothelial cells (**Figure 7B**; n=4-5/group; *p<0.05 vs naïve vehicle, # p<0.05 vs HDM vehicle).

DISCUSSION

Although lymphatic vessels are the major conduit for extravascular fluid clearance in most organs and are important for immune cell transit, little is known about the specific role of lung lymphatics in asthma. Nor has the process of lymphangiogenesis been widely studied in the lung. The major findings of the current study demonstrate that lymphangiogenesis accompanies angiogenesis in a rat asthma model. Furthermore, this proliferative phenotype was retained in lymphatic endothelial cells that were isolated from sensitized rats and studied in vitro. VEGF receptor blockade prevented proliferation in both naïve and HDM lymphatic endothelial cells yet similar levels of VEGF-C, VEGF-D, and VEGFR3 were measured in the two cell types. Results suggest that in vivo antigen sensitization leads to changes in other growth factors, co-receptors, or VEGFR downstream signaling that promote lymphangiogenesis and network expansion.

Numerous studies have confirmed that lymphangiogenesis in the adult organism occurs primarily through the binding of VEGF-C and VEGF-D to lymphatic endothelial cell VEGFR3 (24, 27, 28). The cells responsible for the secretion of these two ligands varies among models yet CD11b⁺ macrophages have been shown to play a critical role in growth factor release (25, 29, 30). The process of lymphatic endothelial cell proliferation, chemotaxis, and tube formation has been extensively studied predominantly in context with

tumor growth and metastasis (31). Within the lung, however, few studies have explored this process. The McDonald laboratory, in a series of elegant studies, have documented the process of lymphangiogenesis in the trachea after ovalbumin sensitization (12) and more extensively, bacterial infection (32-34). During these inflammatory conditions, new lymphatic vessels arise in conjunction with new tracheal blood vessels, and their growth appears to be dependent on VEGF-C/D and VEGFR3 (32), TNF- α (35) and IL- β (36). Morphologic changes in new lymphatics during inflammation suggested an impaired capacity to clear fluid (37). New and remodeled initial lymphatics, the site of interstitial fluid absorption, showed continuous tight junctions normally seen predominantly in collecting lymphatics (38). Thus, contrary to what might be predicted, this dysfunctional lymphangiogenesis in the trachea did not promote enhanced edema clearance (37). Kretschmer and colleagues, using precision cut lung slices from mice, labeled the lymphatic network and showed the drainage pathways important for T-cell transit after HDM sensitization (13). However, they saw no difference in the distribution and morphology of lymph vessels after three HDM challenges. To put their work in context with the present study, we showed lymphangiogenesis in the rat after twice the HDM exposure these authors used.

With regard to lymphangiogenesis in human subjects, relatively little information exists regarding the manifestation of lymphatic vessels and their function in patients with lung disease. Ebina showed that in cases of fatal asthma, there was an overall decrease in lymphatic area compared to normal subjects (10). Increased number and altered phenotype of lymphatic vessels was seen in peripheral lung compartments of patients with airway inflammation in COPD (39). The role of lymphangiogenesis has also been investigated more recently with regard to interstitial lung disease (40, 41), but there was no definitive functionality of new lymphatic vessels apparent. In patients after lung transplant, results suggested that stimulation of lymphangiogenesis improved allograft outcome due to clearance of low MW hyaluronan fragments in patients after lung transplant (42).

Thus, the limited available information regarding lung lymphatic vessels in allergic asthma provided the premise for this study. Our past work demonstrated both bronchial angiogenesis and airway hyperreactivity in Brown Norway rats after house dust mite exposure (15). Therefore, we repeated the same antigen exposure regimen and studied lymphangiogenesis. We confirmed increased bronchial vascularity after 3 weeks of twice-weekly antigen exposure and showed increased lymphatic numbers. Morphometric determination of lymphatic vessels required fidelity of immunostaining with LYVE-1 antibody, which binds to hyaluronan receptors on lymphatic endothelial cells (20). Both frozen and fixed lung sections demonstrated positive staining with this approach. The morphometry of pulmonary lymphatic vessels in the rat has been carefully documented by the seminal work of Leak (43). He showed that collecting lymphatics and lymphatic capillaries are located largely within peribronchial and perivascular connective tissue, with sparse distribution in the pleura. Additional work confirming rat lymphatic anatomy has been performed by Ohtani (44). Within our fixed tissue specimens, LYVE-1⁺ staining was seen predominantly in the peribronchial and periarterial interstitial space but also in the airway wall and surrounding some pulmonary venules. Yet, neither the onset of lymphangiogenesis nor whether this process continues with longer periods of antigen

exposure could be discerned from the current study design. Additional experiments are required to confirm whether the magnitude of the increase in lymphatic vessel number (34%) would stabilize after 3 weeks of antigen exposure. However, in vitro work demonstrated a sustained change in proliferative capacity of isolated lymphatic endothelium.

We isolated lymphatic endothelial cells from lung digest using several different markers of lymphatic endothelial cells. Our extraction methods utilized positive selection of endothelial cells with the pan endothelial cell marker *Lycopersicon esculentum* agglutinin (20), followed by use of an antibody to the lymphatic receptor LYVE-1. Verification of these passaged endothelial cells, was performed by flow cytometry where we relied on the intracellular marker Prox-1, which has been shown to reside exclusively in lymphatic endothelium (45-47). With the additional digestive process required for flow cytometry, use of the intracellular marker Prox-1 proved more robust and consistent than surface markers such as VEGFR3 and LYVE-1. Whether examining Prox-1 expression as a proportion of all live cells or all endothelial cells, greater than 90% was achieved up through 8 passages of in vitro cell culture. This approach was used as exclusion criteria for acceptable cell cultures. Traditional characterization of proliferation, chemotaxis, and tube formation, showed that the cells isolated from antigen-challenged rats demonstrated enhanced proliferative capacity compared to cells isolated from naïve rats.

To further probe the differences between naïve and HDM cells, we studied protein expression of the predominant lymphatic growth factors, VEGF-C and VEGF-D, and their primary receptor VEGFR3 in cell cultures. Cells showed significant levels of VEGF-C and VEGF-D, as well as secreted VEGF-D protein and VEGFR3. However, these did not differ between naïve and HDM cells.

Since VEGF receptors are known to be involved in the studied proliferative characteristics, we used a broad spectrum VEGF receptor antagonist (axitinib) to determine whether the hyper-proliferative responses of HDM exposed cells could be altered. Axitinib has been shown to block VEGFR1-3 (22). Enhanced proliferation and chemotaxis of HDM cells were blocked by this VEGFR antagonist. However, naïve cells were similarly affected. These studies showed the essential nature of VEGF signaling pathways in lymphatic endothelial cell proliferation and chemotaxis. Yet, the in vitro results do not distinguish between mechanisms responsible for the hyper-proliferative state of the HDM cells. We speculate that the functional differences observed in the HDM cells could be due to changes in receptor-binding kinetics, altered co-receptors, additional secondary growth factors, signaling components downstream from VEGFR3, or other factors. Although lymphatic endothelial cells from antigen sensitized rats retained a hyper-proliferative phenotype in vitro, further experiments are required to determine the specific mechanism.

Whether lymphangiogenesis is linked to angiogenesis is not known. Our results demonstrate an increase in bronchial blood vessels at the time when we observed an increase in lymphatic vessel numbers. Perhaps relevant to this observation are results of Sweat and colleagues who showed that in the mesentery, angiogenesis always preceded lymphangiogenesis (28, 48). Treatment with VEGF-C, the most prominent lymphangiogenic factor, led first to angiogenesis followed by subsequent lymphangiogenesis. These limited

studies are consistent with a growing understanding that angiocrines, growth factors released by blood vessel endothelium, can play an important role within a vascular niche by directing proliferation of other cell types. This conjecture is supported by results suggesting potential endothelial cell crosstalk in this model (49).

With regard to the functional importance of allergen-induced lymphangiogenesis in the lung, we can only speculate as to whether this process is a part of the pathologic sequelae of antigen sensitization or is a homeostatic response to clear excess edema fluid, inflammatory cells and antigen burden. The primary physiologic function of lung lymphatics is for edema fluid clearance. In chronic airways disease, such as asthma, the low level of inflammation caused by allergic sensitization leads to increased airway vascularity (1, 2, 3) and as our results show, increased lymphatic vessels. Both factors suggest the presence of excess fluid in and around the airway wall. In the airways, however, where systemic bronchial vessels predominate, details about how this critically important function is handled are not well understood, particularly about how the lymphatics adapt with chronic disease.

Another vital function of lung lymphatics is for immune surveillance. Inflammatory cells in the lung are taken up by lymphatic vessels and transit to draining lymph nodes where antigens are processed. However, more recent data indicates that lymphatics are more than a transportation system and lymphatic endothelial cells can recruit and activate inflammatory cells (30, 50). Lymphatic endothelial cells from bronchus-associated lymphoid tissue were shown to be Thy1⁺ IL-7⁺ IL-33⁺ and these cells were critical in supporting pathogenic Th2 cells during chronic allergen inflammation in human and mice (51). Results suggest that pathogenic lymphatic endothelial cells promote maintenance of T-cells at the local inflammatory sites within airways. At apparent odds with this concept is that lymphatic vessel density was decreased when the Th2 cells or their cytokines IL-4 and IL-13 were co-cultured with lymphatic endothelial cells (14). Thus, it is unclear whether the hyper-proliferative lymphatic network functions more as a conduit for fluid and cell movement or whether inflammatory cell recruitment and activation by lymphatic endothelial cells serve the more prominent function.

In summary, we have shown an increase in the number of lymphatic vessels that parallels the increase in bronchial blood vessels after antigen sensitization with house dust mite in rats. Lymphatic endothelial cells isolated from sensitized rats demonstrate a hyper-proliferative phenotype in culture, and this increase is likely due to signaling pathways downstream from VEGFR3. Although an increased lymphangiogenesis in asthmatic sensitized airways is now well documented, its functional consequences remain to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Research Support: HL10342 and HL113392

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Figure 1. Representative example showing FACS verification of lymphatic endothelial cells

Cells for in vitro culture were positively selected with *Lycopersicon esculentum* agglutinin (endothelial cells), and LYVE-1 (lymphatic endothelial cells) during the proliferation phase. Cells were characterized by flow cytometry after digestion and staining. In this representative example, 94.2% of cells were LIVE cells. Of that population, 97% were endothelial cells (CD141⁺) and not leukocytes (CD45⁻). Of this population 94.2% were Prox-1⁺, a unique intracellular lymphatic endothelial cell marker. This example was from 8th passage lymphatic endothelial cells.

Figure 2. H&E stained histologic sections of the bronchovascular interstitial space from control (PBS) and HDM exposed rats

In the HDM exposed rats, bronchial-associated lymphoid tissue is marked by the mass of black staining cells located adjacent to increased numbers of small bronchial arteries (red arrows). Enlarged lymphatic vessels (green arrows) are also found in this space with HDM exposure compared to control. Bar = 100 μ m

A

Figure 3A. Cross sections of frozen lung from control (PBS) and HDM rats

Green staining is anti-LYVE-1 (lymphatics), red is *Griffonia simplicifolia* lectin (pulmonary artery; PA), and blue is DAPI (all cell nuclei). White arrows indicate lymphatic vessels. In the control rats, lymphatics are seen in the interstitial space between airways and pulmonary arteries, whereas in lungs from HDM sensitized rats, additional lymphatics appear all around the airway wall. Bar = 50 μ m

B

Figure 3B. Confocal images from naïve and HDM rats

White arrows in left panels indicate region of enhanced magnification in right panels. Arrows in high magnification panels on right indicate sparse anti-LYVE-1 lymphatic endothelial staining (green) in the interstitial area between a pulmonary artery (PA; red: *Griffonia simplicifolia* lectin) and an airway outlined by DAPI (blue). The HDM section shows more abundant anti-LYVE-1 lymphatic endothelial staining. Left panel bars = 100 μ m; Right panel bars= 50 μ m

Figure 4A. Fixed lung sections used for lymphatic quantification

Red, chromagen stained LYVE-1⁺ vessels (arrows) are sparse in naïve airways (upper images) compared to HDM lung (lower panels). Right panels are increased magnification of left panel regions indicated by arrows. Bar = 100 μm

B

Figure 4B. Quantification of lymphatic vessel number in naïve and HDM lungs

A significant increase in the number of LYVE-1⁺ lymphatic vessels was observed in HDM treated rats compared to naïve rats. Each point represents the number (#) of lymphatic vessels from one rat (n=3 rats/group). * indicates p=0.036

A

Figure 5A. In vitro LEC proliferation (Ki67⁺) of cells isolated from naïve versus HDM exposed rats

Lymphatic endothelial cell isolated from HDM rats and cultured in 2% serum as well as 20% serum, demonstrated significantly enhanced proliferation compared to lymphatic endothelial cells isolated from naïve rats. * indicates p=0.02 vs naive

B

Figure 5B. In vitro LEC chemotaxis of cells isolated from naïve versus HDM exposed rats
Chemotaxis (cell #/ field) of HDM lymphatic endothelial cells was markedly greater than naïve lymphatic endothelial cells. * indicates $p=0.003$

Figure 5C. Example of tube formation of LEC isolated from naïve versus HDM exposed rats
Bar = 50 µm

D

Figure 5D. Quantification of tube formation

Both the sum (Σ) of all tube lengths/high power field and the number of tubes/well were significantly greater in HDM lymphatic endothelial cells compared to those from naïve rats. * indicates $p < 0.05$

A

Figure 6A. VEGF-C and VEGF-D protein in cultured LEC

The levels of VEGF-C and VEGF-D protein in cell pellets as well as secreted in culture media are summed for naïve and HDM lymphatic endothelial cells normalized for total cell protein (pg/mg protein). No difference in VEGF-C or VEGF-D was seen between naïve and lymphatic endothelial cells.

B

Figure 6B. VEGFR3 protein in cultured LEC

No differences were observed in VEGFR3 normalized to GAPDH in naïve and HDM lymphatic endothelial cells.

Figure 7A. Effects of anti-VEGF receptor blockade on LEC proliferation

Axitinib, a VEGFR1-3 receptor antagonist, had a significant anti-proliferative effect on both naïve and HDM lymphatic endothelial cells and eliminated the differences observed in HDM-induced proliferation at both inhibitor concentrations.

*p<0.05 vs naïve vehicle

Figure 7B. Effects of anti-VEGF receptor blockade on LEC chemotaxis

Axitinib had a significant effect on cell chemotaxis in both naïve and HDM lymphatic endothelial cells and eliminated the differences observed in HDM-induced proliferation.

* $p < 0.05$ vs naïve vehicle, # $p < 0.05$ vs HDM

Table 1

List of antibodies used for immunostaining, flow cytometry, Western analysis, and vendor source.

Antibody	Fluorochrome/Clone	Source
<i>Histology:</i>		
LYVE-1 (fixed)	ab11-034 Permanent Red	Angiobio; Dako
LYVE-1 (frozen)	Alexa Fluor 488, rabbit polyclonal ab10278	Abcam
Prox-1	Rabbit polyclonal ab37128	R&D Systems
<i>Griffonia simplicifolia</i> lectin	Fluorescein, cat # ZA1010	Vector laboratories
<i>Lycopersicon esculentum</i> lectin biotinylated	cat # B-1175	Vector Laboratories
<i>Flow cytometry:</i>		
CD141 (thrombomodulin)	FITC, mouse monoclonal clone LS17-9 , 53-1411-82	eBioscience
CD45	APC-Cy7, clone OX-1, cat# 202216	Biolegend
LYVE-1	Alexa Fluor 647, rabbit polyclonal, bs-1311R0A647	Bioss
Prox-1	PE , rabbit polyclonal, cat# 040484	USBiological
Ki67	PE, mouse anti-human clone B56	BD Pharmingen
<i>Western:</i>		
VEGFR3	Pacific Blue, rabbit polyclonal	Bioss