Suggested Title: Recruited macrophages are preferentially associated with the regulation of lymphangiogenic activity, over resident macrophages, in a murine bleomycin-induced lung injury model.

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Abstract: Pending till last (allowed 300 words).

INTRODUCTION:

The pathology of lymphatic vessels and their endothelial cells continues to be a rapidly growing field of vascular biology. Lymphatic vessels are involved in important biological functions such as drainage of interstitial fluids and proteins to the blood stream and the transport of immune cells and nutrients. Lymphangiogenesis, the process of growth and formation of new lymphatic vessels, It can occur when lymphatic endothelial cells (LECs) are stimulated by inflammation- or tumor-associated factors. It occurs in in various pathological conditions, including wound healing, tissue and organ regeneration, in acute and chronic inflammation, autoimmunity, and tumor growth and tumor metastasis [1-4]. Lymphangiogenesis is regulated by multiple signaling pathways which
coordinate proliferation, sprouting and migration of lymphatic endothelial cells (LECs). Though the molecular mechanisms regulating the growth and function of lymphatic vessels are not yet clearly defined, some of the milestone findings in this field include the findings of the lymphatic endothelial cell–specific growth factor VEGF-C, its tyrosine kinase receptor, VEGFR-3 (Flt4) [5-7] and a second VEGF-C receptor, VEGFR-2 (KDR), which is predominantly expressed by activated endothelia of blood vessels [6, 8]. Structurally related to VEGF-C is VEGF-D, which also binds and activates VEGFR-2 and VEGFR-3, demonstrating a function in the stimulation of lymphangiogenesis [9].

Podoplanin, a membrane mucoprotein is a selective marker expressed abundantly in lymphatic endothelial cells [10]. Podoplanin is expressed in various human tumor cells [10-15] and promotes tumor cell spreading, migration and invasion [16-19]. As well as in carcinogenesis, a role of podoplanin in tissue development and repair has been suggested [18]. Differences between the expression of podoplanin between normal and disease associated lymphatics, however, are yet to be elucidated. The transcription factor prospero-related homeobox 1 (Prox-1) [20, 21] is considered one of the most specific lineage markers for lymphatic endothelium and is exclusively detected in lymphatic vessels of adult tissues and tumors [22]. Vascular structures expressing podoplanin are also positive for Prox-1 [19]. Finally, Lymphatic endothelium–specific hyaluronic acid receptor 1 (LYVE-1), a homologue of the blood vascular endothelium-specific hyaluronan receptor CD44 [23] has also been identified as a specific cell surface protein of LECs and macrophages.

VEGF-C can also up regulate the production of the chemokine CCL21 on lymphatic vessels [24]. CCL21 is believed to play an important role in the initiation of an immune
response by colocalizing naive T cells with DC-presenting antigen [25]. The receptor for CCL21, CCR7, is expressed on all naive T cells, some memory T cells, B cells, and mature dendritic cells and plays a central role in lymphocyte trafficking and homing to lymph nodes [26]. The structure and function of lymphatic vessels may be moderated by inflammation induced by harmful external or internal stimuli including pathogens, damaged cells and irritants [27], as such, lymphangiogenesis may have various functional consequences on inflammation depending on the circumstances and timing of its occurrence.

Macrophages may also play a role in lymphangiogenesis. VEGF-C and its receptor VEGFR-3 have been described to be produced and expressed by activated macrophages [28, 29] and a subpopulation of these cells may be reprogrammed to produce large amounts of VEGF-C and thus induce the development of preexisting lymphatic endothelial cells [29]. It is known that CD11b+ macrophages produce VEGF-C and -D [30] and that these cytokines on conjunctival lymphatic vessels via VEGFR-3 leads to the induction of lymphangiogenesis in the injured cornea [31]. Depletion of macrophages in multiple systems, demonstrate that macrophages are indispensable in driving lymphangiogenesis in inflamed peripheral tissues [32, 33].

It still remains to be clarified whether during lymphangiogenesis, endothelial cells in new vessels are derived from circulating progenitors [34], or whether they are derived from local preexisting vessels by cell division and growth [35]. Lymphangiogenesis is associated with multiple inflammatory diseases and may represent an attractive therapeutic target for inflammatory diseases. A better understanding of how
Lymphangiogenesis is regulated and its contribution to inflammation may benefit clinical research that targets chronic inflammatory diseases.

Intratracheal instillation of bleomycin into mice is widely used as an in vivo model to study inflammation and fibrosis in the lung interstitium that are present in interstitial pneumonias such as UIP and NSIP [36-39]. Despite it's limitations, the bleomycin model of pulmonary fibrosis is the best characterized murine model in use today. The development of fibrosis in this model can be seen by day 14, however, the response to bleomycin is variable. Intratracheally may induce fibrosis that progresses or persists (Thrall RS, Bleomycin-induced pulmonary fibrosis in the rat: inhibition by indomethacin. Am J Pathol 95: 117–130, 1979) Goldstein RH, Lucey EC, Franzblau C, Snider GL. Failure of mechanical properties to parallel changes in lung connective tissue composition in bleomycin-induced pulmonary fibrosis in hamsters. Am Rev Respir Dis 120: 67–73, 1979), on the other hand, a self-limiting response that begins to resolve may also occur (Lawson WE. Increased and prolonged pulmonary fibrosis in surfactant protein C-deficient mice following intratracheal bleomycin. Am J Pathol 167: 1267–1277, 2005) (Phan SH. A comparative study of pulmonary fibrosis induced by bleomycin and an O2 metabolite producing enzyme system. Chest 83: 44S– 45S, 1983). The resolving nature of this model does not mimic human disease. In humans, the disease is slow and progressive disease, taking 10 –20 years to present, while in animal models presentation occurs around 21–28 days. This feature of the animal model provides an opportunity for studies of fibrotic resolution at later time points that are not straightforwardly feasible in human studies.
To further describe the characteristics of lymphangiogenesis, we used a time course model of bleomycin induced lung fibrosis in C57BL/6 mice to examine the relationship between inflammation, the pathological processes and tissue macrophages in the immune response. Using flow cytometry on whole lung digests and stereology on formalin-fixed and paraffin-embedded lung tissues respectively, we assessed inflammation and lymphatic vascular expansion by following the expression of VEGFR2, VEGFR3 and podoplanin on lymphatic endothelial cells over time. We also investigated the tissue macrophage population to determine which sub-set of macrophages was predominantly involved in the regulation of lymphangiogenic activity in bleomycin-induced lung fibrosis mouse model.

**MATERIALS AND METHODS:**

**Animal Studies**

Mouse studies were approved by the Institutional Animal Care and Use Committee at the University of Colorado Denver. The C57BL/6 mice (male, 6-8 weeks old) used were purchased from The Jackson Laboratory (The Jackson Laboratory, California, USA). A total of five mice were used per experimental/test group. An untreated mouse was added for control purposes.

**Bleomycin Treatment**

Bleomycin sulphate (company) was dissolved in sterile PBS/saline and administered as a single dose of 60IU/kg per animal. All animals in the experimental groups received intratracheal (IT) instillations of bleomucin on day 0. Mice were randomly assigned to
four IT bleomycin treatment groups: Day 7, Day 10, Day 14 and Day 21 with 4-5 mice assigned for each time point.

**Bronchoalveolar Lavage**

On days 7, 10, 14 and 21 respectively, mice were sacrificed and the thoracic cavity opened to expose the lungs. Bronchoalveolar lavage was performed through a tracheal cannula with 1ml of PBS (pH 7.4). This procedure was repeated three times. Cold sterile PBS (1ml) was used to inflate the lung, and the lavage fluid was recovered. Total viable cell counts were determined on a hemocytometer using trypan blue exclusion. Differential counts of neutrophils and macrophages were determined on cytospin smears of BAL samples. In brief, 150 μL of cell suspension was centrifuged in a Shandon Single Cytofunnel (Thermo Scientific, USA) at 650 rpm for 3 minutes. The slide produced was air dried, fixed and stained using 3 Hema 3 step stain set (Fisher Scientific, USA). The slides were rinsed in water twice and allowed to dry, then cover glasses were applied with mounting medium (Fisher Scientific) and cells were scored. Results were expressed as total cell number/mL. BAL fluid collection was then flash frozen in liquid nitrogen prior to storage at -80°C.

**Lung Tissue Harvesting**

A cannula was inserted into the trachea and lungs were flushed by perfusing 20 ml of PBS through the right ventricle. A pre-warmed solution of low melting point agarose (1% agarose in PBS) was introduced into the left lung under constant pressure (20 cm H₂O), transferred into a plastic fixation cassette and placed in 10% formalin overnight, prior to being paraffin embedded. The superior and inferior lobes of the right lung were flash
frozen in liquid nitrogen. The middle lobe of the right lung was transferred into 3 ml conical tube, and kept at 4°C for 5-20 min prior to being processed. The larger lobe of the right lung was placed in 60 mm tissue culture dishes dissected and minced into 1-2 mm diameter fragments containing pre-warmed dissociation solution. The left lungs were immediately frozen at −80 °C for protein extraction and the small right lung lobes were immediately stored in RNAlater at −80 °C and used later for RNA extraction.

**Flow Cytometry**

The large right lobe of the lung was digested using dissociation solution. Lung tissue dissociation solution contained Dispase solution (0.6U/mL; Life Technologies), DNase I (60U/ml), and collagenase I (0.3U/mL; Gibco Invitrogen). Lung fragments were disintegrated in this solution (30-60 min; 37°C) until a complete cell suspension was obtained. Cell suspension was filtered through a 40 μm cell strainer (Fisher Scientific, USA), followed by centrifugation (10 min, 1000rpm). Cell pellets were washed twice with PBS/2% FBS (10 min, 1000rpm) and resuspended in 0.5mL PBS/2% FBS and used as needed.

Immunostaining and flow cytometry analyses were performed according to standard procedures. Following lung tissue dissociation described above, single cell suspensions were stained with the following antibodies at concentrations of 1ug/mL: Anti-Mouse Podoplanin-FITC (eBioscience), VEGF-C PE (Bioss), VEGF-D Biotin (Bioss), VEGFR3-APC (R and D Systems), VEGFR2-Pacific Blue (BioLegend), CD11c-PerCP-Cy5.5 (eBioscience), CD11b-AF700 (eBioscience), F4/80 PE-Cy5 (eBioscience), CCL21 PE-Cy7, Siglec-F PE-CF594 (BD Horizon) and Strep-eFluor 605NC (eBioscience). Single
cell suspensions of digested lung tissue were subsequently analyzed on an LSRII flow cytometer (BD). Cell suspensions were incubated in CD16/CD32 (Mouse Fc Block, BD) diluted in FACS buffer at 1:50 ration, for 20 minutes on ice. Samples were then centrifuged at 1500rpm for 5 minutes and the supernatant was discarded. Cells were fixed and permeabilized by re-suspending the cell pellets in 200μl of BD Cytofix/Cytoperm Buffer and incubated for 20-30 minutes on ice. Cells were washed in 1x Perm Wash Buffer (BD). Staining was performed by re-suspending the cell pellet in 100μl of the appropriate antibody master mix dilution in 1xPerm Wash Buffer and incubate for 30 minutes at 4°C in the dark. Cells were washed in 1x Perm Wash Buffer, 1500rpm for 5 minutes and the supernatant was discarded. Cells were re-suspended in 250μl Facs buffer and acquired on the cytometer. For each mouse sample, up to 2500000 total events were collected on the LSRII flow cytometer and analyzed for the presence of resident macrophages (F4/80+ CD11c+ Siglec F+), recruited macrophages (F4/80+ CD11b+ Siglec F-), lymphatic endothelial cells (LECs, Podoplanin+ VEGFR3+ VEGFR2-) and for the expression of VEGF-C, VEGF-D and CCL21.

**Preparation of Whole-Lung Lysates**

Mouse lungs were homogenized in mammalian protein extraction reagent containing Protein Stabilizing Cocktail, Halt Protease Inhibitor Cocktail, Halt Phosphatase Inhibitor Cocktail (Thermo Scientific, Rockford, IL, USA), 150 mM NaCl, and 1 mM EDTA. Lysates were centrifuged (14,000 rpm, 20 min, 4°C), and the supernatant was harvested and transferred to an ultrafree-MC-GV 0.22μM filter unit (Millipore) and centrifuged at 10000g at 4°C for 10-20 minutes. The supernatant collected was termed
“whole-lung lysate”. Protein concentration was measured by means of the Micro BCA protein assay (Thermo Scientific) according to the manufacturers’ protocol.

Sircol Assay

Total lung collagen levels were determined by measuring collagen protein accumulation in 15-30mg of flash frozen lung tissue using the Sircol Assay (Biocolor, UK) and following the manufacturer’s protocol. Total lung collagen levels and compared with a standard curve using collagen standards supplied by the manufacturer of 5, 10, 15, 20, 30, 40, and 50μg according to manufacturer’s instructions. Data were reported as μg of collagen per mg of lung tissue.

Morphometric Measurements

Estimation of lung volume and length and volumes of Lymphatic vessels in pulmonary tissue compartments.

Isotropic Orientation: Prior to any sectioning and performing morphometric measurement the lung was sectioned into isotropic uniform random (IUR) sections in order to remove biases in sampling and sectioning of the tissue [40, 41]. Briefly, the lung was placed in cylindrical paraffin blocks. Blocks were randomly spun on a flat surface for about 5 seconds and the spinning was manually stopped. The vertical axis of the block marked the top and the bottom of the paraffin block. This was then the orientation that was used for sample sectioning and the lung was cut parallel to this axis into slices (3mm thick) with a sharp blade. About 4-6 sections of total serial sections of the tissue were obtained.
Estimation of total lung volume: To determine the volume of the lung, an image of the surface area of each slice was obtained by using an Epson Perfection V700 scanner. The images were digitized and displayed on screen using Adobe Photoshop, stored in eight-bit (256 level) format, and then imported into Adobe Photoshop for determination of surface area. The total lung volume was then calculated by applying the Cavalieri method at the final magnification of 600 pixels/inch [42]. A grid of points was laid out over the sampled lung sections, and the surface area was calculated. The volume was estimated using the following formula \( V = \text{area of section} \times \text{section thickness} \). Lung slices were then serially aligned, embedded in paraffin wax in tissue processing and embedding cassettes. 4-μm-thick consecutive sections were prepared using a microtome and were mounted on glass slides for further staining. Sections were air-dried before the staining procedure.

I was trying to see if Joshay could get us a figure like this one:

Stereological assessment of lung tissue

Double immunofluorescence staining: Paraffin sections were deparaffinized and rehydrated in xylene and a descending series of ethanol, 100% ethanol twice for 3 min followed by hydration with 95% ethanol for 1 min and finally rinsed in distilled water. Antigen retrieval was performed by immersing the slides in 10 mM Sodium Citrate Buffer and boiling the samples in a steamer for 30 minutes. After cooling for 10 minutes, sections were rinsed in dH2O and blocked with 250mL of blocking solution containing 10% goat serum and 10% rabbit serum in superblock, for one hour at room temperature in a stain tray, in the dark. The blocking solution was aspirated and 250mL of a mixture
of two primary antibodies, 1: dilution of goat anti-mouse VEGFR3 (R&D) and 1:500 dilution of rat-anti-mouse thrombomodulin were added and incubated for one hour at room temperature. Slides were washed with TBST. Followed by the addition of a mixture of two secondary antibodies, 1:200 dilution AlexaFluor 488 donkey anti-goat, 1:200 dilution AlexaFluor 594 rabbit anti-rat (1:200) and 1:500 dilution of DAPI. Slides were incubated for 30 minutes at room temperature in a stain tray, in the dark. Slides were washed with TBST followed by a wash with PBS. Using mount coverslips, slides were sealed with a drop of mounting medium, and stored in the dark at -20°C or 4°C.

**Statistical Analysis**

Data are reported as mean and standard deviation of the mean. Statistical analysis was performed using unpaired Student t test or ANOVA with tukey. Statistical difference was accepted at P< 0.05.

**RESULTS:** (for some, data are shown on both a scatter plot and a bar graph)

**Assessment of pulmonary macrophages in sequential bronchoalveolar lavage aspirates following acute lung injury.**

Bronchoalveolar lavage was performed at each harvest time point. Inflammatory responses were assessed ex vivo by determining the total cell counts of cells retrieved by BAL. Total cell counts increased from baseline, 2.2 x 10⁵/mL up to 2.99 x 10⁶/mL by day 28, indicating an influx of macrophages and neutrophils over time (Figure 1). Though the influx failed to reach significance when compared with baseline cell counts, the total cell counts in the treated groups were greater in than at baseline, indicating
evidence of cellular inflammation in the airway, which is consistent with previous observations. (42b).

**Collagen accumulation is increased over time in response to bleomycin-induced pulmonary fibrosis.**

IT instillation of bleomycin is a well described model of pulmonary fibrosis [43]. Administration of bleomycin leads to a patchy fibrotic process in several strains of mice and rats, inflammatory cell migration and accumulation of collagen, [44-46].

Total collagen content in the right lung of each animal was determined by using the Sircol assay (**Figure 2**). The amount of collagen in lung tissue increased over time with prolonged exposure to bleomycin. Baseline levels of untreated mice was 1.49\(\mu\)g/mg of tissue reaching a peak of 2.44\(\mu\)g/mg of tissue at day 10 and then subsequently levels decreased over time to 1.82\(\mu\)g/mg of tissue by day 28 (p=n.s). Total collagen content in bleomycin-treated lungs was not significantly greater, by one-way ANOVA, than the total collagen content in lungs obtained from control mouse lungs. The patchy distribution of fibrosis that is normally observed following IT bleomycin treatment, is likely the reason for the underestimation of the changes in collagen content over time.

**Analysis of total lung volume over time in bleomycin induced fibrosis**

Mario is putting together the materials and methods for this section.

Joshay is working on getting this data together. I asked if he could tabulate and/or create a graph (a possible additional figure??)
Inflammatory cell profiles of single cell suspensions from lung tissue

The inflammatory cell profile was analyzed using single-cell suspensions freshly obtained from collagenase-digested right lung tissue and labelled for flow cytometry. Resident macrophages were identified as F4/80+ CD11c+ Siglec F+ populations, while recruited macrophages were identified as F4/80+ CD11b+ Siglec F- populations (Figure 3). The number of resident macrophages remained fairly consistent over the course of bleomycin treatment (Figure 4), when evaluated as either the number of cells (28000 cells ± 9700 cells) or as the percent of the F4/80+ population (29% ± 9% cells of the F4/80+ population, p=n.s).

Recruited macrophages (F4/80+ CD11b+ Siglec F-) did however, increase over the course of bleomycin treatment when analyzed as either the number of cells (4000 cells at baseline/untreated cells, increasing to 18700 cells by Day 10 and decreasing to back to near baseline levels of 4500 cells), Figure 4, or as the percent of the F4/80+ population (3.8% of the F4/80+ population at baseline/untreated cells, increasing to 23.8% of the F4/80+ population by Day 10 and decreasing to back to near baseline levels of 4.8% of the F4/80+ population).

Expression of Podoplanin on Lymphatic Endothelial Cells increases over time in Bleomycin-induced lung fibrosis

Podoplanin was used for the identification of lymphatic endothelial cells and their discrimination from the vascular endothelium [10]. We analyzed the expression and distribution of podoplanin using tissue dissociation of sample preparations obtained from large right lobe of the lung Figure 5. Absolute counts of lymphatic endothelial cells,
identified by flow cytometry as Podoplanin+ events, was significantly increased from baseline (32000) to 127000 by day 10 of bleomycin treatment (SD=34000, p<0.01 ). Expression and number of Podoplanin+ cells returned to baseline levels by Day 21.

Next, using flow cytometric analysis and antibodies directed against Lymphatic Endothelial Cells markers we compared the expression of VEGFR3 and VEGFR2 on the podoplanin+ populations described above Figure 6. The expression of VEGFR3 on Podoplanin+ cells was markedly increased by Day 10 (40.9%, p<0.001) and Day 14 (43.7%, p<0.0001) compared baseline levels of VEGFR3 in untreated control samples (19.7%, SD=12.6%). Comparable changes in the levels of expression of VEGFR2 on Podoplanin+ cell populations were not observed (11.7% at baseline to 13.9% by Day 14, SD=2.3%, p=ns).

**Stereological assessment of lung tissue.**

**Lymphatic vessel length and vessel volume increases in parallel with increased severity of lung injury.**

Systematic uniform random sampling was applied to each lung specimen to ensure that every part of the section had an equal chance of being included in the analysis. Lung sections were stained using VEGFR3, Thrombomodulin was included to differentiate the vascular endothelial cells Figure 7. Using a computer-assisted stereology tool [47] to determine the length and the volume density of lymphatic endothelial cells, revealed an increase in lymphatic vessel length and volume density that peaked at day 14 harvest (Figure 8). Lymphatic vessel length increased over time from 2.44 x 10^-8mm/mm3 at baseline to 5.94 x 10^-8mm/mm3 at Day 14 (SD = 1.25 x 10^-8mm/mm3, p=ns) and
decreased to $3.61 \times 10^{-8}$ mm$^3$/mm$^3$ by Day 21. Correspondingly, lymphatic vessel volume also increased over time from $3.59 \times 10^{-3}$ mm$^3$/mm$^3$ at baseline to $8.63 \times 10^{-3}$ mm$^3$/mm$^3$ by Day 14 (SD = $1.81 \times 10^{-3}$ mm$^3$/mm$^3$, p=ns) and decreased to $5.29 \times 10^{-3}$ mm$^3$/mm$^3$ by Day 21.

**Lymphatic Length and lymphatic volume preferentially correlates with CD11b+ Siglec F+ recruited macrophages.**

The length and volume of lymphatic and blood vessels quantified using immunostained lung tissue were correlated with the quantification of CD11c+SiglecF+ resident and CD11b+Siglec F- recruited macrophages. The volume of lymphatic vessels correlated with CD11b+Siglec F- recruited macrophages (Spearman’s rank correlation coefficient = 0.54, p=0.0063) Figure 9. In contrast, the volume of lymphatic vessels did not correlate with CD11c+Siglec F+ resident macrophages (Spearman’s rank correlation coefficient = 0.13, p=0.56). Similarly, the length of lymphatic vessels correlated with CD11b+Siglec F- recruited macrophages (Spearman’s rank correlation coefficient = 0.44, p=0.032). The length of lymphatic vessels also did not correlate with CD11c+Siglec F+ resident macrophages (Spearman’s rank correlation coefficient = 0.13, p=0.55) respectively. Expansion of lymphatic vessels occurred in correlation with increased infiltration of recruited macrophages.

**DISCUSSION**

Lymphatic vessels are present in nearly all vascularized tissues. They are mainly involved in the transportation of interstitial fluid from the peripheral tissues. Recent advances in lymphatic vessel biology research have revealed that lymphatic vessels
actively sense and respond to the tissue environment [48]. The formation of lymphatic structures has been implicated in multiple lung diseases, including pulmonary fibrosis. Since lymphatic vessels are significantly influenced by physiological and/or pathological modifications of their environment [27], inflammatory conditions can affect lymphatic vessels, but conversely, it is also possible and the lymphatic vessels can influence the inflammatory response.

In the study presented here, we sought to describe the pathogenesis and the sequential changes observed following lung fibrosis that occurs in response to administration of bleomycin. Using primarily flow cytometry and morphometry techniques, we assessed inflammation, fibrosis and lymphatic vascular expansion by following the expression of VEGFR2, VEGFR3 and podoplanin on lymphatic endothelial cells over time. We sought to determine if a particular sub-set of macrophages was predominantly involved in the regulation of lymphangiogenic activity in the bleomycin-induced lung fibrosis. Our results demonstrate that F4/80+CD11b+Siglec F- recruited macrophages rather than the resident macrophages appear to be preferentially involved in the regulation of lymphangiogenic activity during the onset of fibrosis in the mouse lung.

The role of tumor- associated macrophages in promoting angiogenesis and acquiring an angiogenic phenotype has been demonstrated (Grimshaw, M. J., (2002) Endothelin-2 is a macrophage chemoattractant: implications for macrophage distribution in tumors). It has been proposed that thioglycolate-activated CD11b+ macrophages were able to transdifferentiate into lymphatic endothelial cells in cornea inflammation model [49]. In addition, observations that CD11b-expressing tumor- associated macrophages acquire lymphatic specific markers including Prox1, Lyve1, Podoplanin and VEGFR3
and a down-regulation of myeloid markers, and their ability to integrate into growing lymphatic vessels in an experimental mouse tumor model (Hall KL, (2012) New model of macrophage acquisition of the lymphatic endothelial phenotype. PLoS One 7: e31794) have led to tumor- associated macrophages being proposed as a potential source of lymphatic endothelial cells and contributung to tumor lymphangiogenesis (Zumsteg A, (2009) Myeloid cells contribute to tumor lymphangiogenesis. PLoS One 4: e7067). Conversely, other studies [32, 50, 51] have reported that CD11b+ macrophages were critical for lymphangiogenesis during inflammation and cancer and that they regulated lymphatic endothelial cell proliferation did not differentiate into lymphatic endothelial cells (Gordon EJ, (2010) Macrophages define dermal lymphatic vessel calibre during development by regulating lymphatic endothelial cell proliferation. Development 137: 3899-3910). The origin of lymphatic endothelial cells is still in question, since in cancer and inflammation, newly formed vessels may arise from existing ones [52].

The role and function of the lymphatic circulation in the fibrotic process remains unclear and under explored [53]. We show here that recruited macrophages play a role in the setting of fibrosis, that correlates with lymphangiogenic activity in the injured lung. Our results corroborate finding in human IPF (El-Chemaly. Abnormal lymphangiogenesis in idiopathic pulmonary fibrosis with insights into cellular and molecular mechanisms. Proc Natl Acad Sci U S A. 2009). It is generally agreed that the VEGF-C–VEGF receptor-3 interaction in lymphatic endothelial cells is the most important signaling interaction for inflammatory lymphangiogenesis, however, further studies are needed to elucidate the biological effects under various inflammatory conditions.
No therapies are currently in place to manipulate lymphatic vessel for inflammatory disorders, further research on lymphatic vessel biology needs to focus on the signals and factors that drive or limit lymphangiogenesis. More studies are needed to clarify the biological role of inflammatory lymphangiogenesis in order to merit it as a possible therapeutic target in chronic inflammatory disorders. In addition, research on novel targets that specifically manipulate lymphangiogenesis are needed.
FIGURES

Figure 1 Total cell count in BAL of mice treated by intratracheal treatment of Bleomycin and sacrificed on different days. Total cell counts increased from $2.2 \times 10^6$ in untreated mice to $3 \times 10^6$ in bleomycin treated mice, consistent with an influx of macrophages and neutrophils (p=ns).

Figure 2 Total collagen content in the right lung of each animal determined by the Sircol Collagen Assay. Total collagen increased from $1.5\text{mg/mg}$ of tissue in untreated mice to $2.24\text{mg/mg}$ in bleomycin treated mice by day 10 (p=ns), that returned to baseline levels.
Figure 3 Flow cytometric analysis of macrophage populations. Resident macrophages were identified as F4/80+ CD11c+ Siglec F+ populations. Recruited macrophages were identified as F4/80+ CD11b+ Siglec F- populations.

Figure 4 Proportion of Resident and Recruited Macrophages following IT bleomycin treatment. The proportion of Resident Macrophages (F4/80+ CD11c+ Siglec F+), were reduced by Day 7 (p=0.5) compared to untreated mice, but returned to baseline levels.
by Day 14. The proportion of recruited macrophages (F4/80+ CD11b+ Siglec F-) increased from baseline to 23.9\% by day 10 (SD=6.8\%, p<0.01) and returned to baseline by day 21.

**Figure 5** Levels of lymphatic endothelial cells, identified by flow cytometry using Podoplanin, increased between baseline day 10 (SD=34000, p<0.01) and returned to baseline levels by Day 21. The expression of VEGFR3 on Podoplanin+ cells was markedly increased by Day 10 (40.9\%, *** p<0.001) and Day 14 (43.7\%, **** p<0.0001) compared to untreated control samples (19.7\%, SD=12.6\%). Changes in the levels of expression of VEGFR2 on Podoplanin+ cell populations were not observed (11.7\% at baseline to 13.9\% by Day14, SD=2.3\%, p=ns).
Figure 6 Expression of VEGFR3 on Lymphatic Endothelial Cells preferentially increases over time in Bleomycin-induced lung inflammatory conditions in comparison to the expression of VEGFR2.

Figure 7 Double immunofluorescence staining was performed on 4-mm-thick formalin-fixed and paraffin-embedded tissue samples. Slides were dewaxed and hydrated. Lymphatic vessels were identified by staining with VEGFR3 and Thrombomodulin was
used to indicate blood vessels (magnification X 20, white arrows indicate the presence of lymphatic vessels).

Figure 8. Lung sections stained using VEGFR3 and Thrombomodulin (Figure 7), were stereologically assessed to determine the length and the volume density of Lymphatic endothelial cells. Length and volume density of lymphatic vessels were quantified at 20X magnification and by using a computer-assisted stereology tool (STEPanizer, USA). Systematic uniform random sampling was applied to ensure that every part of the section had an equal chance of being included in the analysis.
Figure 9

**Correlation between Resident Macs and Lymphatic vessel length density**

Spearman’s Correlation: $r = -0.318$, $p = 0.14$
Linear Regression $r^2 = 0.62$, $p = 0.24$

**Correlation between resident macrophages and lymphatic vessel volume density**

Spearman’s Correlation: $r=0.46$, $p=0.0225$
Linear Regression $r^2 = 0.166$, $p = 0.048$

**Correlation between Recruited Macs and Lymphatic vessel length density**

Spearman’s Correlation: $r=0.34$, $p=0.108$
Linear Regression $r^2 = 0.11$, $p = 0.108$

**Correlation between recruited macrophages and lymphatic vessel volume density**

Spearman’s Correlation: $r=0.55$, $p=0.0049$
Linear Regression $r^2 = 0.28$, $p = 0.0077$

Possible additional data for inclusion:

1. Expression of podoplanin on VEGFR2 and VEGFR3 populations: i.e Using flow cytometry, the VEGFR2 and VEGFR3 populations were gated and individually analyzed for their expression of podoplanin. This is different from Figure 7, where the podoplanin population was selected and evaluated for expression of VEGFR2 and VEGFR3. The populations below were much lower percentages,
for the most part, less than 0.2% of the F4/80 population and so the number of events/cells per mouse, may not be very meaningful??
REFERENCES:

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Additional: