THE UNIVERSITY OF CHICAGO

PULMONARY LYMPHANGIOGENESIS IN CHRONIC ALLERGIC ASTHMA

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Abbreviations

ACKR4	Atypical chemokine receptor 4
AF	Alexa fluor
BALF	Bronchoalveolar lavage fluid
BALT	Bronchus associated lymphoid tissue
BLC	B lymphocyte chemoattractant
CCL11	CC motif chemokine ligand 11
CCL19	CC motif chemokine ligand 19
CCL2	CC motif chemokine ligand 2
CCL20	CC motif chemokine ligand 20
CCL21	CC motif chemokine ligand 21
CCL3	CC motif chemokine ligand 3
CCL4	CC motif chemokine ligand 4
CCL5	CC motif chemokine ligand 5
CCR7	CC motif chemokine receptor 7
CCSP	Club cell secretory protein
CD	Cluster of differentiation
cDNA	complementary deoxyribonucleic acid
COPD	Chronic obstructive pulmonary disease
CreERT2	Cre recombinase-estrogen receptor T2 domain
CXCL12	CXC motif chemokine ligand 12
CXCL13	CXC motif chemokine ligand 13
CXCR4	CXC motif chemokine receptor 4
CXCR5	CXC motif chemokine receptor 5
DAPI	4′,6-diamidino-2-phenylindole
DBE	Dibenzyl ether

DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EtOH	ethyl alcohol
FACS	Fluorescence-Activated Cell Sorting
FlowSOM	Flow cytometry self-organizing map
FoxP3	Forkhead box P3
FPKM	Fragments Per Kilobase of transcript per Million mapped reads
H&E	Hematoxylin and eosin
HAPC	Hepatic antigen presenting cell
HDM	House dust mite extract
hIgE	human imunoglobulin isotype E
i.t.	Intratracheal instillation
iBALT	inducible bronchus associated lymphoid tissue
iDISCO	Immunolabelling- enabled 3D Imaging of Solvent-Cleared Organs
IFNγ	Interferon gamma
IgE	Immunoglobulin isotype E
IgG1	Immunoglobulin isotype G1
IL-13	Interleukin 13
IL-33	Interleukin 33
IL-4	Interleukin 4
IL-4Rα	Interleukin 4 receptor alpha chain
IL-5	Interleukin 5
IL-5Rα	Interleukin 5 receptor alpha chain
IL-7	Interleukin 7
LAG	lymphangiogenesis
LEC	Lymphatic endothelial cell
LGEA	Lung gene expression analysis

NALT ..... Nasal associated lymphoid tissue

OCT ..... Optimal cutting temperature compound

OVA ..... Ovalbumin

PBS ..... Phosphate buffered saline

PCNA ..... Proliferating cell nuclear antigen

pFA ..... Para formaldehyde

pLEC ..... Pulmonary lymphatic endothelial cell

$pMan_{OVA}$ ..... Mannosylated form of OVA

Prox1 ..... Prospero homeobox 1

qPCR ..... quantitative polymerase chain reaction

RANTES ..... Regulated upon activation, normal T cell expressed, and secreted

RNAseq ..... Ribonucleic acid sequencing

rtTA ..... Reverse tetracycline transactivator

SDF-1 ..... Stromal derived factor 1

TBS ..... Tris buffered saline

$T_{FH}13$ ..... IL-13-producing T follicular helper cell

T_H2 ..... Type II T lymphocyte

T-PER ..... Tissue protein extraction reagent

TRE ..... Tetracycline responsive element

tSNE ..... t-distributed stochastic neighbor embedding

VEGF ..... Vascular endothelial growth factor

VEGFR ..... Vascular endothelial growth factor receptor

αCXCL13 ..... anti-CXC motif chemokine ligand 13

αSMA ..... Alpha smooth muscle actin

αVEGFR-3 ..... anti-vascular endothelial growth factor receptor 3

Abstract

In asthmatics, the lung stroma undergoes significant remodeling in response to chronic airway inflammation. Nonetheless, there are limited studies characterizing the pulmonary lymphatic vasculature in asthma, nor whether these vessels expand or regress due to disease. In this thesis, I aim to address the role the pulmonary lymphatics undertake in allergic airway inflammation and provide a reconciliation between such behavior and current literature. Furthermore, how pulmonary lymphatics can exacerbate allergic airway disease, by facilitating induction of bronchus associated lymphoid tissue (BALT) and raising local and systemic IgE levels. Finally, we explore the use of a tolerogenic platform which leverages the natural tolerance inducing mechanisms of the body to drive allergen specific tolerance and ameliorate allergic airway disease.

In these studies, we utilize human lung samples and the house dust mite (HDM) model of allergic airway inflammation to address the role of the pulmonary lymphatic endothelium in

response to allergic airway disease. Here, we found that in asthmatic lungs, the pulmonary lymphatic density is increased, a phenomenon that was recapitulated in our HDM mouse model of allergic airway disease. These findings are not in conflict with recent literature that suggests that two cytokines central to the allergic response, IL-4 and IL-13, have potent anti-lymphangiogenic capacity, as lymphatic expansion was temporally decoupled from allergen inhalation. Furthermore, we found that by determining the levels of the pro-lymphangiogenic growth factors, VEGF-C and VEGF-D, and gauging these against the anti-lymphangiogenic cytokines, IL-4 and IL-13, we can get a measurement that coincides with the state of the pulmonary lymphatic vasculature and correlates with the numbers of pulmonary lymphatic endothelial cells. Based on lung protein analysis, we posit that when there is a balance between the pro-lymphangiogenic growth factors and the anti-lymphangiogenic cytokines, the pulmonary lymphatic vasculature will be constrained. However, an imbalance between these molecules can drive pulmonary lymphangiogenesis once the allergen-mediated inflammation subsides along with the anti-lymphangiogenic cytokines. Given this temporal decoupling between lymphangiogenesis and allergen-mediated inflammation, we pondered what roles could the pulmonary lymphatic vasculature mediate if it were to expand during allergic airway disease.

Our cohort of human asthmatic lung samples exhibited BALT, and specifically in asthmatic sera, the levels of VEGF-C correlated with the levels of IgE. Such correlation was recapitulated in mice undergoing chronic allergic inflammation, as well as the induction of BALT. Interestingly, we found that when we introduce VEGF-C into mice undergoing chronic HDM-mediated inflammation, their local and systemic IgE levels were raised, as well as the extent of BALT. In allergic mice, VEGFR3 signaling was both necessary and sufficient to mediate induction of BALT, and its expression by lymphatic endothelial cells was required for induction and for the VEGF-C

mediated exacerbation. Mice with an expanded lymphatic vasculature where particularly susceptible to developing BALT and IgE upon allergen inhalation, suggesting that the pulmonary lymphatic vasculature could promote a lung microenvironment susceptible to allergic disease. Finally, we found that pulmonary lymphatics could upregulate CXCL13 in response to allergic airway inflammation, and expression of CXCL13 was required for the VEGF-C mediated BALT exacerbation. Following this train of thought, CXCL13 blocking antibodies showed promise as a potential therapeutic in allergic asthma, as their use ameliorated BALT in mice undergoing allergic airway disease. Altogether, our data suggests that in the context of allergic airway disease, the pulmonary lymphatic vasculature undertakes active roles in mediating BALT through CXCL13 dependent mechanisms.

Finally, we assessed the possibility of using a tolerogenic platform to generate allergen specific tolerance and ameliorate allergic airway disease. In collaboration with the Hubbell laboratory, we utilized a synthetically glycosylated form of a model allergen to leverage the tolerance inducing mechanisms of the liver microenvironment to ameliorate the phenotypes associated with allergic airway inflammation. Prophylactic treatment with a glycosylated model allergen reduced the effects of allergen inhalation, including airway and lung eosinophilia, systemic IgE and allergen specific IgG1 levels, as well as mucus hypersecretion. Prophylactic treatment with this tolerogenic platform induced allergen specific regulatory T cells, which inversely correlated with hallmarks of allergic inflammation, and restricted allergic T cell responses. Last, we found that therapeutic intervention with the glycosylated allergen in previously sensitized mice could still ameliorate some features of allergic airway disease, highlighting the potential for this tolerogenic platform in desensitizing former allergic individuals, including those suffering from allergic asthma.

Chapter I: Introduction

Asthma and the allergic type2 endotype

Asthma is a clinical syndrome of chronic airway inflammation characterized by recurrent, recursive, airway obstruction (GINA, 2015; Lange 1998). Current studies estimate that asthma affects more than 300 million people worldwide, including 25 million in the U.S. alone (Braman, 2006). There is no cure for asthma. Current treatments involve the use of oral and inhaled corticosteroids which, while they rapidly reduce airway obstruction caused by an asthma attack, they also broadly suppress the immune system and have debilitating long-term side effects (NHS, 2021). While these treatments are somewhat effective at disease management, because of the heterogeneity of the disease, they are not appropriate for every patient (Vijverberg, 2018).

Type 2 high allergic asthma, caused by exaggerated type II immune responses to innocuous inhaled allergens is the most common asthma endotype, and its prevalence is on the rise (Bousquet, 2005). Type II asthma is a severe endotype characterized by induction of allergen-specific type II T lymphocytes (T_H2), airway eosinophilia and the presence of allergen-specific IgE (Wenzel, 1999). Upon allergen inhalation, Th2 cells produce cytokines whose signaling orchestrate the main cellular effectors to the allergic response. Interleukin (IL)-4 is certainly necessary for the class switch recombination of B cells to the IgG1 and IgE isotypes, and production of allergen-specific IgE can coat eosinophils, basophils and mast cells and promote their degranulation upon allergen encounter (Fischer, 2002; Finkelman, 1988; Galli, 2012) IL-5 supports eosinophil granulopoiesis and is a potent eosinophil activator and chemoattractant, driving lung and airway eosinophilia in response to allergen inhalation (Denburg, 2001; Warringa, 1992; Pelaia, 2019). Finally, IL-13 acts on the lung stroma and triggers changes to the airway epithelium driving goblet cell metaplasia and mucus hypersecretion, as well as, smooth muscle hypertrophy leading to airway smooth muscle thickening which contributes to airway hyperresponsiveness (Kondo, 2006; Risse, 2011; Eum 2005; Manson, 2020) Altogether, cytokines made by allergen specific T_H2 cells mediate the cellular immune effectors responsible for the symptoms associated with type 2 allergic asthma.

Spatial coordination in allergic asthma: chemokines and chemokine receptors

An essential aspect to mount a robust immune response is the spatial coordination and recruitment of specific immune cell subsets to distinct areas of the lung microenvironment (Lambrecht, 2015). To achieve such a task, the immune system relies on a set of chemotactic cytokines (chemokines) that act in a coordinated manner to direct, recruit and organize leukocytes in response to an insult or challenge (Palomino, 2015). Chemokines are broadly classified based

on their primary protein structure of their conserved cysteine residues, thus generating four families: CC, CXC, C and CX3C (Mélik-Parsadaniantz, 2008). These chemokines are recognized by chemokine receptors in a complex manner, as there is no one-to-one correspondence between an individual chemokine and an individual receptor (Hughes, 2018). A single chemokine receptor can recognize and act upon different chemokines, and a single chemokine can act on different receptors, nonetheless, studies in animal models have shown that chemokines act in a coordinated manner to orchestrate an immune response (Lukacs, 2006). Furthermore, identification of an atypical set of chemokine receptors that recognize chemokines but fail to transduce their signal into a migratory effect add a layer of regulation into the cellular spatial coordination (Ulvmar, 2011). Altogether, expression, production, recognition, and regulation of the chemotactic axis play a central role in directing an immune response, such as the ones associated with chronic allergic asthma.

In allergic asthma, chemokine production plays important roles in leukocyte recruitment, exodus, and coordination in response to an allergen exposure (Lukacs, 2006). The airway epithelium, the lung resident alveolar macrophages and lung mast cells act as lung sentinels, and, upon allergen encounter, can produce significant levels of chemokines to drive initial leukocyte recruitment into the lung tissue (Liu, 2018; Draijer, 2017). These chemokines include monocyte recruitment factors such as CCL2 and CCL3 (Deshmane 2009; Sherry 1988), eosinophil recruitment factors such as CCL11/eotaxin-1 (Williams, 2001), lymphocyte recruitment factors such as CCL4 and CCL20 (Guilliams 2013; Shen, 2021), as well as pan-leukocyte chemokines such as RANTES/CCL5 (Koya, 2006). Similarly, upon inflammatory stimuli, vascular endothelial cells upregulate chemokine expression as well as adhesion factors to facilitate leukocyte capture, rolling, arrest and transmigration into the lung tissue (McEver, 2013). Altogether, in response to

allergen encounter, chemokine production by the lung stroma and the lung sentinel cells mediates the initial recruitment of circulating leukocytes into the lung tissue thus initiating allergic inflammation.

Lung tissue egress is coordinated by specific chemokines produced by pulmonary lymphatic vessels, which provide and exit route from the lung to the draining lymph nodes. In order to mount a robust immune response, dendritic cells must capture allergen in the lung and migrate to the draining lymph nodes where they can educate and mount an allergen-specific T cell response (Martín-Fontecha, 2009). This migration event is mainly driven by lymphatic derived CCL21, which acts on CCR7 bearing dendritic cells, guiding these to the draining lymph nodes (Johnson, 2010; Riol-Blanco, 2005). Furthermore, the CCL21:CCR7 migration tightly regulated by production of CCL19 (Rot and von Andrian, 2004) (another CCR7 agonist) and its removal by ACKR4 bearing stromal cells (Bryce, 2016). Together, these chemokines and chemokine receptors coordinate leukocyte egress from the lung to the draining lymph nodes, facilitating a critical step in priming a robust allergic response.

Finally, a different set of chemokines coordinate the induction of specific, organized tertiary lymphoid structures, such as bronchus associated lymphoid tissue (BALT) which play a significant role in the pathogenesis of allergic asthma (Randall, 2010). In secondary lymphoid organs, the lymph node stroma uses chemokines to spatially coordinate T cells and B cells into an organized structure known as a germinal centre (Allen and Cyster, 2008). Indeed, germinal centres facilitate B cells processes such as affinity maturation and somatic hypermutation which are critical in raising high affinity antibody responses (Victora and Nussenzweig, 2012). Production of CXCL12/SDF-1, which acts on CXCR4, facilitates B cell trafficking to the dark zone area of the germinal centre, where B cells can proliferate and undergo somatic hypermutation (Rodda,

2015). In contrast, CXCL13/BLC attracts T follicular helper cells and B cells in the light zone of the germinal centre, facilitating affinity maturation to select for the high affinity B cell clones (Pikor, 2020). In cases of chronic inflammation, structures that resemble secondary lymphoid organs are induced in the injured tissue and are termed tertiary lymphoid structures (Luo, 2019). In the case of chronic allergic asthma, induction of bronchus associated lymphoid tissue, a tertiary lymphoid structure in the lung parenchyma, facilitates the organization of germinal centres in the lung, thus driving a local antibody response. However, there is limited knowledge on the roles that CXCL12/SDF-1:CXCR4 and CXCL13/BLC:CXCR5 play in the induction and maturation of bronchus associated lymphoid tissue, and whether these roles may be context dependent (Foo and Phipps, 2010).

Stromal cells and lung remodeling in allergic asthma

The allergic immune response can drive significant lung tissue remodeling which in turn causes the symptoms associated chronic allergic asthma. Upon allergen inhalation, the airway epithelium acts as primary barrier, and is remodeled in response to the inflammatory insult (Bergeron, 2010). In response to the environmental insult, the airway epithelium can undergo apoptosis, which upon chronic damage, can result in an aberrant tissue repair response (Chan, 2016). As previously mentioned, IL-13 signaling can drive goblet cell metaplasia and airway smooth muscle thickening, both of which can remodel the lung airways and promote mucus plugging and airway bronchoconstriction (Kondo, 2006; Manson, 2020). Furthermore, in combination with IL-4, IL-13 can also drive subepithelial fibrosis and enhanced collagen deposition, having detrimental effects on the airway tissue (Gour and Wills-Karp, 2015). Additionally, other cytokines such as IL-6, IL-8 and TSLP are known to induce cellular

senescence, and in particular senescent fibroblasts can reduce pulmonary compliance and trigger airway remodeling (Wu, 2013; Wang, 2020). Altogether, the airway epithelium can drive significant changes to the lung microenvironment in response to inhaled allergens and the immune response to such insults.

Significant vascular endothelial remodeling has been reported to be associated with chronic allergic asthma (Elias, 2003; Detoraki, 2010). Bronchoscopy samples from patients suffering from asthma have revealed increased vascularity and higher vessel density when compared to control samples (Li, 1997; Vrught, 2000; Wilson, 2002). Similarly, there is enrichment of the vascular endothelial growth factor (VEGF) -A in asthmatic samples, and its cellular sources have been extensively characterized (Hoshino, 2001; Lee, 2001; Feltis, 2006; Chetta, 2005; Zanini, 2007). In contrast, there is modest work in characterizing lymphatic vessels in the context of asthma. Chronic inflammatory diseases are associated with lymphatic vessel growth, termed lymphangiogenesis, and these include pulmonary diseases such as COPD and idiopathic pulmonary fibrosis (Poto, 2022; El-Chemaly, 2009). Furthermore, some studies have suggested that human lung mast cells can be a source of the lymphangiogenic growth factors, VEGF-C and VEGF-D, and these in turn could potentially promote lymphangiogenesis in asthmatic patients (Detoraki, 2009). However, a single study on fatal asthmatic samples suggests that the distribution of lymphatic vessels in the airway walls was decreased in these patients, providing a counterpoint to whether lymphangiogenesis occurs in chronic asthma (Ebina, 2008).

Summary

Animal models of allergic airway disease have furthered our understanding of lymphatic vessel biology as it pertains to allergic airway inflammation. Similar to other models of chronic airway inflammation, a rat model of house dust mite (HDM) mediated allergic airway inflammation revealed that pulmonary lymphangiogenesis occurs after the allergen insult, and that these pulmonary lymphatic endothelial cells exhibit a higher chemotactic and proliferative phenotype (Moldobaeva, 2017). However, recent work has introduced that IL-4 and IL-13 can have potent anti-lymphangiogenic activity, and thus confounds our understanding of how lymphatic vessels may interact to expand or not in response to chronic allergic airway inflammation (Shin, 2015; Savetsky, 2015). In this thesis, I aim to utilize mouse models of allergic airway disease to further understand whether pulmonary lymphangiogenesis occurs in response to chronic allergic airway inflammation, and if such, reconcile the interplay between the anti-lymphangiogenic cytokines, IL-4 and IL-13, and the pro-lymphangiogenesis growth factors VEGF-C and VEGF-D. Furthermore, we are interested in investigating whether pulmonary lymphatics may play a role in orchestrating the induction of tertiary lymphoid structures in chronic allergic asthma, as recent work has suggested a causative role for lymphatic endothelial cells in the development of secondary lymphoid organs. Altogether, the work in this thesis may provide insight into whether pulmonary lymphatics may play a protective or a pathogenic role with respect to chronic asthma, and whether we could promote or prevent pulmonary lymphangiogenesis as a potential therapeutic in asthmatic patients.

Chapter II: Material and Methods

Human lung samples. Non-transplatable lungs were obtained from the Regional Organ and Tissue Donor Network, an organ procurement organization that provides services regionally to 12 million people withing the national donation system, and through the Gift of Hope Organ Tissue & Donor Network.

Mice. C57BL6/J (B6) mice were purchased from The Jackson Laboratory and bred in-house to 6 – 12 weeks of age. All transgenic mice are kept in a C57BL6/J background. Prox1 CreERT2 (Prox1) mice were provided by Taija Makinen (Uppsala Universitet, Uppsala, Sweden). VEGFR3lox.lox mice were generously provided by Kari Alitalo (University of Helsinki, Helsinki, Finland) to Anne Eichmann (Yale University, New Haven, CT) and bred in our facility to generate Prox1CreERT2 mice in the VEGFR3lox.lox homozygous background (Prox1VEGFR3). CCSP-rtTA and TRE-VEGFd mice were kindly provided by Joseph Rutkowsky (Texas A&M University,

College Station, TX) and were bred to generate CCSP^{rtTA}VEGF-D^{TRE} mice. CXCL13$^{+/-}$ (CXCL13Het) mice were provided by Jason Cyster (University of California at San Francisco, San Francisco, CA) and were bred to generate CXCL13$^{-/-}$ (CXCL13KO) mice. All mice were bred and housed in a specific pathogen-free facility maintained by the University of Chicago Animal Resource Center. In all experiments, mice were age and sex matched and their genotypes blinded through ear-tagging. The studies performed conform to the principles set forth by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of laboratory animals in biomedical research. The experiments and treatments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Chicago.

Histology & epifluorescence microscopy

Sample preparation and tissue fixation. Mouse lungs were perfused with 5mL of phosphate buffered saline (PBS) (Catalog #10010049, Thermo Fischer Scientific, Rockford, IL) prior to removal and lobe dissection. Cranial lobes were fixed through overnight submersion in a 1% paraformaldehyde (pFA) solution prepared through dilution of a 4% pFA solution (Catalog #AAJ19943K2, Thermo Scientific, Rockford, IL) in PBS. Fixed lobes were changed into a PBS solution prior to histologic processing.

Tissue sectioning and immunofluorescence staining. Lung lobes were paraffin embedded, cut into 8 µm sections, and stained with hematoxylin and eosin by the University of Chicago Human Tissue Resource Center. For immunofluorescence staining, slides were submerged in xylene for 10 min and then washed through an ethanol (EtOH) solution hydration gradient (100%, 100%, 96%, 96%, 70%, H_2O, H_2O) prior to heat induced epitope retrieval. Slides were then immersed in a sodium citrate solution prepared from a 10-fold dilution of Citrate buffer concentrate (Catalog #005000, Thermo Fischer Scientific, Rockford, IL) in deionized water and heated to 85 – 95 °C

for 45 minutes. Slides were washed with Tris-buffered saline (TBS), treated with 10% dimethylsulfoxide (DMSO) (Catalog #D2650, Sigma-Aldrich, St. Louis, MO) in TBS, and blocked for at least 30 minutes with a 0.5% casein (Catalog #B6429, Sigma-Aldrich, St. Louis, MO) solution in TBS. Sections were immunostained with primary antibodies overnight at 4 °C. Slides were then washed thrice with 0.1% Tween 20 (Catalog #TWN510.100, Bioshop Canada, Burlington, Ontario) in TBS before being counterstained with the appropriate AlexaFluor conjugated secondary antibodies for 90 minutes at room temperature. Slides are then washed thrice with 0.1% Tween 20 in TBS prior to incubation with fluorophore-conjugated primary antibodies overnight at 4 °C. Slides are washed thrice with 0.1% Tween 20 in TBS before being stained with the nucleic acid stain Hoechst 33342 (Catalog #H1399, Thermo Fischer Scientific, Rockford, IL) for 10 minutes in the dark at room temperature. Slides were then subjected to an ethanol solution dehydration gradient (H_2O, H_2O, 70%, 96%, 96%, 100%, 100%) and incubated in xylene for 10 minutes before being mounted with No. 1.5 thickness coverslips and DePex (Catalog #50-980-371, Thermo Fischer Scientific, Rockford, IL).

Epifluorescence microscopy. Slide imaging was performed with a Leica DMI8 microscope (Leica Microsystems, Inc., Buffalo Grove, IL) using a 25x oil-immersion objective and LAS_X Leica Acquisition software. Its motorized stage enabled for whole section tiling and imaging. Excitation/emission filters are available to enable four-color imaging including, DAPI, AF488, AF555, and AF647. Images were collected with a CCD camera (CoolSnap Myo) that has high quantum efficiency in visible light. Further image processing was performed with ImageJ software (National Institutes of Health, Bethesda, MD).

3-Dimensional confocal microscopy

Sample preparation, tissue fixation and cryopreservation. Mouse lungs were perfused with 5mL of PBS and inflated to physiological volume with a 1% pFA solution instilled through the tracheae. After tying down the tracheae, lungs are removed from the chest cavity and fixed by overnight submersion in a 1% pFA solution. For cryopreservation, lobes are dissected and incubated twice in a fresh 15% sucrose solution overnight. Lung lobes are then allowed to equilibrate in a 30% sucrose solution until they sank to the bottom of the reservoir. At this stage, lung lobes were embedded in Optimal Cutting Temperature compound (OCT 4586), frozen with liquid nitrogen and stored at -80 °C until sectioning.

300 um sectioning and immunofluorescence staining. 300 µm sections were sliced from frozen lung lobe blocks using a cryostat. Sections were incubated in room temperature TBS to allow for the OCT to dissolve prior to blocking and immunofluorescent immunostaining. Tissue sections were then treated with 10% DMSO in TBS, and permeabilized and blocked for at least 60 minutes with a 0.25% casein/0.05% Triton x100 (Catalog #9400, VWR International, Radnor, PA) solution in TBS. Sections are then immunostained with primary antibodies in a 0.25% casein/0.05% Triton x100 solution overnight at 4 °C. Tissue sections are washed thrice with 0.1% Tween 20 in TBS prior to counterstaining with the appropriate AlexaFluor conjugated secondary antibodies overnight at 4 °C. Tissue sections are washed thrice with 0.1% Tween 20 in TBS prior to staining with fluorophore-conjugated primary antibodies overnight at 4 °C.

Modified iDISCO (Immunolabelling-enabled 3D Imaging of Solvent-Cleared Organs). Tissue sections are subjected to an ethanol solution dehydration gradient consisting of 10-minute sequential incubation steps in (50%, 70%, 80%, 100%, 100%)EtOH before being immersed into dibenzyl-ether (Catalog #33630, Sigma-Aldrich, St. Louis, MO) or ethyl cinnamate (Catalog

#W243000, Sigma-Aldrich, St. Louis, MO) overnight. Tissue sections are then mounted onto slides with No. 1.5 thickness coverslips and DePex.

Laser scanning confocal microscopy. Slide imaging was performed with the Caliber I.D. RS-G4 upright laser scanning confocal microscope at the University of Chicago Integrated Light Microscopy Faciliity. Images were captured using a 20x water immersion objective. The motorized XY-Z stage enables for high speed, high quality tiling of 3-Dimensional samples. Excitation/emission filters are available to enable four-color imaging including, DAPI, AF488, AF594, and AF647. Further image processing was performed using ImageJ software.

Whole-mount microscopy

Sample preparation and tissue fixation. Mouse lungs were perfused with 5mL of PBS and inflated to physiological volume with a 1% pFA solution instilled through the tracheae. After tying down the tracheae, lungs are removed from the chest cavity and fixed by overnight submersion in a 1% pFA solution.

Immunofluorescence staining. Cranial and post-caval lung lobes are blocked and permeabilized in blocking solutions including 0.1% Triton X overnight at 4 °C. Lobes are then immunostained with fluorophore-conjugated primary antibodies for 5 days with rocking at 4 °C.

Modified iDISCO. Lobes are then subjected to an ethanol solution dehydration gradient consisting of 1-hour sequential incubation steps in (50%, 70%, 80%, 100%, 100%) EtOH before being immersed into dibenzyl-ether or ethyl cinnamate overnight. Samples are rocked in dibenzyl-ether or ethyl cinnamate with these reagents being replenished with fresh reagents every other day for a period not shorter than a month.

Lightsheet whole-mount microscopy. Whole mount imaging was performed using the LaVision Ultramicroscope II Large Format Upright Lightsheet at the University of Chicago Integrated Light Microscopy Faciliity. Images were captured with a 4x NA 0.3 objective, with refractive index matching collar set to 1.55 to match dibenzylether and/or ethyl cinammate. Five-color imaging is possible through five solid state lasers at 440, 488, 561, 640 and 785 nm, and emission filters at 480/40, 525/50, 620/60, 680/30 and 845/55. Image deconvolution was performed using MatLab software and further image processing was performed using ImageJ software.

Mouse models of allergic airway disease

Sensitization and chronic challenge models. House dust mite (HDM) (Stallergenes Greer Laboratories, Lenoir, NC) was resuspended in sterile, endotoxin-free PBS. In an acute sensitization and challenge model, mice were administered 100ug HDM via intratracheal instillation (i.t.) on day 0, and further challenged with 25ug HDM on days 7, 8, 9 and 10, and were euthanized on d13. In a chronic model of allergic airway disease, mice were administered 100ug HDM via intratracheal instillation on day 0, and further challenged with 25ug HDM on days 7, 8, 9 ,10, 13, 16, 19, 22, 25, 28, and euthanized on d31.

Intratracheal allergen instillation. Mice are weighed one day prior to intratracheal instillation to determine appropriate anesthetic dose. In brief, mice are lightly anesthetized using a ketamine/xylazine solution that renders them immobile but lightly responsive. Mice's tongues are grasped to prevent ingestion of the allergen. After 50 uL of allergen solution is instilled through the pharyngeal route, mice are allowed to aspirate the fluid prior to tongue release.

Immunomodulatory factors: VEGF-C, VEGF-C$_{C156S}$, αVEGFR-3 blocking/isotype antibodies. To modulate the allergic airway inflammatory response, growth factors and antibodies

were resuspended in endotoxin-free PBS and kept sterile until administration. In all instances, the immunomodulatory factor is added to the individual allergen concoction seconds prior to intratracheal instillation.

Bronchoalveolar lavage. After mice are euthanized, bronchoalveolar lavage is isolated by using sterile PBS to wash the airways four times via a trachea-cannula for a total recovery of approximately 3mL. Cells are centrifuged and resuspended in an appropriate volume for flow cytometric analysis.

Lung cell processing. After euthanasia, lungs are dissected into its individual lobes and set for differential processing. The medial and caudal lobes are dissociated by mechanical mincing followed by a 1-hour long collagenase digestion with Collagenase IV (Catalog #LS004188, Worthington Biochemical, Lakewood, NJ) and Collagenase D. (Catalog #11088882001, Sigma-Aldrich, St. Louis, MO). Samples are then filtered through a 70-μm filter prior to red blood cell lysis using ACK lysing buffer (Catalog #A1049201, Fisher Scientific, Rockford, IL). Mediastinal lymph node samples are mechanically filtered through a 70-μm filter prior to resuspension in an appropriate final volume. All samples are then resuspended into single-cell suspensions and counted prior to flow cytometric analysis.

Blood processing & IgE ELISA. 20 – 40 uL blood samplings are collected through cheek-puncture of the submandibular vein. Blood drops are isolated in EDTA coated tubes (Catalog #22-040-200, Fisher Scientific, Rockford, IL) and centrifuged for 10 minutes at 15000 rpm at 4 °C in a tabletop conventional centrifuge. Plasma samples are carefully isolated and frozen at -80 °C for further analysis. To assess presence of distinct antibody isotypes, the plasma samples are thawed on ice, and resuspended to an appropriate volume for enzyme-linked immunoassay (ELISA) analysis. Anti-IgE ELISA was performed following the directions and reagents provided by

Invitrogen's IgE Mouse Uncoated ELISA kit (Catalog #50-112-5120, Invitrogen, ThermoFischer, Rockford, IL).

Protein isolation & ELISA. Post-caval lobes are dissected, placed in pre-weighed Eppendorf tubes, and weighed in an analytical scale prior to submersion into liquid nitrogen. To isolate protein, lobes are placed into lysing matrix D tubes (Catalog #116913100, MP Biomedicals, Santa Ana, CA) pre-filled with 600 uL of T PER Tissue Protein Extraction Reagent (Catalog# 78510, Thermo Fischer Scientific, Rockford, IL). After mechanical lysis of the lobe, protein solution is aliquoted and frozen for further ELISA analysis. To assess presence of distinct cytokines and growth factors in the protein lysate, samples are thawed on ice and resuspended in the appropriate volume for ELISA analysis. Anti-IL-4, IL-13, VEGF-C and VEGF-D ELISAs were performed following the respective directions and reagents provided in the ELISA kits. (Catalog# DY404, DY413, DY752B, DY469, R&D Systems, Minneapolis, MN)

Flow cytometry analysis. Lung single cell suspensions were resuspended in 50 uL FACS buffer (PBS containing 2% fetal bovine serum) and stained with fluorescently labeled antibodies. Intracellular transcription factor staining was performed using the FoxP3/Transcription Factor Staining Buffer Set (eBioscience #00-5523-00) and following the manufacturer's instructions. Samples were anlayzed using an LSR Fortessa (BD Biosciences) and the data analyzed using the FlowJo software. Cell sorting was performed using a BD FACSAriaIIIu (BD Biosciences). All cytometers are maintained by the Flow Cytometry and Antibody Technology Core Facility at the University of Chicago.

RNA sequencing. Lung single cell suspensions were prepared from mice that were sensitized and challenged with HDM or saline control. Lymphatic endothelial cells were stained and sorted, and RNA was extracted in Trizol following the manufacturer's instructions. The University of Chicago

Genomics Facility generated cDNA and sequencing libraries using Next-Gen Illumina Sequencing. Quality of the raw sequencing reads was assessed through FastQC. RNAreads were mapped onto the mm10 mouse reference genome.

Statistics. Statistical analyses were performed using the GraphPad Prism software. P-values less than 0.05 were considered as significant (* $p<0.05$, ** $p<0.01$, *** $p<0.0001$, **** $p<0.00001$). Comparisons between two groups were analyzed by unpaired two-tailed student's t-test (for equal variance), student's t-test with a Welch's correction (for unequal variance) or Mann-Whitney when appropriate. Comparisons between three groups or more were analyzed using 1-way ANOVA with Sidak

Study approval. All animal studies were approved by the University of Chicago Institutional Animal Care and Use Committee.

Chapter III: Pulmonary lymphangiogenesis is temporally decoupled from allergen inhalation

Abstract

In asthmatics, the lung stroma undergoes significant remodeling in response to chronic airway inflammation. Nonetheless, there are limited studies characterizing whether pulmonary lymphatics undergo growth or regression in this disease. In this chapter, we assessed the density of pulmonary lymphatics in a cohort of human lung samples obtained from donors with familial reports of asthma, or from age and sex-matched controls. We found that the asthmatic samples exhibited a higher pulmonary lymphatic vessel density compared to their control counterparts. Furthermore, we utilized a mouse mode of allergic airway inflammation to study the behavior of pulmonary lymphatics in the context of type II immunity. We found that pulmonary

lymphangiogenesis occurs late in the allergic response, once allergen inhalation ceases. Furthermore, chronic allergic inflammation blocked lymphatic expansion, suggesting that these two processes are not occurring independently from each other. Measurements of the pro-lymphangiogenic growth factors VEGF-C and VEGF-D, as well as the anti-lymphangiogenic cytokines, IL-4 and IL-13, revealed that an imbalance between these factors coincided with lymphatic vessel expansion and correlated with the numbers of pulmonary lymphatic endothelial cells. Altogether, these data suggest that pulmonary lymphangiogenesis is temporally decoupled from allergen-mediated inflammation, potentially through mechanisms involving the signaling molecules VEGF-C and VEGF-D as well as, IL-4 and IL-13.

Introduction

Chronic inflammatory diseases such as asthma, chronic obstructive pulmonary disease, and chronic pneumonia are associated with significant remodeling of the lung microvasculature (Detoraki, 2010; Hardavella, 2012; Baluk, 2009). Upon inflammation, pulmonary angiogenesis, the formation of newly blood vessels from preexisting ones, occurs and mediates traffic of immune effectors into the interstitium and subsequently the airway space (Detoraki, 2010). Chronic airway inflammation also drives lymphatic vessel proliferation and expansion via an analog process termed lymphangiogenesis, however, this vasculature provides an exit route to promote fluid drainage and immune cell exit from the lung tissue (Baluk, 2005). While lymphatic vessels proliferate in response to chronic inflammation, there is limited knowledge on whether there is communication or regulation between the immune system and lymphatic vessel remodeling.

Significant vascular remodeling has been reported as associated to patients suffering from bronchial asthma(Elias, 2003; Detoraki, 2010). As previously mentioned, current reports have provided evidence that the vascular endothelium proliferates and that its main angiogenic factor VEGF-A is enriched in asthmatic samples. In contrast, there is limited work determining whether pulmonary lymphatics undergo lymphangiogenesis in asthma, with few reports determining the sources of VEGF-C and VEGF-D (Detoraki, 2009) and analyzing lymphatics in fatal asthmatic samples (Ebina, 2008). Similarly, animal work studies on pulmonary lymphatics in allergic airway inflammation are limited, with only a couple of studies suggesting that pulmonary lymphangiogenesis occurs in a rat model of allergic airway inflammation (Moldobaeva, 2017) or in a mouse model of allergic airway inflammation (Maisel, 2021).

Recent work has introduced that two pro-inflammatory cytokines, IL-4 and IL-13, that play critical roles in type2 allergic asthma, have additional potent anti-lymphangiogenic activity (Shin, 2015; Savetsky, 2015). Both in vitro and in vivo studies revealed that IL-4 and IL-13 can impair lymphatic vessels through downregulation of their master transcription factor Prox1 (Shin, 2015; Savetsky, 2015), required to maintain the lymphatic endothelial cell identity (Johnson, 2008). Functionally, treatment with these anti-lymphangiogenic cytokines resulted in impaired lymphatic tube formation and reduced lymphatic endothelial cell migration. In mouse models of corneal lymphangiogenesis and airway inflammation, treatment with blocking antibodies against IL-4 and/or IL-13 or its common receptor chain IL-4Rα resulted in increased lymphatic vessel density, suggesting that the activity of these cytokines prevent lymphatic vessel growth in vivo (Shin, 2015; Savetsky, 2015). Taken together, these results highlight a novel role for the type II cytokines IL-4 and IL-13 in regulating lymphatic vessel proliferation and activity.

The goal of this chapter is to provide a reconciliation to the conundrum introduced by the latest mechanistic studies in animal models of allergic airway disease. Human studies have shown that the pulmonary vasculature undergoes significant remodeling in asthmatics, and animal models of allergic airway disease recapitulate this vessel growth. Nonetheless, it is now clear that two central cytokines to the allergic inflammatory response, IL-4 and IL-13, oppose the growth of pulmonary lymphatic vessels.

Here we utilize a mouse model of allergic airway inflammation utilizing the clinically relevant allergen house dust mite extract (HDM) to study the behavior of pulmonary lymphatic vessels in-vivo without pharmacological perturbations on the system. We found that pulmonary lymphangiogenesis occurs late in the allergic response, once allergen challenge ceases. Furthermore, chronic allergen instillation blocks the previously observed lymphatic expansion, suggesting that pulmonary lymphangiogenesis is not occurring independently from the allergic inflammatory response. Similarly, once chronic allergen instillation ceases, pulmonary lymphangiogenesis ensues. Measurements of the pro-lymphangiogenic growth factors, VEGF-C and VEGF-D, and the anti-lymphangiogenic cytokines, IL-4 and IL-13, present in the lungs at different time points in the response could not explain the lymphatic vessels behavior. However, defining a lymphangiogenesis score, which incorporated both the pro-lymphangiogenic growth factors and the anti-lymphangiogenic cytokines coincided with lymphatic vessel expansion and correlated with lymphatic endothelial cell numbers in the lungs of these mice. Altogether, these data suggest that pulmonary lymphangiogenesis in response to allergic airway inflammation is temporally decoupled from allergen challenge, potentially through a mechanism involving IL-4 and IL-13.

Results

Lung samples from asthmatic patients present with high lymphatic vessel density

Previous literature suggests that asthmatics tend to undergo significant airway and vessel remodeling in response to allergic inflammation associated with allergen encounter. To determine whether pulmonary lymphangiogenesis occurs in allergic airway inflammation, we interrogated human samples collected from non-transplantable lungs obtained through an organ procurement organization that provides regional services to circa 12 million people within the national donation system. From this set of tissues, we determined two cohorts of lung samples: one consisting on samples coming from patients with familial reports of asthma, as well as those whose cause of death was listed as an asthma attack, and a second one coming from patients with no familial history of asthma nor any medical report of pulmonary disease or immunological disorder. Control lung samples were selected to age and sex-match the asthmatic cohort.

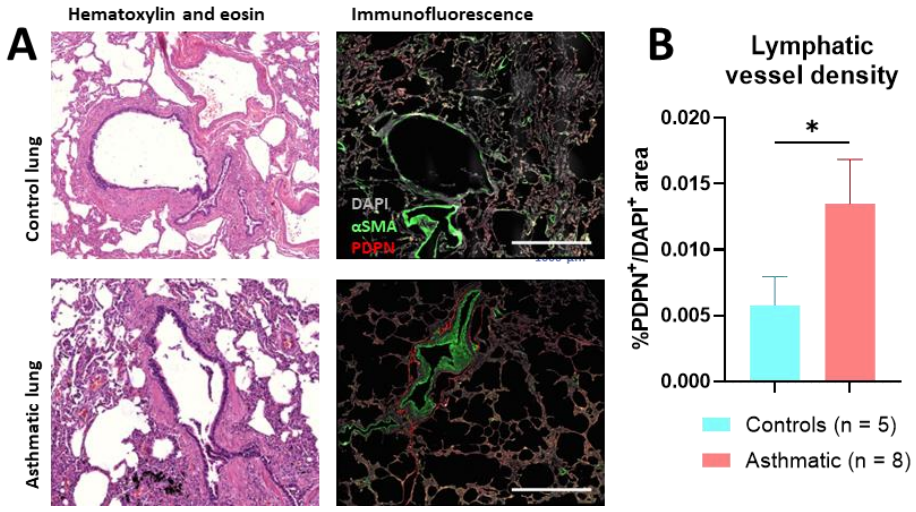

Figure 3-1. Asthmatic samples exhibit a high pulmonary lymphatic density

(A) Lung samples obtained from donors with familial reports of asthma, as well as, age and sex matched controls were stained with hematoxylin and eosin, or with immunofluorescent antibodies.
(B) Podoplanin quantification over 4 independent fields of view per human donor

Immunofluorescence antibody staining for podoplanin identifies human pulmonary lymphatic endothelial cells while staining for αSMA depicts expression surrounding airways as well as in the blood vasculature. (Figure 3.1 A) By using unbiased, automated, computational approaches, we were able to quantify the area determined by podoplanin positive pixels as well as DAPI positive pixels and determine a measure of lymphatic vessel density per $DAPI^{+}$ tissue per field of view. By averaging four fields of view over two non-serial section per patient, the data shows that lung samples from patients with familial reports of asthma present with a higher pulmonary lymphatic vessel density than that of control lungs. (Figure 3.1 B) This finding is in line with the results obtained from animal models of allergic airway disease (Moldobaeva, 2017; Maisel 2021).

A HDM mouse model of allergic airway inflammation recapitulates pulmonary lymphangiogenesis

Dust mite allergy is a prevalent disease that is roughly estimated to affect about 1-2% of the world's population, that is from 65 to 130 million people worldwide (Colloff, 2009). It has been estimated that up to 60% of patients with allergic asthma develop IgE antibodies against house dust mite allergens (Basagaña, 2004) and therefore, upon exposure to the mites, these patients develop respiratory symptoms that when mismanaged can lead to life-threatening events (Mayo Clinic, 2021).[18] The ability to culture the dust mites have provided an invaluable clinically relevant allergen as repeated instillation of the HDM extract to the upper or lower airways of mice results in an asthmatic-like disease mediated by an allergic immune response to the extract. We therefore implemented a sensitization and challenge model in mice through repeated intratracheal

instillation of the HDM allergen or PBS (as its vehicle control) to ask whether pulmonary lymphatic vessels underwent remodeling in response to allergic inflammation.

Mice were intratracheally dosed with either HDM or vehicle PBS control following the acute sensitization and challenge model before being euthanized early (at d13) or late (at d31) after the challenges to assess the state of the lymphatic vasculature. Through surface marker expression of CD31 and podoplanin (gp38) we can identify lymphatic endothelial cells from lung single cell suspensions (Figure 3.2 A). Quantification of the lymphatic and blood vascular endothelial cells revealed that pulmonary lymphatic endothelial cell numbers were significantly increased only late (by d31) and not early in the allergic response (Figure 3.2 B). In contrast to lymphatic endothelial cells, blood vascular endothelial cells showed no significant changes in our model of HDM sensitization and challenge as assessed by flow cytometry. This late increase in pulmonary lymphatic endothelial cells was corroborated by an unbiased, automated quantification of scanning confocal microscopy of 300 μm slices of the lungs from these mice (Figure 3.2 C). Finally, representative z-projections from the tiled, hyper-stacks depict the airways (αSMA^{+}) and the pulmonary lymphatic vasculature ($VEGFR3^{+}$) of the mice subjected to the acute HDM sensitization and challenge model (Figure 3.2 D).

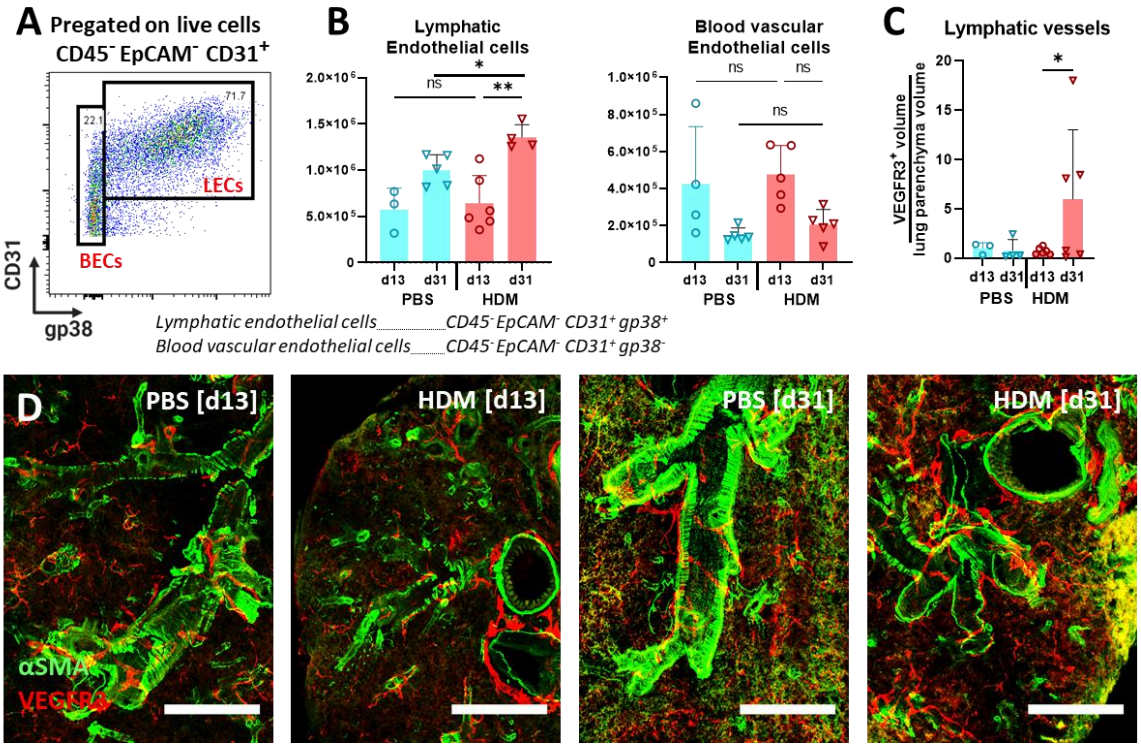

Figure 3-2. Pulmonary lymphangiogenesis occurs late in a mouse model of HDM sensitization and challenge

(A) Mice were sensitized and challenged following the acute sensitization and challenge schedule. Lung samples were dissociated into single cell suspensions and analyzed by flow cytometry. Representative gating identifying pulmonary endothelial cells.
(B) Pulmonary endothelial cell quantification. Immunofluorescence analysis of pulmonary lymphatic vessels.
(C) Pulmonary lymphatic vessel quantification.
(D) Representative lung micrographs.

Chronic HDM-mediated allergic airway inflammation prevents pulmonary lymphatic expansion

It has been demonstrated that T_H2 cells, through production of IL-4 and IL-13 cytokines central to the allergic airway pathology, restrict lymphatic endothelial cell expansion in both in-vitro models of lymphatic tube formation and in-vivo models of allergic airway inflammation and corneal lymphangiogenesis (Shin, 2015; Savetsky, 2015). In contrast to this study, we observed an expansion of the pulmonary lymphatic vasculature in response to a T_H2-mediated model of allergic

airway inflammation, albeit late in the response (Figure 2). We proposed two hypotheses to try and explain this phenomenon of late pulmonary lymphatic expansion. On one hand, it could be possible that HDM-mediated inflammation instigates pulmonary lymphangiogenesis, however the latter phenomenon is a slow process that occurs independently of allergic inflammation such that we can only observe differences late after the allergic insult. On the other hand, we could posit that HDM-mediated inflammation instigates pulmonary lymphangiogenesis while T_H2 cells restricts this expansion through production of IL-4 and IL-13. In such a scenario, we do not observe lymphatic expansion early in the response (as T_H2 cells are prevalent in responding to the allergen), but we observe lymphatic expansion late as allergen wanes and T_H2 reduce production of IL-4 and IL-13.

To test these hypotheses, we implemented a chronic model of HDM-mediated allergic airway inflammation (Figure 3.3 A). If our first hypothesis is correct and lymphatic expansion occurs independently from allergic HDM-mediated inflammation, we would expect to observe an expansion of the lymphatic vasculature by d31 from sensitization, regardless of whether the mice were subjected to an acute sensitization and challenge or to chronic allergen challenge. In contrast, if the second hypothesis is correct and pulmonary lymphangiogenesis is coupled to allergic HDM-mediated inflammation, we would expect that the lymphatic expansion observed by d31 of the sensitization and challenge model would be restricted in the chronic allergen challenge model.

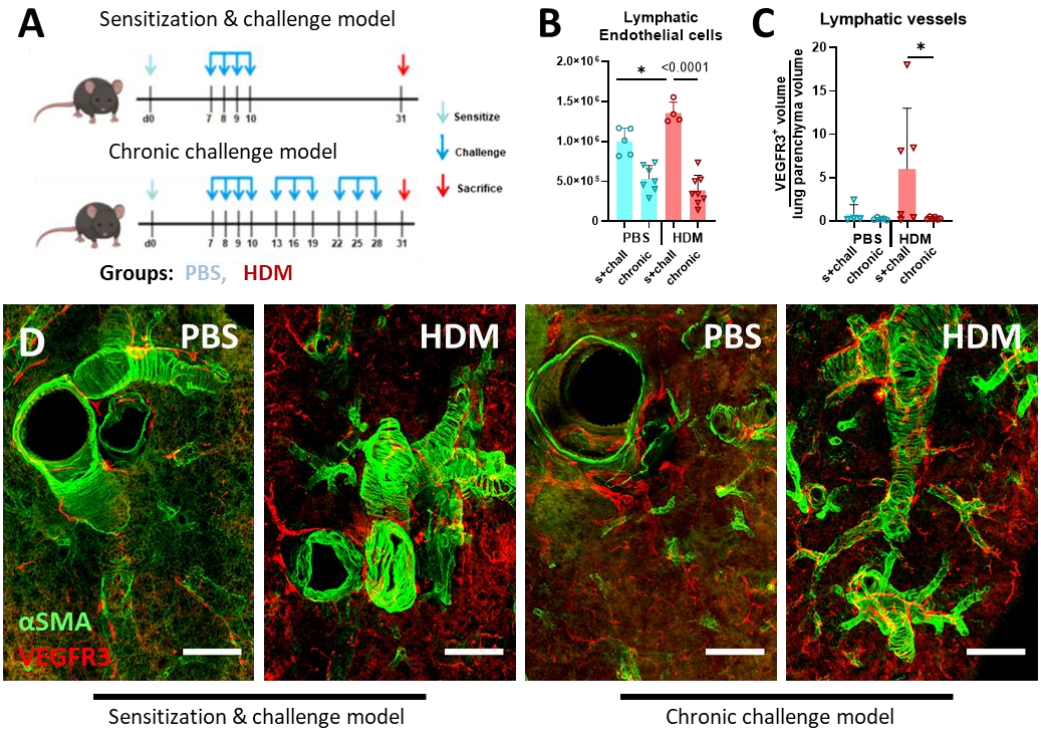

Figure 3-3. Chronic HDM-mediated inflammation prevents pulmonary lymphangiogenesis.

(A) Mice were sensitized and challenged following the acute sensitization and challenge schedule or the chronic schedule. Lung samples were dissociated into single cell suspensions and analyzed by flow cytometry.
(B) Pulmonary endothelial cell quantification. Immunofluorescence analysis of pulmonary lymphatic vessels.
(C) Pulmonary lymphatic vessel quantification.
(D) Representative lung micrographs.

Similar to previous experiments, we utilized flow cytometry to identify and quantify lymphatic endothelial cells from the lungs of mice subjected to the acute sensitization and challenge model or the chronic allergen challenge model. We observed a significant increase in pulmonary lymphatic endothelial cells in mice that were acutely challenged with HDM. In contrast, mice that were chronically challenged with HDM did not depict an increase in pulmonary lymphatic endothelial cells neither by flow cytometry nor by scanning confocal microscopy

(Figure 3.3 B & C). This was also depicted by representative z-projections of lung micrographs from mice subjected to the different allergic airway inflammation models stained for αSMA^+ depicting airways and $VEGFR3^+$ lymphatic vessels (Figure 3.3 D).

Cessation of allergen challenge results in pulmonary lymphangiogenesis in a chronic model of HDM-mediated allergic airway inflammation

The comparison between the sensitization and challenge model and the chronic allergen model suggests that pulmonary lymphangiogenesis is not happening independently from the allergic inflammatory response. If that is the case, we would expect that once allergen challenge subsides in the chronic HDM-inflammation model pulmonary lymphangiogenesis would ensue. To test this hypothesis, we subjected mice to the chronic HDM model and euthanized the mice shortly after the last challenge (d31) or late (d50) after the last challenge.

To identify lymphatic endothelial cells from lung single cell suspensions, we subjected the lung stromal compartment ($CD45^-$) to two unsupervised, unbiased, clustering algorithms: tSNE (Figure 3.4 A) and FlowSOM (Figure 3.4 B). The FlowSOM algorithm identified six differential cell populations based on the molecular marker expression of the lung single cell suspensions (Figure 3.4 B). By overlaying the FlowSOM populations on the tSNE plot, we found similar clustering on populations 0, 1, 3 and 4 by the two algorithms (Figure 3.4 A). Populations 2 and 5 were identified differently by FlowSOM and tSNE, and thus were disregarded for subsequent analysis. Based on molecular marker expression and population proportion in the lung stromal compartment, we identified population 1 ($CD31^+$ $Prox1^+$ $gp38^+$) as lymphatic endothelial cells, and population 4 ($CD31^+$ $Prox1^-$ $gp38^-$) as blood vascular endothelial cells. Quantification of these

populations revealed that pulmonary lymphatic endothelial cells significantly increase late by d50, in contrast to blood vascular endothelial cells (Figure 3.4 C). The increase in pulmonary lymphatic endothelial cells by d50 was corroborated by scanning confocal microscopy depicting pulmonary lymphatic vessels through staining for VEGFR3^{+} in 300 µm lung sections (Figure 3.4 D) and quantified using unbiased, automated methods (Figure 3.4 E).

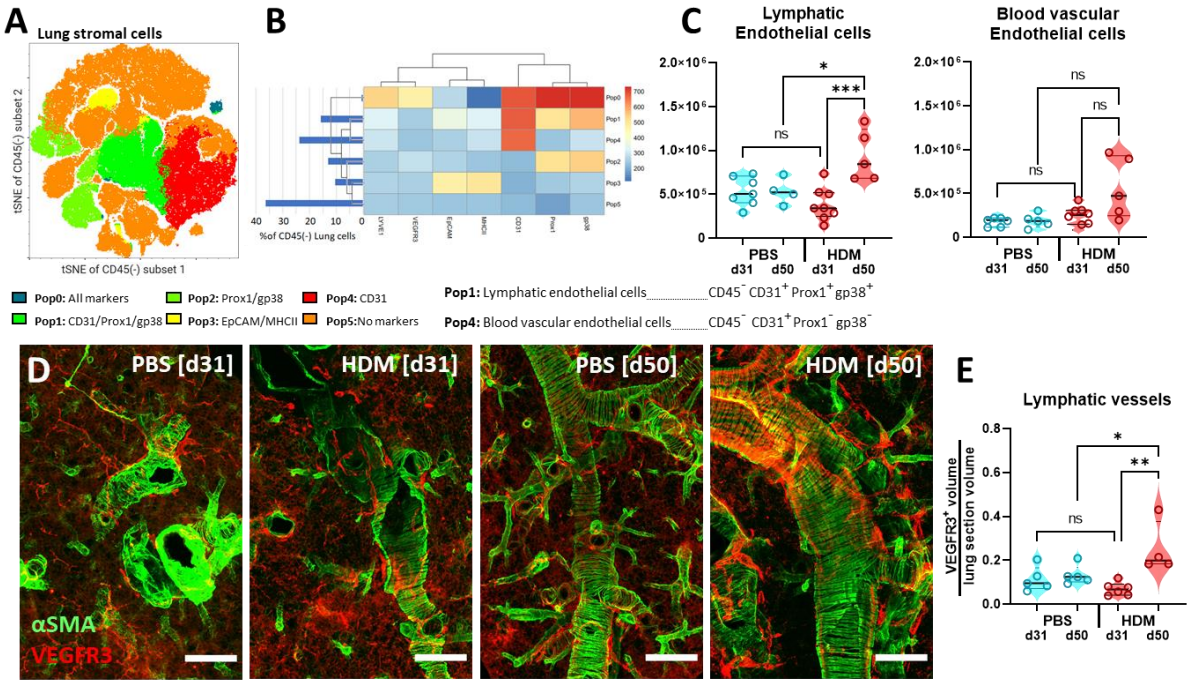

Figure 3-4. Pulmonary lymphangiogenesis ensues after chronic allergen inhalation subsides.

(A) Mice were sensitized and challenged following the chronic schedule. Lung samples were dissociated into single cell suspensions and analyzed by flow cytometry. FlowSOM stromal populations superimposed on a TSNE plot.
(B) Surface marker expression on FlowSOM cell populations.
(C) Pulmonary endothelial cell quantification.
(D) Representative lung micrographs.
(E) Pulmonary lymphatic vessel quantification.

In chronic allergy, an imbalance between pro-lymphangiogenic growth factors and anti-lymphangiogenic cytokines temporally decouples pulmonary lymphangiogenesis from allergen inhalation

Given the behavior that pulmonary lymphatic endothelial cells exhibit in response to chronic allergic airway inflammation, we turned to the lymphatic growth factors VEGF-C and VEGF-D as an attempt to explain its regulation. We generated protein isolates through mechanical dissociation of the post-caval lobe from the lungs of mice that were subjected to chronic allergic airway inflammation and had been euthanized early or late after the last challenge. By performing ELISA against the pro-lymphangiogenic growth factors VEGF-C and VEGF-D, we found that these were significantly increased with allergic inflammation regardless of the timepoint the samples were collected at (Figure 3.5 A & B). Given that the increases in the lymphatic growth factors did not coincide with the previously observed increase in pulmonary lymphatic endothelial cells (Figure 3.4 C), it was not surprising that neither growth factor correlated with the number of lymphatic endothelial cells in the lung (Figure 3.5 C). We therefore turned our attention to additional cytokines which may regulate the behavior of the pulmonary lymphatic vasculature in response to allergic inflammation.

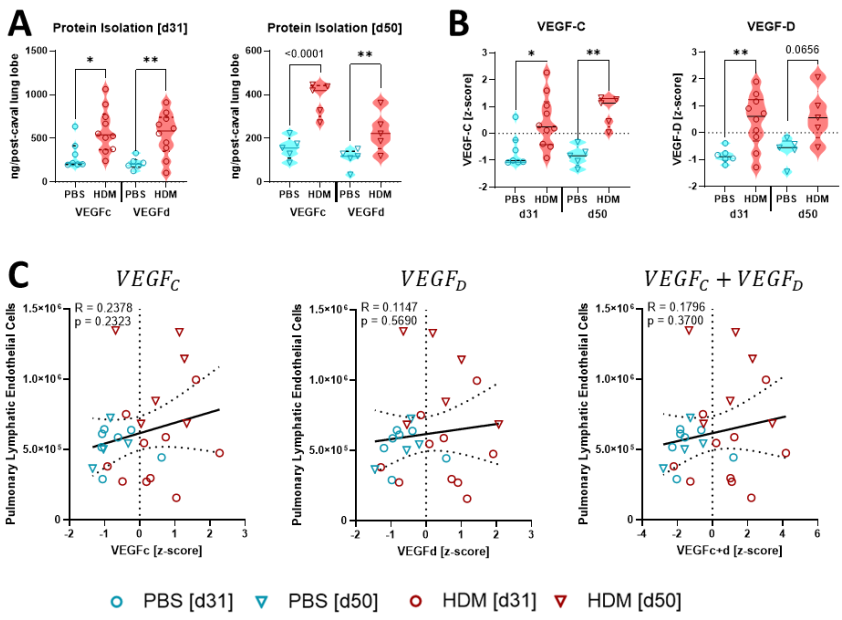

Figure 3-5. The pro-lymphangiogenesis growth factors VEGF-C and VEGF-D are increased in response to HDM-mediated chronic allergic airway inflammation.

(A) Lung samples were obtained from mice subjected to the chronic schedule of allergic airway inflammation. Lung VEGF-C and VEGF-D levels were assessed by ELISA. Lung VEGF-C and VEGF-D levels at d31 or d50.
(B) Lung VEGF-C and VEGF-D z-scores.
(C) Correlation plots between pulmonary lymphatic endothelial cell numbers and the lymphangiogenic growth factor z-scores.

Recently, it has been demonstrated that T_H2 cells, through expression of IL-4 and IL-13, can regulate lymphangiogenesis in the context of airway inflammation and corneal lymphatic expansion.[13, 14] If such is the case, we could posit that a balance between the pro-lymphangiogenic growth factors and the anti-lymphangiogenic cytokines would result in growth stasis in pulmonary lymphatic endothelial cells, while an imbalance in favor of the growth factors would lead to lymphangiogenesis. To test this hypothesis, we assessed the levels of IL-4 and IL-13 by performing the corresponding ELISA on the protein isolates acquired from the post-caval lobes. We found that the levels of IL-4 and IL-13 were significantly increased in the

lung samples from mice that were euthanized close to the last HDM challenge (Figure 3.6 A). In contrast, the levels of IL-4 and IL-13 were no longer significantly increased in lung samples from mice that were euthanized late after the last HDM challenge (Figure 3.6 B).

Given that each growth factor and cytokine acts at different concentration levels in-vivo, we relied on z-scores to perform a head-to-head comparison between the pro-lymphangiogenic growth factors to the anti-lymphangiogenic cytokines. Following this rationale, we defined a lymphangiogenesis z-score ($LAG_{z\text{-}score}$) which ratios the sum of the VEGF-C and VEGF-D z-scores against the z-scores from IL-4 and IL-13 (Figure 3.6 C). A linear transformation prevented the emergence of undetermined forms as the original z-distribution is centered at the origin. In mice that were subjected to chronic allergy and euthanized close to the last allergen challenge, the $LAG_{z\text{-}score}$ was close to one suggesting that the growth factor activity was balanced to the anti-lymphangiogenic cytokine activity (Figure 3.6 C). In contrast, in mice were subjected to chronic allergy but euthanized late after the last allergen challenge, the $LAG_{z\text{-}score}$ was higher than one, in some cases more than double, suggesting that the activity of the pro-lymphangiogenic growth factors may overcome the regulation from the anti-lymphangiogenic cytokines (Figure 3.6 C). Furthermore, the $LAG_{z\text{-}score}$ significantly correlated well with the number of pulmonary lymphatic endothelial cells coming from the mice subjected to chronic allergy, but not with the number of blood vascular endothelial cells (Figure 3.6 D), suggesting that the regulation mediated through the imbalance of VEGF-C, VEGF-D, IL-4, and IL-13 may be specific to the lymphatic endothelium.

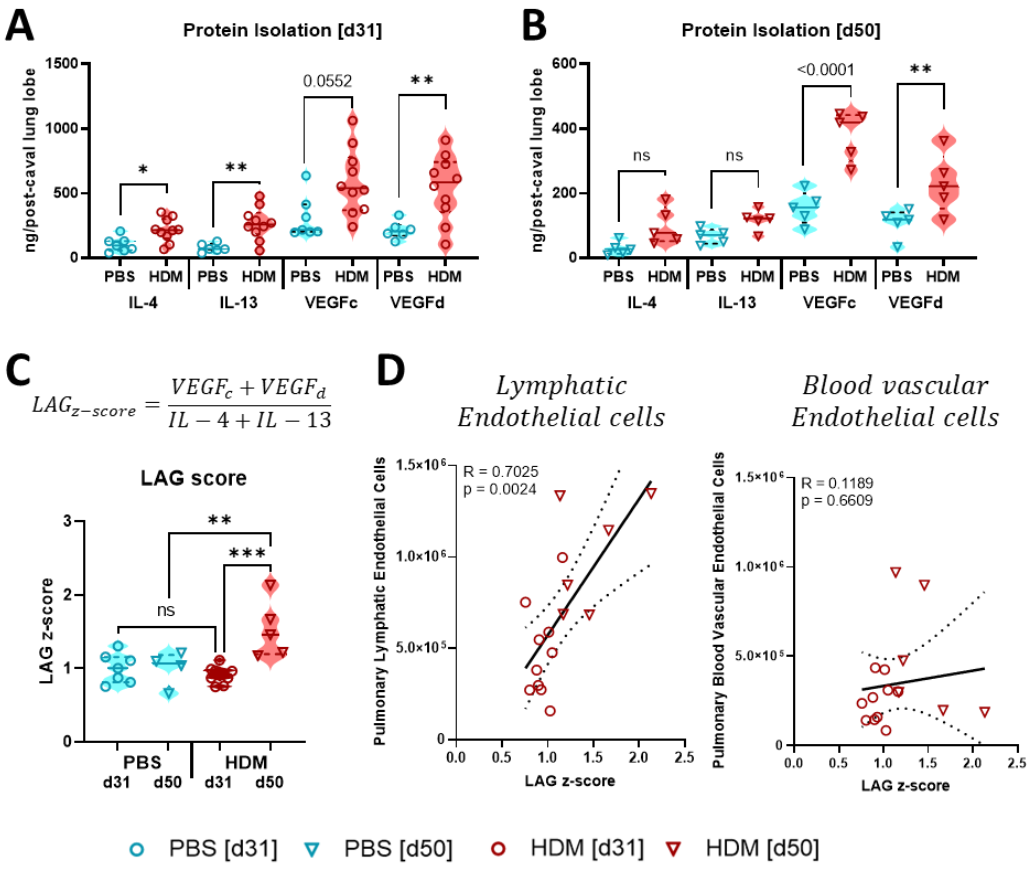

Figure 3-6. A lymphangiogenesis score, that incorporates both the pro- lymphangiogenesis growth factors VEGF-C and VEGF-D as well as the anti-lymphangiogenesis cytokines IL-4 and IL-13, coincides with pulmonary vessel expansion and correlates with pulmonary lymphatic endothelial cell numbers.

(A) Lung samples were obtained from mice subjected to the chronic schedule of allergic airway inflammation. Lung IL-4, IL-13, VEGF-C and VEGF-D levels were assessed by ELISA. Lung IL-4, IL-13, VEGF-C and VEGF-D levels at d31
(B) Lung IL-4, IL-13, VEGF-C and VEGF-D levels at d50
(C) Lymphangiogenesis z-score.
(D) Correlation plots between pulmonary endothelial cell numbers and the lymphangiogenesis z-score.

Human studies have shown that the pulmonary vasculature of asthmatic patients undergoes significant remodeling, and both angiogenesis and lymphangiogenesis have been recapitulated in animal models of allergic airway disease.[5-8,11] The finding the IL-4 and IL-13

prevent the growth of pulmonary lymphatic vessels provided a mechanistic challenge to the previous observations that pulmonary lymphatics may expand in response to allergic inflammation.[12-14] By utilizing a mouse model of HDM-mediated allergic airway inflammation, we studied the behavior of pulmonary lymphatic vessels in-vivo without pharmacological or genetic perturbations. Altogether, our results suggest that pulmonary lymphangiogenesis is temporally decoupled from allergen challenge, only occurring once allergen challenge ceases. While we cannot provide a causative role without the use of genetic mouse models, we can provide a mechanistic reconciliation by which an imbalance between the pro-lymphangiogenic growth factors, VEGF-C and VEGF-D, and the anti-lymphangiogenic cytokines, IL-4 and IL-13, could explain why pulmonary lymphangiogenesis ensues once allergen ceases (Figure 3.7). We also introduced the notion of gauging the pro-lymphangiogenic growth factors against the anti-lymphangiogenic cytokines through the definition of a lymphangiogenesis z-score, which, in our mouse model of HDM-mediated chronic allergic inflammation, correlated well with the numbers of pulmonary lymphatic endothelial cells. Finally, while this model could provide an attractive reconciliation to the conundrum of whether pulmonary lymphatics expand or not in patients suffering from allergic asthma, only future mechanistic studies, through the use of genetic tools, could test and refine it, as its purpose is to serve as a first step in understanding the behavior of the pulmonary lymphatic endothelium in the context of allergic asthma.

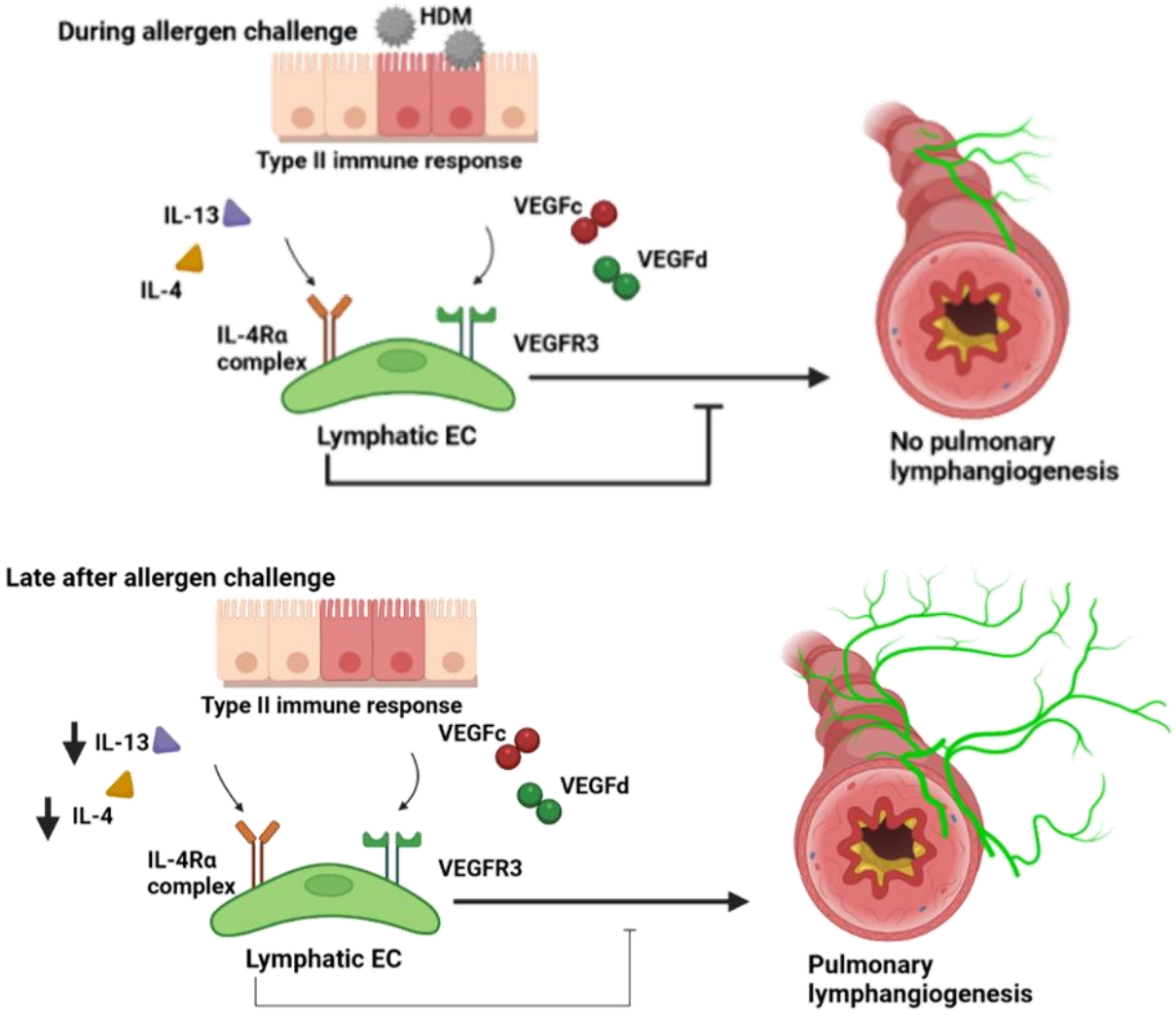

Figure 3-7. An imbalance between the prolymphangiogenic growth factors and the antilymphangiogenic type II cytokines temporally decouple pulmonary lymphangiogenesis from allergen inhalation.

Discussion

In this section, we found that lung samples coming from patients with familial reports of asthma, including patients whose cause of death was listed as an asthma attack, tend to have a higher pulmonary lymphatic density compared to control samples coming from patients with no familial history of asthma nor any medical report of pulmonary disease or immunological

disorder. We then implemented HDM-mediated mouse models of acute and chronic allergic airway inflammation to study the behavior of pulmonary lymphatics in response to the allergic immune response in the absence of pharmacological perturbations. In both models we found that pulmonary lymphatics do expand in response to allergic airway inflammation, albeit they do so late after allergen challenge has ceased. This temporal decoupling of allergen-induced type 2 inflammation and pulmonary lymphangiogenesis is consistent with the current literature, including that which suggests that animal models of allergic airway inflammation exhibit pulmonary lymphangiogenesis, as well as with the reports that suggest that the type two cytokines prevent this phenomenon from happening. This study, therefore, provides initial understanding into the behavior pulmonary lymphatics undertake in response to allergic airway inflammation and aims at reconciling apparent conflicting views in the current literature.

Previous literature suggested a role for pulmonary lymphatics in allergic airway disease, as samples from asthmatics were enriched in the VEGF growth factors and animal models recapitulated both angiogenesis and lymphangiogenesis in response to a clinically relevant allergen HDM. The recent finding that the T_H2 cytokines, IL-4 and IL-13, inhibit lymphangiogenesis posed a conundrum with the previous literature. The authors in the recent study had utilized both pharmacological inhibition of IL-4 and/or IL-13 as well as their common high affinity receptor chain IL4Rα, as well as genetic deletion of IL4Rα to study the behavior of pulmonary lymphatics in models of airway disease. While the authors provided mechanistic understanding through in-vitro cocultures and pharmacological perturbations in this system, the in-vivo models were limited by the fact that IL-4, IL-13, and IL4Rα play central roles in mediating allergic airway inflammation and therefore the pharmacological and genetic perturbations were not specific in targeting solely the lymphatic vasculature. In contrast, we

decided to study the behavior of the pulmonary lymphatic vasculature in the context of allergic airway disease without the use of pharmacological or genetic perturbation as to not hinder the immune response itself. In these models of allergic airway disease, we found a temporal decoupling of pulmonary lymphangiogenesis from allergen induced inflammation. This behavior could not solely be explained by the lymphatic growth factors VEGF-C and/or VEGF-D, as these were enriched in the lungs of mice receiving the allergen regardless of the timepoint or schedule. In agreement with the mechanism posited by the recent literature, late decreases in IL-4 and IL-13 coincided with increases in pulmonary lymphatic endothelial cells. Furthermore, by gaging both the pro-lymphangiogenesis growth factors, VEGF-C and VEGF-D, against the anti-lymphangiogenic cytokines, IL-4 and IL-13, allowed us to determine a lymphangiogenesis score which coincided with the state of the lymphatic vasculature and correlated with the number of pulmonary lymphatic endothelial cells. Taken together, these data suggest that while mechanistically IL-4 and IL-13 restrict pulmonary lymphangiogenesis, lymphatic vessel growth can happen in response to allergic airway inflammation albeit late after allergen challenge, presumably when the increases in IL-4 and IL-13 subside.

Given how allergic airway inflammation and pulmonary lymphangiogenesis are regulated in such a way that both phenomena will not occur concurrently, it is tempting to posit that there could be an evolutionary benefit to preserve such temporal regulation. If such were the case, one could ponder about the detrimental consequences that an individual could undergo if both phenomena were to occur concurrently. These questions drive the motivation for the subsequent studies presented in the following chapter.

Chapter IV: VEGFR3-driven, pulmonary lymphangiogenesis exacerbates induction of bronchus associated lymphoid tissue in a CXCL13 dependent manner.

Abstract

Asthma is a debilitating chronic inflammatory disease that causes airway obstruction and diminishes lung functionality. Patients suffering from allergic asthma exhibit chronic airway inflammation due to immune hyperresponsiveness to innocuous respiratory allergens. In asthmatic sera, vascular endothelial growth factor (VEGF) -C levels correlated with IgE levels and fatal asthmatic lung samples present with a higher amount of bronchus associated lymphoid tissue

(BALT), however, it is unclear whether these two phenomena are interconnected. In this study, we used a model of house dust mite extract (HDM) mediated chronic allergic airway inflammation to probe whether VEGF-C signaling could be causative in raising IgE levels in chronic allergy. We have here demonstrated that VEGF-C driven pulmonary lymphangiogenesis promotes induction of BALT and production of IgE. We determined that the VEGF-C mediated exacerbation acts through VEGF receptor (VEGFR)-3 expressed by lymphatic endothelial cells (LEC), and that specific pharmacological stimulation and blocking of VEGFR-3 coincides with an exacerbated and reduced induction of BALT respectively. Next, using a genetic mouse model of pulmonary lymphangiogenesis, we showed that an expanded lymphatic vasculature predisposed the lung microenvironment to develop BALT. In response to HDM-mediated chronic airway inflammation, pulmonary LECs upregulate CXCL13, and furthermore CXCL13 levels in the lung positively correlated with the extent of BALT. Consistent with this finding, CXCL13 heterozygous and knock-out (but not wild-type) mice were protected from an exacerbated induction of BALT in response to co-instillation of VEGF-C with the HDM allergen. Finally, pharmacological blocking during the challenge phase of the chronic allergy model reduced induction of BALT and correlated with reduced levels of lung IgE levels. Altogether, these results suggest a causative role for pulmonary LECs in mediating induction of BALT in chronic allergic airway inflammation and identifies the CXCL13 CXCR5 chemotactic pathway as a potential therapeutic target for patients suffering from chronic allergic asthma.

Introduction

More than 300 million people worldwide live with asthma, with more than 25 million asthma patients residing in the U.S. alone (GINA, 2015). It has long been recognized that asthmatic

lungs acquire a baseline state of chronic airway inflammation, and that this inflammation in turn causes significant remodeling of the airways and the pulmonary vasculature (Detoraki 2010). Nonetheless, the roles the pulmonary blood and lymphatic vasculatures play with respect to allergic asthma have been fairly understudied. Certainly, asthmatic lungs undergo significant vessel remodeling, as bronchoscopy biopsies have revealed increased vascularity and higher vessel density in asthmatics compared to control samples (Vrugt, 2000, Wilson, 2002, Li, 1997). Similarly, previous studies have identified potential cellular sources of the vascular endothelial growth factors (VEGF) in-vitro (Detoraki, 2009), however there is modest work in identifying the role of VEGFs in asthma (Hoshino, 2001; Feltis, 2006; Lee, 2001). In contrast, a different study suggested that there may be reduced numbers of airway associated lymphatic vessels in samples from patients who died from an asthma attack, however this study had a limited sample cohort overrepresented with older subjects (Ebina 2006). The discrepancies in these observations may be due to most of these studies not differentiating between the blood and lymphatic vasculatures when performing their observations, nor distinguishing between major blood vessels and lymphatic collectors from the lung microvasculature. Because of these discrepancies, we have limited our understanding of the roles the blood and lymphatic pulmonary vasculatures may play in mediating allergic airway disease.

Animal models of allergic disease have modestly improved our understanding of the pulmonary vasculature in allergic airway disease. Currently, there is moderate work aiming to delineate the role for the blood and lymphatic vasculature and their respective growth factors by modeling allergic airway inflammation in animal models of disease. Certainly, there is extensive work on determining the role of the blood vasculature and its main growth factor VEGF-A in mediating allergic disease, and most of these studies have identified a pathological role for blood

vessels and for VEGF-A driven angiogenesis (Lee, 2004; Shen 2018, Meyer and Akdis 2013). In contrast to the blood vasculature, there is limited focus on the role for the lymphatic vasculature in allergic airway inflammation and there seems to be little consensus for its role in mediating the disease. Work from Shin *et al* and Savetsky *et al* have demonstrated that IL-4 and IL-13, two of the central cytokines that mediate type II allergic inflammation, restrain lymphatic growth and prevent lymphangiogenesis in mouse models of allergic airway inflammation and corneal lymphangiogenesis respectively (Shin, 2015; Savetsky, 2015). In contrast to these, Moldobaeva *et al* reported that pulmonary lymphangiogenesis is triggered in a rat model of house dust mite mediated allergic airway inflammation, and that the pulmonary LECs isolated from these rats displayed a higher proliferative phenotype (Moldobaeva, 2017). We have previously reported that in a mouse model of HDM-mediated allergic airway inflammation, pulmonary lymphangiogenesis occurs, albeit, after the initial inflammatory stimulus is long gone (Maisel, 2021). Thus, we posit that the role for pulmonary lymphatics in allergic airway disease remains controversial, as its behavior is highly dependent on the temporal state of the disease at which this vasculature is analyzed.

In this current study, we investigated serum samples from asthmatics and controls and found a positive correlation between the lymphatic growth factor, VEGF-C and a mediator of the allergic disease, hIgE. We therefore proceeded to study the role of the lymphatic growth factor VEGF-C and its cognate receptor VEGFR-3 in pulmonary lymphatics to assess whether these play a role in raising the IgE levels in a chronic model of HDM-mediated allergic airway disease. Through pharmacological stimulation and inhibition of VEGFR3, the main mitotic receptor for the lymphatic endothelium, we found that in chronic allergic airway inflammation, expansion of the pulmonary lymphatic vasculature presented with higher induction of bronchus associated

lymphoid tissue and higher levels of local lung and systemic IgE levels, while inhibition reduced such induction. Using genetic mouse models, we tracked the phenotype to VEGFR3-bearing pulmonary lymphatic endothelial cells, and these in turn significantly change their chemokine transcriptome in response to HDM-mediated chronic allergic airway inflammation. We focused on expression of CXCL13, the B lymphocyte chemoattractant, as this was an attractive factor for mediating induction of bronchus associated lymphoid tissue and an attractive target to ameliorate such induction. Our findings suggest a mechanism where in response to HDM-driven allergy, pulmonary lymphatic endothelial cells can upregulate CXCL13, and mediate induction of bronchus associated lymphoid tissue. Furthermore, as bronchus associated lymphoid tissue was primarily found in samples from asthmatics who died due to an asthma attack, CXCL13 may be a novel therapeutic target to ameliorate chronic inflammation associated to allergic asthma.

Results

Chronic allergic airway inflammation associated to asthma presents with bronchus associated lymphoid tissue in mouse and human samples.

To assess whether lung samples from asthmatics tend to develop bronchus associated lymphoid tissue, we examined lung tissue sections from donors who died from an asthma attack, as well as those with familial reports of asthma who died from unrelated causes. All lung samples were procured through the Gift of Hope Organ Bank of Illinois. Control lungs with no reported lung disease were selected to match the asthmatic samples based on their age, sex, race, and use of noxious substances.

To assess the presence of bronchus associated lymphoid tissue, we performed immunofluorescence to identify major structures in the lung tissue utilizing antibodies against podoplanin, CD20 and αSMA. (Figure 4-1A, B). As previously shown (Al-Muhsen, 2017), we found that, when compared to their matched control cohort, lung samples from asthmatics tend to have higher amounts of $CD20^{+}$ area per field of view which identified structures reminiscent of bronchus associated lymphoid tissue (Figure 4-1C). When the asthmatic samples were segregated by their cause of death, we found that the samples from asthmatics who died from an asthma attack significantly differed from their control cohort (Figure 4-1D). Consistent with their familial reports, sera from the asthmatic cohort contained higher levels of IgE compared to the matched control cohort, and these IgE levels did not differ when segregated by their cause of death (Figure 4-1E, F). Finally, we found a borderline significant correlation between the serum levels of IgE and the lymphatic growth factor VEGF-C in the asthmatic cohort but not in the control cohort. (Figure 4-1G).

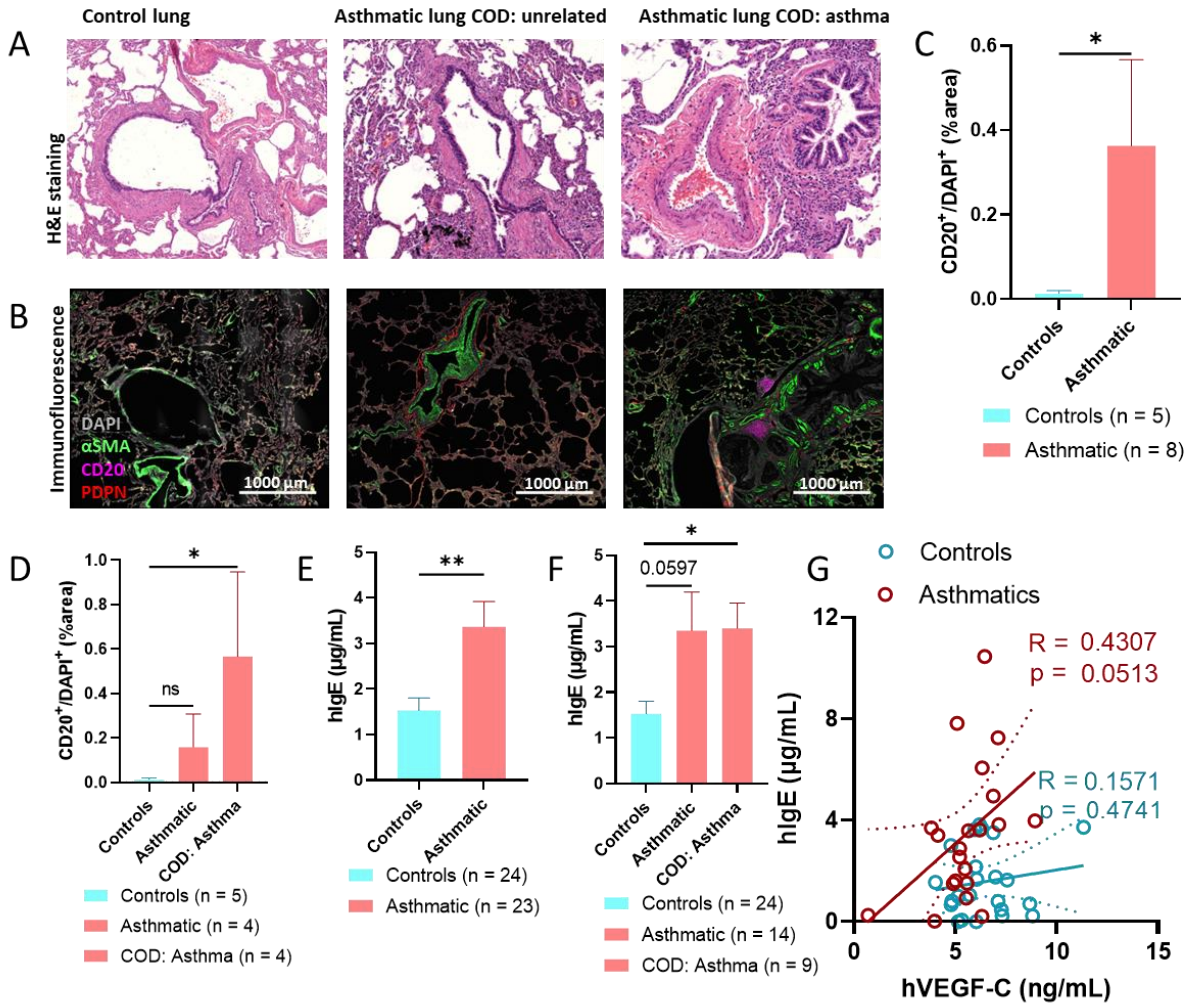

Figure 4-1. In asthmatic sera, VEGF-C levels positively correlate with IgE levels.

(A) H&E staining of lung samples from control subjects, asthmatics whose cause of death (COD) is unrelated to asthma, and asthmatics where the COD was an asthma attack.
(B) Immunofluorescence staining of the previous human lung sample cohorts for αSMA, CD20 and podoplanin.
(C) Quantification for CD20+ area in asthmatic samples compared to control samples.
(D) Quantification for CD20+ area, in controls and asthmatic samples segregated by COD.
(E) Levels of serum human IgE (hIgE) in asthmatic samples compared to control samples as measured by ELISA.
(F) Levels of serum hIgE in controls and asthmatic samples segregated by COD.
(G) Levels of serum hIgE and VEGF-C from controls subjects (depicted in blue) and asthmatic subjects (depicted in red). R Pearson coefficient.

To study the induction of bronchus associated lymphoid tissue, we implemented a house dust mite (HDM) model of chronic allergic airway inflammation. One month after their initial sensitization, mice that were chronically subjected to the allergen developed tertiary lymphoid structures, reminiscent of bronchus associated lymphoid tissue (Figure 4-2A, B), as well as higher levels of local IgE. (Figure 4-2C). Furthermore, in HDM-treated mice (but not PBS-treated controls) the VEGF-C levels positively correlated with the IgE levels isolated from the lung tissue. (Figure 4-2D). Additionally, mice that were chronically challenged with the allergen developed lung and airway allergic inflammation, including lung and airway eosinophilia, increased $CD4^+$ T cell infiltration, and airway B cell infiltration (Figure 4-2E, F). Together, these data show that induction of bronchus associated lymphoid tissue occurs in asthmatics, particularly in those undergoing an asthma attack, and that this phenomenon can be recapitulated in a mouse model of chronic allergic airway inflammation utilizing the clinically relevant human allergen HDM extract.

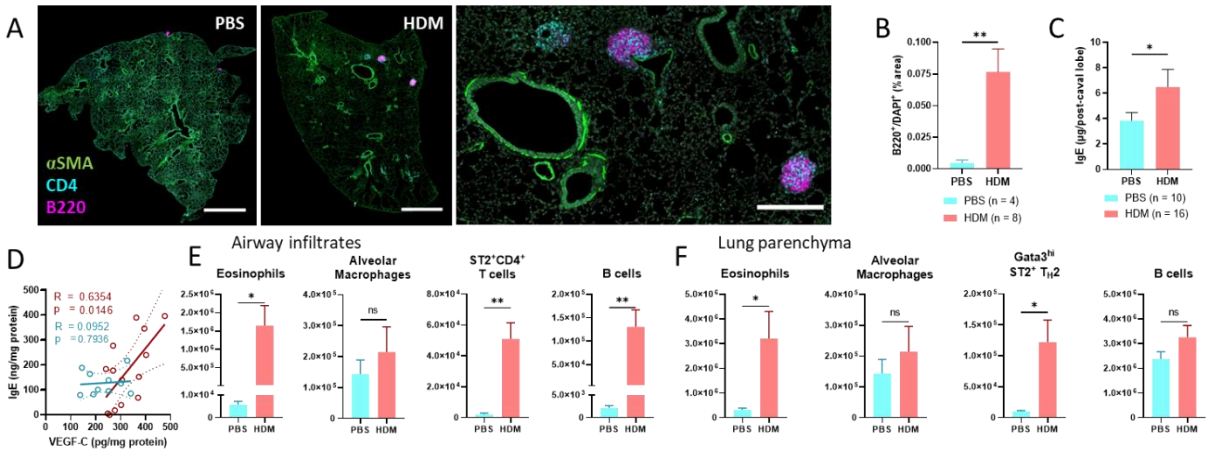

Figure 4-2. In mice subjected to chronic allergic airway inflammation, lung VEGF-C levels positively correlate with lung IgE levels.

(A) Immunofluorescence staining for αSMA, CD4 and B220 in lungs from mice that were subjected to chronic allergic airway inflammation with HDM or with PBS vehicle control. Scale bar 1mm for the tiled sections, 300um for the higher magnification micrograph.
(B) Quantification for B220+ area over whole lung sections.
(C) Levels of lung IgE protein as determined by ELISA.

Figure 4-2. - continued.

(D) Levels of lung IgE and VEGF-C from PBS-treated mice (depicted in blue) and HDM-treated mice (depicted in red). R Pearson coefficient.
(E) Airway immune infiltrates procured from bronchoalveolar lavage fluid (BALF) as identified by flow cytometry.
(F) Lung immune infiltrates as identified by flow cytometry.

In chronically HDM-inflamed allergic mice, stimulation of the VEGF-C or the VEGFR3 signaling axis exacerbates induction of bronchus associated lymphoid tissue and local IgE levels.

Given that asthmatic sera exhibited a positive correlation between VEGF-C and IgE levels, we proceeded our study by asking what the role for VEGF-C is in chronic allergic airway inflammation. To address the role of VEGF-C signaling, we modulated our HDM-model of chronic airway inflammation by co-administering VEGF-C with allergen through intra-tracheal instillation. When compared to HDM-inflamed control mice, mice that received HDM and VEGF-C displayed hyperplastic pulmonary lymphatic vessels (Figure 4-3A) and increased numbers of lung lymphatic endothelial cells (Figure 3B). Strikingly, mice that were inflamed with HDM and VEGF-C, or alternatively with HDM and VEGF-C_{C156S} (a VEGFR3 specific agonist), displayed significantly higher levels of lung IgE (Figure 4-3C). Furthermore, mice that were modulated with the lymphatic growth factors displayed a higher amount of bronchus associated lymphoid tissue (Figure 4-3D, E) assessed through $B220^{+}$ area, and this measure significantly correlated with the levels of lung IgE. The increase of bronchus associated lymphoid tissue was corroborated by whole-mount lightsheet microscopy of post-caval lung lobes (Figure 4-3G, H). Finally, mice that were subjected to chronic HDM-inflammation modulated with VEGF-C or with VEGF-C_{C156S} overall tend to have higher levels of plasma IgE compared to their HDM-inflamed controls (Figure 4-3I). Altogether, these changes suggest that in chronically allergic mice, stimulation of the VEGF-C and VEGFR3 signaling axis leads to the development of bronchus associated lymphoid tissues and IgE production.

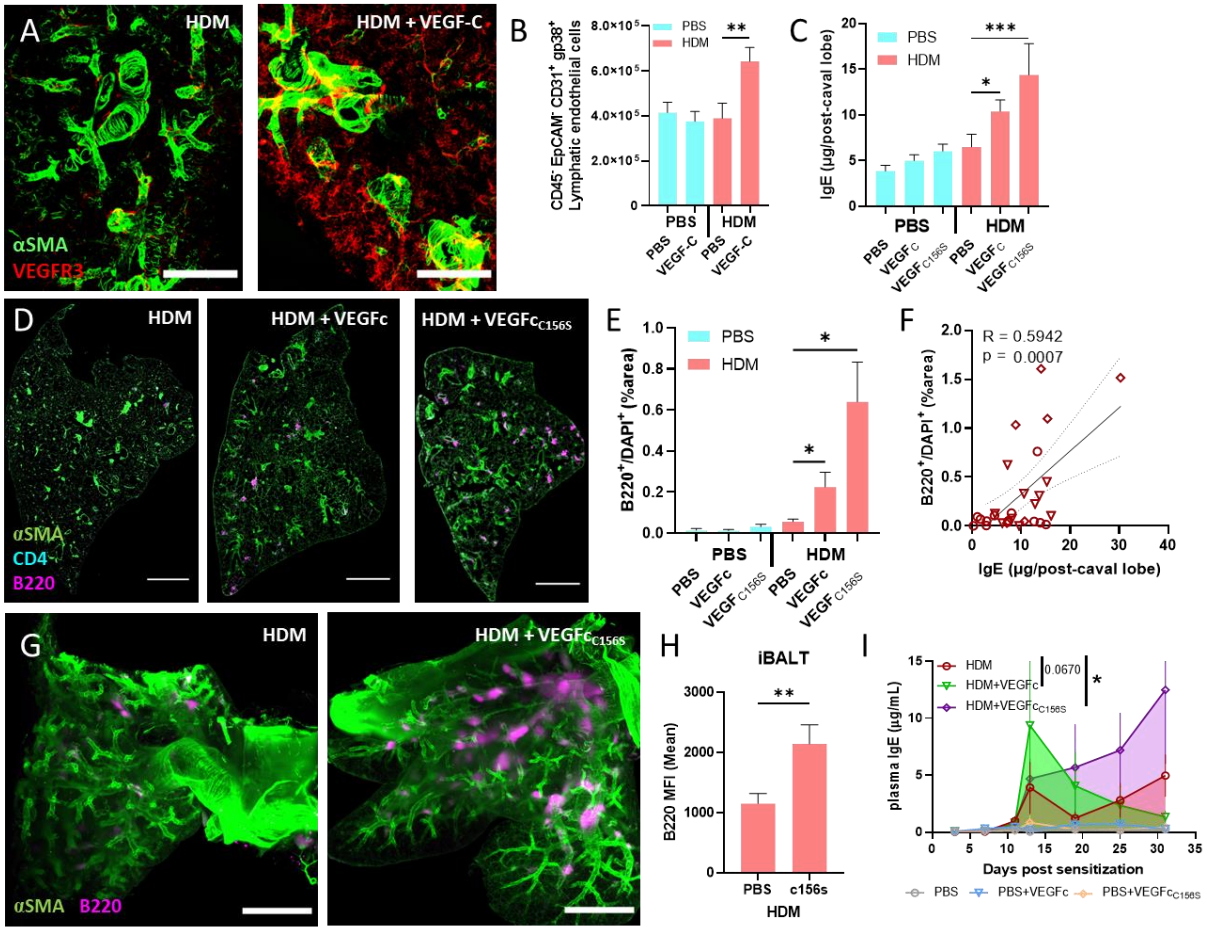

Figure 4-3. In chronically allergic mice, VEGF-C signaling drives lung IgE levels and iBALT.

(A) Scanning confocal immunofluorescent micrographs for αSMA, VEGFR-3 of 300um thick lung sections from mice that were subjected to chronic allergy with HDM modulated with VEGF-C or with PBS vehicle control. Scale bar 300um.
(B) Pulmonary lymphatic endothelial cells as identified by flow cytometry.
(C) Levels of lung IgE protein as determined by ELISA.
(D) Immunofluorescence staining for αSMA, CD4 and B220 in lungs from mice that were subjected to chronic allergy with HDM modulated with VEGF-C, with the VEGFR-3 specific agonist VEGF-CC156S or with PBS vehicle control. Scale bar 1mm.
(E) Quantification for B220+ area over whole lung sections.
(F) Paired analysis of lung levels of IgE with the levels of bronchus associated lymphoid tissue as determined by lung section B220+ area. R Pearson coefficient.
(G) Whole-mount immunofluorescent microscopy of iDISCO-cleared post-caval lung lobes from mice subjected to chronic allergy with HDM modulated with the VEGFR-3 specific agonist VEGF-CC156S or with PBS vehicle control. Scale bar 1mm.
(H) Quantification for B220+ signal.
(I) Levels of plasma IgE throughout the allergic response as determined by ELISA. Statistics compare area under the curve as determined through Riemann sums.

Induction of bronchus associated lymphoid tissue requires VEGFR3 expression in lymphatic endothelial cells

Given that specific stimulation of VEGFR3 (through the use of VEGF-C_{C156S}) was sufficient to exacerbate induction of bronchus associated lymphoid tissue in chronically HDM-inflamed mice, we pondered whether VEGFR3 blocking would diminish induction of bronchus associated lymphoid tissue. To address this question, we utilized a VEGFR3-functionally blocking antibody (clone mF4-31C1 kindly provided by Eli Lilly) to block VEGFR3 signaling throughout the chronic challenge phase of our HDM-mouse model. We found that systemic blocking of VEGFR3, through intraperitoneal injection of the antibody failed to decrease the levels of bronchus associated tissue in mouse lungs (Figure 4-4A, B). In contrast to systemic injections, local blocking of VEGFR3, through intra-tracheal co-administration of the blocking antibody (or isotype control) with the allergen, significantly diminished the levels of bronchus associated lymphoid tissue (Figure 4-4C, D). Together with previous results, in chronically HDM-inflamed mice, modulation of the VEGFR3 signaling pathway through stimulation with VEGF-C_{C156S} or blocking with mF4-31C1, increases and diminishes respectively the induction of bronchus associated lymphoid tissue, suggesting a causative role for VEGFR3 signaling in mediating induction of bronchus associated lymphoid tissue.

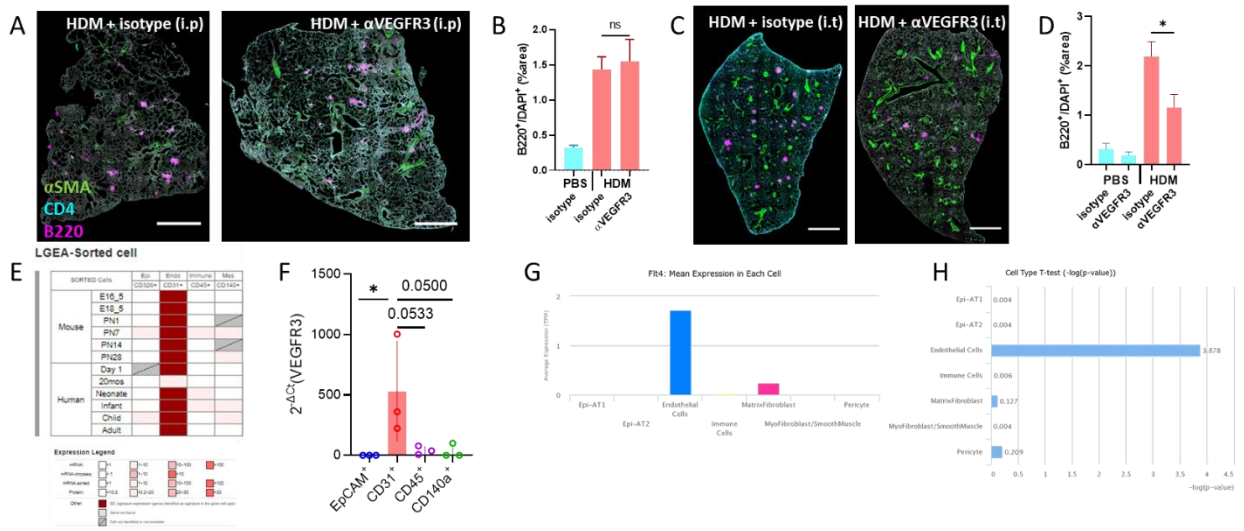

Figure 4-4. Induction of bronchus associated lymphoid tissue requires local VEGFR3 signaling; VEGFR3 is expressed by lung endothelial cells

(A) Immunofluorescence staining for αSMA, CD4 and B220 in lungs from mice that were subjected to chronic allergy with HDM modulated with intraperitoneal (i.p.) injection of αVEGFR-3 functionally blocking antibodies or control isotype antibodies. Scale bar 1mm.
(B) Quantification for B220+ area over whole lung sections.
(C) Immunofluorescence staining for αSMA, CD4 and B220 in lungs from mice that were subjected to chronic allergy with HDM modulated with intratracheal (i.t.) instillation of αVEGFR-3 functionally blocking antibodies or control isotype antibodies. Scale bar 1mm.
(D) Quantification for B220+ area over whole lung sections.
(E) FLT4 expression as determined by bulk RNA sequencing (RNAseq) from FACSorted mouse and human lung cells from the LGEA publicly available datasets attained from LungGENS.
(F) FLT4 expression as determined by bulk RNA sequencing (RNAseq) from FACSorted mouse lung cells.
(G) FLT4 expression as determined by single cell RNAseq mouse lung cells from the LGEA publicly available datasets attained from LungGENS.
(H) Cell type t-test of FLT4 expression as determined by single cell RNAseq from the LGEA publicly available datasets attained from LungGENS.

Given the importance of the VEGFR3 signaling pathway in the development of bronchus associated lymphoid tissue, we turned to identify which lung cell type would be the source of VEGFR3 responsible for the modulation of bronchus associated lymphoid tissue. We suspected that pulmonary lymphatic endothelial cells would be a major source of VEGFR3 in the lung tissue,

nonetheless, recent literature has suggested VEGFR3 expression by other cell types including alveolar macrophages and blood endothelial cells (Yamashita, 2022 ; Asosingh, 2018). To assess expression of VEGFR3 in lung cells, we turned to publicly available RNA sequencing (RNAseq) datasets from the lung gene expression in single cell (LungGENS) database (Du, 2015; Du 2017). In the lung gene expression analysis (LGEA) web portal, we interrogated RNAseq datasets from mouse and human lung FACSorted cells for expression of VEGFR3 (FLT4). These datasets placed expression of VEGFR3 primarly to the mouse lung endothelial cell compartment, with little to no expression in the epithelial ($CD326^+$), immune ($CD45^+$) or mesenchymal ($CD140a^+$) compartments (Figure 4-4E). Similarly, we FACSorted lung cells using the same gating strategy and isolated RNA from the epithelial ($CD326^+$), immune ($CD45^+$), mesenchymal ($CD140a^+$) and endothelial ($CD31^+$) lung cell compartments. After performing reverse transcription and quantitative polymerase chain reaction (qPCR), we determined that VEGFR3 expression is found in the lung endothelial cell compartment, with little to no expression in the other cell compartments. Furthermore, single cell RNAseq datasets from mouse lung tissue corroborate that VEGFR3 is primarily expressed by endothelial cells, with low expression in the matrix fibroblast and pericyte compartments (Figure 4-4G, H). Altogether, these data suggest that in the lung, VEGFR3 is primarily expressed by endothelial cells.

To determine whether VEGFR3 expression by lymphatic endothelial cells plays a role in mediating bronchus associated lymphoid tissue in chronic allergic airway disease, we subjected $Prox1^{CreERT2}VEGFR3^{lox/lox}$ mice and their $VEGFR3^{lox/lox}$ littermate control mice to our HDM-mediated schedule of allergic inflammation. Prior to instigating chronic allergy, transgenic and littermate control mice received systemic administration of tamoxifen to excise the VEGFR3 floxed alleles in Prox1-expressing lymphatic endothelial cells effectively implementing a LEC-

specific, inducible conditional VEGFR3 knockout mouse model (LEC^{VEGFR3KO}) (Haiko, 2008). In contrast to VEGFR3$^{lox/lox}$ littermate controls, the LEC^{VEGFR3KO} mice exhibited a borderline significant reduction in their induction of bronchus associated lymphoid tissue (Figure 4-5A, B) in response to HDM-mediated chronic inflammation. This phenotype was accompanied by reduced levels of plasma IgE throughout the response (Figure 4-5C).

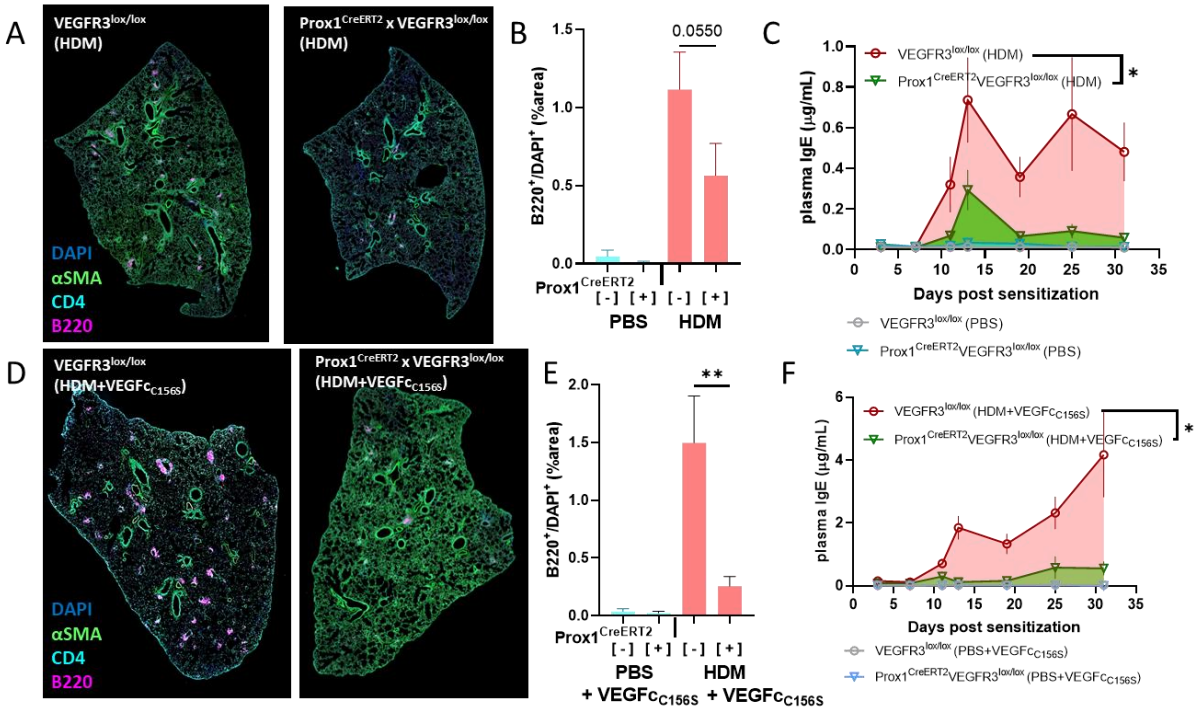

Figure 4-5. Induction of bronchus associated lymphoid tissue requires VEGFR3 expression by lymphatic endothelial cells.

(A) Immunofluorescence staining for αSMA, CD4 and B220 in lungs from VEGFR3lox/lox mice or Prox1CreERT2VEGFR3lox/lox mice subjected to chronic allergy with HDM or with PBS vehicle control. Scale bar 1mm.
(B) Quantification for B220+ area over whole lung sections.
(C) Levels of plasma IgE throughout the allergic response as determined by ELISA. Statistics compare area under the curve as determined through Riemann sums.
(D) Immunofluorescence staining for αSMA, CD4 and B220 in lungs from VEGFR3lox/lox mice or Prox1CreERT2VEGFR3lox/lox mice subjected to chronic allergy with PBS or HDM modulated with the VEGFR-3 specific agonist VEGF-CC156S. Scale bar 1mm.
(E) Quantification for B220+ area over whole lung sections.
(F) Levels of plasma IgE throughout the allergic response as determined by ELISA. Statistics compare area under the curve as determined through Riemann sums.

Next, we wanted to assess whether the VEGF-C mediated, exacerbated bronchus associated lymphoid tissue response was acting through VEGFR3 expressed by lymphatic endothelial cells. After tamoxifen induction to excise the VEGFR3 floxed alleles, tranasgenic $LEC^{VEGFR3KO}$ mice and $VEGFR3^{lox/lox}$ littermate control mice were subjected to chronic allergic airway inflammation with co-administration of HDM and the VEGFR3-specific agonist $VEGF\text{-}C_{C156S}$. Littermate control mice exhibited an exacerbated bronchus associated response, which was not raised in the $LEC^{VEGFR3KO}$ mice (Figure 4-5D, E). Furthermore, $LEC^{VEGFR3KO}$ mice raised lower levels of systemic IgE throughout the allergic response. (Figure 4-5F) Altogether, these results suggest that in response to chronic allergy, VEGFR3 in lymphatic endothelial cells play an important role in mediating bronchus associated lymphoid tissue and that its expression in lymphatic endothelial cells is necessary to mediate the VEGF-C driven exacerbations.

An expanded pulmonary lymphatic vasculature predisposes the lung microenvironment to develop bronchus associated lymphoid tissue in response to chronic allergic airway inflammation.

Given that modulation of the VEGF-C and VEGFR3 signaling axis modulates the extent of bronchus associated lymphoid tissue in chronic allergic inflammation, and that this phenomenon is acting through lymphatic endothelial cells, we asked whether an overrepresentation of the lung lymphatic vasculature could recapitulate the exacerbated bronchus associated lymphoid tissue response in the absence of pharmacological intervention. To determine whether pulmonary lymphatic endothelial cells could mediate induction of bronchus associated lymphoid tissue, we utilized a mouse model which allowed us to overrepresent lymphatic endothelial cells in the lung tissue (Lammoglia, 2016). The $CCSP^{rtTA}VEGF\text{-}D^{TRE}$ mouse allows for doxycycline controlled ectopic expression of VEGF-D in club cells in the lung. When subjected to a doxycycline

containing diet, CCSP^{rtTA}VEGF-D^{TRE} mice exhibited an expanded pulmonary lymphatic vasculature (Figure 4-6A) and higher numbers of lymphatic endothelial cells in their lungs (Figure 4-6B). Similarly, pulmonary lymphatic endothelial cells isolated from CCSP^{rtTA}VEGF-D^{TRE} mice exhibited higher expression of the proliferating cell nuclear antigen (Figure 4-6C, D). Despite this significant lung remodeling, the lung immune microenvironment did not exhibit significant changes in either innate cells, antigen presenting cells, or their T cell compartment (Figure 4-7A, B). Because in the CCSP^{rtTA}VEGF-D^{TRE} mice the expanded lung lymphatic vasculature persists for up to ninety days post doxycycline induction (Lammoglia, 2016), we utilized these mice to assess whether having an overrepresentation of the pulmonary lymphatic vasculature would predispose the lung microenvironment to develop bronchus associated lymphoid tissue in response to chronic allergic airway inflammation.

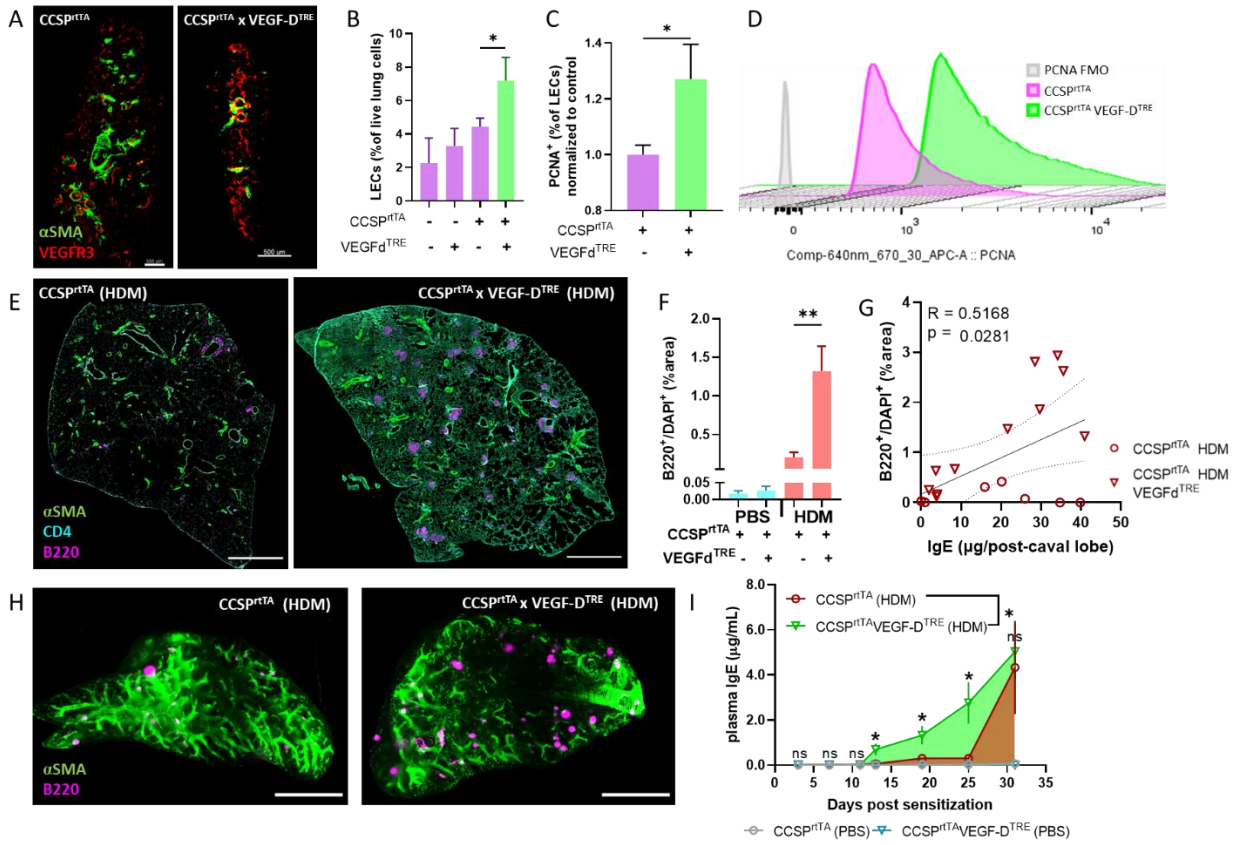

Figure 4-6. An expanded pulmonary lymphatic vasculature predisposes the lung microenvironment to develop bronchus associated lymphoid tissue in response to chronic allergy.

(A) Scanning confocal immunofluorescent micrographs for αSMA, VEGFR-3 of 300um thick lung sections from CCSP^{rtTA}VEGF-D^{TRE} transgenic mice or littermate CCSP^{rtTA} control mice after doxycycline treatment.
(B) Pulmonary lymphatic endothelial cells as identified by flow cytometry.
(C) Proliferating cell nuclear antigen (PCNA)+ pulmonary lymphatic endothelial cells as quantified by flow cytometry.
(D) PCNA expression in pulmonary LECs as determined by flow cytometry.
(E) Immunofluorescence staining for αSMA, CD4 and B220 in lungs from doxycycline activated CCSP^{rtTA}VEGF-D^{TRE} transgenic mice or littermate CCSP^{rtTA} control mice subjected to chronic allergy with HDM or with PBS vehicle control. Scale bar 1mm.
(F) Quantification for B220+ area over whole lung sections.
(G) Paired analysis of lung levels of IgE with the levels of bronchus associated lymphoid tissue as determined by lung section B220+ area. R Pearson coefficient.

Figure 4-6. - continued

(H) Whole-mount immunofluorescent microscropy of iDISCO-cleared cranial lung lobes from doxycycline activated CCSP^{rtTA}VEGF-D^{TRE} transgenic mice or littermate CCSP^{rtTA} control mice subjected to chronic HDM allergy. Scale bar 1mm.

(I) Levels of plasma IgE throughout the allergic response as determined by ELISA. Statistics compare area under the curve as determined through Riemann sums.

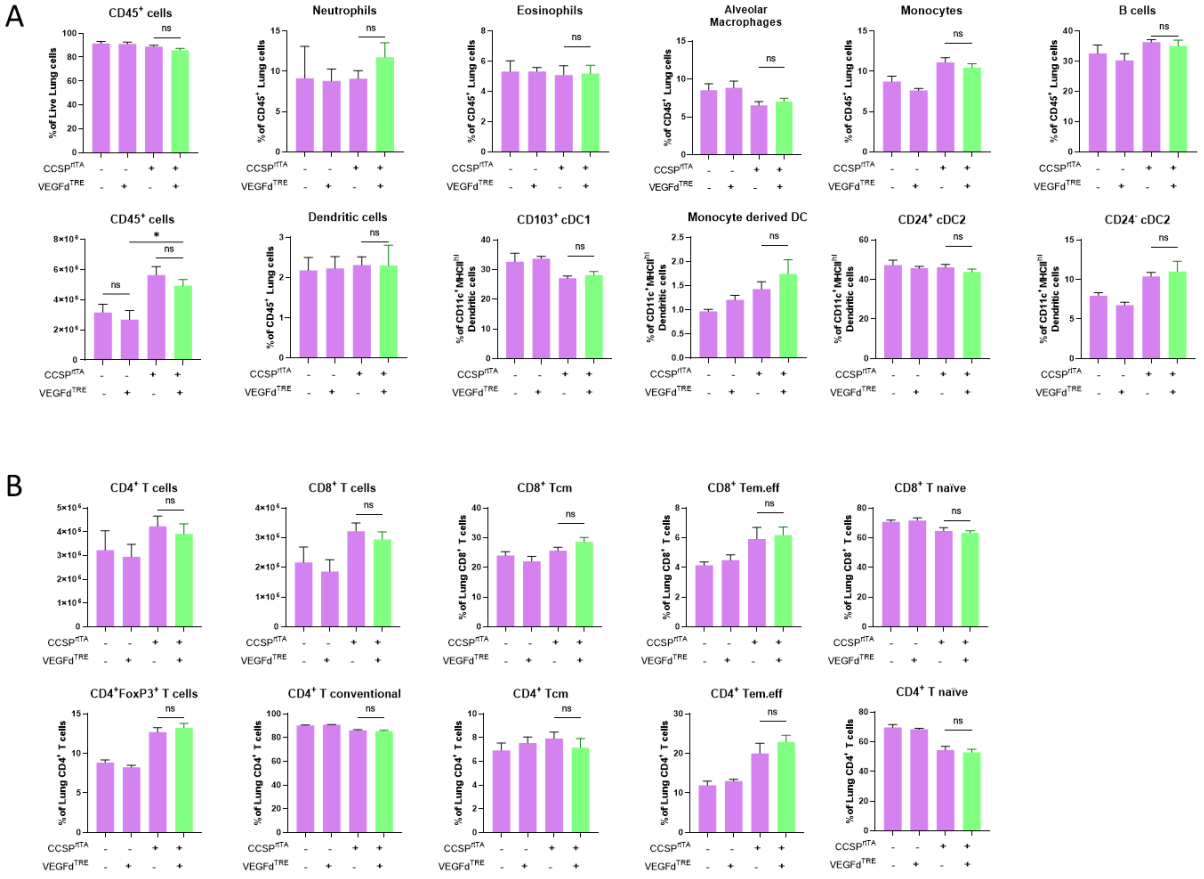

Figure 4-7. Expansion of the pulmonary lymphatic vasculature minimally changes the lung immune microenvironment.

(A) Lung immune infiltrates as identified by flow cytometry from CCSP^{rtTA}VEGF-D^{TRE} transgenic mice or littermate CCSP^{rtTA} single positive, VEGF-D^{TRE} single positive, or double negative control mice after doxycycline treatment.

(B) Lung T cell compartment as identified by flow cytometry from CCSP^{rtTA}VEGF-D^{TRE} transgenic mice or littermate CCSP^{rtTA} single positive, VEGF-D^{TRE} single positive, or double negative control mice after doxycycline treatment.

To determine whether an expanded pulmonary lymphatic vasculature would predispose the lung microenvironment to develop bronchus associated lymphoid tissue, we subjected $CCSP^{rtTA}$VEGF-D^{TRE} transgenic mice or $CCSP^{rtTA}$ control mice to chronic allergic airway inflammation after their initial doxycycline induction. In contrast to their single transgene control counterparts, $CCSP^{rtTA}$VEGF-D^{TRE} mice developed high levels of bronchus associated lymphoid tissue in response to chronic allergen challenge (Figure 4-6E, F). Furthermore, the local levels of IgE significantly correlated with the levels of bronchus associated lymphoid tissue (Figure 4-6G). The iBALT increase was corroborated by whole mount immunofluorescence lightsheet microscopy (Figure 4-6H), and the $CCSP^{rtTA}$VEGF-D^{TRE} mice exhibited higher systemic IgE levels throughout the response compared to their single transgene control counterparts (Figure 4-6I). Altogether, these results suggest that having an expanded pulmonary lymphatic vasculature predisposes the lung microenvironment to develop bronchus associated lymphoid tissue and higher systemic levels of IgE in response to chronic allergic airway inflammation.

In chronic allergy, pulmonary lymphatic endothelial cells upregulate CXCL13 which is critical for the VEGF-C mediated exacerbated induction of bronchus associated lymphoid tissue.

Given that in the context of chronic allergy, an expanded pulmonary lymphatic vasculature could exacerbate the induction of bronchus associated lymphoid tissue in the absence of VEGFR3-stimulating pharmacological agents, we posited that induction of bronchus associated lymphoid tissue could be partially mediated by a lymphatic endothelial cell response to chronic allergic airway inflammation. To determine the changes in the pulmonary lymphatic endothelial cell transcriptome in response to chronic allergic airway inflammation, we performed RNAseq on pulmonary lymphatic endothelial cells isolated from HDM-treated or saline control treated mice.

RNAseq revealed a modest set of differentially expressed genes between pulmonary lymphatic endothelial cells coming from HDM-treated mice compared to their saline treated control counterparts (data not shown). Further analysis of the RNAseq dataset revealed differential expression on chemokine transcripts in pulmonary lymphatic endothelial cells in response to HDM-mediated inflammation (Figure 4-8A, B). Of notice, the B lymphocyte chemoattractant, CXCL13, showed a substantial and significant upregulation from its expression levels at baseline in control mice (Figure 4-8C). Furthermore, the protein levels of CXCL13 in the lungs of chronically HDM-inflamed mice significantly correlated with the levels of bronchus associated lymphoid tissue (Figure 4-8D). Similarly, when mice were co-treated with allergen and VEGF-C, they exhibited increased lung infiltration by cells bearing the CXCL13-cognate receptor CXCR5, as well as infiltration by lymphocytes bearing expression of this receptor (Figure 4-8E). Together, these data suggest that pulmonary lymphatic endothelial cells upregulate CXCL13 in response to chronic allergy and that the CXCL13 and CXCR5 chemotactic axis plays an important role in mediating induction of bronchus associated lymphoid tissue in response to chronic allergic airway inflammation.

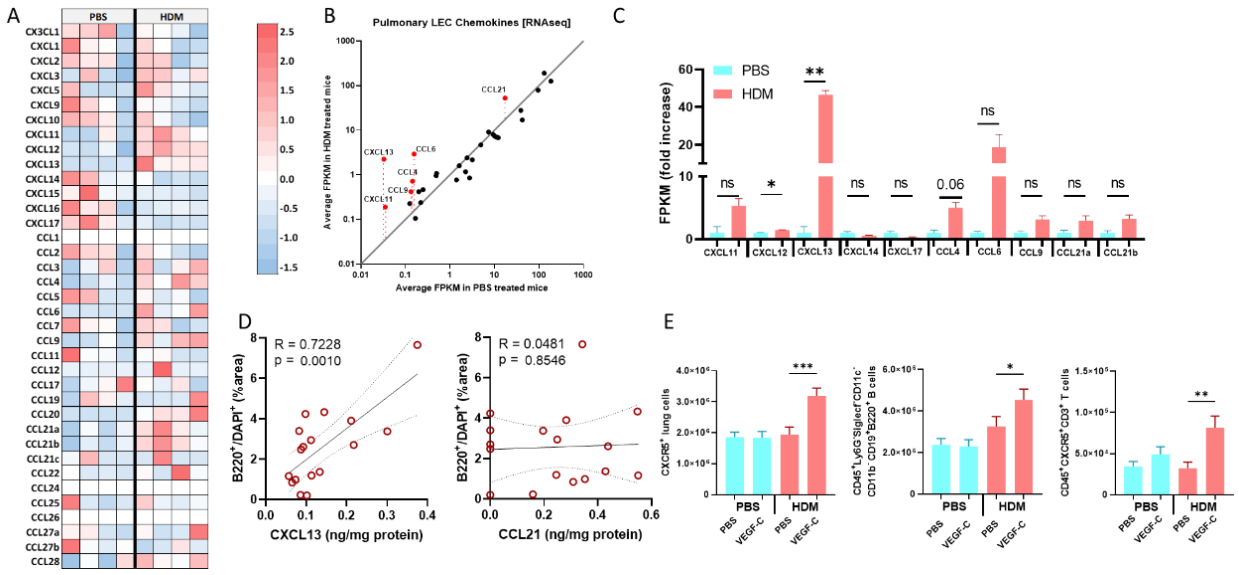

Figure 4-8. In response to chronic allergy, pulmonary LECs express CXCL13, and lung CXCL13 positively correlates with bronchus associated lymphoid tissue.

(A) FPKM of chemokines expressed by pulmonary lymphatic endothelial cells isolated from mice subjected to chronic HDM mediated allergy or to PBS vehicle control.
(B) Average FPKM quantification. Chemokines in red reach independent statistical significance when compared between the two groups.
(C) Average chemokine FPKM expressed as fold increase.
(D) Paired analysis of lung protein levels of CXCL13 or CCL21 with the levels of bronchus associated lymphoid tissue as determined by lung section B220+ area. R Pearson coefficient.
(E) Lung infiltrating CXCR5+ cells as identified by flow cytometry in mice subjected to chronic HDM-mediated allergy modulated with VEGF-C or with PBS vehicle control.

To address the role of CXCL13 in the VEGF-C mediated exacerbated induction of bronchus associated lymphoid tissue, we subjected $CXCL13^{-/-}$ (CXCL13 KO), $CXCL13^{+/-}$ (CXCL13 Het) and $CXCL13^{+/+}$ (CXCL13 WT) mice to our chronic model of HDM ± VEGF-C allergic airway inflammation. Of note, while CXCL13 KO mice exhibit severe defects in lymph node organogenesis (Ansel, 2000), including failure at developing the mediastinal lung draining lymph nodes, CXCL13 Het mice overcome this limitation, and the mediastinal lymph node is present in adult mice. In contrast to CXCL13 WT mice, which exhibited an exacerbated induction

of bronchus associated lymphoid tissue in response to co-administration of the allergen with VEGF-C, CXCL13 Het and CXCL13 KO mice failed to do so (Figure 4-9A, B). Similarly, while CXCL13 WT mice exhibited overall higher levels of systemic IgE when treated with the allergen and VEGF-C, CXCL13 Het and CXCL13 KO mice failed to recapitulate such phenomena (Figure 4-9C). Together, these data suggests that the VEGF-C mediated exacerbation of bronchus associated lymphoid tissue is dependent on CXCL13.

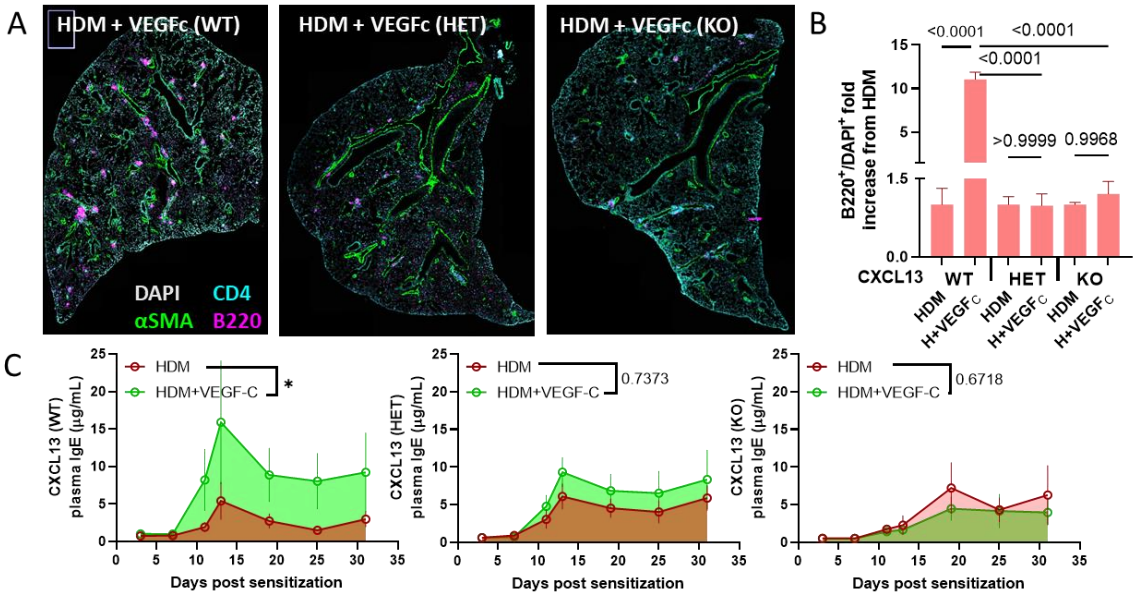

Figure 4-9. CXCL13 is necessary to mediate the VEGF-C associated BALT exacerbated response.

(A) Immunofluorescence staining for αSMA, CD4 and B220 in lungs from CXCL13$^{+/+}$, CXCL13$^{+/-}$, or CXCL13$^{-/-}$ mice subjected to chronic HDM-mediated allergy modulated with VEGF-C, or with PBS vehicle control. Scale bar 1mm.
(B) Quantification for B220+ area over whole lung sections.
(C) Levels of plasma IgE throughout the allergic response as determined by ELISA. Statistics compare the area under the curve as determined through Riemann sums.

Treatment with anti-CXCL13 blocking antibodies protects against induction of bronchus associated lymphoid tissue in a model of chronic allergic airway inflammation.

Given that CXCL13 played a crucial role in mediating the exacerbated induction of bronchus associated lymphoid tissue, we speculated that blocking CXCL13 in the lung could have a protective effect in chronic allergic airway inflammation. Mice were sensitized and challenged with HDM following our chronic schedule of allergic airway inflammation, while treatment with the anti-CXCL13 (αCXCL13) blocking antibody, MAB470, or control isotype antibodies occurred only during the challenge phase of the model. Consistent with our findings, mice that were treated with the αCXCL13 blocking antibody exhibited a reduced induction of bronchus associated lymphoid tissue in response to chronic allergic inflammation (Figure 4-10A, B). This reduction was likely due to a reduced bioavailability of CXCL13 in the lung tissue from the treatment with the αCXCL13 blocking antibody (Figure 4-10C). Finally, the local levels of IgE significantly correlated with the extent of bronchus associated lymphoid tissue (Figure 4-10D). Altogether, these data suggest that CXCL13 could be a novel therapeutic target in limiting the extent of bronchus associated lymphoid tissue and local IgE production in patients suffering from chronic allergic airway inflammation associated with asthma.

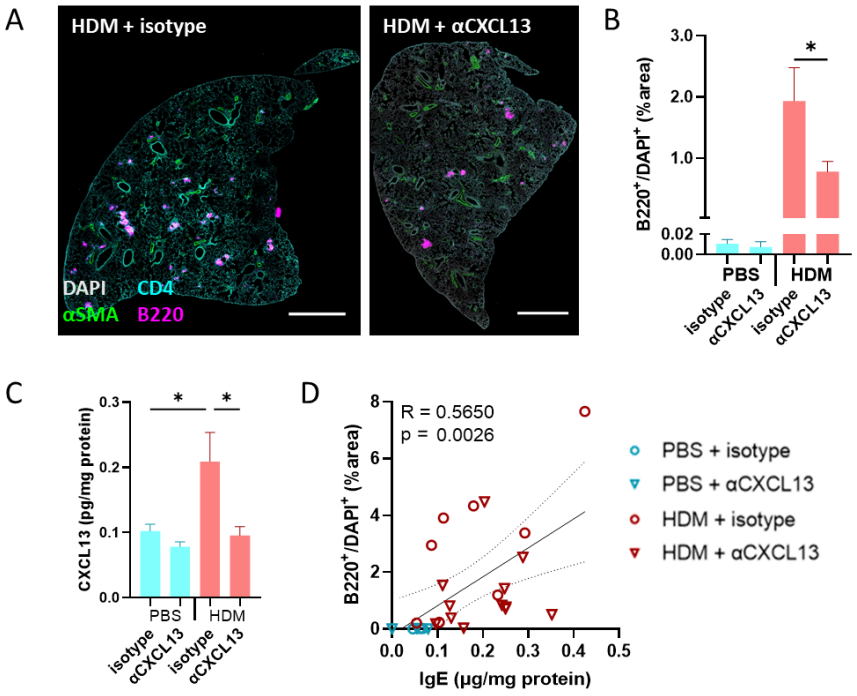

Figure 4-10. Treatment with αCXCL13 blocking antibodies ameliorates induction of bronchus associated lymphoid tissue.

(A) Immunofluorescence staining for αSMA, CD4 and B220 in lungs from mice that were subjected to chronic allergy with HDM modulated with intratracheal (i.t.) instillation of αCXCL13 blocking antibodies or control isotype antibodies. Scale bar 1mm.
(B) Quantification for B220+ area over whole lung sections.
(C) Levels of lung CXCL13 as determined by ELISA.
(D) Paired analysis of lung levels of IgE with the levels of bronchus associated lymphoid tissue as determined by lung section B220+ area. R Pearson coefficient.

Discussion

Our study demonstrates that in chronic allergic airway inflammation, pulmonary lymphatics can mediate induction of bronchus associated lymphoid tissue, and this phenomenon appears to be acting through expression of the B lymphocyte chemoattractant, CXCL13. We found that stimulation with VEGF-C, which drives pulmonary lymphatic growth when delivered locally into the lungs, exacerbated induction of bronchus associated lymphoid tissue, while local blocking

of VEGFR3 reduced such induction. This phenomenon acted through VEGFR3 expressed by lymphatic endothelial cells, as $Prox1^{CreERT2}VEGFR3^{lox/lox}$ mice were protected from the VEGF-C mediated exacerbations. Furthermore, transgenic mice with an expanded lymphatic vasculature were susceptible to an exacerbated induction of bronchus associated lymphoid tissue (in the absence of exogenous pharmacological stimulation of the VEGF-C VEGFR3 signaling axis) suggesting that pulmonary lymphangiogenesis may predispose the lung microenvironment to develop bronchus associated lymphoid tissue upon allergen inhalation. Given the role that we have identified for pulmonary lymphatic endothelial cells in mediating induction of bronchus associated lymphoid tissue in mice, it may become important to re-assess the involvement of the pulmonary lymphatic vasculature in asthmatic patients, as current human studies propose a modest role, if any, for pulmonary lymphatics in mediating the allergic disease.

Current literature has suggested a role for lymphatic endothelial cells as lymphoid tissue organizer cells in the context of lymph node organogenesis in the sterile mouse embryo (Onder, 2017). Following their findings, the authors posited that the mechanisms in play at mediating lymph node organogenesis may be recapitulated in the course of chronic inflammatory diseases resulting in the induction of tertiary lymphoid structures, such as bronchus associated lymphoid tissue. Indeed, previous studies have identified an association between pulmonary lymphangiogenesis, and induction of bronchus associated lymphoid tissue in a model of chronic microbial airway inflammation, and the author's attributed such association to immune cells from the BALT producing the lymphatic growth factors VEGF-C and VEGF-D, thus driving preferential growth of lymphatic vessels towards the BALT (Baluk, 2014). Our study builds upon their proposed model, as our data suggest that pulmonary lymphatic endothelial cells, through the production of chemokines such as CXCL13, can recruit immune cells to the BALT and these cells

in turn can stimulate lymphatic growth through production of VEGF-C and VEGF-D, thus potentially completing a positive feedback loop that promotes the induction of bronchus associated lymphoid tissue. Furthermore, Shinoda *et al* proposed that pulmonary lymphatics can support the induction of iBALT through the production of cytokines, such as IL-7 to promote lymphocyte survival, as well as IL-33 to promote type-II inflammation (Shinoda, 2016). Certainly, induction of bronchus associated lymphoid tissue is a complex phenomenon, with multiple mechanisms at play in supporting its development. Our study's objective was to highlight the importance for pulmonary lymphatics in mediating induction of bronchus associated lymphoid tissue in order to reinvigorate investigation of pulmonary lymphatic endothelial cells as inflammatory mediators in a complex disease such as asthma.

Previous studies in patients with allergic rhinitis and/or asthma have identified that tertiary lymphoid structures, such as nasal associated lymphoid tissue and bronchus associated lymphoid tissue, are attractive sites for B cell priming and class switching towards the IgE isotype. Indeed, significant work has been done in identifying IgE^+ B cells in local mucosal environments (KleinJan, 2000), confirming that NALT and BALT can biologically support class-switching towards the IgE isotype (Smurthwaite, 2001; Chvatchko 1996), uncovering evidence that IgE class switching does occur in local mucosal environments (Durham, 1997; Ying, 2001), and that tertiary lymphoid tissue environments are particularly poised to support class-switching towards IgE (Chudakov, 2020). Nonetheless, the recent identification of an IL-13 producing T follicular helper cell ($T_{FH}13$) subset have provided strong evidence that class switching towards the IgE isotype can also occur in secondary lymphoid organs, specifically generating anaphylactic-inducing, high affinity, allergen specific IgE (Gowthaman, 2019). In our study, we observed either raised levels of systemic IgE or raised levels of local lung IgE when the mice experienced an exacerbated

bronchus associated lymphoid tissue response and these high levels of IgE correlated with the extent if bronchus associated lymphoid tissue. Interestingly, in any of these cases, we did not observe a heightened T or B cell response in the mediastinal lymph nodes, which may support the idea that the bronchus associated lymphoid tissue is contributing to the IgE local and systemic response. We look forward to future studies that focus on determining the location for B cell class switching towards the IgE isotype (Nelson and Wu, 2022), as these will provide invaluable knowledge that will better guide therapeutic development if we are to stop production of this potent antibody isotype in patients suffering from allergic asthma.

Asthmatics tend to maintain a state of chronic allergic airway inflammation, and the latter in turn drives significant changes to the lung microenvironment. Our mouse studies revealed that in response to chronic allergic inflammation, pulmonary lymphatics undergo significant changes in their transcriptome, including upregulation of chemokines such as CCL21 and CXCL13. While CCL21 did not correlate with the extent of BALT in the lungs of mice subjected to HDM-mediated chronic inflammation, CXCL13 protein levels did, and we observed increased cellular infiltration of cells bearing its cognate receptor CXCR5 when we exacerbated the response utilizing exogenous VEGF-C. Following these findings, CXCL13 heterozygous and CXCL13 knock-out mice were protected from the VEGF-C mediated exacerbations of bronchus associated lymphoid tissue, and its associated higher levels of systemic IgE. A previous study identified that bronchoalveolar lavage fluid samples collected from asthmatics have higher CXCL13 protein levels, and that the cells isolated from such samples express higher levels of CXCL13 transcripts. Furthermore, they explored the possibility of utilizing a CXCL13 blocking antibody in the alum OVA model of experimental allergic airway inflammation to ameliorate hallmarks of the disease (Baay-Guzman, 2012). Similarly, we can corroborate that by utilizing the house dust mite extract,

a clinically relevant human allergen, in a mouse model of chronic allergic airway inflammation, the use of the same CXCL13 blocking antibody ameliorated induction of bronchus associated lymphoid tissue, and this in turn correlates with the local levels of lung IgE. Understanding the mechanisms by which bronchus associated lymphoid tissue may be an ideal microenvironment for the production of IgE will prove essential if we are to develop BALT disrupting therapeutics, such as anti-CXCL13-therapies, which could be beneficial in controlling the IgE response in asthma and other atopic diseases.

Chapter V: Prophylactic and therapeutic use of synthetically glycosylated antigens reduces T cell mediated immunity and limits allergic airway disease.

Abstract

Asthma is a disease of chronic airway inflammation affecting more than 300 million people worldwide, including 25 million in the U.S. alone. There is currently no cure for asthma. While effective, current treatments cause detrimental side effects. Corticosteroids cause broad immune suppression and long-term debilitating disease, including diabetes. Novel immune modulatory therapeutics predispose patients to infections, particularly helminth infections. Therefore, there is a current need for new therapeutics that target the underlying causes of the disease without

triggering detrimental side effects. The Hubbell laboratory has developed a platform that leverages the liver's intrinsic immune tolerance-inducing mechanisms that, in an antigen specific manner, limits inflammatory T cell priming, while priming regulatory T cell responses. Through synthetic glycosylation, the Hubbell lab modifies antigens to target C-type lectin receptors in hepatic antigen presenting cells (HPAC). Through presentation in a non-inflammatory manner, in this chapter we aim to limit the allergic T cell responses causing airway disease without causing broad immunosuppression.

Introduction

Asthma is a disease of chronic airway inflammation affecting more than 300 million people worldwide, including 25 million in the U.S. alone (GINA, 2015). Type 2 high allergic asthma, caused by exaggerated type II immune responses to innocuous inhaled allergens is the most common asthma endotype, and its prevalence is on the rise (Bousquet, 2005). There is currently no cure for asthma. Current treatments involve the use of oral and inhaled corticosteroids which, while they rapidly reduce airway obstruction caused by an asthma attack, they also broadly suppress the immune system and have debilitating long-term side effects. Indeed, the use of corticosteroids have been associated with increased risk of infections as well as physiological dysregulation resulting in high blood pressure, mood and behavioral changes, and in some instances type 2 diabetes (Hwang, 2014). Therefore, there is a current need for alternative therapeutics that can target the underlying causes of the disease without causing broad immunosuppression or raising the risk of long-term side effects.

Immune modulatory therapies have been an attractive alternative to the use of steroids in treating allergic asthma, however its use leaves patients vulnerable to infections, particularly helminth-caused parasitic infections (Papathanassiou, 2016). Current research in allergic asthma

have uncovered the immune effector pathways responsible for most of the symptoms associated with the disease, and these novel therapeutics aim to target these pathways to reduce the inflammatory response. For example, Xolair (omalizymab) targets human IgE and prevents its binding with its high affinity receptor found in eosinophils, basophils, and mast cells, thus preventing the cell from releasing its immune mediators responsible for the allergic symptoms (Di Domenico, 2011). In contrast, Cynqair(reslizumab) and Nucala(mepolizumab) target IL-5 which prevent its interactions with eosinophils, among other IL-5 receptor-bearing cells, and reduces eosinophil recruitment and activation (Hom, 2017; Choy, 2016). Fasenra(benralizumab) binds the IL-5Ra and targets eosinophils for their removal, thus eliminating some of the cellular effectors of the disease (Harrison, 2021). While these novel therapies rationally target specific effectors of the immune system, they do not treat the underlying orchestrators of the type II immune response, and they still cause unwanted side-effects including immune suppression which predisposes patients to subsequent infections.

The Hubbell laboratory has developed a platform that leverages the body's intrinsic immune tolerance inducing mechanisms that both control antigen-specific T cell development, while priming antigen-specific regulatory T cell responses (Wilson, 2019). The liver microenvironment has demonstrated its ability to drive immune tolerance to a variety of food-derived antigens, through physiological antigen-presentation by liver resident hepatic antigen presenting cells (HAPC), including dendritic cells, Kupffer cells, hepatocytes, and liver-sinusoidal endothelial cells (Li, 2013; Horst, 2016). To target these variety of HAPCs, the Hubbell laboratory uses glycosylated antigens to target the C-type lectin receptors which play central roles in the clearance of apoptotic cell debris in a non-inflammatory manner. By endowing antigens and potential allergens with synthetic glycosylations, we aim to leverage the tolerance-inducing

mechanisms in the liver to control allergen-specific T cells and promote induction of allergen specific tolerance, thus controlling the central effectors of allergic inflammation without triggering broad immunosuppression.

In this chapter, we explore the possibility of introducing glycosylated allergens to leverage the tolerance promoting environment of the liver to assess whether these tolerogenic processes can ameliorate allergic airway disease. Previous studies from the Hubbell laboratory have determined that intravenous injection of a glycosylated form of chicken ovalbumin (OVA), a model antigen in mouse models of immune disease, effectively targets the liver microenvironment where it is taken up and presented by HAPCs (Wilson, 2019). Prophylactic intervention using glycosylated OVA resulted in a reduced immune response upon intradermal challenge in a mouse model of OVA-driven inflammation. We found that prophylactic intervention by intravenous injection of theglycosylated forms of OVA resulted in an ameliorated form of allergic airway disease, characterized by low systemic type II antibody responses, reduced lung and airway eosinophilia, and reduced mucus production and lung pathology. Furthermore, prophylactic treatment with the glycosylated OVA induced antigen specific regulatory T cells, and their lung cells had a lower capacity at producing type II cytokines upon allergen-specific restimulations. Finally, we explored the use of the glycosylated OVA in a therapeutic model of allergic airway disease. Strikingly, mice that were previously sensitized against OVA, and thus had already developed OVA specific IgG_1 antibodies and OVA specific T_H2 cells, exhibited reduced airway and lung infiltration upon therapeutic intervention with the glycosylated allergen. Altogether, these results highlight the potential of utilizing glycosylated forms of respiratory allergens to target the tolerogenic liver microenvironment and potentially ameliorate allergic airway disease by targeting the orchestrators of the allergic response.

Results:

In previous studies, the Hubbell laboratory has utilized glycosylated antigens in a prophylactic manner to ameliorate, in an antigen-specific manner, inflammatory models of immune disease (Wilson, 2019). To assess whether this platform could ameliorate symptoms of allergic disease, we implemented a mouse model of allergic airway inflammation with OVA as our model respiratory allergen. In brief, mice are sensitized through intraperitoneal injections of an alum/OVA formulation, which drives induction of OVA specific type II immunity, including IgG_1^+ B cells and T_H2 cells. Upon OVA inhalation, mice that were previously sensitized exhibit symptoms of allergic airway disease, including systemic production of IgE and OVA specific IgG_1, lung and airway eosinophilia, and mucus hypersecretion. Therefore, we can use this mouse model model of allergic airway inflammation to test whether intervention with glycosylated forms of the respiratory allergen could ameliorate allergic disease.

To assess whether the presentation of a glycosylated allergen could drive allergen-specific tolerance, we prophylactically introduced a mannosylated form of OVA ($pMan_{OVA}$) intravenously. Mice received two injections with either $pMan_{OVA}$, endotoxin-free OVA, or saline vehicle as control prior to sensitization and challenge with the model allergen (Fig 5-1A). Compared to saline and OVA treated mice, mice that were treated with $pMan_{OVA}$ exhibited reduced levels of systemic IgE (Fig 5-1B) and OVA specific IgG1 (Fig 5-1C) throughout the model, suggesting that the prophylactic treatment was ameliorating the B cell aspect of the allergic response. Furthermore, mice treated with $pMan_{OVA}$ had reduced lung and airway eosinophilia (Fig 5-1D, E), a hallmark of allergic airway inflammation. Finally, mice that had been treated with $pMan_{OVA}$ displayed significantly reduced mucus production in their airways as determined by periodic acid-Schiff staining (Figure 5-1F). Altogether, these data suggest that

prophylactic treatment with pMan$_{OVA}$ ameliorates lung phenotypes associated with allergic airway disease.

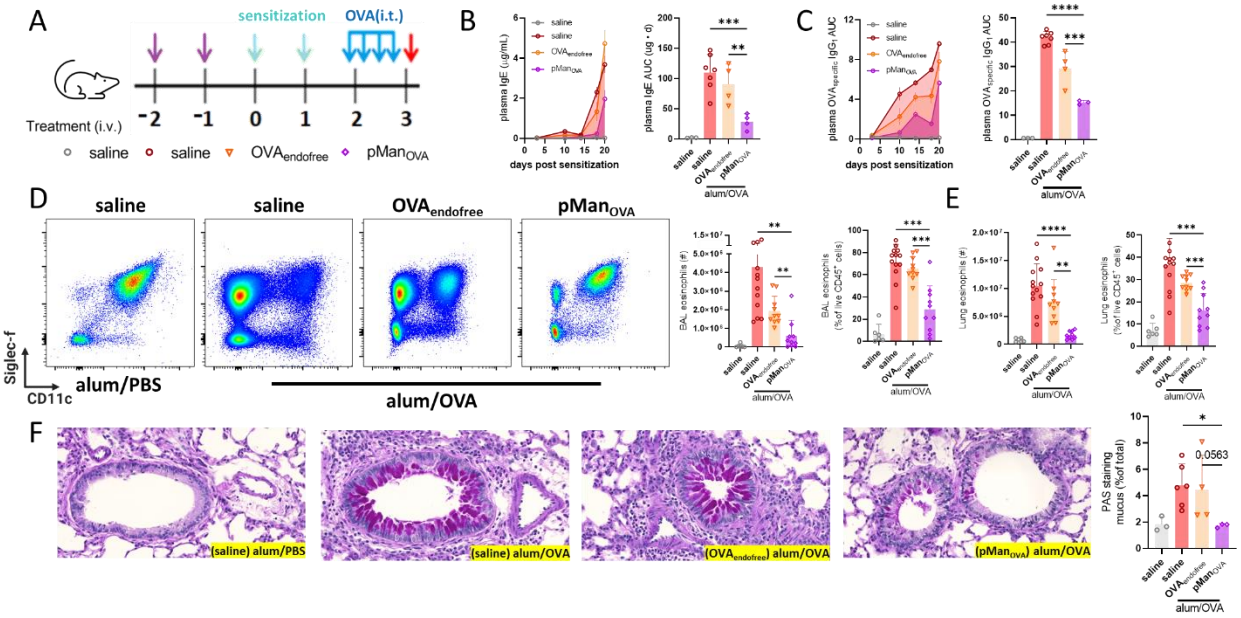

Figure 5-1. Prophylactic intravenous intervention with a mannosylated antigen reduces the symptoms of allergic airway disease

(A) Depiction of the prophylactic intervention in a model of allergic airway disease.
(B) Plasma IgE levels as determined by ELISA.
(C) Plasma OVA-specific IgG1 levels as determined by ELISA.
(D) Airway eosinophils as identified by flow cytometry from BALF.
(E) Lung eosinophils as identified by flow cytometry from lung tissue.
(F) Periodic-Acid Schiff staining on lung tissue sections.

Given that treatment with pMan$_{OVA}$ ameliorated the effector pathways associated with type II cytokine signaling, we followed by asking whether prophylactic treatment with the glycosylated allergen had reduced T cell immunity. Analysis of bronchoalveolar lavage fluid (BALF) and lung samples, revealed reduced infiltration by CD4 T cells, the main T cell effectors in allergic airway disease (Fig 5-2A). There were no specific changes on the proportions of bulk FoxP3+ or T_H2 cells (Fig 5-2A), but because of the reduced T cell infiltration, both subsets

exhibited reduced infiltration as well (data not shown). Not surprisingly, the levels of airway or lung eosinophilia did not correlate with the levels of FoxP3+ cells (Fig 5-2B). To assess changes to the antigen specific T cell compartment, we infused the mice, prior to prophylactic treatment, with OT-II transgenic T cells which recognize the **$OVA_{323-339}$** peptide sequence in the context of $I\text{-}A^b$. Analysis on OT-II cells isolated from the lungs of $pMan_{OVA}$ treated mice revealed that while there is a significant reduction in the numbers of OT-II cells recovered, these specifically acquire a FoxP3+ signature (Fig 5-2C). Furthermore, the proportion of these antigen-specific regulatory T cells negatively correlated with the levels of lung and airway eosinophilia (Fig 5-2D). Finally, upon performing OVA specific in-vitro restimulations, we found that the lung cells coming from mice treated with $pMan_{OVA}$ exhibited reduced production of the type II cytokines, IL-4, IL-5 and IL-13 (Fig 5-2E, F). Importantly, in $pMan_{OVA}$ treated mice, amelioration of type II cytokine production was not coupled with production of other cytokines, such as IFNγ, suggesting that prophylactic treatment with the glycosylated allergen was reducing rather than shifting the polarization of the immune response (Fig 5-2E, F). Together, these data suggest that prophylactic treatment with $pMan_{OVA}$ induces antigen-specific regulatory T cells while reducing type II cytokine production by lung cells upon allergen rencounter, and the latter in turn could ameliorate the phenotypes associated with allergic airway inflammation.

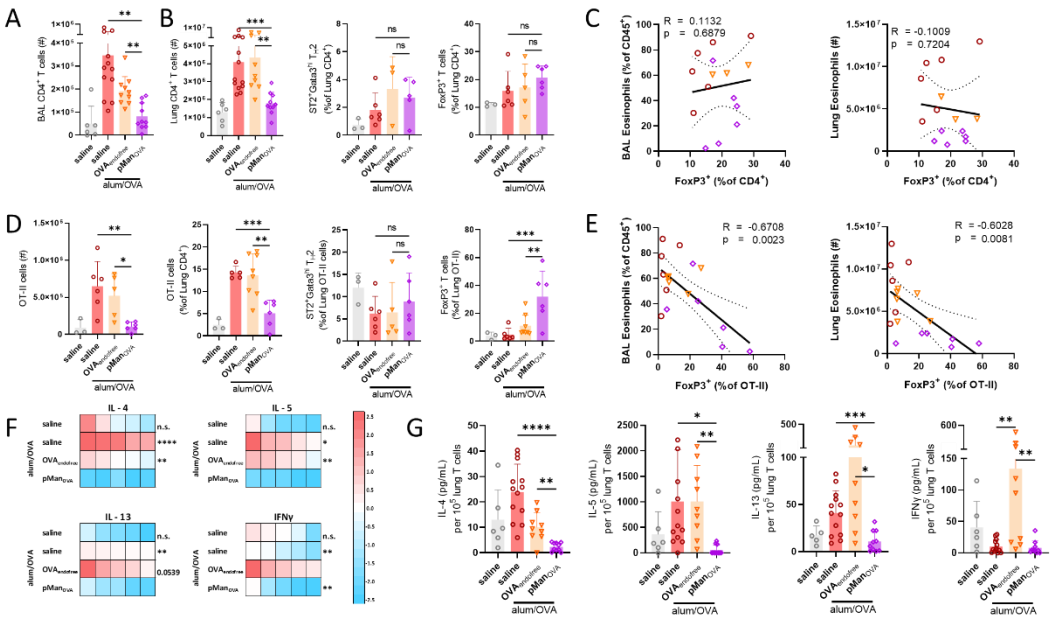

Figure 5-2. Prophylactic intervention with a mannosylated antigen induces antigen specific regulatory T cells and reduces type II cytokine production.

(A) Airway T cells as identified by flow cytometry.
(B) Lung T cells as identified by flow cytometry.
(C) Paired analysis of lung and airway eosinophilia with FoxP3+ T cells.
(D) Lung OT-II cells as identified by flow cytometry.
(E) Paired analysis of lung and airway eosinophilia with FoxP3+ OT-II cells.
(F) Heat map depicting cytokine production by lung cells upon allergen specific restimulation.
(G) Cytokine production by lung cells upon allergen specific restimulation.

Finally, because patients suffering from allergic asthma present with previously developed allergen-specific type II immunity, we wanted to assess whether therapeutic intervention with $pMan_{OVA}$ could ameliorate phenotypes associated with allergic airway disease. To address this question, we sensitized mice to raise type II immunity, in the form of OVA specific IgG_1 antibodies and T_H2 cells, prior to treatment with $pMan_{OVA}$, unmodified OVA, or saline vehicle control (Fig 5-3A). Therapeutic intervention had limited effect on changing the levels of OVA specific IgG1 (Fig 5-3B). Upon allergen inhalation, mice that were therapeutically treated with $pMan_{OVA}$ displayed reduced immune cell airway infiltration, as determined by analysis of BALF samples

(Fig 3C) and lung samples (Fig 5-3D). Such reduced inflammation included reduced eosinophilia, T cell and B cell infiltration (Fig 5-3C, D). Furthermore, therapeutic treatment with $pMan_{OVA}$ reduced induction of IgE upon allergen inhalation (Fig 5-3E). Upon allergen specific restimulation, lung cells isolated from $pMan_{OVA}$ treated mice released reduced levels of IL-5 (Figure 5-3F), consistent with the reduced airway and lung eosinophilia (Figure 5-3C, D). Altogether, these data suggest that even in previously sensitized mice, therapeutic intervention with $pMan_{OVA}$ can significantly reduce both lung and airway immune infiltration as well as systemic IgE levels, highlighting a potential role at ameliorating allergic airway disease.

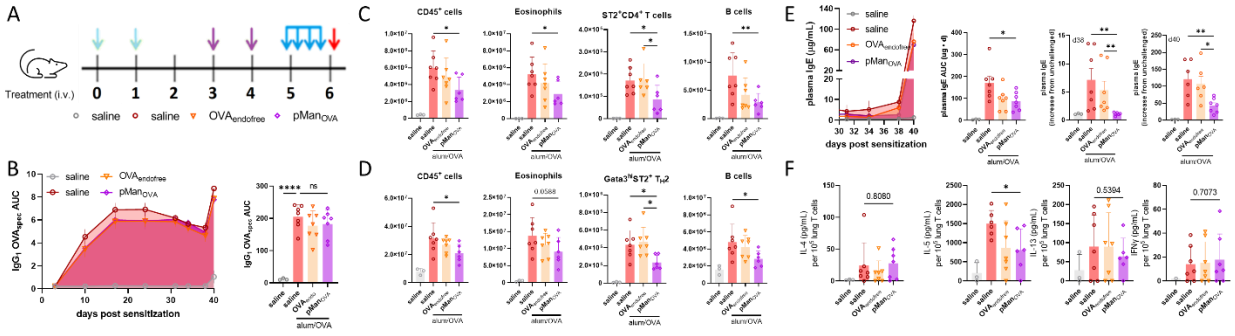

Figure 5-3. Therapeutic intravenous intervention with a mannosylated form of antigen reduces lung and airway immune infiltration.

(A) Depiction of the therapeutic intervention in a model of allergic airway disease.
(B) Plasma OVA-specific IgG1 levels as determined by ELISA.
(C) Airway immune infiltrataes as identified by flow cytometry from BALF.
(D) Lung immune infiltrataes as identified by flow cytometry from lung tissue.
(E) Plasma IgE levels as determined by ELISA.
(F) Cytokine production by lung cells upon allergen specific restimulation.

Discussion:

While current treatments in allergic asthma are effective at suppressing the allergic response, they have severe side effects that debilitate the patient experiencing the disease. The

use of oral or inhaled corticosteroids have been associated with a number of side-effects, including an increased risk of infections as well as physiological dysregulation which results in high blood pressure, behavioral mood changes, and in some instances type 2 diabetes (Hwang, 2014). Novel antibody immunotherapies perform a more targeted approach at controlling the allergic disease symptoms by targeting the effector molecules driving the response, however, these also make the patient more vulnerable to infections, specifically, parasitic infections. Because of these limitations and side-effects, there is a need for novel therapeutics that can restrain the immune response against allergens without causing total immune suppression.

The Hubbell laboratory developed a platform that leverages the immune tolerance generating mechanisms of the liver microenvironment to drive antigen specific tolerance. By using synthetical glycosylation of antigens, they can target these antigens to HAPCs, where antigen presentation can educate the immune system to tolerate such molecules (Wilson, 2019). In this chapter, we explored the possibility of using this platform to tolerize against potential allergens that could drive allergic airway inflammation. Indeed, prophylactic treatment with a synthetically mannosylated model allergen, reduced all facets of allergic airway inflammation, including airway and lung eosinophilia, mucus hypersecretion, and systemic IgE and allergen specific IgG1 antibodies. Prophylactic treatment with $pMan_{OVA}$ reduced the CD4 effector T cell response, while inducing allergen specific regulatory T cells. Furthermore, we tested whether therapeutic treatment with a glycosylated antigen could potentially ameliorate the allergic airway disease. In this setting, treatment with $pMan_{OVA}$ reduced airway and lung immune infiltration, as well as systemic IgE levels. Upon allergen specific restimulation, we observed an amelioration in IL-5 production in lung cells isolated from mice treated with $pMan_{OVA}$, consistent with the reduced airway and lung eosinophilia. Altogether, our studies suggests that the mannosylated

antigen platform that the Hubbell lab developed can be utilized to drive allergen specific tolerance in a prophylactic manner and ameliorate airway inflammation when used in a therapeutic manner.

Our studies in this chapter, highlight the potential of allergen immunotherapies to restrain type II immune responses in the context of allergic asthma. By harnessing the endogenous tolerogenic mechanisms, these immunotherapies can drive allergen specific T cell tolerance which can then ameliorate the features of allergic airway disease. One main feature that highlights the importance of the synthetic glycosylations can be found in Figure 5-2, where allergen treatment can boost the production of IL-13 in an allergen specific restimulation setting. Furthermore, treatment with the native allergen may polarize the nature of the immune response, as depicted by the induction of IFNγ (Figure 5-2E, F). In contrast, treatment with the glycosylated allergen dampened the response rather than changing the nature of the response or boosting the allergic immune response. Altogether, these studies provide a proof of concept that the use of synthetically glycosylated allergens can become a novel therapeutic that drives allergen specific tolerance and suppresses the features of allergic airway disease.

Chapter VI: Discussion

Throughout these studies, we have elucidated novel immunological roles for the lymphatic endothelium in the context of allergic airway disease. Specifically, I have established that in the context of chronic allergic airway inflammation, pulmonary lymphatics can mediate the induction of bronchus associated lymphoid tissue, which may in turn exacerbate the local and systemic levels of IgE. Driving lymphangiogenesis, through local growth factor signaling or genetic mouse models exacerbated iBALT, while blocking lymphangiogenesis through local pharmacological agents or through inducible conditional gene knock-out models ameliorated iBALT. Furthermore, I have identified CXCL13 expression in pulmonary lymphatic endothelial cells as a novel factor that may prove central to BALT induction in asthmatics and a potential therapeutic target. Indeed, the VEGF-C driven BALT exacerbations required CXCL13 signaling, as genetic mouse models

lacking this chemokine failed to exhibit such exacerbation, and therapeutic blocking of CXCL13 reduced the extent of BALT. Taken together, these data support the idea that pulmonary lymphatics can mediate induction of bronchus associated lymphoid tissue in response to chronic allergic airway inflammation, and thus its role could be central in the pathogenicity of asthma.

Historically, BALT and NALT, the respiratory tissue tertiary lymphoid structures, have been attractive sites for the induction of allergen specific IgE (Berdal, 1954). Ever since then, IgE has been a contentious antibody isotype, raising controversies on its site of induction (Janeway, 2001; Smurthwaite 2002), whether IgE+ B cells can form part of germinal centres (Chvatchko, 1996; He, 2013), whether IgE+ B cells can form memory cells (Laffleur, 2017), as well as which signals are needed to drive the different natures of IgE (Hong, 2019; Gowthaman, 2019; Looney, 2016; Shen, 2021). There is now incontrovertible evidence that IgE switching can occur in tertiary lymphoid structures, as previous work have shown that NALT and BALT can support B cell class-switching towards IgE (Durham, 1997; KleinJan, 2000; Ying, 2001; Smurthwaite, 2002) and that IgE is locally produced in response to allergen challenge (Chvatchko, 1996). In our work, we found that in a model of chronic allergy, when inducing or preventing pulmonary lymphatic growth, iBALT followed. Furthermore, lung levels of IgE positively correlated with the extent of iBALT, and in severe cases of exacerbated iBALT we observed raised systemic levels of IgE. Interestingly, there were no outstanding differences in the mediastinal lymph nodes that could suggest an exacerbated B cell response in the secondary lymphoid site. In contrast, most B cell changes were found when analyzing the lung tissue, including a higher number of B cells that exhibited a germinal centre phenotype. These phenomena were in line with the concept that tertiary lymphoid structures can support class-switching towards IgE on instances of allergic disease, and thus we suggest that these structures should be the focus of further investigation.

Recent findings have identified that lymphatic endothelial cells direct the initial steps driving lymph node organogenesis (Onder, 2017). It is clear that a common feature in multiple organogenesis mechanisms is the need for positive feedback signaling loop, and this feature may be necessary in the induction of tertiary lymphoid structures. Previous studies noted an association between pulmonary lymphatics and iBALT, and they attributed this preferential growth to the immune cells in iBALT producing the lymphatic growth factors (Baluk, 2014). We would like to build upon this model, pulmonary lymphatic vessels can upregulate several chemokines in response to chronic allergic inflammation, and thus attract the immune cells that can seed iBALT. Through both of these processes, lymphatic endothelial cells can mediate a positive feedback loop by attracting the immune cell types that can in turn provide the lymphatic growth factors and engage lymphatic receptors to further activate the vessels. This feedback loop may prove necessary to seed the site of the tertiary lymphoid structure, and, in an analog pattern to lymph node organogenesis, lymphatic vessels may control the initiation site and the initial steps that drive the formation of tertiary lymphoid structures. Nonetheless, iBALT development is a complex process, and while lymphatics may play a role in the initiation steps, we expect other stromal cell subsets to contribute on the latter steps driving the expansion and maturation of the BALT.

In chapter 3, we provided a reconciliation between the literature that suggests that pulmonary lymphangiogenesis occurs in response to allergic airway inflammation, and the fact that IL-4 and IL-13 can inhibit lymphangiogenesis. Using HDM as a model allergen, we found that pulmonary lymphangiogenesis occurs, albeit temporally decoupled from allergen inhalation. Additionally, an imbalance between the pro-lymphangiogenesis factors, VEGF-C and VEGF-D, and the anti-lymphangiogenesis cytokines, IL-4 and IL-13, coincided and correlated with the state of the pulmonary vasculature in mice subjected to chronic allergic airway inflammation. It is

interesting to ponder on the evolutionary selection that may have occurred that preserved such a regulatory signaling pathway to prevent temporal coupling of lymphatic growth and allergen inhalation. Certainly, temporally coupling of lymphangiogenesis and allergen inhalation exacerbated the allergic immune response via induction of BALT and raising local and systemic IgE levels (Chapter IV). Furthermore, our data suggested that pulmonary lymphatic growth predisposes the lung microenvironment to develop BALT, thus making it susceptible to develop an exacerbated immune response to allergen inhalation. Our data would suggest that there is benefit in preventing temporal coupling of lymphatic vessel growth and a type II allergic response, and thus we would like to highlight the importance of lymphatic expression of IL-4Rα to restrain lymphangiogenesis in the context of allergic inflammation.

Genomic studies have identified single nucleotide polymorphisms that are associated with asthmatics and that also fall within the IL-4Rα locus (Beghé, 2003; Isidoro-García, 2005; Knutsen, 2010). Some of these polymorphisms enhance IL-4 signaling through IL-4Rα by either enhancing the sensitivity of the receptor for IL-4 (Risma, 2002) or by enhancing the persistence of the receptor signaling capabilities (Ford, 2006). Given the central importance of IL-4Rα in transducing IL-4 and IL-13 signaling, we posit that expression of this receptor in lymphatic endothelial cells may be critical in restraining lymphangiogenesis during allergic inflammation. Thus, we can ponder on the possibility that some of the polymorphisms associated with asthma and with IL-4Rα may downregulate or interfere with IL-4Rα signaling in lymphatic endothelial cells and thus dysregulate the temporal decoupling of lymphangiogenesis and allergic inflammation. If that were the case, we could envision that pulmonary lymphangiogenesis may ensue regardless of the allergic inflammatory state of the lung microenvironment and thus drive BALT formation to promote IgE production and facilitate the individual's sensitization. In such a scenario, restraining

the pro-lymphangiogenesis growth factors, VEGF-C and VEGF-D, or its common receptor VEGFR-3 may become an attractive therapeutic option. Altogether, we pose that the pulmonary lymphatic vasculature can actively participate in mediating immune processes during allergic airway inflammation that in turn could potentially exacerbate the disesase, and thus warrant significant interest in its study.

Conclusion

In allergic asthma, pulmonary lymphatic endothelial cells are an understudied cell type and its role in the disease pathogenesis is underappreciated. In inflammatory settings that drive a type II immune response, signaling from IL-4 and IL-13 can restrain pulmonary lymphatic growth, nonetheless, dysregulation of this signaling axis may prove detrimental to the disease. In this thesis, I found that under normal conditions, in the context of allergic airway disease, pulmonary lymphangiogenesis is restrained, potentially by IL-4 and IL-13. Furthermore, we performed experiments that tested a scenario where pulmonary lymphangiogenesis occurred during an allergic response and found that these vessels exacerbated the immune response by inducing BALT and raising local and systemic IgE levels. Given that chronic inflammatory insults can drive pulmonary lymphangiogenesis and that these vessels persist in their expanded state long after the initial stimuli is gone, we posed that such a lung microenvironment would be predisposed to develop BALT upon allergen inhalation. Indeed, my studies using genetic mouse models supported this hypothesis, and we narrowed down on a chemokine, CXCL13, that is central to the induction of tertiary lymphoid structures. We found that lymphatic endothelial cells can upregulate expression of CXCL13 upon allergic inflammation, and also that CXCL13 blocking could be a potential therapeutic avenue in chronic asthma, as this ameliorated BALT induction. Finally, we posit that dysregulation of IL-4Rα in lymphatic endothelial cells may pose a potential

susceptibility in the context of allergic asthma, as such a scenario could promote temporal coupling of allergic inflammation and pulmonary lymphangiogenesis thus exacerbating the response through BALT induction. Altogether, the studies in this thesis highlight how pulmonary lymphatic vessels can participate in allergic airway inflammation and contribute to novel mechanistic understanding in the induction of tertiary lymphoid structures associated to chronic immunopathologies, which may eventually drive the identification and implementation of innovative immunotherapies.

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