MECHANISMS OF ASTHMA PROTECTION BY MICROBIAL AGENTS

by

Sydney VanLinden

A Dissertation Submitted to the Faculty of the

GENETICS GRADUATE INTERDISCIPLINARY PROGRAM

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2025

THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Dissertation Committee, we certify that we have read the dissertation prepared by: Sydney VanLinden, titled: Mechanisms of Asthma Protection by Microbial Agents, and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Casey Romanoski
Casey Romanoski, PhD
Casey Romanoski (Aug 22, 2025 09:09:36 PDT)
Date: 08/22/2025

Donata Vercelli
Donata Vercelli, MD
Date: 08/22/2025

Deborah A Meyers, PhD
Deborah Meyers, PhD
Deborah A Meyers, PhD (Aug 22, 2025 15:35:24 PDT)
Date: 08/22/2025

Darren Cusanovich, PhD
Date: 08/22/2025

Vadim Pivniouk
Vadim Pivniouk, PhD
Vadim Pivniouk (Aug 22, 2025 17:27:57 PDT)
Date: 08/22/2025

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copies of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Casey Romanoski
Casey Romanoski, PhD
Casey Romanoski (Aug 22, 2025 09:09:36 PDT)
Date: 08/22/2025
Dissertation Committee Chair
Genetics Graduate Interdisciplinary Program

ACKNOWLEDGEMENTS

I would like to thank my advisor, Donata Vercelli, for her endless support and patience while mentoring me in my graduate studies. Thank you for all the tough conversations and never-ending encouragement, I wouldn't be here without you. I would also like to thank all the members of the Vercelli lab, past and present. Thank you all so much for everything you taught me, all your words of encouragement, and all your help throughout my journey. Finally, thank you to each of my committee members for your support, guidance, constructive criticisms, and feedback, which have been invaluable.

LAND ACKNOWLEDGEMENT

We respectfully acknowledge the University of Arizona is on the land and territories of Indigenous peoples. Today, Arizona is home to 22 federally recognized tribes, with Tucson being home to the O'odham and the Yaqui. Committed to diversity and inclusion, the University strives to build sustainable relationships with sovereign Native Nations and Indigenous communities through education offerings, partnerships, and community service.

DEDICATION

This is dedicated to my loving family and friends. To my parents for always seeing my potential, encouraging me to be my best, and helping me every step of the way. To my husband Kyle, who is always there for me, through the best and the worst of it all. And to my friends, for making sure I had fun along the way.

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

Asthma is the most common chronic disease of childhood and global prevalence has increased significantly in recent decades, coinciding with a shift in exposure to microbes present in the environment. Our lab previously showed that airway administration of microbial agents, such as Amish farm dust extracts (AFDE) or the bacterial lysate OM-85 (Broncho-Vaxom), protects against experimental asthma and has profound transcriptional effects in an ovalbumin murine model of experimental asthma. However, transcriptional mechanisms underlying the asthma protective effects of AFDE and OM-85 require further investigation. The work described in this dissertation sought to characterize the cellular and transcriptional modifications induced in the lung by AFDE and OM-85 using an *Alternaria alternata* extract-induced experimental asthma mouse model. Considering the phenotypic similarities, a comparison was performed to determine whether AFDE and OM-85 confer asthma protection by similar changes in transcription.

Lung function and bronchoalveolar lavage (BAL) cellularity were measured in BALB/c wild-type-mice treated intranasally (i.n.) with either AFDE or OM-85 in the presence or absence of *Alternaria* extracts. Lung tissue collected from these mice was used to perform flow cytometry, bulk RNA-seq, and single nucleus (sn)RNA-seq to profile and compare the responses to AFDE and OM-85.

This work showed that the protection conferred by AFDE and OM-85 involves the differential expression of several shared genes. Both microbial agents suppress airway hyperresponsiveness (AHR) and eosinophilia, downregulate genes involved in type-2 inflammation, and induce a strong, activated, polyclonal B cell signal. Ongoing studies in B cell

deficient mice seek to determine whether B cells are required for the asthma protective effects of AFDE.

ABBREVIATIONS

AFDE: Amish farm dust extracts

AHR: airway hyperresponsiveness

BAL: bronchoalveolar lavage

BCR: B cell receptor

DE: differentially expressed

GEM: gel in-bead emulsion

GEX: gene expression

HDM: house dust mite

Ig: immunoglobulin

Il: interleukin

ILC2: group 2 innate lymphoid cell

I.n.: intranasal

I.p.: intraperitoneal

NET: neutrophil extracellular traps

OVA: ovalbumin

PC: principal component

PCA: principal component analysis

R.o.: retro-orbital

RTI: respiratory tract infection

scRNA-seq: single cell RNA-sequencing

snRNA-seq: single nucleus RNA-sequencing

CHAPTER 1: INTRODUCTION

1.1 – Asthma

Asthma is a complex and heterogeneous, chronic inflammatory airway disorder characterized by recurrent, and typically reversible airway obstruction estimated to affect over 300 million people worldwide [1]. The prevalence of asthma and other allergic diseases has increased globally in recent decades [2, 3]. While various treatments are available for managing the various symptoms of asthma, there is no cure, underscoring the need for focused research aimed at preventing the onset of this complex disease.

1.1.1 Asthma pathogenesis: The innate and adaptive immune responses

Asthma encompasses a spectrum of immune responses that result in airway inflammation, airway hyperresponsiveness (AHR), and reversible airway obstruction [4]. Chronic respiratory inflammation causes a reduction in the diameter of the airways, resulting in shortness of breath, wheezing, and chest tightness [5] which can eventually contribute to tissue fibrosis and smooth muscle cell thickening that permanently remodel the airways, reducing respiratory function.

The pathogenesis of asthma is complex, involving both innate and adaptive immune responses to initiate and perpetuate inflammation [6, 7] and our understanding of these responses is constantly evolving. As there are many diverse phenotypes of asthma, it is common to categorize them into endotypes, or groups of distinct biological mechanisms underlying the disease traits. One of the primary asthma endotypes often described is “type-2- (T2) high” asthma [8, 9]. T2-high asthma is characterized by the predominant Th2 cell and Th2 cytokine

response that drives airway inflammation and is estimated to account for approximately 50% of asthma cases [10].

The epithelium lining the airways serves as the primary barrier that encounters the external environment carrying airborne pathogens, allergens, and pollutants. Cells involved in innate immunity including airway epithelial cells, macrophages, dendritic cells, neutrophils, and eosinophils have pattern recognition receptors (PRRs) for the identification of these external insults [5, 11]. Activated by the recognition of external stimuli and damage to the airway barrier, epithelial cells produce alarmins, including thymic stromal lymphopoietin (TSLP), IL-25, and IL-33. The secreted alarmins sustain the activation of the innate immune system by recruiting eosinophils, neutrophils, mast cells, basophils, dendritic cells, and activating type-2 innate lymphoid cells (ILC2s) [5, 12-16]. ILC2s play a key role in asthma pathogenesis as a source of the inflammatory cytokines interleukin (IL)-4, IL-5, and IL-13 [17].

IL-5 is responsible for the maturation, activation, proliferation, migration, and survival of eosinophils [18]. In addition to IL-5, eosinophils respond to chemokines including CCL7 (MCP3), CCL5 (RANTES), CCL13 (MCP-4), CCL11 (Eotaxin-1), CCL15, CCL26, and CCL24 (Eotaxin-2) to migrate to the lung [19]. Eosinophil degranulation releases tissue damaging granule proteins, including eosinophil cationic protein (ECP), major basic protein (MBP), eosinophil-derived neurotoxin (EDN), and eosinophil peroxidase (EPX), as well as eosinophilic extracellular traps (EETs) comprised of granule proteins tangled in the cell's mitochondrial DNA, causing inflammation and bronchial hyperreactivity [20, 21]. Another Th2 cytokine, IL-13, acts as an effector molecule causing smooth muscle cell contraction and airway obstruction, while also signaling for epithelial cell transdifferentiation into mucus-producing goblet cells

[22]. Overall, increased secretion of IL-13 results in AHR, goblet cell hyperplasia, and mucus overproduction which contribute to airway remodeling [5, 23-26].

Additionally, alarmins and type-2 inflammatory cytokines play a role in the activation of the adaptive immune response by signaling for naïve T cell differentiation to effector T cells [12, 27]. T2-high asthma is characterized by a predominant response of Th2 cells which differentiate in response to the alarmins TSLP, IL-25, IL-33, but also to the inflammatory cytokine IL-4. The resulting Th2 cytokine response includes the production and release of IL-4, IL-5, and IL-13 [17, 28, 29]. IL-4 and IL-13 also stimulate the activation and class switching of B cells to produce immunoglobulin (Ig)E, which binds to high-affinity IgE receptors (FcεRI) present on mast cells [30]. Upon subsequent exposure to the allergen, the IgE will bind the recognized antigen resulting in the degranulation of the mast cell and release of vasoactive mediators that elicit local inflammation via increased vascular permeability and eosinophilic recruitment [31, 32]. Although asthma pathogenesis has classically been characterized as a T helper 2-cell dependent, IgE-mediated disease associated with eosinophilic inflammation, alternative immunological pathways have been described [5, 33, 34].

1.2 – Genetic and environmental factors influence asthma susceptibility

Asthma is a complex disease, meaning an individual's susceptibility to develop asthma is shaped by genetics, environmental factors, and the interaction of the two during pivotal developmental stages. From a genetic perspective, numerous genome-wide association studies (GWAS) have been performed using cohorts of children and adults with asthma to identify locations in the genome associated with asthma [35-37]. Most of the genes present at the locations associated with asthma play key roles in the immune response to ***environmental***

exposures, including allergens, bacteria, and viruses or they are related to type-2 inflammation. Some of the genes identified encode the toll-like receptors (TLRs) for the recognition of pathogens, the alarmins TSLP and IL-33, other inflammatory cytokines like IL-4, IL-5, and IL-6, and genes involved in the signaling for type-2 inflammation like SMAD3 and SMAD6 [35].

However, the increase in the incidence of asthma and other allergic diseases in developed countries over the last few decades has occurred too rapidly for widespread changes in the genome to have contributed to this effect [3]. One theory to explain this increased incidence is the "Hygiene Hypothesis" [38], which posits that improved overall hygiene, the wide use of antibiotics, a shift to urban living conditions, and smaller family size has reduced the opportunities for microbial exposure, leading to improper immune system development that erroneously targets allergens and beneficial microbes [39]. Continuing with this framework, the "Old Friends Hypothesis" explains that the improper development of the immune system is due to an inadequate exposure to the variety of pathogenic and non-pathogenic microbes that humanity evolved alongside, which served to drive the expansion of the immune system's repertoire and proper regulation [39, 40]. The development of the immune system from the prenatal period, through infancy, and into early childhood is vulnerable to many factors that have been found to influence the development of childhood asthma, including but not limited to exposures to allergens, air pollution, respiratory viral infections and microbial exposures [41, 42]. Altogether, this emphasizes a strong role for environmental exposures in asthma pathogenesis.

1.2.1 The Farm Effect and the traditional Amish farming environment

Several epidemiological studies throughout Europe have described the "Farm Effect", an observation that children from rural areas that grow up on farms with traditional farming practices have significantly lower risk for developing asthma [43-48]. Through these studies, several elements of traditional farming were identified as asthma-protective factors, including exposure to farm animals and their microbes, consumption of unprocessed cow's milk, and contact with hay and straw [47, 49, 50].

A compelling, natural comparison of the effects of the traditional farming environment on asthma prevalence can be found in two U.S. farming communities with similar genetic ancestry, yet divergent farming practices: the Amish and the Hutterites. The Amish maintain smaller traditional farms with barns and animals close to the home, while the Hutterites practice industrialized farming with large barns separated from the community's homes. In studies from our group, Stein et al. [51] found that, despite similarities in genetic background and lifestyle choices such as diet, family size, and pet ownership that would otherwise alter susceptibility to asthma, Amish children develop asthma 4 to 6 times less frequently than Hutterite children. Dust collected from the homes of these children revealed a significant difference in the household microbial load, as airborne endotoxin levels from Amish homes were 6.8 times higher than those of the Hutterites. This corresponded with enhanced innate immune responses in Amish children, indicated by an increase in the number of neutrophils in the PBMCs and altered expression of genes involved in innate immune pathways, including *TNF* and *IRF7*. Mechanistic studies in an ovalbumin (OVA)-driven experimental asthma mouse model showed that intranasal administration of extracts made from dust collected from the Amish *homes*, but not the Hutterite's, conferred protection against cardinal asthma phenotypes: AHR, bronchoalveolar

lavage (BAL) eosinophilia and expression of type-2 cytokines in the lung. In the same paper [51], our lab established that the asthma protective effects of the Amish house dust extracts required the innate immune system. The proteins MyD88 and Trif are involved at the crossroads of many innate immune signaling pathways and knockout mice lacking both MyD88 and Trif were no longer protected from AHR or airway eosinophilia by the Amish house dust extracts. This is a key finding, as the innate immune system is the first line of defense against microbes, which the Amish home environment is especially rich in.

Interestingly, a follow up experiment comparing extracts made from dust collected in Amish and Hutterite *barns* in the same murine model found both to be asthma protective [52]. Although the environmental factors in Hutterite barns show the same capacity for asthma protection as the Amish barns and homes, it is thought that Hutterite children are kept far from this beneficial exposure in early life.

In a recent paper, our lab and collaborators sought to characterize the composition of extracts derived from Amish cowsheds and identify the asthma-protective agents within [53]. While the Amish farming environment likely harbors a diverse array of live microbes, the studies outlined in the paper found that even autoclaved farm dust extracts retained strong asthma protective properties. After identifying bioactive, asthma-protective fractions of the dust, an analysis of the proteins revealed the presence of transport proteins Bos d 2 and OBP, which carried metabolite cargo originating from plants and microbes. It is hypothesized that these metabolites, when delivered to mucosal surfaces via the transport proteins, may regulate airway immune responses in a way that protects against asthma development. These findings highlight the potential of environmental microbes and the metabolites they produce to shape the immune function in the airways for asthma protection.

1.2.2 The bacterial lysate, OM-85 for immunomodulation

Our lab's studies investigating the traditional Amish farming environment established that microbe-rich dust extracts can protect against allergic airway inflammation in mice. However, these extracts are comprised of a complex and heterogenous mixture of environmental microbes and metabolites, which poses significant challenges for their standardization and reproducible use in the prophylaxis of asthma. There has been a longstanding scientific interest in the use of microbes to naturally modulate the immune system and promote resistance to other microbial infections and in the last few decades, standardized preparations of lysed bacteria have been investigated for the use in prevention of respiratory infection and disease [54-57].

One such preparation is **OM-85** (Broncho-Vaxom), a standardized, pharmaceutical grade, low-endotoxin alkaline lysate of 21 bacterial strains across 5 genera (*Moraxella, Hemophilus, Klebsiella, Staphylococcus,* and *Streptococcus*) that are associated with human upper respiratory infections [58, 59]. OM-85 has been used empirically worldwide, apart from in the United States, for over three decades for the prevention of recurrent respiratory infections (RTIs) in children and adults. Numerous studies have suggested that OM-85 significantly reduces the frequency, duration, and severity of RTIs in children, while also reducing the time of antibiotic use and hospitalization [60-64]. Within these studies, investigations into immunological mechanisms revealed that OM-85 increases serum and secretory IgA levels [65], induces the maturation of T cell stimulating dendritic cells [66], and shifts the Th1/Th2 responses by upregulation of IFN-γ and downregulation of IL-4 [67]. However, the results regarding the effectiveness of oral administration of OM-85 for the prophylaxis of respiratory infections are mixed [68].

Based on the results from the intranasal administration of Amish farm dust extracts published in Stein et al. [51], our lab was approached to study the effects of intranasal

administration of OM-85 in an experimental asthma mouse model. In the recent paper published by our lab, Pivniouk et al. [69] established that airway administration of OM-85 suppresses allergic asthma in both OVA- and *Alternaria alternata*-induced experimental asthma mouse models. OM-85 significantly suppressed AHR, eosinophilic inflammation in the BAL, goblet cell metaplasia, and elevated lung expression of IL-5 and IL-13. Transcriptomic analysis revealed a downregulation of genes involved in type-2 inflammation and ILC2 activation. At the same time, genes associated with tolerogenic dendritic cells and epithelial barrier integrity were upregulated. These findings suggest that OM-85 strengthens the airway epithelial barrier and reduces the secretion of IL-33 and activation of ILC2s in the lung, blocking the early initiation of type-2 inflammation. These protective effects also require the innate immune system, as OM-85-conferred protection was lost in MyD88$^{-/-}$ Trif$^{-/-}$ mice [69]. Together, these results demonstrate that airway administration of OM-85 can effectively dampen allergic airway inflammation.

CHAPTER 2: MECHANISMS UNDERLYING ASTHMA PROTECTION: A COMPARISON OF AMISH FARM DUST EXTRACTS AND THE BACTERIAL LYSATE, OM-85

2.1 – INTRODUCTION

The studies investigating AFDE published in Stein et al. [51] and those investigating OM-85 published in Pivniouk et al. [69] established that airway administration of these microbe-rich agents confer protection against experimental allergic asthma in mouse models. Both AFDE and OM-85 inhibit AHR, airway eosinophilia, and the expression of type-2 inflammatory cytokines in the lungs, while improving the function of the airway epithelial barrier. Moreover, the protective effects of both AFDE and OM-85 are dependent on the innate immune system. Though the biological mechanisms underlying these protective effects have been studied, they are not completely understood.

The protective effects of AFDE were previously studied in an OVA-induced experimental asthma mouse model [51]. OVA is the major protein found in chicken egg whites, which has been isolated and standardized for use as an antigen in immunological studies of food allergy and experimental allergic asthma. For use in a mouse model of allergic asthma, OVA is first administered by intraperitoneal (i.p.) injection with an adjuvant to elicit a systemic type-2 inflammatory response, which is then localized acutely to the airways by intranasal OVA challenge [70]. Although this ultimately mimics the allergic inflammatory responses of asthma in humans – specifically AHR and the recruitment of inflammatory lymphocytes and eosinophils – the route of administration is not physiologically relevant to human exposure. By comparison, *Alternaria alternata* is a protease-rich fungus commonly found both outdoors and indoors

particularly in arid environments. Sensitization to *Alternaria* is commonly associated with childhood asthma [71-73]. Extracts made from *Alternaria* are heterogeneous, comprised of membrane components, metabolites, and proteins including proteases and the major allergen Alt a 1 [74], making standardization difficult. However, intranasal sensitization and challenge with *Alternaria* extracts recapitulates the route of exposure for humans, eliciting type-2 inflammation locally in the airways without the need for an adjuvant. For these reasons, the research presented here made use of a previously published *Alternaria* extract-driven experimental asthma mouse model [69].

For my project, I aimed to further elucidate candidates responsible for the protective effects of AFDE and OM-85 by investigating the cellular and transcriptional changes induced by these microbial agents in the airways of an *Alternaria*-induced experimental asthma mouse model. Given that both microbial agents elicit similar phenotypic responses, I designed an experiment that allowed me to directly compare the transcriptional modifications induced by AFDE and OM-85 to determine whether they achieve asthma-protection by similar mechanisms.

2.2 - METHODS

Amish farm dust extracts and OM-85 preparation

A colleague in the lab generated aqueous extracts of AFDE by weighing the dust material and adding a volume of cold, sterile, endo-toxin free water based on the weight of the dust (1 g/100 mL H_2O) before shaking at 400 rpm at room temperature for 1 hour. After centrifugation at 2500 rpm at 4°C for 1 hour the supernatant was collected in a sterile hood. The aqueous AFDE were aliquoted and stored in cryovials at -80°C until my use.

OM-85 concentrate (lot # 1622001, 23.1 mg/ml of dry residue**)** was provided by OM PHARMA and is the soluble supernatant obtained after bacterial lysis. It represents the drug substance prior to its lyophilization and final manufacturing as Broncho-Vaxom™. The concentrate was stored at 4°C and diluted to 1 mg/50 µl with saline immediately before use.

Mice and experimental asthma model

Seven- to eight-week old, male BALB/c wild-type-mice (Charles River Laboratories) were sensitized intranasally with *Alternaria alternata* extracts (Greer Laboratories: 50 µg of dry weight in 50 µl of saline) on days 0 and 1, and challenged intranasally (25 µg of dry weight in 50 µl of saline) on days 17, 18 and 19, as shown in Fig. 2.1A. From day -10 to day 16, AFDE (5 mg in 50 µl of saline) or OM-85 (1 mg in 50 µl saline, 50 µl per treatment) were intranasally instilled every other day for a total of 14 treatments, using the protocol described in Pivniouk et al [69]. Terminal assessments on day 20 included invasive lung function measurements and the collection of BAL for cell differentials and protein quantification, serum for immunoglobulins by ELISA, and lung tissue for histology, RNA extraction, and flow cytometry.

In preparation for single nucleus (sn)RNA-seq, 8-week-old, male BALB/c mice received intranasal instillations of AFDE (5 mg in 50 µl of saline) or OM-85 (1 mg in 50 µl saline, 50 µl per treatment) every other day between days -10 and 0 for a total of 5 treatments, as shown in Fig. 2.6A. On day 0 total, unmanipulated lung tissue was collected, sealed in a cryovial, flash frozen in liquid nitrogen, and stored at -80°C.

BAL analysis

BAL was obtained by administering cold 1% BSA in 1X PBS (1 ml, twice) through a tracheal cannula into the airways and slowly aspirating the fluid. After counting the total cells in the volume recovered, cell differentials were calculated by an operator blinded to the mouse treatment group from Hema 3 stained slides where at least 400 cells/slide were counted. The total cell count was adjusted for the volume of BAL recovered on a per mouse basis. In selected experiments, BAL IgA or IgG levels were measured by ELISA (Invitrogen).

Serum analysis

Whole blood was collected and allowed to clot at room temperature for at least 30 minutes before centrifugation at 12,000 x g. The clear top layer of serum was collected and in select experiments, serum IgG levels were measured by ELISA (Invitrogen).

Cell phenotyping by flow cytometry

Lung cell suspensions were prepared by digestion with Liberase TM (Roche Life Science) and DNase I (Sigma). Tissue debris was removed by passing cell suspensions through a 70 µm cell strainer and red blood cells were lysed with ACK buffer. To block nonspecific

staining by fluorochrome-conjugated antibodies, cells were preincubated with Fc block (BD Bioscience: 10 μg/ml) for 10-15 min on ice. The following anti-mouse surface antigen antibodies were then used to perform flow cytometry and characterize the cellular composition of the lung: anti-CD3-FITC (553065), anti-CD4-PE-Cy7 (561099), anti-CD8-APC-Fire750 (560182), anti-CD11b-APC-Fire750 (561039), anti-CD11c-PE-Cy7 (558079), anti-CD170 (Siglec-F)-AF647 (562680, all from BD Biosciences); anti-CD19-APC-Fire750 (115557), anti-CD45R/B220-PE (103207), anti-TCR γ/δ-BV510 (118131), anti-Ly6G-BV421 (127627, all from BioLegend).

To stain, cells were incubated for 30 minutes on ice in the dark with appropriate antibodies (0.5-5 μg/1x10^6 cells in a final volume of 100 μl; using the concentration recommended by each manufacturer). The gating strategy used to characterize the immune cells of the lung (macrophages, eosinophils, neutrophils, B cells, T cells – both αβ T cells and γδ T cells) is shown in Fig. 2.3 based on the definition of each immune cell type-of the lung according to Van Hoecke et al. [75]. Approximately 30,000-50,000 events were captured per sample using a BD Fortessa flow cytometer (BD Biosciences) and analyzed with FlowJo software (Version 10.6.0, Becton Dickinson, 2019).

Bulk RNA-sequencing

RNA was extracted from mouse unfractionated lung tissue (n=6-10 mice/treatment group). RNA isolation was performed with RNeasy Mini kit (Qiagen) according to the manufacturer's protocol, including the steps for DNase I digestion. Isolated RNA was sent to the University of Chicago Genomics Facility for RNA quality control, library preparation, and sequencing on Illumina's NovaSeq 6000, 100 basepair paired end reads. Reads were mapped to the mouse genome (version mm10/GRCm38) using STAR [76]. HOMER [77] was used to select

uniquely aligning reads, to filter PCR clones (up to a maximum of 3 clones per read), and to generate the read count matrix for 32,285 transcripts (Gencode version M23). Transcripts whose expression levels exceeded 0.5 transcripts per million in at least 10% of samples were retained for downstream analyses (n=16,488). DESeq2 (version 1.38.3) [78] was used to normalize for differences in library size and latent factors were identified using RUVSeq (version 1.32.0) [79]. Finally, normalized counts were generated using an rlog transformation (DESeq2), accounting for estimated variability from experimental variables not directly measured.

The retained, normalized transcripts were used as input for principal component analysis (PCA). PCA was performed on the retained, normalized transcripts. To determine if mice cluster by treatment group independently, the package "factoextra" (version 1.0.7) was used to determine the optimal number of clusters by Silhouette statistic and Gap statistic graphs and to carry out K-means clustering by Euclidean distance [80]. Differential expression analyses compared the gene expression of *Alternaria*+OM-85 (*Alt*/OM-85)- to *Alternaria*-treated mice, *Alternaria*+AFDE (*Alt*/AFDE)- to *Alternaria*-treated mice, OM-85- to saline-treated mice, and AFDE- to saline-treated mice. Significantly differentially expressed (DE) genes were defined as having a $|\log2(\text{fold change})| > 0.5$ and an FDR<0.05. Genes DE in the comparison of OM-85- to saline-treated mice and the comparison of AFDE- to saline-treated mice were further investigated for the direction and size effect of the change in gene expression. In a similar analysis, genes DE in the comparison of *Alt*/OM-85- to *Alternaria*-treated mice and the comparison of *Alt*/AFDE- to *Alternaria*-treated mice were further investigated for the direction and size effect of the change in gene expression, before and after the exclusion of immunoglobulin variable region genes.

Single nucleus RNA-sequencing

Nuclei isolation from mouse lung tissue was achieved following the methods outlined by Zhang et al. [81]. After isolation, nuclei were additionally filtered through a 40 µm Flowmi cell strainer and stained with DAPI (Invitrogen NucBlue™ Fixed Cell ReadyProbes™ Reagent) according to the manufacturer's protocol. Nuclei for use in the protocol outlined in the "Chromium Next GEM Single Cell 5' v2 (Dual Index) User Guide" (10X Genomics) were prepared by sorting 20,000 viable, intact nuclei per condition directly into the Reaction Master Mix, without RT Enzyme C using a BD FACSAria III cell sorter (BD Biosciences). After determining the total volume of nuclei in Reaction Master Mix, the appropriate amount of RT Enzyme C and nuclease-free water was added according to the "Cell Suspension Volume Calculator Table" provided in Section 1.1. The remainder of the protocol was followed exactly for GEM (Gel Bead-In Emulsions) generation & barcoding, post GEM-RT cleanup & cDNA amplification, library construction, and 5' Gene expression (GEX) dual index library construction. The final libraries were submitted to the University of Chicago where library-size distribution was measured with the Agilent 5300 Fragment Analyzer System and sequencing was performed on Illumina's NovaSeq 6000 with 200 bp paired end reads, according to 10X Genomics' recommendations.

The paired-end snRNA-seq FASTQ files were processed with 10X Genomics CellRanger (version 7.0.0), producing a sparse read matrix for each sample. Prior to quality control and filtering, the RNA profiles for the nuclei of an estimated 138,791 cells were captured. Further analysis was conducted using the R (version 4.2.2) package Seurat (version 5.0.3) [82], following the Satija lab's Guided Clustering Tutorial (https://satijalab.org/seurat). The default parameters from the tutorial were used, however the cutoff for minimum reads per cell was

increased to 1,000 to remove low quality/dying cells. The package Scrublet (version 0.2.3) [83] was used to estimate barcodes assigned to 2 or more cells (doublets) and predicted doublet barcodes were excluded from further analysis. The remaining 107,507 cells were clustered and the package Harmony (1.2.0) [84] was used to integrate the data before normalization, scaling, PCA, and clustering via Seurat. Marker genes were identified for each cluster and cell type-annotation was achieved by label transfer referencing bulk RNA-seq data from Koenitzer et al. [85] and RNA-seq datasets from the Immunological Genome Project (ImmGen) [86]. Once cell types were established, a pseudo-bulk differential expression analysis was performed. The package SingleCellExperiment (version 1.28.1) [87] was used to convert the data for the package Scater (version 1.34.1) [88] to aggregate the total counts per cell by the final cell type. The final cell type-data was filtered for lowly expressed genes and individuals with low library size, and edgeR (version 4.4.2) [89] was used to identify DE genes ($P<0.05$) per cell type-between OM-85- and saline-treated mice and between AFDE- and saline-treated mice. DE genes shared between these comparisons in the B cell population were further investigated.

To further characterize the B cells, the cluster of cells annotated as B cells was selected from the final dataset. As no further quality control or filtering were required, these cells were passed through normalization, scaling, PCA, and clustering using Seurat's default parameters with the nearest neighbor clustering resolution set to 0.1. Using the default parameters of the Seurat function "FindAllMarkers", marker genes with expression that defines one cluster of cells apart from the others were identified and the expression of these marker genes was visualized using Seurat's "FeaturePlot" function.

Statistics

Unless otherwise stated, R version 4.4.0 was used. To determine statistical significance, pairwise Student's t-tests were performed, unless otherwise stated. The package "lmerTest" (version 3.1.3) [90] was used to perform a linear mixed effects model considering treatment group and dosage of acetylcholine as independent variables (fixed effects), while the identifier for each mouse was used to account for repeated measures for the subject (random effects). P-values for the overall interaction between group and acetylcholine dose were reported. Nominal P-values < 0.05 were considered significant for all terminal measurements and false discovery rates (FDR) were used to adjust for multiple testing for -omics analyses. Figures throughout this dissertation were generated using the R package "ggplot2" (version 3.5.1) [91], GraphPad Prism (version 8.0.2 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com) and Adobe Illustrator (version 29.7.1, 2025).

2.3 – RESULTS

2.3.1 - *Airway administration of OM-85 and AFDE protects against Alternaria-induced asthma*

To directly compare the effects of intranasal administration of OM-85 and AFDE in an *Alternaria*-induced experimental asthma mouse model, I used a previously published protocol in which Balb/c mice were sensitized with *Alternaria alternata* extracts (50 µg dry weight in 50 µl saline) on days 0 and 1 and challenged with the same extracts (25 µg dry weight in 50 µl saline) on days 17, 18 and 19. Treatment with either OM-85 (1 mg in 50 µl saline) or AFDE (5 mg in 50 µl saline) occurred every other day between day -10 and day 16. Terminal assessments were made on day 20 (Fig. 2.1A).

The results from OM-85 administration were similar to those published in Pivniouk et al. [69]. OM-85 treatment significantly suppressed *Alternaria*-induced total airway resistance (P=0.002), comparable to treatment with AFDE (P=0.0003, Fig. 2.1B). OM-85 and AFDE both significantly inhibited *Alternaria*-induced BAL eosinophilia (P=3.6e-6 and P=3.9e-5, respectively) and increased the number of airway macrophages (P=0.001 and P=0.0004, respectively), regardless of *Alternaria* exposure. An increase in airway neutrophils induced by *Alt*/AFDE treatment was not observed in mice treated with OM-85 (Fig. 2.1C). In the lung tissue, both OM-85 and AFDE significantly reduced *Alternaria*-induced expression of *Il5* (P=0.004 and P=0.005, respectively) and *Il13* (P=0.005 and P=0.005, respectively), with no change in expression of *Ifng*. AFDE, but not OM-85, increased the expression of *Il17* (Fig. 2.1D).

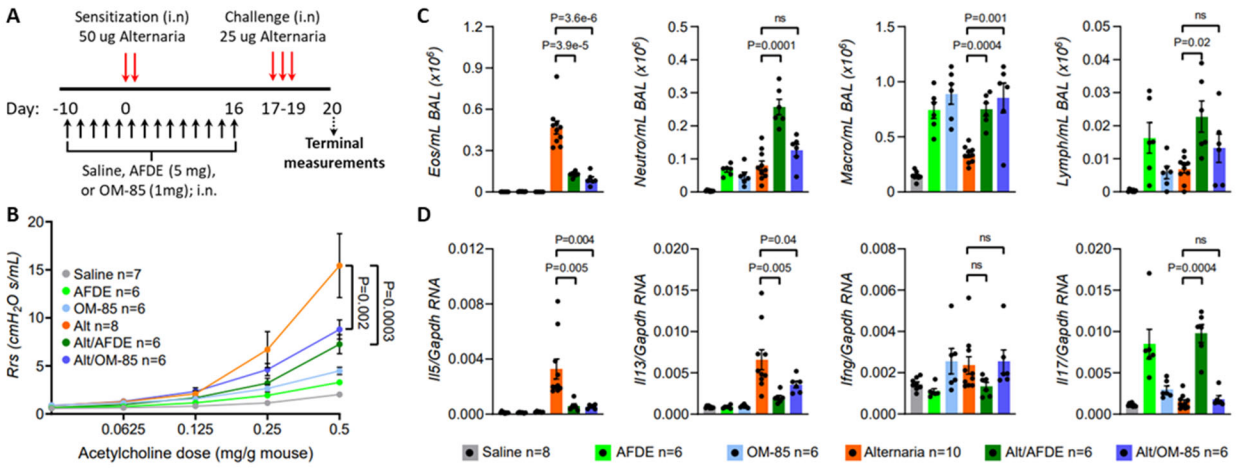

Fig. 2.1. Intranasal administration of OM-85 protects against *Alternaria*-induced asthma. A: Balb/c mice were treated i.n. with OM-85 (1mg) or AFDE (5mg) between days -10 and 16 and sensitized i.n. with *Alternaria* (50µg/50µl) on days 0 and 1. Intranasal challenge with *Alternaria* (25µg/50µl) on days 16-19 preceded terminal measurement collection on day 20. **B:** Invasive lung function measurements. Symbols denote mean and bars denote SEM. Statistical significance was assessed by repeated measures mixed models. **C:** Total BAL cellularity with differentials. **D:** Cytokine mRNA levels in lungs relative to Gapdh expression. Symbols denote individual mice and bars denote mean ± SEM. Unpaired, two-tailed t-tests were used for statistical significance.

2.3.2 - *OM-85 and AFDE induce similar modifications to gene expression profiles in the cells of the lung*

In view of the similar protective phenotypes elicited by OM-85 and AFDE, I sought to examine the changes in transcription associated with these phenotypes. The transcriptomes from mice treated with saline, *Alternaria*, OM-85, AFDE, *Alt*/OM-85 or *Alt*/AFDE (Fig. 2.1A) were profiled by RNA-seq performed on unfractionated lung tissue. The expression of 16,553 genes was measured and used to perform PCA. Principal component (PC)1 captured 47.12% of the variance in the total dataset and distinctly separated the mice by treatment group, except for the mice given only OM-85 or AFDE which had overlapping 95% confidence intervals. In contrast, PC2 captured 22.24% of the variance, separating the mice that received either microbial agent from those that did not (Fig. 2.2A). Although mice treated with *Alt*/OM-85 and *Alt*/AFDE

appeared close, k-means clustering performed on the PCA normalized data revealed they are distinct groups, unlike mice treated with only OM-85 or AFDE which cluster together (Fig. 2.2B). An additional PCA was performed using gene expression data from saline-, OM-85-, or AFDE-treated mice, which confirmed that the gene expression profiles of mice that did not receive *Alternaria* are indistinguishable (data not shown).

2.3.3 – *Differential expression analysis reveals similar modifications in expression induced by OM-85 and AFDE*

To capture the changes in gene expression induced by OM-85 and AFDE alone, I performed two differential expression analyses comparing the gene expression of OM-85- to saline-treated mice (OM-85 vs. Saline) and AFDE- relative to saline-treated mice (AFDE vs. Saline). In the OM-85 vs. Saline comparison, 1,912 genes were significantly DE and 1,618 genes were significantly DE in AFDE vs. Saline. Of these DE genes, 1,343 genes were shared between OM-85 vs. Saline and AFDE vs. Saline and most of these genes were similarly DE, both in terms of magnitude and direction (Fig. 2.2C).

The genes upregulated in both comparisons included genes involved in immunoglobulin isotype-switching or genes encoding the heavy chains of several immunoglobulin isotypes. These comprised *Aicda*, which codes for activation-induced cytidine deaminase or AID and plays a key role in class-switch recombination allowing for B cells to produce immunoglobulins with different effector functions. *Ighg2b* and *Ighg2c* both code for the IgG2 subclass of IgG, responsible for the activation of the complement cascade and the neutralization of toxins and viruses. The polymerization and secretion of IgA, encoded by *Igha*, requires the J-chain (*Jchain*) to perform a critical role in mucosal immunity as it neutralizes or prevents toxins, viruses, and

bacteria from binding to the mucosal surface. Interestingly *Ighe,* which encodes the IgE heavy chain and is associated with allergic inflammation and parasitic infection, is upregulated by *Alternaria* and its expression is amplified by OM-85 and AFDE [92]. Also among the genes upregulated in both OM-85- vs. Saline- and AFDE- vs. Saline-treated mice were 214 immunoglobulin variable region genes marking B cells with unique antigen specificity, though we do not know which antigens these regions are specific for.

Also upregulated by both microbial agents was expression of *Nuggc*, which codes for the nuclear GTPase SLIP-GC expressed in germinal center B cells and likely regulates the activity of AID [93]; *Rgs13*, which encodes RGS13, a negative regulator of G protein-coupled receptors that recognize secreted microbial products [94, 95]; and *Il17a*, a proinflammatory cytokine involved in type-3 inflammation which has been implicated in neutrophilic inflammation and airway remodeling in severe asthma [96]. Two of the genes most strongly downregulated by both OM-85 and AFDE were *Cd209f* and *Cd209g*, which code for SIGNR7 and SIGNR8, respectively, mouse-specific family members of the CD209/DC-SIGN protein family [97]. Though SIGNR7 and SIGNR8 have not been deeply studied, other members of the CD209 family are expressed on the surface of dendritic cells and macrophages functioning to attach antigen presenting cells to T cells and as pathogen binding receptors [97, 98].

Of the 569 genes uniquely DE in the OM-85 vs. Saline comparison, almost all the upregulated genes were Ig variable region genes. Amid them were *Ccl1*, responsible for eosinophil recruitment [99]; *Cxcl10*, which encodes the type-1 inflammatory IFN-gamma-inducible protein 10 also upregulated in allergic pulmonary inflammation [100]; the innate B cell marker, *Cd5* [101]; and *Il12b*, a subunit of the cytokine IL-12 that induces IFN-γ production and differentiation of Th1 cells [102]. The genes strongly downregulated in OM-85 vs. Saline

included the pathogen binding macrophage receptor *MARCO* [103]; *Ear6*, a member of the eosinophil-associated ribonuclease A family [104], and *Mcidas*, which encodes multicilin, a regulator of ciliated cell differentiation [105]. In the AFDE vs. Saline comparison, 275 genes were uniquely DE, including several immunoglobulin variable region genes. Several γδ T cell-related genes, including variable regions for the T cell receptor delta chain (*Trdv5*) and gamma chain (*Trgv2*) were upregulated by AFDE only [106].

To determine the effects of OM-85 and AFDE in the context of *Alternaria*, differential expression analyses were performed comparing *Alt*/OM-85- to *Alternaria*-treated mice (*Alt*/OM-85 vs. *Alt*) and *Alt*/AFDE- to *Alternaria*-treated mice (*Alt*/AFDE vs. *Alt*). The *Alt*/OM-85 vs. *Alt* comparison identified 1,891 significantly DE genes ($|\log2(\text{fold change})| > 0.5$ and FDR < 0.05) and in the comparison of *Alt*/AFDE vs. *Alt*, 1,357 genes met these criteria. Of these DE genes, 1,047 genes were shared between *Alt*/OM-85 vs. *Alt* and *Alt*/AFDE vs. *Alt,* and most of these genes were similarly DE, in terms of both magnitude and direction (Fig. 2.2D).

Interestingly, the genes most strongly upregulated by both OM-85 and AFDE in the context of *Alternaria* included the same immunoglobulin heavy chain and class-switching genes (*Aicda, Ighg2b, Ighg2c, Igha, Jchain,* and *Ighe*) seen in the comparisons without *Alternaria*. Also upregulated by both microbial agents were *Nuggc*, *Rgs13*, and 201 immunoglobulin variable region genes. Among the 1,047 shared DE genes, 262 were downregulated by both OM-85 and AFDE in the context of *Alternaria*. The genes downregulated most strongly included the previously mentioned *Cd209f* and *Cd209g*, *Cd163*, a marker of alternatively activated macrophages [107], and *Retnlb*, which encodes resistin-like molecule β (RELMβ)/found in inflammatory zone 2 (FIZZ2) and is expressed by lung epithelial cells induced by type-2 cytokines or bacterial infection [108]. Also strongly downregulated were genes related to

Alternaria-induced type-2 inflammation. These included the previously mentioned *Ear6* and *Mcidas*, as well as the type-2 inflammatory cytokine *Il5*; the *Il13* receptor, *Il13ra*; and the eosinophil granule protein, eosinophil peroxidase (*Epx*). Also downregulated were *Chil4*, which encodes the chitinase-like protein Chil3 (also known as Ym2) upregulated in the lung during type-2 inflammation [109]; *Aldh1a3*, the expression of which is elevated in asthma patients, perhaps because of its role in retinoic acid signaling [110]; and *Sox9*, which promotes goblet cell differentiation and goblet cell hyperplasia [111].

Of the 310 genes uniquely DE in *Alt*/OM-85 vs. *Alt*, the strongest increases in expression belonged to immunoglobulin variable region genes and *Csn2*, which encodes the protein casoparan found to activate macrophages for phagocytosis [112]. The 844 genes DE only in the *Alt*/AFDE vs. *Alt* comparison included strongly upregulated genes related to γδ T cells, like the previously mentioned *Trdv5,* the γδ T cell receptor gene *Tcrg-V4*, and two of the T cell receptor gamma joining genes, *Trgj1* and *Trgj2* [106]. Also upregulated were a small number of immunoglobulin variable region genes.

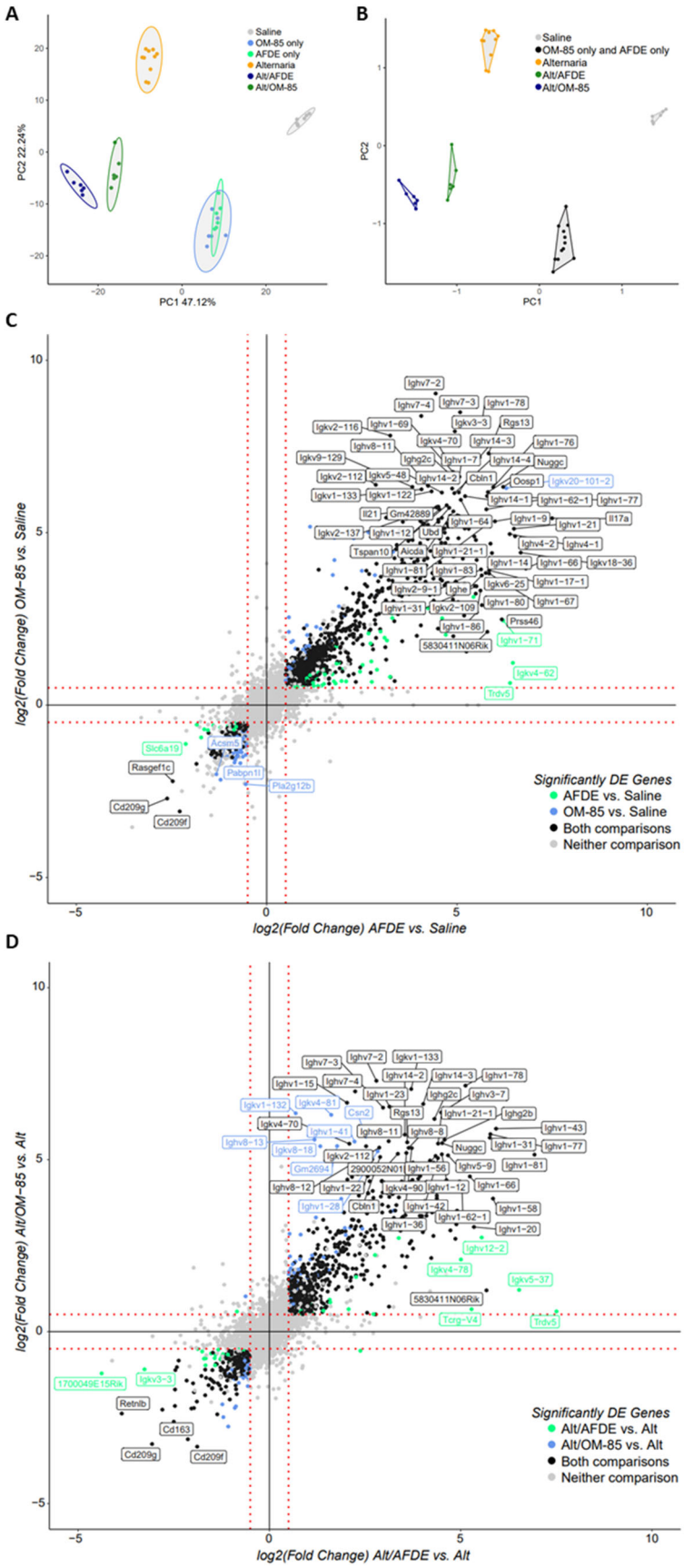

Fig. 2.2. Transcriptomic analysis reveals OM-85 and AFDE induce similar changes in expression.

A: Clustering of mouse lung transcriptome profiles by PCA. Each symbol represents the transcriptomic profile of a single mouse. The distance between 2 symbols indicates how similar the profiles are to one another. Ellipses represent the 95% confidence intervals for each treatment group: Saline, n=8; OM-85, n=6; AFDE, n=6; Alternaria, n=10; Alt/OM-85, n=6; and Alt/AFDE, n=6. **B:** K-means clustering performed on PCA-transformed data using k=5. Each symbol represents the transcriptomic profile of a single mouse and concentration ellipses indicate where the data points are most dense. **C and D:** Scatterplots zoomed in to depict the relationship between DE genes in **C:** OM-85 vs. Saline and AFDE vs. Saline, or in **D:** Alt/OM-85 vs. Alt and Alt/AFDE vs. Alt. Each symbol represents the log2(fold change) in gene expression for a single gene. Genes significantly DE (|log2(fold change)|>0.5 and FDR<0.05) by OM-85 and AFDE relative to their controls are shown in black. Genes significantly DE by AFDE, but not OM-85, are shown in green – and vice versa, in blue. Non-significant genes are illustrated in grey. The red dashed lines indicate |log2(fold change)| = 0.5. **C:** Genes that reached -2<log2(fold change)>5.5 are labelled, and 1 gene is excluded from view: Igkv4 in AFDE vs. Saline (log2(fold change)=17.06, FDR=7.94e-21). **D:** Genes that reached -3<log2(fold change)>5 are labelled, and 2 genes were excluded from view: GM29328 in Alt/OM-85 vs. Alt (log2(fold change)=-20.99, FDR=4.87e-63) and Sds in Alt/AFDE vs. Alt (log2(fold change)=-14.03, FDR=5.92e-17).

2.3.4 – *Flow cytometry reveals a B cell expansion induced by AFDE and OM-85*

To evaluate the effects of AFDE and OM-85 on the immune cell landscape of the lungs, flow cytometry was performed to characterize the major immune cell types present in the lung tissue. From the mice sensitized and challenged as described above (Fig. 2.1A), 2-4 mice per treatment group were randomly selected and immune lung cells were phenotyped by flow cytometry following a gating strategy adapted from Van Hoecke et al. [75] (Fig. 2.3).
The results of these analyses suggested that treatment with OM-85, but not AFDE, increased the number of $CD11c^{high}SiglecF^{+}$ macrophages regardless of *Alternaria* exposure (Fig. 2.4A). OM-85 and AFDE both appeared to have modestly suppressed *Alternaria*-induced increase in $CD11c^{low}CD3^{-}B220^{-}CD11b^{+}Ly6G^{-}$ eosinophils and $CD11c^{low}CD3^{-}B220^{-}CD11b^{+}Ly6G^{+}$ neutrophils (Fig. 2.4B-C). A nearly 2-fold increase in the number of $CD19^{+}B220^{+}$ B cells was observed in mice given *Alt*/OM-85 or *Alt*/AFDE when compared to mice given *Alternaria* alone. This increase was not present in mice treated with OM-85 or AFDE alone (Fig. 2.4D). *Alt*/AFDE treatment induced a nearly 2-fold increase in lung $CD3^{+}B220^{-}$ T cells relative to mice that received *Alternaria* (Fig. 2.4E). Further characterization of these T cells revealed an AFDE-specific increase in the number of γδT cells ($CD3^{+}B220^{-}$ γδTCR^{+}, Fig. 2.4F-G). We note that the conclusions that could be drawn from this analysis were limited by the relatively low number of mice assayed.

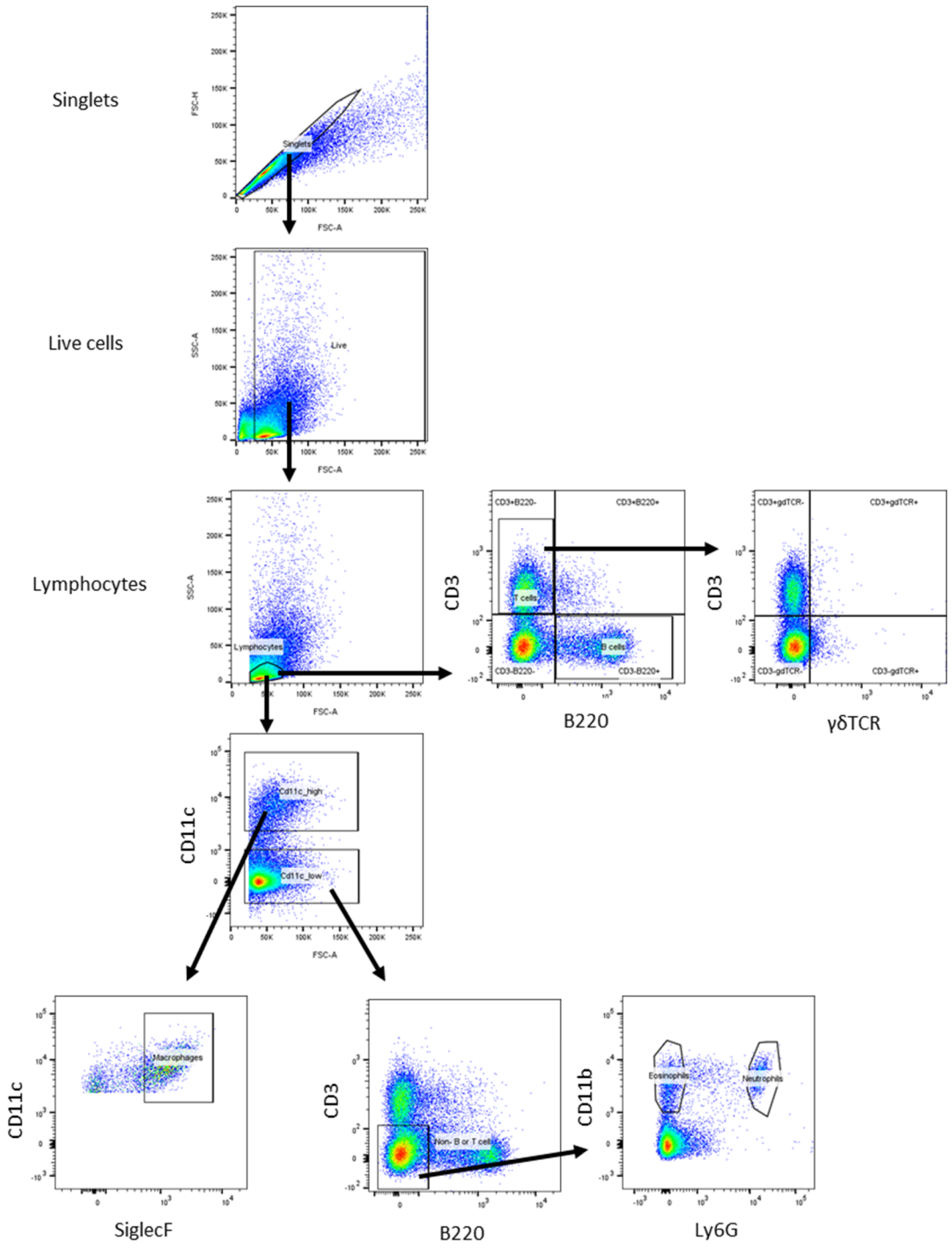

Fig. 2.3. Flow cytometry gating strategy for lung immune cell identification and phenotyping. Cells from OM-85-, AFDE-, *Alt*/OM-85-, and *Alt*/AFDE-treated mice were used as controls.

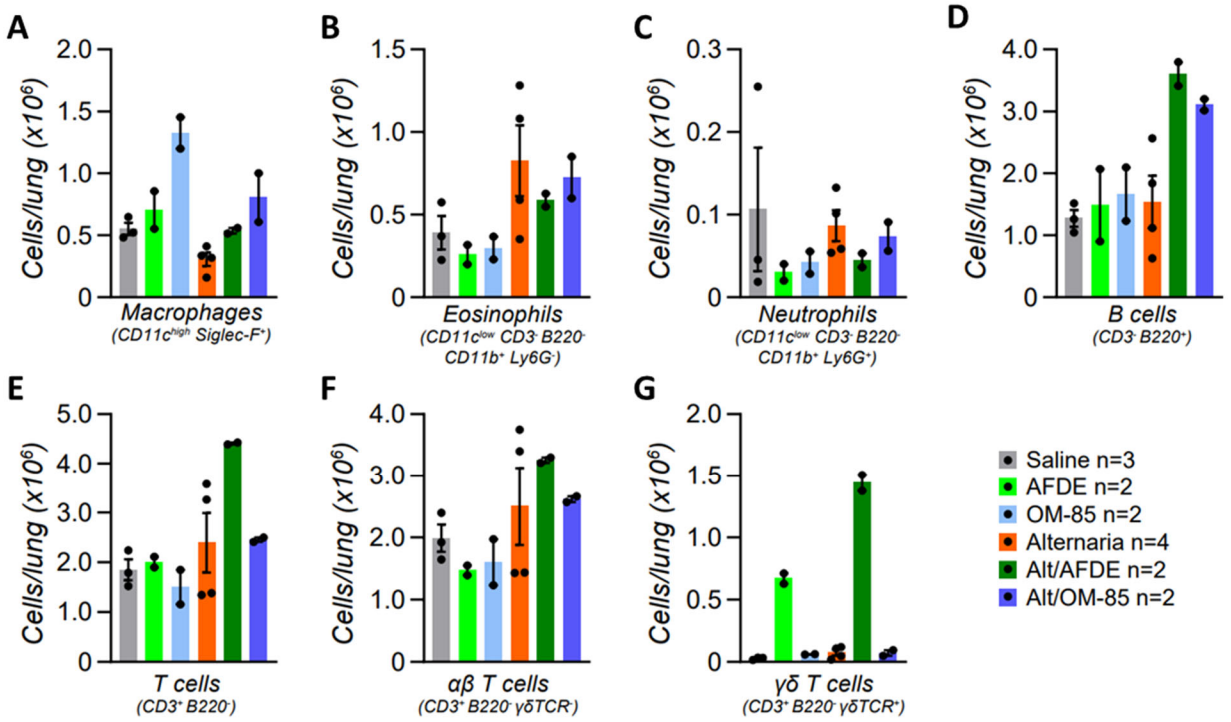

Fig. 2.4. Airway administration of OM-85 and AFDE induces an expansion of B cells in the lung. Lung cells were isolated at day 20 from BALB/c mice treated with saline, OM-85, AFDE, *Alternaria*, *Alt*/OM-85, or *Alt*/AFDE, as in Fig. 3.1A. Total numbers of cells per lung identified by flow cytometry: **A:** Macrophages, **B:** eosinophils, **C:** neutrophils, **D:** B cells, and **E:** T cells as well as **F:** αβT cells and **G:** γδT cells. Data are from 2-4 mice per group from 2 experiments. Symbols denote individual mice and bars denote mean ± SEM. Statistical significance could not be calculated provided the number of mice.

2.3.5 - *OM-85 and AFDE induce an increase in total BAL IgA and IgG and serum IgG*

Having observed an OM-85- and AFDE-induced upregulation of the heavy chain genes for IgA (*Igha*) and IgG (*Ighg2b* and *Ighg2c*) and an expansion of B cells in the lung, protein quantification was performed to determine if these antibodies were being produced in response to our microbial agents. Treatment with either *Alt*/OM-85 or *Alt*/AFDE significantly increased the concentration of IgA in BAL (P=6.4e-5 and P=0.009, respectively; Fig. 2.5A). Total BAL IgG was significantly increased in *Alt*/AFDE, and my results suggest this was also true for

Alt/OM-85. However, the exact IgG concentration for the *Alt*/OM-85 treatment group could not be determined as I did not dilute the samples enough and many ELISA values were above the assay's upper limit of detection (2,000 ng/mL, Fig. 2.5B). Total serum IgG levels were also significantly higher in *Alt*/OM-85- and *Alt*/AFDE- relative to *Alternaria*-treated mice (P=0.009 and P=0.002, respectively; Fig. 2.5C). Increases in BAL IgA and IgG, as well as serum IgG were also observed in mice given OM-85 or AFDE alone (Fig. 2.5).

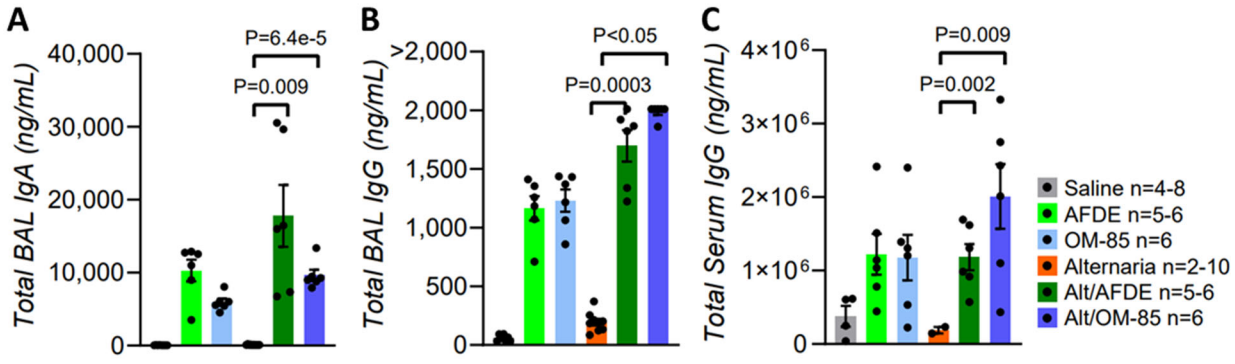

Fig. 2.5. OM-85 and AFDE induce an increase in total BAL IgA and IgG and serum IgG. BAL and serum were collected at day 20 from BALB/c mice treated with saline, OM-85, AFDE, *Alternaria*, *Alt*/OM-85, or *Alt*/AFDE, as in Fig. 1a. **A-B:** Total BAL IgA and IgG levels measured in BAL and **C:** total serum IgG levels. Data are from 2-10 mice per group from 2 experiments. Symbols denote individual mice and bars denote mean ± SEM. Unpaired, two-tailed t-tests were used for statistical significance.

2.3.6 - *Single nucleus analysis reveals early modifications in the cellular composition and transcriptional profile of the lung after OM-85 or AFDE treatment*

To assess the cellular and transcriptional changes induced by OM-85 and AFDE in the lungs before the introduction of an allergen, snRNA-seq was performed to profile gene expression in individual lung cells. To capture the early events of the response, mice were treated intranasally with saline, OM-85, or AFDE (n=5-6 mice/treatment group) every other day for 10

days, for a total of 5 treatments, as shown in Fig. 2.6A. One day after the last treatment, unmanipulated lung tissue was collected and flash frozen until processed to single nuclei. snRNA-seq captured the transcriptional profiles of 107,507 nuclei across all 3 treatment groups, representing both immune and structural lung cell populations (Fig. 2.6B). Treatment with OM-85 and AFDE significantly modified the proportion ($P<0.05$) of many of the cell types identified by this analysis, these modifications being nearly identical in magnitude and direction relative to saline (Fig. 2.6C). Two pseudo-bulk DE analyses were performed at the level of individual cell types, comparing gene expression between OM-85- and saline-treated mice (OM-85 vs. Saline) and AFDE- and saline-treated mice (AFDE vs. Saline). Following only 5 treatments, both OM-85 and AFDE induced significant differential expression ($FDR<0.05$) in thousands of genes across nearly every cell type-assayed (Fig. 2.6D).

Based on the bulk RNA-seq data shown in section 2.3.3, the flow cytometry analyses presented in section 2.3.4, and the notable early increase in B cells captured by snRNA-seq, I focused my further analyses on the B cell cluster. Within this cluster, a total of 1,871 genes were significantly DE ($FDR<0.05$) in the AFDE vs. Saline comparison and 1,941 genes met these criteria in the OM-85- vs. Saline comparison (Fig. 2.6D). 844 genes were shared between these comparisons. The FDR for each gene was ranked in each DE comparison and the sums of these ranks were used to sort for the most significant shared genes.

Among them, I found upregulated genes related to B cell development, activation, and regulation. These included *Fcrl5*, which codes for an IgG receptor expressed in memory B cells during chronic infections [113]; *Ccr6*, a marker gene for memory B cell precursors [114]; *Rasgrp3,* responsible for regulating B cell proliferation [115]; and *Cd83*, a maturation marker for dendritic cells that is also expressed in activated B cells [116]. Also upregulated were genes

associated with other immune functions. For example, *Il9r*, which signals for immune development and inflammation [117]; *Cfp,* also known as properdin, which promotes the activation of complement [118]; *Sp140*, a regulator of other immune-related genes [119]; *Cybb,* involved in macrophage polarization [120]; *Mpeg1*, a macrophage and neutrophil-expressed pore forming protein involved in microbe clearance; and the previously mentioned *Cd300lf* were all upregulated by both OM-85 and AFDE. A few of the most significant DE genes shared by OM-85 and AFDE were downregulated, including *St6gal1,* which regulates immunoglobulin production [121]; *Cd55*, also known as DAF or decay-accelerating factor, which regulates the complement system while activating dendritic cells, T cells, and B cells [122]; and *Samhd1*, which regulates innate immunity [123].

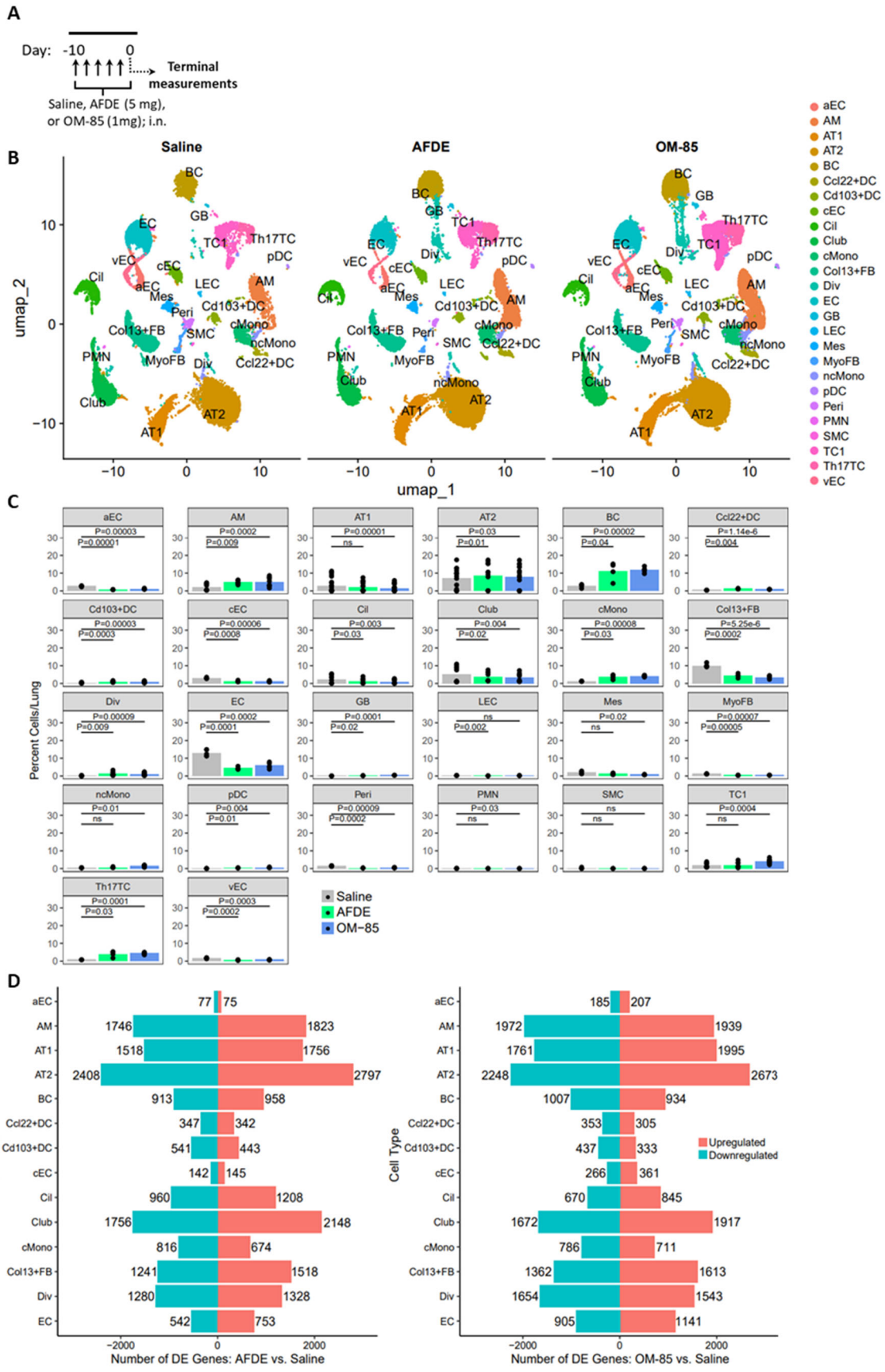
A
Day: -10 0
Terminal measurements
Saline, AFDE (5 mg), or OM-85 (1mg); i.n.
B
Saline
AFDE
OM-85
umap_2
umap_1
aEC
AM
AT1
AT2
BC
Ccl22+DC
Cd103+DC
cEC
Cil
Club
cMono
Col13+FB
Div
EC
GB
LEC
Mes
MyoFB
ncMono
pDC
Peri
PMN
SMC
TC1
Th17TC
vEC
C
Percent Cells/Lung
Saline
AFDE
OM-85
D
Cell Type
Number of DE Genes: AFDE vs. Saline
Number of DE Genes: OM-85 vs. Saline
Upregulated
Downregulated

Fig. 2.6. snRNA-seq analysis shows OM-85- and AFDE-triggered early transcriptional modifications in the lung. A: BALB/c mice were treated intranasally with saline, AFDE (5mg), or OM-85 (1mg) 5 times from day -10 to day -1 and lung tissue was collected for single nucleus RNA-seq on day 0. **B:** Unbiased clustering of mouse lung cell transcriptomes in a UMAP plot, shown by treatment group. Each symbol represents the summarized gene expression of a single nucleus (n=107,507) and the distance between any 2 symbols indicates how similar the profiles are to one another. Cell type was predicted by the average expression profile and clusters were annotated. **C:** The percent of each cell type in the treated lung. Symbols denote individual mice and bars denote mean. Unpaired, two-tailed t-tests were used for statistical significance. **D:** The number of DE genes in AFDE- relative to Saline-treated mice (left) and OM-85- relative to Saline-treated mice (right) by cell type as determined by psuedobulk RNA-seq analysis. Blue bars denote the number of downregulated genes and red bars denote the number of upregulated genes with a P-value < 0.05. aEC = arterial endothelial cells; AM = alveolar macrophages; AT1 = alveolar type 1 epithelial cells; AT2 = alveolar type 2 epithelial cells; BC = B cells; Ccl22+DC = CCL22+ dendritic cells; Cd103+DC = CD103+ dendritic cells, cEC = capillary endothelial cells, Cil = ciliated cells, Club = club cells, cMono = classical monocytes, Col13+FB = collagen type XIII positive fibroblasts, Div = dividing T and B cells, EC = endothelial cells, LEC = lymphatic endothelial cells, Mes = mesothelial cells, MyoFB = myofibroblasts, ncMono = non-classical monocytes; pDC = plasmacytoid dendritic cells; Peri = pericytes, PMN = polymorphonuclear cells; SMC = smooth muscle cells; TC1 = T cells, Th17TC = T helper 17 cells, vEC = venous endothelial cells.

2.3.7 – *Re-clustering B cells reveals sub-populations differentially induced by AFDE and OM-85*

To further explore the transcriptional modifications induced by treatment with OM-85 or AFDE specifically in lung B cells, I selected only the cells in the cluster annotated as B cells and re-clustered them. This analysis unveiled 4 B cell sub-clusters (Fig. 2.7A), few of which originated from saline-treated mice. The cells represented in Cluster 0 were induced by both AFDE and OM-85, while the cells of Clusters 1, 2, and 3 were more strongly induced by OM-85 (Fig. 2.7B). To better understand the identity of the B cell sub-populations induced by OM-85 and AFDE, I sought out marker genes whose expression separated a given cluster from all the other clusters.

Among the top 10 marker genes called for Cluster 0 and 1 were *Satb1, Ighd, Bach2, Poxp1*, and *Fcer2a* (CD23), which are related to early B cell development, survival, and maturation [124-128]. The marker genes identified for Cluster 2, such as *Mtss1* [129]; *Siglecg* [130]; and the ubiquitin ligase genes *Klhl14* and *Cb1b* [131, 132] are related to regulation of

BCR specificity. The top 10 marker genes for Cluster 3 included Ig light chain genes (*Iglc1, Iglc2,* and *Iglc3*) and genes related to different B cell effector functions (*Fcrl5, Mir142hg, Cd79a,* and *Cd37*) [133-136] and their differentiation to plasma cells (*Ms4a1*, also known as CD20) [137]. Although the expression of these marker genes was used to define a given cluster relative to the others, I found that the expression of most of these genes (except *Klhl14* and *Iglc1)* could be detected in all 4 clusters, (Fig. 2.7C).

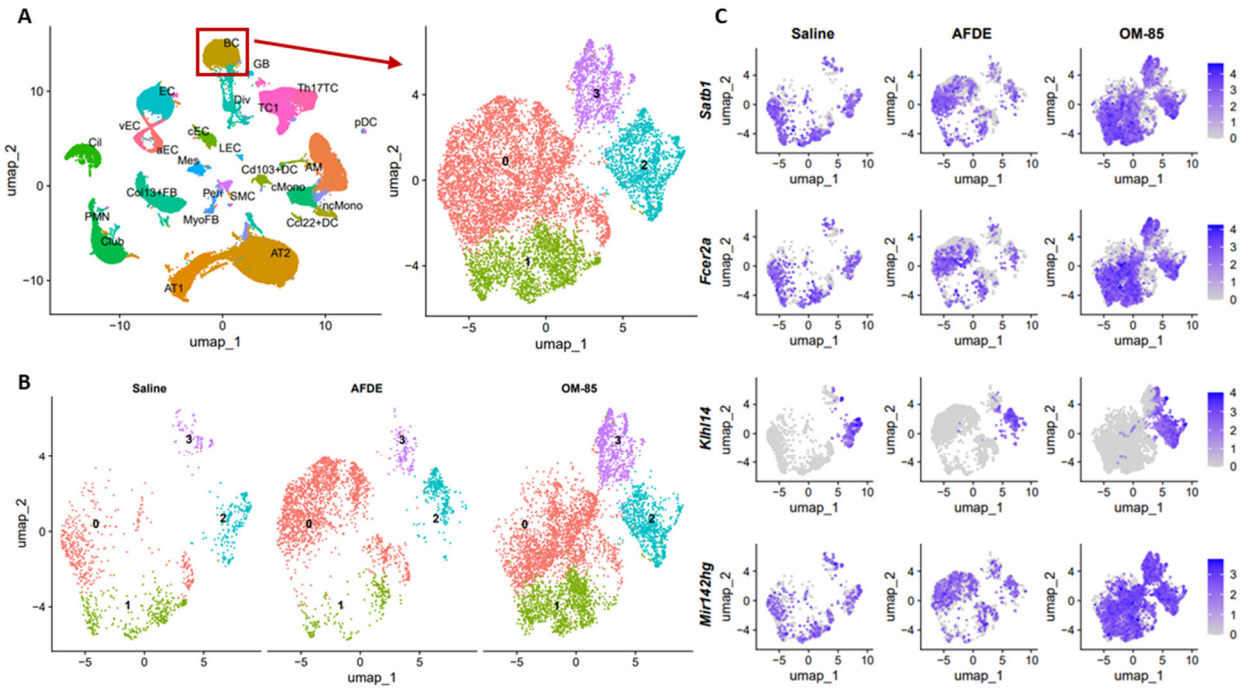

Fig. 2.7. Sub clustering reveals 4 B cell sub-populations. A: Clustering of the mouse lung cell transcriptome profiles from both treatment groups in a UMAP plot. Each symbol represents the transcriptomic profile of a single nucleus (n= 26,604, left). B cells, highlighted in red, were re-clustered (right). **B:** Re-clustered B cells, shown by treatment. **C:** Feature plots displaying expression of *Satb1, Fcer2a, Klhl14,* or *Mir142hg*, shown by treatment group. Mice per group: Saline, n=6; AFDE, n=5; OM-85, n=6.

2.4 - DISCUSSION

Studies previously published by our lab established that the bacterial lysate, OM-85 and extracts made from the microbe-rich dust of traditional Amish farms protect against experimental allergic asthma when administered intranasally to mice. Both OM-85 and AFDE inhibit type-2 inflammation and require the innate immune system for these protective effects. In the work outlined in this chapter, I aimed to better understand the cellular and transcriptional responses underlying the asthma-protective effects of OM-85 and AFDE and to compare the effects of these microbial agents so as to identify core candidates responsible for asthma protection.

Both OM-85 and AFDE inhibited *Alternaria*-induced type-2 inflammation, as shown by a robust suppression of AHR and airway eosinophilia, and the downregulation of *Il5* and *Il13* in the lung. Bulk RNA-seq analysis on unfractionated lung tissue revealed that OM-85 and AFDE induce similar modifications to gene expression patterns of the lung, as most of the genes DE in response to OM-85 or AFDE were the same genes and changed expression in the same direction, with nearly the same magnitude. Specifically in the context of *Alternaria*, treatment with AFDE or OM-85 downregulated the expression of several genes involved in *Alternaria*-induced type-2 inflammation such as *Il5*, *Il13ra, Retnlb, Ear6, Mcidas, Chil3, Aldh1a3*, [104, 105, 108-110]; as well as genes involved in goblet cell hyperplasia (*Sox9*) [111] and macrophage activation (*Cd163*) [107]. Regardless of *Alternaria*, both OM-85 and AFDE induced a robust B cell activation signal indicated by the strong upregulation of several immunoglobulin heavy chain and immunoglobulin variable region genes. Flow cytometry on a limited number of mice suggested the induction of B cell-related genes coincided with an expansion of the number of B cells in the lung, though this increase in B cell number was only observed in mice that received *Alt*/OM-85 or *Alt*/AFDE. Protein quantification of total IgA in the BAL and total IgG in the BAL

and serum aligned with gene expression data, as antibody levels were increased in mice that received OM-85, *Alt*/OM-85, AFDE, and *Alt*/AFDE

snRNA-seq was performed to characterize the cellular and transcriptional modifications initiated by OM-85 and AFDE before introduction of the allergen and better understand how these changes might prevent allergic inflammation. This analysis revealed that after only 5 treatments with OM-85 or AFDE, the cellular landscape of the lung began to change, and widespread transcriptomic modifications were induced by both microbial agents. A closer investigation of B cells identified B cell sub-clusters that were differentially induced by AFDE and OM-85. Based on the marker genes for each cluster, Cluster 0 induced by both AFDE and OM-85 likely consists of B cells playing a role in innate immunity. Clusters 1, 2, and 3 were strongly induced by OM-85 and expressed genes related to B cell regulation and immunoglobulin class switching, perhaps representing a more activated, antigen-specific B cell response.

In conclusion, OM-85 and AFDE confer asthma protection in phenotypically similar ways. The results of my comparison suggest that this is the case because treatments with OM-85 and AFDE modify the expression of the same genes with the same direction and magnitude of effect. Many of these genes are related to an activated, polyclonal B cell response, which prompted me to investigate the role of B cells in the asthma protective effects of OM-85 and AFDE.

CHAPTER 3: WORK IN PROGRESS: ARE B CELLS REQUIRED FOR THE ASTHMA PROTECTION CONFERRED BY AFDE?

3.1 – INTRODUCTION

Three independent, unbiased analyses characterizing the cellular and transcriptional mechanisms that underlie the asthma protective effects of AFDE and OM-85 identified a robust, polyclonal B cell activation signal.

B cells play a critical role in immunity with the primary function of producing antigen specific antibodies known as immunoglobulins. Immunoglobulins are comprised of two heavy chain and two light chain molecules, each with a constant and a variable region. The constant regions of the heavy chains determine the class and function of the antibody, while the variable regions of both chains determine the antibody's specificity. The first antibody class produced by B cells is IgM, comprised of the μ heavy chain. As B cells mature from hematopoietic stem cells to pro-B cells and subsequently pre-B cells, the rearrangement of the immunoglobulin heavy and light chain V(D)J genes occurs and results in a unique antigen binding site [138]. This membrane bound IgM acts as the B cell receptor (BCR) on immature B cells. Without the BCR, B cells cannot mature.

B cell knockout mice have been developed to investigate the role of B cells in immune responses. Using a targeted disruption of the immunoglobulin μ heavy chain, Kitamura et al. developed μMT (B6.129S2-*Ighm*^{tm1Cgn}/J) mice with B cells arrested in the pre-B cell stage of maturation and unable to successfully synthesize IgM [139]. In comparison, the Jh$^{-/-}$ (C.Cg-*Igh-J*^{tm1Dhu}) mice developed by Huszar et al. [140] lack all 4 JH gene segments of the immunoglobulin heavy chain, resulting in mice that cannot produce any mature B cells and

exhibit total ablation of antibodies. In $Jh^{-/-}$ mice, type-2 inflammatory responses induced by exposure to a cocktail of allergens, including OVA and fungal allergens, were attenuated [141].

Using these $Jh^{-/-}$ mice lacking mature B cells, I aimed to assess whether B cells are required for the asthma protective effects of AFDE. Of note, the three experiments I performed provided inconsistent results. Therefore, the work presented in this chapter is still in progress and so far, inconclusive.

3.2– METHODS

Mice and experimental asthma

Across 3 experiments, eight- to nine-week old, male and female BALB/c wild-type-mice (Taconic Biosciences and Charles River Laboratories) and BALB/c $Jh^{-/-}$ mice deficient in mature B cells [140] (Taconic Biosciences, were sensitized and challenged intranasally with *Alternaria alternata* extracts as described in Chapter 2 and shown in Fig. 3.1A. AFDE (5 mg in 50 µl of saline) were administered intranasally every other day from day -10 to day 16 for a total of 14 treatments, following the protocol outlined in Pivniouk et al [69]. Terminal assessments on day 20 included invasive lung function measurements and the collection of BAL for cell differentials, and lung tissue for RNA extraction and flow cytometry.

Of note, wild-type-mice and $Jh^{-/-}$ mice used in experiment 1 were ordered from Taconic Biosciences and housed in our local mouse facility for 1 week prior to the beginning of the experiment. Wild-type-mice and $Jh^{-/-}$ mice used in experiment 2 were also ordered from Taconic Biosciences and housed in our local mouse facility for 1 week prior to the beginning of the experiment. Wild-type-mice used in experiment 3 were ordered from Charles River Laboratories and housed in our local mouse facility for 1 week prior to the beginning of the experiment, while the $Jh^{-/-}$ mice were bred from mice originally ordered from Taconic Biosciences and housed in our local mouse facility until the experiment began.

BAL analysis

BAL was collected and cell differentials were calculated as described in Chapter 2.

Phenotyping by flow cytometry

Lung cell suspensions were prepared as described in Chapter 2. For flow cytometry, non-specific staining by fluorochrome-conjugated antibodies was blocked by preincubating cells with Fc block (BD Bioscience: 10 µg/ml) for 10-15 min on ice. Anti-CD19-APC-Fire750 (115557) and anti-CD45R/B220-PE (103207) antibodies, both from BioLegend, were used to perform flow cytometry and determine whether B cells could be detected in the lungs of WT and Jh-/- mice. Cells were incubated for 30 minutes on ice in the dark with these antibodies (0.25 µg/1x10^6 cells in a final volume of 100 µl, as recommended by the manufacturer). The gating strategy to identify B cell populations is shown in Fig. 3.4A. Approximately 50,000 events were captured per sample using a BD Fortessa flow cytometer (BD Biosciences) and analyzed with FlowJo software (Version 10.6.0, Becton Dickinson, 2019).

3.3 – RESULTS

3.3.1 – *Experiment #1: Wild-type controls exhibited a deficient response to Alternaria*

To investigate whether B cells are required for the asthma protective effects of AFDE, male, wild-type-and $Jh^{-/-}$ BALB/c mice from Taconic Biosciences were sensitized and challenged with *Alternaria* extracts and treated with AFDE exactly as described in Chapter 2 (n=2-4 mice/treatment group, Fig. 3.1A). In *Alternaria*-treated wild-type mice, invasive lung function measurements revealed an approximately 5-fold increase in AHR relative to saline-treated controls - a response much lower than the expected ~15-fold *Alternaria*-induced increase in AHR we typically see (section 3.2.1). Treatment of wild-type mice with AFDE significantly inhibited the modest *Alternaria*-induced AHR (P=0.002). In contrast, *Alternaria* induced brisk AHR in $Jh^{-/-}$ mice and this response was not suppressed by AFDE (Fig. 3.1B).

Both wild-type and $Jh^{-/-}$ Alt/AFDE-treated mice exhibited significant inhibition of airway eosinophilia (P=0.049 and P=0.02, respectively, Fig. 3.1C) and a non-significant downregulation of *Il5* and *Il13* expression in the lung, compared to the mice given *Alternaria*. Notably, treatment with AFDE significantly increased the expression of *Il17* in the lung of both wild-type and $Jh^{-/-}$ mice (P=0.04 and P=0.001, respectively, Fig. 3.1D). *Ifng* expression was slightly increased in the $Jh^{-/-}$ mice given *Alternaria* and *Alt*/AFDE (Fig. 3.2C). Conclusions could not be drawn from this experiment because responses in control mice were unusual and seemingly deficient.

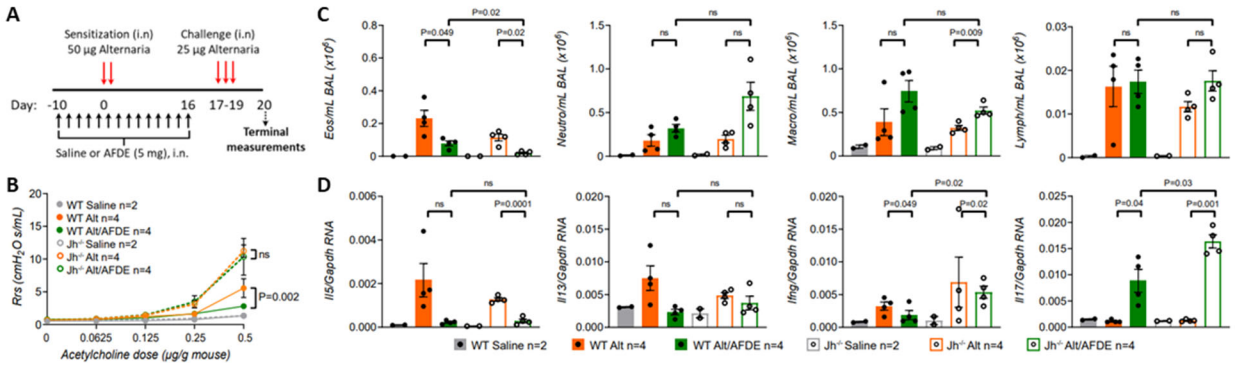

Fig. 3.1. In the first experiment, *Alternaria*-treated wild-type mice exhibit a deficient response. A: Wild-type and Jh$^{-/-}$ BALB/c mice from Taconic Biosciences were treated i.n. with AFDE (5 mg) between days -10 and 16 and sensitized i.n. with Alternaria (50 µg/ 50 µl) on days 0 and 1. Intranasal challenge with Alternaria (25 µg/ 50 µl) on days 17-19 preceded terminal measurement collection on day 20. **B:** Invasive lung function measurements. Symbols denote mean and bars denote SEM. Statistical significance was assessed by repeated measures mixed models. **C:** Total BAL cellularity with differentials. **D:** Cytokine mRNA levels in lungs relative to Gapdh expression. Symbols denote individual mice and bars denote mean ± SEM. Unpaired, two-tailed t-tests were used for statistical significance.

3.3.2 – *Experiment #2: AFDE amplify BAL neutrophilia and fail to suppress AHR in Alternaria-treated mice lacking B cells*

Experiment #1 was repeated, again using male, wild-type and Jh$^{-/-}$ BALB/c mice from Taconic Biosciences (n=2-4 mice/treatment group, Fig. 3.1A). In this experiment, in wild-type mice *Alternaria* induced strong AHR that was significantly (P=5.3e-7) inhibited by treatment with AFDE. In contrast, in B cell-deficient Jh$^{-/-}$mice, AFDE treatment did not suppress *Alternaria*-induced AHR (Fig. 3.2A). Interestingly, treatment with AFDE abrogated BAL eosinophilia in both wild-type- and Jh$^{-/-}$ mice (P=4.03e-5 and P=0.0003, respectively). In Jh$^{-/-}$ mice, but not in wild-type controls, treatment with *Alt*/AFDE induced a noticeable, though not significant, increase in BAL neutrophilia relative to *Alternaria*-treated mice (Fig. 2.12B). Compared to *Alternaria*-treated mice, expression of *Il5* and *Il13* was decreased by AFDE in both wild-type and Jh$^{-/-}$ mice, though also not significantly. *Il17* expression was modestly increased by

Alt/AFDE treatment compared to *Alternaria* alone in both wild-type-(P=0.04) and Jh$^{-/-}$ mice (P=0.06) and *Ifng* expression did not significantly change (Fig. 3.2C).

Chen et al. [142] described an inhibitory interaction between the B cell protein RELMα (encoded by the gene *Retnla*) and IL-17-producing γδ T cells that exacerbates neutrophilic inflammation. To assess whether the lack of B cells affected the expression of RELMα, lung expression was assayed by qPCR in unfractionated lung tissue and found to be suppressed by AFDE treatment relative to *Alternaria* – significantly in the wildtype-mice (P=0.04). No significant differences in *Retnla* mRNA levels were detected between wild-type- and Jh$^{-/-}$ mice (Fig. 3.2C). The results of this experiment, and particularly the increase in AHR and the (modest) amplification of BAL neutrophilia detected in Jh-/- mice treated with AFDE and *Alternaria*, raised the possibility that B cells might play a role in AFDE-induced asthma protection.

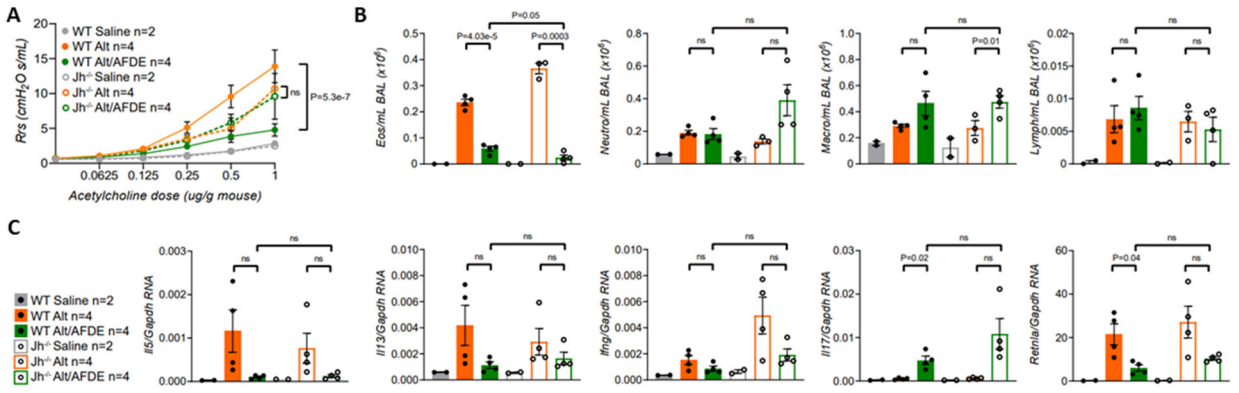

Fig. 3.2. In experiment #2, AFDE amplify BAL neutrophilia and fail to suppress AHR in Alternaria-treated mice lacking B cells. A: Invasive lung function measurements. Symbols denote mean and bars denote SEM. Statistical significance was assessed by repeated measures mixed models. **B:** Total BAL cellularity with differentials. **C:** Cytokine and *Retnla* mRNA levels in lungs relative to *Gapdh* expression. Symbols denote individual mice and bars denote mean ± SEM. Unpaired, two-tailed t-tests were used for statistical significance.

3.3.3 – Experiment #3: AFDE protect in-house bred Jh$^{-/-}$ mice against Alternaria-induced experimental asthma

In the Vercelli lab, experiments of interest are performed at least twice, ideally by different researchers. Therefore, in order to confirm the results of experiment #2, a third experiment was performed by one of my colleagues following the treatment protocol used before (Fig. 3.1A). However, because of cost considerations, we used wild-type male and female mice from Charles River Laboratories as controls, and Jh$^{-/-}$ mice that had been originally ordered from Taconic Biosciences and were bred and maintained in our mouse facility (n=1-3 mice/treatment group).

Invasive lung function measurements showed significant suppression of *Alternaria*-induced AHR in *both* wild-type- (P=1.7e-6) and Jh$^{-/-}$ mice (P=7.0e-10, Fig. 2.15A). In both wild-type- and Jh$^{-/-}$ mice, airway eosinophilia was almost completely abrogated by AFDE treatment compared to mice given *Alternaria*, and airway neutrophilia was similarly induced by AFDE (Fig. 2.15B). The expression of *Il5* and *Il13* was reduced in *Alt*/AFDE-treated mice with no significant difference between mouse strains, and *Il17* expression was increased by AFDE treatment equally in wild-type- and Jh$^{-/-}$ mice. *Ifng* expression was significantly increased in *Alt*/AFDE Jh$^{-/-}$ mice compared to *Alt*/AFDE wild-type-mice. Thus, the results of experiment #3 did not confirm those of experiment #2.

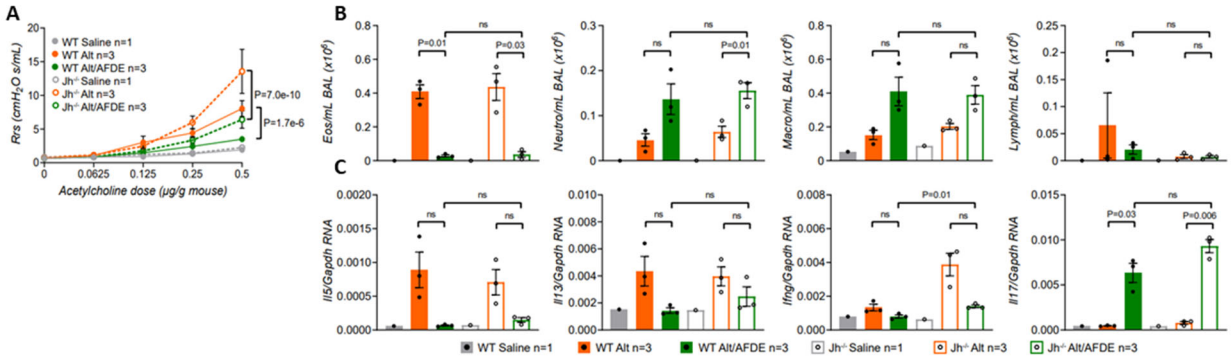

Fig. 3.3. In the third experiment, AFDE protect in-house bred Jh$^{-/-}$ mice against *Alternaria*-induced experimental asthma. A: Invasive lung function measurements. Symbols denote mean and bars denote SEM. Statistical significance was assessed by repeated measures mixed models. **B:** Total BAL cellularity with differentials. **C:** Cytokine mRNA levels in lungs relative to Gapdh expression. Symbols denote individual mice and bars denote mean ± SEM. Unpaired, two-tailed t-tests were used for statistical significance.

3.3.4 – Jh$^{-/-}$ mice bred in-house are B cell deficient

To confirm that the Jh$^{-/-}$ mice bred and housed in our mouse facility maintained the mature B cell-deficient phenotype, I performed flow cytometry targeting CD19^{+}B220^{+} lymphocytes (gating strategy shown in Fig. 3.4A) among the lung cells from the mice assayed in section 3.2.3. In the wild-type-mice, AFDE significantly increased the number of B cells relative to those induced by *Alternaria*, and the Jh$^{-/-}$ mice lacked any B cells regardless of treatment (Fig. 3.4B-C). Therefore, discrepancies among my experiments could not be explained by issues with mouse B cell phenotype.

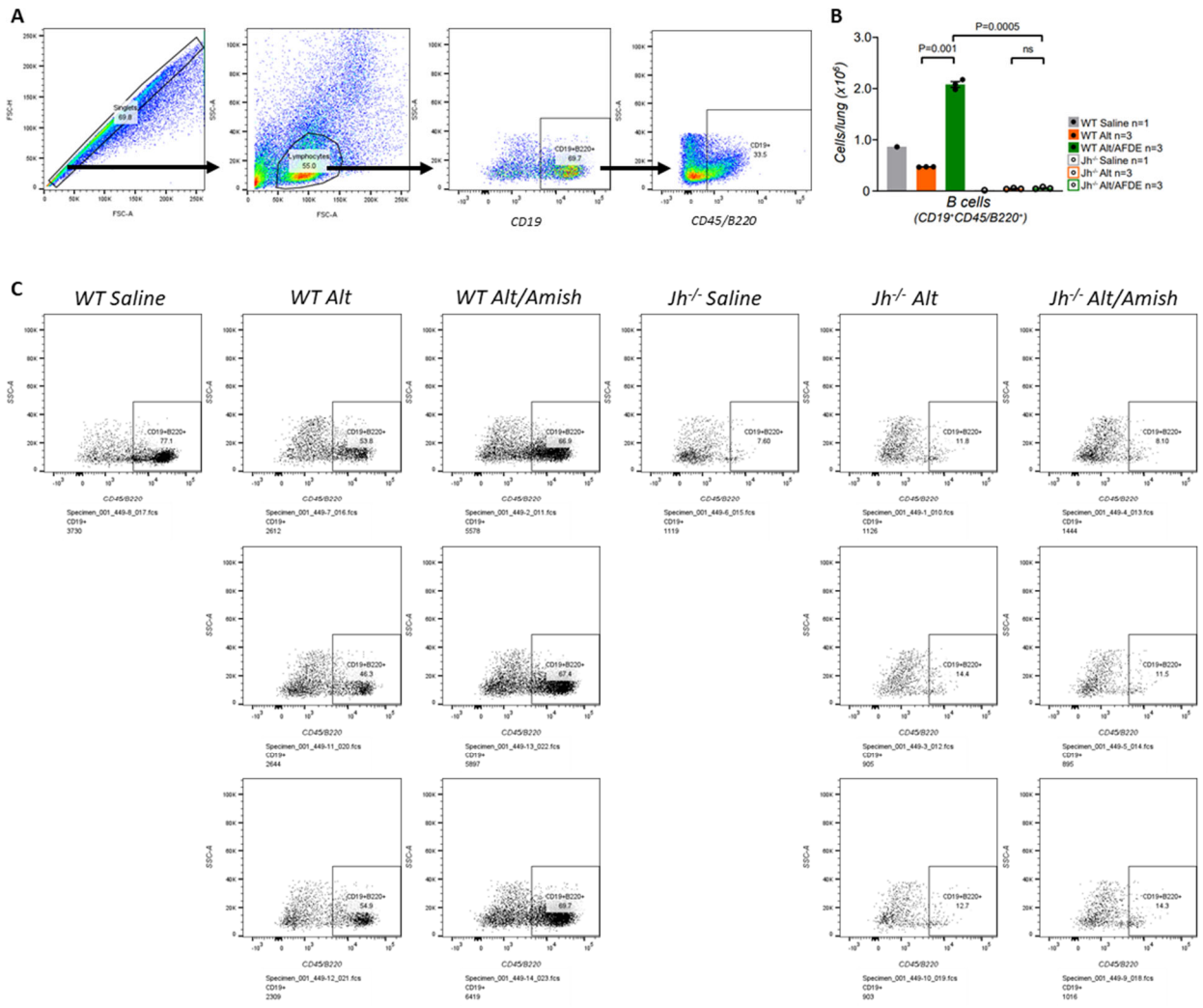

Fig. 3.4. Jh$^{-/-}$ mice bred in house remain B cell deficient. Lung cells were isolated at day 20 from wildtype and Jh$^{-/-}$ BALB/c mice treated with saline, *Alternaria*, or *Alt*/AFDE, as in Fig. 2.1A. **A:** Flow cytometry gating strategy for CD19^{+}CD45/B220^{+} cells. Cell mixtures from *Alt*/AFDE-treated mice were used as controls. **B:** From the total lung cells, B cells (CD19^{+}CD45/B220^{+}) were analyzed by flow cytometry and represented as total cell numbers per lung. Data are from 1-3 mice per group from 1 experiment. Symbols denote individual mice and bars denote mean ± SEM. Statistical significance could not be calculated provided the number of mice. **C:** Dot plots depicting all CD19^{+} cells, and within the gate, cells that are also CD45/B220^{+} (x-axis). Each panel represents a single mouse, dots represent individual cells, and values within the gate denote percent of CD19^{+} cells that are also CD45/B220^{+}.

3.4 – DISCUSSION

My characterization of the immune response to the asthma-protective microbial agents, AFDE and OM-85 (Chapter 2) identified a robust upregulation of genes related to B cell activation and an expansion of B cells in the lungs. Following up on this robust B cell signal, the experiments presented in this chapter illustrate the work performed to determine whether B cells are required for the asthma-protective effects induced by AFDE.

Across the three experiments conducted using Jh-/- mice, the results were variable and remain, so far, difficult to interpret. In the first experiment, the wild-type-mice did not exhibit the expected response to *Alternaria*, possibly because of suboptimal intranasal administration. As a result, the data from the $Jh^{-/-}$ mice in this experiment were inconclusive. In the second experiment, the wild-type-mice responded appropriately, even though high variability was detected in the $Jh^{-/-}$ mice treated with Alt/AFDE. A third experiment was performed by a different operator using $Jh^{-/-}$ mice bred and housed in our mouse facility and wild-type controls from a different company. Flow cytometry confirmed these $Jh^{-/-}$ mice bred in-house remain B cell-deficient. While the second experiment suggested B cells might be required for the asthma-protective effects of AFDE, the third experiment did not support this hypothesis: both wild-type and $Jh^{-/-}$ mice were protected from *Alternaria*-induced airway inflammation following AFDE treatment. However, different housing conditions and microbial exposures of the mice prior to treatment with AFDE add an additional variable that is difficult to assess at this time.

These findings are preliminary, and further experiments are necessary to explore the role of B cells in the asthma protective effects of AFDE. Future studies should also test the response to OM-85 in this context.

CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

The work presented in this dissertation provides a comparison of the cellular and transcriptional changes in the lung underlying the asthma-protective effects of exposure to the microbe-rich AFDE and the bacterial lysate, OM-85. Intranasal administration of AFDE or OM-85 in the previously published *Alternaria*-driven experimental asthma mouse model [69] suppressed airway inflammation as measured by airway resistance, BAL cellularity, and expression of type-2 cytokines. AFDE, but not OM-85, also significantly induced airway neutrophilia, an increase in the number of γδT cells in the lungs, and an increase in the expression of *Il17* in this treatment protocol.

The asthma protection conferred by both microbial agents is observable by similar phenotypic responses, which led to the question of whether these effects were the result of similar modifications to the transcriptome. Perhaps not surprisingly, the DE analyses performed on gene expression profiled by bulk RNA-seq revealed that the majority of DE genes are modified by both AFDE and OM-85, in the same direction with the same magnitude of effect, regardless of *Alternaria* exposure.

The DE analysis comparing AFDE vs. Saline and OM-85 vs. Saline identified many immunoglobulin heavy chain and class switch recombination genes strongly upregulated by both microbial agents, including *Ighg2b, Ighg2c Igha, Ighe*, *Jchain,* and *Aicda* [92]. Additionally, several immunoglobulin variable region genes were upregulated by both microbial agents. The variable regions of an antibody are responsible for determining which antigens the antibody will be able to bind/recognize. Any given antibody has a single, highly specific target, however it has been described that there can be over 5,000 different antibody clones that recognize a single

antigen [143]. Though we do not know which antigens these variable region genes are specific for, their upregulation in both AFDE vs. Saline and OM-85 vs. Saline implies some shared specificity in the polyclonal effector B cell responses to these microbial agents. While the standardized bacterial lysate and the dust collected to create AFDE are likely comprised of different microbes that each elicit a unique antibody response – as indicated by the treatment-unique upregulation of immunoglobulin variable region genes – there are likely common antigens present among the microbes and microbial products in AFDE and OM-85 responsible for the shared immunoglobulin variable region genes.

Interestingly, when comparing the effects of AFDE and OM-85 in the context of *Alternaria*, the same immunoglobulin heavy chain and class switch recombination genes were strongly upregulated by both microbial agents, as well as many of the immunoglobulin variable region genes, indicating a robust B cell activation signal. Uniquely shared in the *Alt*/AFDE vs. *Alt* and *Alt*/OM-85 vs. *Alt* comparison was a downregulation of several genes related to type-2 inflammation, providing insight as to which pathways induced by *Alternaria* are key to suppress for asthma protection.

In view of the strong B cell activation signal, I sought to characterize the immune cell landscape of the lung by flow cytometry. Unfortunately, lung cells were only collected from 2 mice per group treated with the microbial agents, limiting the conclusions that could be drawn from the results. Therefore, lung cells from additional mice should be characterized in future studies. With these limitations in mind, flow cytometry suggested that both AFDE and OM-85 increased the number of B cells in the context of *Alternaria*. Protein concentration levels support the results of the bulk RNA-seq, as AFDE and OM-85 increased production of total IgA in BAL and IgG in BAL and serum, regardless of *Alternaria* exposure.

snRNA-seq showed that just 5 treatments with either AFDE or OM-85 significantly altered the cellular landscape of the lung and the transcriptional profiles of those lung cells. Considering the robust polyclonal B cell activation signal identified by bulk RNA-seq, the expansion of B cells in the lung tissue, and the notable increase in B cells early in the response to AFDE and OM-85, my investigation of the snRNA-seq data focused on the B cell population. Re-clustering the B cells established 4 sub-clusters of B cells induced early in the response to AFDE and OM-85. Marker genes for each cluster suggested that clustering reflected different stages of B cell maturation. The marker genes for Clusters 0 and 1 included *Satb1, Ighd, Bach2, Poxp1*, and *Fcer2a*, which are related to early B cell development, survival, and maturation [124-128]. Cluster 2 marker genes, such as *Mtss1* [129], *Siglecg* [130], and the ubiquitin ligase genes *Klhl14* and *Cb1b* [131, 132] are related to regulation of the BCR specificity. Cluster 3 marker genes included immunoglobulin light chain genes (*Iglc1, Iglc2,* and *Iglc3*), genes related to the different effector functions of B cells (*Fcrl5, Mir142hg, Cd79a,* and *Cd37*) [133-136] and their differentiation to plasma cells (*Ms4a1*/*Cd20*) [137]. However, the expression of these marker genes is not specific to a single sub-cluster. In fact, most of the genes were expressed across all 4 sub-clusters, suggesting that the cells of each cluster likely represent a mix of naïve, regulatory, and mature B cells, rather than simply different stages of B cell development. It is possible, then, that the clusters are distinguished by a subtle change in expression of, for example, immunoglobulin variable regions and the specific antibody response of that B cell sub-population. A differential expression analysis comparing the changes in expression between AFDE vs. Saline and OM-85 vs. Saline may shed some light on these subtle differences, though changes in expression will be influenced by the total number of cells expressing a given gene.

A major limitation of this comparison is that most of the measurements and analyses were performed to characterize the immune response to AFDE or OM-85 in an allergic asthma model, following treatments, sensitizations, and challenges. However, the snRNA-seq analysis was performed on mice treated only with AFDE or OM-85 before *Alternaria* would be administered because an attempt to perform snRNA-seq on mice treated according to the protocol outline in Fig. 1A resulted in uninterpretable data from the mice given *Alt*/OM-85. It is possible that the failure of this assay relates to the process of isolating nuclei rather than leaving the cells intact. During allergic inflammation, granulocytes like eosinophils and neutrophils are recruited to the lungs in high numbers. Studies published since our attempt at snRNA-seq have found that the lysis of eosinophils and neutrophils not only makes them difficult to profile, but also reduces the quality of all other nuclei assayed [144, 145].

To profile eosinophils and neutrophils, which play major roles in allergic inflammation, and to maintain the quality of the results from all the other cell types of the lung, I optimized the protocol. In data not shown, leaving cells intact for single cell RNA-seq (scRNA-seq) using 10X Genomics' Chromium platform greatly improved the data quality from all cells while allowing for the characterization of neutrophils. However, even with the use of this optimized protocol eosinophils were not captured. A final pilot experiment to troubleshoot the capture of eosinophils was designed based on methods for capturing eosinophils in single cell analyses published by Gurtner et al. [146]. Whereas the Chromium controller makes use of microfluidic pumps to isolate individual nuclei or cells for profiling, the BD Bioscience's Rhapsody platform uses gravity to settle cells into individual microwells of a cartridge. Once again, I saw improved data quality from the lung cells over that from the snRNA-seq analysis and was able to capture neutrophils. However, despite the gentler handling of the fragile eosinophils, no expression of

eosinophil marker genes was detected. A complete comparison of the biological mechanisms underlying the asthma protective effect of AFDE and OM-85 will require profiling transcriptional alterations in the context of an allergen. Though this assay has been improved, there is still room for improvement in the capture of eosinophils as they are critical in the pathways of type-2 inflammation.

Each of the studies discussed in Chapter 2 identified a robust B cell activation signal induced by both AFDE and OM-85. Based on these findings, I chose to further investigate the role of B cells in the asthma protective effects of AFDE and OM-85, specifically whether B cells are required for protection or are immune bystanders activated as a consequence of microbial exposure in the lung.

As of now, three experiments have been conducted using *Alternaria* extracts to induce experimental asthma in $Jh^{-/-}$ mice that lack all mature B cells. The initial experiment used wild-type- and $Jh^{-/-}$ BALB/c mice ordered from Taconic Biosciences. Unfortunately, the wild-type-mice did not elicit a strong response to *Alternaria*, likely due to inadequate intranasal administration, making the other results indeterminate. An exact repeat of the first experiment showed that, compared to wildtype-mice, *Alternaria*-treated $Jh^{-/-}$ mice exposed to AFDE exhibited comparable suppression of BAL eosinophilia, amplification of BAL neutrophilia paralleled by a robust increase in lung *Il17* expression, and no significant inhibition of AHR. This led to the hypothesis that in the absence of B cells, allergen-dependent eosinophilic airway inflammation shifts towards a more severe eosinophilic/neutrophilic inflammatory response.

How does the absence of B cells influence neutrophils and γδ T cells, as well as the expression of *Il17* in the lungs? Regulatory B cells are a subpopulation of B cells that maintain immune tolerance, limit the response of other immune cells, and re-establish homeostasis after

the response is resolved [147]. Although there is no definitive set of biomarkers for regulatory B cells, they suppress immune response primarily through the secretion of IL-10, which has often been used to identify them experimentally [148]. To evaluate the role of regulatory B cells in the activity of AFDE, I checked for *Il10* expression in our qPCR, bulk RNA-seq, and snRNA-seq analyses. However, *Il10* expression did not change with ADFE administration in any of these assays, suggesting that regulatory B cells are not part of the AFDE-induced B cell expansion and probably do not play a role in the protective effects of AFDE (data not shown).

In their 2018 paper, Chen et al. [142] describe a helminth-induced emphysema model using BALB/c $Jh^{-/-}$ mice also from Taconic Biosciences, which saw a marked increase in IL-$17A^{+}$ γδ T cells and neutrophils in the lungs of mice 7 days after inoculation with *Nippostrongylus brasiliensis*. The authors found that the protein Resistin-like molecule α (RELMα), produced by B cells in response to type-2 inflammation, inhibits IL-17-producing γδ T cells and downregulates IL-17 driven neutrophilic inflammation. They reported that the gene that codes for RELMα, *Retnla*, was significantly downregulated in the $Jh^{-/-}$ mice relative to wildtype-mice. In my model, the differential expression analysis on the bulk RNA-seq data showed that, relative to saline-treated mice, *Alternaria* exposure increased *Retnla* expression over 8-fold (P-value = 7.02e-29). Treatment with AFDE, with or without *Alternaria* exposure, did not change the expression of *Retnla* in the bulk RNA-seq data or early in the response as captured by snRNA-seq. Based on these data I hypothesized that AFDE suppresses the type-2 eosinophilic airway inflammation while simultaneously inducing a type-3 inflammatory response, specifically an increase in IL-17 producing γδT cells. Furthermore, I hypothesized that *Retnla* produced by B cells in response to *Alternaria* inhibits this AFDE-induced increase in γδ T cells to a degree, allowing for the suppression of the type-2 inflammatory response without a

shift into IL-17 -mediated inflammation. However, in the absence of *Retnla* producing B cells, the AFDE-induced γδT cells could produce IL-17 unchecked. In the AFDE-treated $Jh^{-/-}$ mice, this would manifest with an increase in *Il17* expression that recruits neutrophils [149] and enhances contractility of smooth muscle cells [150] causing AHR despite the suppression of eosinophilia and *Il5* and *Il13* expression. To test this hypothesis, I checked the *Alt*/AFDE-treated $Jh^{-/-}$ mice for a decrease in *Retnla* expression by qPCR, however there was no difference in *Retnla* expression between the $Jh^{-/-}$ and wild-type-mice.

The results from the Alt/AFDE $Jh^{-/-}$ mice in experiment #2 showed high variability between mice, prompting another experiment performed by another member of the lab. Previously, the wild-type-and $Jh^{-/-}$ mice ordered from Taconic would arrive and acclimate in our mouse facility for one week prior to treatment with AFDE. In this case, wild-type-controls were ordered from Charles River Laboratory and acclimated for the usual one week, however, the $Jh^{-/-}$ mice were bred and housed in our mouse facility for their entire lives (8-9 weeks) before treatments began. In this repeat experiment, the original phenotype– a lack of asthma protection conferred by AFDE in the absence of B cells – was not observed. In both $Jh^{-/-}$ and wild-type-mice, BAL eosinophilia and expression of *Il5* and *Il13* was suppressed by treatment with AFDE. There was no significant difference in the AFDE-induced neutrophilia or *Il17* expression.

Flow cytometry confirmed that the $Jh^{-/-}$ mice bred in-house were still B cell deficient. One explanation for the differences between these mice could be related to the environments the mice were housed in. Published studies done with various strains of B cell deficient mice have found that a lack of B cells, and specifically the loss of mucosal IgA, can cause microbial dysbiosis possibly due to the fact that the small intestine is home to more than 80% of all plasma cells, most of which produce secretory IgA at the mucosal surfaces [151]. Other published

research has shown that in mice lacking activation-induced cytidine deaminase ($AID^{-/-}$), an essential enzyme for B cell class switch recombination and somatic hypermutation, gut bacteria expand aberrantly and elicit a drastic accumulation of IgM plasma cells that enlarge the lymphoid tissues [152-154]. It was reported that in B10.A mice with disrupted expression of the Ig heavy chain μ (μMT) and $Jh^{-/-}$ BALB/c mice, the absence of IgA did not alter the total amounts of bacterial DNA in the gut. However, serum lipopolysaccharide (LPS) concentrations were higher than in wild-type control mice, suggesting the bacterial composition is modified – likely due to a redirection of epithelial cell function for the upregulation of IFN-dependent innate immune genes [155]. Since $Jh^{-/-}$ mice are immunocompromised it is plausible that the $Jh^{-/-}$ mice bred and housed in our mouse facility had different exposure to microbes and their products prior to treatment with AFDE, resulting in the varied responses relative to mice obtained directly from Taconic.

Additional experiments using the B cell deficient $Jh^{-/-}$ mice are currently planned to continue investigating whether B cells are required for the asthma protective effects of AFDE, as well as OM-85.

REFERENCES

1. GA, N., *The Global Asthma Report, Auckland, New Zealand.* 2018.
2. Beasley, R., et al., *Prevalence and etiology of asthma.* J Allergy Clin Immunol, 2000. **105**(2 Pt 2): p. S466-72.
3. Bach, J.F., *The effect of infections on susceptibility to autoimmune and allergic diseases.* N Engl J Med, 2002. **347**(12): p. 911-20.
4. Martinez, F.D. and D. Vercelli, *Asthma.* Lancet, 2013. **382**(9901): p. 1360-72.
5. Hammad, H. and B.N. Lambrecht, *The basic immunology of asthma.* Cell, 2021. **184**(6): p. 1469-1485.
6. Holtzman, M.J., *Asthma as a chronic disease of the innate and adaptive immune systems responding to viruses and allergens.* (1558-8238 (Electronic)).
7. Holgate, S.T., *Innate and adaptive immune responses in asthma.* Nat Med, 2012. **18**(5): p. 673-83.
8. Kuruvilla, M.E., F.E. Lee, and G.B. Lee, *Understanding Asthma Phenotypes, Endotypes, and Mechanisms of Disease.* Clin Rev Allergy Immunol, 2019. **56**(2): p. 219-233.
9. Woodruff, P.G., et al., *T-helper type 2-driven inflammation defines major subphenotypes of asthma.* Am J Respir Crit Care Med, 2009. **180**(5): p. 388-95.
10. Douwes, J., et al., *Non-eosinophilic asthma: importance and possible mechanisms.* Thorax, 2002. **57**(7): p. 643-8.
11. Holgate, S.T., *Innate and adaptive immune responses in asthma.* (1546-170X (Electronic)).
12. Roan, F., K. Obata-Ninomiya, and S.F. Ziegler, *Epithelial cell-derived cytokines: more than just signaling the alarm.* J Clin Invest, 2019. **129**(4): p. 1441-1451.
13. Cayrol, C. and J.P. Girard, *Interleukin-33 (IL-33): A nuclear cytokine from the IL-1 family.* Immunol Rev, 2018. **281**(1): p. 154-168.
14. Peng, B., et al., *Role of IL-25 on Eosinophils in the Initiation of Th2 Responses in Allergic Asthma.* Frontiers in Immunology, 2022. **Volume 13 - 2022**.
15. West, E.E., M. Kashyap, and W.J. Leonard, *TSLP: A Key Regulator of Asthma Pathogenesis.* Drug Discov Today Dis Mech, 2012. **9**(3-4).
16. Borowczyk, J., et al., *IL-25 (IL-17E) in epithelial immunology and pathophysiology.* J Allergy Clin Immunol, 2021. **148**(1): p. 40-52.
17. Annunziato, F., C. Romagnani, and S. Romagnani, *The 3 major types of innate and adaptive cell-mediated effector immunity.* J Allergy Clin Immunol, 2015. **135**(3): p. 626-35.
18. Pelaia, C., et al., *Interleukin-5 in the Pathophysiology of Severe Asthma.* Front Physiol, 2019. **10**: p. 1514.
19. Nussbaum, J.C., et al., *Type 2 innate lymphoid cells control eosinophil homeostasis.* Nature, 2013. **502**(7470): p. 245-8.
20. Hussain, M. and G. Liu, *Eosinophilic Asthma: Pathophysiology and Therapeutic Horizons.* Cells, 2024. **13**(5).
21. Fulkerson, P.C. and M.E. Rothenberg, *Targeting eosinophils in allergy, inflammation and beyond.* Nature Reviews Drug Discovery, 2013. **12**(2): p. 117-129.
22. Whitsett, J.A. and T. Alenghat, *Respiratory epithelial cells orchestrate pulmonary innate immunity.* Nature Immunology, 2015. **16**(1): p. 27-35.
23. Seibold, M.A., *Interleukin-13 Stimulation Reveals the Cellular and Functional Plasticity of the Airway Epithelium.* Ann Am Thorac Soc, 2018. **15**(Suppl 2): p. S98-s102.
24. Steinke, J.W. and L. Borish, *Th2 cytokines and asthma. Interleukin-4: its role in the pathogenesis of asthma, and targeting it for asthma treatment with interleukin-4 receptor antagonists.* Respir Res, 2001. **2**(2): p. 66-70.

25. Kanda, A., et al., *Multiple Biological Aspects of Eosinophils in Host Defense, Eosinophil-Associated Diseases, Immunoregulation, and Homeostasis: Is Their Role Beneficial, Detrimental, Regulator, or Bystander?* Biol Pharm Bull, 2020. **43**(1): p. 20-30.
26. Cho, J.Y., et al., *Inhibition of airway remodeling in IL-5-deficient mice.* J Clin Invest, 2004. **113**(4): p. 551-60.
27. Rochman, Y., et al., *TSLP signaling in CD4(+) T cells programs a pathogenic T helper 2 cell state.* Sci Signal, 2018. **11**(521).
28. Ballesteros-Tato, A., et al., *T Follicular Helper Cell Plasticity Shapes Pathogenic T Helper 2 Cell-Mediated Immunity to Inhaled House Dust Mite.* Immunity, 2016. **44**(2): p. 259-73.
29. Junttila, I.S., *Tuning the Cytokine Responses: An Update on Interleukin (IL)-4 and IL-13 Receptor Complexes.* Front Immunol, 2018. **9**: p. 888.
30. Keegan, A.D., W.J. Leonard, and J. Zhu, *Recent advances in understanding the role of IL-4 signaling.* Fac Rev, 2021. **10**: p. 71.
31. Oettgen, H.C. and R.S. Geha, *IgE regulation and roles in asthma pathogenesis.* J Allergy Clin Immunol, 2001. **107**(3): p. 429-40.
32. Elieh Ali Komi, D. and L. Bjermer, *Mast Cell-Mediated Orchestration of the Immune Responses in Human Allergic Asthma: Current Insights.* Clin Rev Allergy Immunol, 2019. **56**(2): p. 234-247.
33. Jatakanon, A., et al., *Neutrophilic inflammation in severe persistent asthma.* Am J Respir Crit Care Med, 1999. **160**(5 Pt 1): p. 1532-9.
34. Ray, A., et al., *Current concepts of severe asthma.* J Clin Invest, 2016. **126**(7): p. 2394-403.
35. Pividori, M., et al., *Shared and distinct genetic risk factors for childhood-onset and adult-onset asthma: genome-wide and transcriptome-wide studies.* Lancet Respir Med, 2019. **7**(6): p. 509-522.
36. Han, Y., et al., *Genome-wide analysis highlights contribution of immune system pathways to the genetic architecture of asthma.* Nat Commun, 2020. **11**(1): p. 1776.
37. Ober, C., *Asthma Genetics in the Post-GWAS Era.* Ann Am Thorac Soc, 2016. **13 Suppl 1**(Suppl 1): p. S85-90.
38. Strachan, D.P., *Hay fever, hygiene, and household size.* Bmj, 1989. **299**(6710): p. 1259-60.
39. Rook, G.A.W., *The old friends hypothesis: evolution, immunoregulation and essential microbial inputs.* Front Allergy, 2023. **4**: p. 1220481.
40. von Hertzen, L., I. Hanski, and T. Haahtela, *Natural immunity. Biodiversity loss and inflammatory diseases are two global megatrends that might be related.* EMBO Rep, 2011. **12**(11): p. 1089-93.
41. Raedler, D. and B. Schaub, *Immune mechanisms and development of childhood asthma.* Lancet Respir Med, 2014. **2**(8): p. 647-56.
42. Ober, C. and T.-C. Yao, *The genetics of asthma and allergic disease: a 21st century perspective.* Immunological Reviews, 2011. **242**(1): p. 10-30.
43. Riedler, J., et al., *Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey.* The Lancet, 2001. **358**(9288): p. 1129-1133.
44. Von Ehrenstein, O.S., et al., *Reduced risk of hay fever and asthma among children of farmers.* (0954-7894 (Print)).
45. Braun-Fahrländer, C., et al., *Environmental exposure to endotoxin and its relation to asthma in school-age children.* N Engl J Med, 2002. **347**(12): p. 869-77.
46. Ege, M.J., et al., *Not all farming environments protect against the development of asthma and wheeze in children.* 2007(0091-6749 (Print)).
47. Ege, M.J., et al., *Exposure to environmental microorganisms and childhood asthma.* N Engl J Med, 2011. **364**(8): p. 701-9.
48. Ege, M.J., et al., *Gene-environment interaction for childhood asthma and exposure to farming in Central Europe.* J Allergy Clin Immunol, 2011. **127**(1): p. 138-44, 144.e1-4.

49. von Mutius, E. and D. Vercelli, *Farm living: effects on childhood asthma and allergy.* Nat Rev Immunol, 2010. **10**(12): p. 861-8.
50. Wlasiuk, G. and D. Vercelli, *The farm effect, or: when, what and how a farming environment protects from asthma and allergic disease.* Curr Opin Allergy Clin Immunol, 2012. **12**(5): p. 461-6.
51. Stein, M.M., et al., *Innate Immunity and Asthma Risk in Amish and Hutterite Farm Children.* New England Journal of Medicine, 2016. **375**(5): p. 411-421.
52. Gozdz, J., C. Ober, and D. Vercelli, *Innate Immunity and Asthma Risk.* N Engl J Med, 2016. **375**(19): p. 1898-1899.
53. Marques dos Santos, M., et al., *Asthma-protective agents in dust from traditional farm environments.* Journal of Allergy and Clinical Immunology, 2023. **152**(3): p. 610-621.
54. Cazzola, M., S. Anapurapu, and C.P. Page, *Polyvalent mechanical bacterial lysate for the prevention of recurrent respiratory infections: a meta-analysis.* Pulm Pharmacol Ther, 2012. **25**(1): p. 62-8.
55. Esposito, S., et al., *Nonspecific immunomodulators for recurrent respiratory tract infections, wheezing and asthma in children: a systematic review of mechanistic and clinical evidence.* Curr Opin Allergy Clin Immunol, 2018. **18**(3): p. 198-209.
56. Hancock, R.E., A. Nijnik, and D.J. Philpott, *Modulating immunity as a therapy for bacterial infections.* Nat Rev Microbiol, 2012. **10**(4): p. 243-54.
57. Suárez, N., et al., *Bacterial Lysates as Immunotherapies for Respiratory Infections: Methods of Preparation.* Front Bioeng Biotechnol, 2020. **8**: p. 545.
58. Pasquali, C., et al., *Enhanced Mucosal Antibody Production and Protection against Respiratory Infections Following an Orally Administered Bacterial Extract.* Front Med (Lausanne), 2014. **1**: p. 41.
59. BAUER, J., Alain, et al., *BACTERIAL EXTRACT FOR RESPIRATORY DISORDERS AND PROCES FOR ITS PREPARATION*. 2008.
60. Schaad, U.B., R. Mütterlein, and H. Goffin, *Immunostimulation with OM-85 in Children with Recurrent Infections of the Upper Respiratory Tract: A Double-Blind, Placebo-Controlled Multicenter Study.* Chest, 2002. **122**(6): p. 2042-2049.
61. Zhang, W., et al., *Whether Immunostimulants Are Effective in Susceptible Children Suffering From Recurrent Respiratory Tract Infections: A Modeling Analysis Based on Literature Aggregate Data.* J Clin Pharmacol, 2022. **62**(2): p. 245-253.
62. Yin, J., et al., *Broncho-Vaxom in pediatric recurrent respiratory tract infections: A systematic review and meta-analysis.* Int Immunopharmacol, 2018. **54**: p. 198-209.
63. Jara-Pérez, J.V. and A. Berber, *Primary prevention of acute respiratory tract infections in children using a bacterial immunostimulant: a double-masked, placebo-controlled clinical trial.* Clin Ther, 2000. **22**(6): p. 748-59.
64. Gutiérrez-Tarango, M.D. and A. Berber, *Safety and efficacy of two courses of OM-85 BV in the prevention of respiratory tract infections in children during 12 months.* Chest, 2001. **119**(6): p. 1742-8.
65. Koatz, A.M., et al., *Clinical and Immunological Benefits of OM-85 Bacterial Lysate in Patients with Allergic Rhinitis, Asthma, and COPD and Recurrent Respiratory Infections.* Lung, 2016. **194**(4): p. 687-97.
66. Zelle-Rieser, C., et al., *A clinically approved oral vaccine against pneumotropic bacteria induces the terminal maturation of CD83+ immunostimulatory dendritic cells.* Immunol Lett, 2001. **76**(1): p. 63-7.
67. Huber, M., H. Mossmann, and W.G. Bessler, *Th1-orientated immunological properties of the bacterial extract OM-85-BV.* Eur J Med Res, 2005. **10**(5): p. 209-17.

68. Castro-Rodriguez, J.A., K.N. Turi, and E. Forno, *A critical analysis of the effect of OM-85 for the prevention of recurrent respiratory tract infections or wheezing/asthma from systematic reviews with meta-analysis.* Pediatr Allergy Immunol, 2024. **35**(7): p. e14186.
69. Pivniouk, V., et al., *Airway administration of OM-85, a bacterial lysate, blocks experimental asthma by targeting dendritic cells and the epithelium/IL-33/ILC2 axis.* Journal of Allergy and Clinical Immunology, 2022. **149**(3): p. 943-956.
70. Corazza, N. and T. Kaufmann, *Novel insights into mechanisms of food allergy and allergic airway inflammation using experimental mouse models.* Allergy, 2012. **67**(12): p. 1483-90.
71. Downs, S.H., et al., *Clinical importance of Alternaria exposure in children.* Am J Respir Crit Care Med, 2001. **164**(3): p. 455-9.
72. Neukirch, C., et al., *Is sensitization to Alternaria alternata a risk factor for severe asthma? A population-based study.* Journal of Allergy and Clinical Immunology, 1999. **103**(4): p. 709-711.
73. Salo, P.M., et al., *Exposure to Alternaria alternata in US homes is associated with asthma symptoms.* J Allergy Clin Immunol, 2006. **118**(4): p. 892-8.
74. Aden, E., et al., *Standardization of Alternaria alternata: extraction and quantification of alt a 1 by using an mAb-based 2-site binding assay.* J Allergy Clin Immunol, 1999. **104**(1): p. 128-35.
75. Van Hoecke, L., et al., *Bronchoalveolar Lavage of Murine Lungs to Analyze Inflammatory Cell Infiltration.* J Vis Exp, 2017(123).
76. Dobin, A., et al., *STAR: ultrafast universal RNA-seq aligner.* Bioinformatics, 2013. **29**(1): p. 15-21.
77. Heinz, S., et al., *Simple Combinations of Lineage-Determining Transcription Factors Prime cis-Regulatory Elements Required for Macrophage and B Cell Identities.* Molecular Cell, 2010. **38**(4): p. 576-589.
78. Love, M.I., W. Huber, and S. Anders, *Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2.* Genome Biology, 2014. **15**(12): p. 550.
79. Risso, D., et al., *Normalization of RNA-seq data using factor analysis of control genes or samples.* Nature Biotechnology, 2014. **32**(9): p. 896-902.
80. Kassambara A, M.F., *factoextra: Extract and Visualize the Results of Multivariate Data Analyses*. 2020.
81. Zhang, H., et al., *txci-ATAC-seq: a massive-scale single-cell technique to profile chromatin accessibility.* Genome Biol, 2024. **25**(1): p. 78.
82. Hao, Y., et al., *Dictionary learning for integrative, multimodal and scalable single-cell analysis.* Nature Biotechnology, 2024. **42**(2): p. 293-304.
83. Wolock, S.L., R. Lopez, and A.M. Klein, *Scrublet: Computational Identification of Cell Doublets in Single-Cell Transcriptomic Data.* Cell Systems, 2019. **8**(4): p. 281-291.e9.
84. Korsunsky, I., et al., *Fast, sensitive and accurate integration of single-cell data with Harmony.* Nature Methods, 2019. **16**(12): p. 1289-1296.
85. Koenitzer, J.R., et al., *Single-Nucleus RNA-Sequencing Profiling of Mouse Lung. Reduced Dissociation Bias and Improved Rare Cell-Type Detection Compared with Single-Cell RNA Sequencing.* Am J Respir Cell Mol Biol, 2020. **63**(6): p. 739-747.
86. Heng, T.S. and M.W. Painter, *The Immunological Genome Project: networks of gene expression in immune cells.* Nat Immunol, 2008. **9**(10): p. 1091-4.
87. Amezquita, R.A., et al., *Orchestrating single-cell analysis with Bioconductor.* Nature Methods, 2020. **17**(2): p. 137-145.
88. McCarthy, D.J., et al., *Scater: pre-processing, quality control, normalization and visualization of single-cell RNA-seq data in R.* Bioinformatics, 2017. **33**(8): p. 1179-1186.
89. Chen, Y., et al., *edgeR v4: powerful differential analysis of sequencing data with expanded functionality and improved support for small counts and larger datasets.* Nucleic Acids Res, 2025. **53**(2).

90. Kuznetsova, A., P.B. Brockhoff, and R.H.B. Christensen, *lmerTest Package: Tests in Linear Mixed Effects Models.* Journal of Statistical Software, 2017. **82**(13): p. 1 - 26.
91. Wickham, H., *ggplot2: Elegant Graphics for Data Analysis*. 2016: Springer-Verlag New York.
92. Schroeder, H.W., Jr. and L. Cavacini, *Structure and function of immunoglobulins.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S41-52.
93. Richter, K., et al., *Speckled-like pattern in the germinal center (SLIP-GC), a nuclear GTPase expressed in activation-induced deaminase-expressing lymphomas and germinal center B cells.* J Biol Chem, 2009. **284**(44): p. 30652-61.
94. Xie, Z., E.C. Chan, and K.M. Druey, *R4 Regulator of G Protein Signaling (RGS) Proteins in Inflammation and Immunity.* Aaps j, 2016. **18**(2): p. 294-304.
95. Shi, G.X., et al., *RGS13 regulates germinal center B lymphocytes responsiveness to CXC chemokine ligand (CXCL)12 and CXCL13.* J Immunol, 2002. **169**(5): p. 2507-15.
96. Chesné, J., et al., *IL-17 in severe asthma. Where do we stand?* Am J Respir Crit Care Med, 2014. **190**(10): p. 1094-101.
97. Powlesland, A.S., et al., *Widely divergent biochemical properties of the complete set of mouse DC-SIGN-related proteins.* J Biol Chem, 2006. **281**(29): p. 20440-9.
98. Ortiz, M., et al., *The evolutionary history of the CD209 (DC-SIGN) family in humans and non-human primates.* Genes Immun, 2008. **9**(6): p. 483-92.
99. Bishop, B. and C.M. Lloyd, *CC chemokine ligand 1 promotes recruitment of eosinophils but not Th2 cells during the development of allergic airways disease.* J Immunol, 2003. **170**(9): p. 4810-7.
100. Medoff, B.D., et al., *IFN-gamma-inducible protein 10 (CXCL10) contributes to airway hyperreactivity and airway inflammation in a mouse model of asthma.* J Immunol, 2002. **168**(10): p. 5278-86.
101. Hardy, R.R., *B-1 B cell development.* J Immunol, 2006. **177**(5): p. 2749-54.
102. Trinchieri, G., *Interleukin-12 and the regulation of innate resistance and adaptive immunity.* Nat Rev Immunol, 2003. **3**(2): p. 133-46.
103. Kraal, G., et al., *The macrophage receptor MARCO.* Microbes Infect, 2000. **2**(3): p. 313-6.
104. Panova, V., et al., *Group-2 innate lymphoid cell-dependent regulation of tissue neutrophil migration by alternatively activated macrophage-secreted Ear11.* Mucosal Immunol, 2021. **14**(1): p. 26-37.
105. Boon, M., et al., *MCIDAS mutations result in a mucociliary clearance disorder with reduced generation of multiple motile cilia.* Nat Commun, 2014. **5**: p. 4418.
106. Hu, Y., et al., *γδ T cells: origin and fate, subsets, diseases and immunotherapy.* Signal Transduct Target Ther, 2023. **8**(1): p. 434.
107. Etzerodt, A. and S.K. Moestrup, *CD163 and inflammation: biological, diagnostic, and therapeutic aspects.* Antioxid Redox Signal, 2013. **18**(17): p. 2352-63.
108. Pine, G.M., H.M. Batugedara, and M.G. Nair, *Here, there and everywhere: Resistin-like molecules in infection, inflammation, and metabolic disorders.* Cytokine, 2018. **110**: p. 442-451.
109. Webb, D.C., A.N. McKenzie, and P.S. Foster, *Expression of the Ym2 lectin-binding protein is dependent on interleukin (IL)-4 and IL-13 signal transduction: identification of a novel allergy-associated protein.* J Biol Chem, 2001. **276**(45): p. 41969-76.
110. Defnet, A.E., et al., *Dysregulated retinoic acid signaling in airway smooth muscle cells in asthma.* Faseb j, 2021. **35**(12): p. e22016.
111. Jiang, M., et al., *VEGF receptor 2 (KDR) protects airways from mucus metaplasia through a Sox9-dependent pathway.* Dev Cell, 2021. **56**(11): p. 1646-1660.e5.
112. Lebrun, I., et al., *Effects of 'casoparan', a peptide isolated from casein hydrolysates with mastoparan-like properties.* Mediators Inflamm, 2004. **13**(4): p. 263-8.

113. Li, H., et al., *Fc receptor-like 4 and 5 define human atypical memory B cells.* Int Immunol, 2020. **32**(12): p. 755-770.
114. Suan, D., et al., *CCR6 Defines Memory B Cell Precursors in Mouse and Human Germinal Centers, Revealing Light-Zone Location and Predominant Low Antigen Affinity.* Immunity, 2017. **47**(6): p. 1142-1153.e4.
115. Coughlin, J.J., et al., *RasGRP1 and RasGRP3 regulate B cell proliferation by facilitating B cell receptor-Ras signaling.* J Immunol, 2005. **175**(11): p. 7179-84.
116. Krzyzak, L., et al., *CD83 Modulates B Cell Activation and Germinal Center Responses.* J Immunol, 2016. **196**(9): p. 3581-94.
117. Kauppi, P., et al., *The IL9R region contribution in asthma is supported by genetic association in an isolated population.* Eur J Hum Genet, 2000. **8**(10): p. 788-92.
118. Chen, J.Y., C. Cortes, and V.P. Ferreira, *Properdin: A multifaceted molecule involved in inflammation and diseases.* Mol Immunol, 2018. **102**: p. 58-72.
119. Karaky, M., et al., *SP140 regulates the expression of immune-related genes associated with multiple sclerosis and other autoimmune diseases by NF-κB inhibition.* Hum Mol Genet, 2018. **27**(23): p. 4012-4023.
120. Gerrick, K.Y., et al., *Transcriptional profiling identifies novel regulators of macrophage polarization.* PLoS One, 2018. **13**(12): p. e0208602.
121. Irons, E.E., P.R. Punch, and J.T.Y. Lau, *Blood-Borne ST6GAL1 Regulates Immunoglobulin Production in B Cells.* Front Immunol, 2020. **11**: p. 617.
122. An, F.Q., et al., *KLF4 and CD55 expression and function depend on each other.* Front Immunol, 2023. **14**: p. 1290684.
123. Maharana, S., et al., *SAMHD1 controls innate immunity by regulating condensation of immunogenic self RNA.* Mol Cell, 2022. **82**(19): p. 3712-3728.e10.
124. Ozawa, T., et al., *Special AT-Rich Sequence-Binding Protein 1 Supports Survival and Maturation of Naive B Cells Stimulated by B Cell Receptors.* J Immunol, 2022. **208**(8): p. 1937-1946.
125. Dirks, J., et al., *IgD shapes the pre-immune naïve B cell compartment in humans.* Front Immunol, 2023. **14**: p. 1096019.
126. Muto, A., et al., *Identification of Bach2 as a B-cell-specific partner for small maf proteins that negatively regulate the immunoglobulin heavy chain gene 3' enhancer.* Embo j, 1998. **17**(19): p. 5734-43.
127. Patzelt, T., et al., *Foxp1 controls mature B cell survival and the development of follicular and B-1 B cells.* Proc Natl Acad Sci U S A, 2018. **115**(12): p. 3120-3125.
128. Aranda, C.J., et al., *IgG memory B cells expressing IL4R and FCER2 are associated with atopic diseases.* Allergy, 2023. **78**(3): p. 752-766.
129. Sarapulov, A.V., et al., *Missing-in-Metastasis/Metastasis Suppressor 1 Regulates B Cell Receptor Signaling, B Cell Metabolic Potential, and T Cell-Independent Immune Responses.* Front Immunol, 2020. **11**: p. 599.
130. Hoffmann, A., et al., *Siglec-G is a B1 cell-inhibitory receptor that controls expansion and calcium signaling of the B1 cell population.* Nat Immunol, 2007. **8**(7): p. 695-704.
131. Choi, J., et al., *Regulation of B cell receptor-dependent NF-κB signaling by the tumor suppressor KLHL14.* Proc Natl Acad Sci U S A, 2020. **117**(11): p. 6092-6102.
132. Liu, Q., et al., *E3 ubiquitin ligase Cbl-b in innate and adaptive immunity.* Cell Cycle, 2014. **13**(12): p. 1875-84.
133. Damdinsuren, B., et al., *B cell receptor induced Fc receptor-like 5 expression is mediated by multiple signaling pathways converging on NF-κB and NFAT.* Mol Immunol, 2016. **73**: p. 112-21.
134. Zheng, B., et al., *The Function of MicroRNAs in B-Cell Development, Lymphoma, and Their Potential in Clinical Practice.* Front Immunol, 2018. **9**: p. 936.

135. Huse, K., et al., *Mechanism of CD79A and CD79B Support for IgM+ B Cell Fitness through B Cell Receptor Surface Expression.* J Immunol, 2022. **209**(10): p. 2042-2053.
136. Barrena, S., et al., *Aberrant expression of tetraspanin molecules in B-cell chronic lymphoproliferative disorders and its correlation with normal B-cell maturation.* Leukemia, 2005. **19**(8): p. 1376-83.
137. Pavlasova, G. and M. Mraz, *The regulation and function of CD20: an "enigma" of B-cell biology and targeted therapy.* Haematologica, 2020. **105**(6): p. 1494-1506.
138. Hoehn, K.B., et al., *The Diversity and Molecular Evolution of B-Cell Receptors during Infection.* Mol Biol Evol, 2016. **33**(5): p. 1147-57.
139. Kitamura, D., et al., *A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene.* (0028-0836 (Print)).
140. Chen, J., et al., *Immunoglobulin gene rearrangement in B cell deficient mice generated by targeted deletion of the JH locus.* Int Immunol, 1993. **5**(6): p. 647-56.
141. Drake, L.Y., et al., *B cells play key roles in th2-type airway immune responses in mice exposed to natural airborne allergens.* PLoS One, 2015. **10**(3): p. e0121660.
142. Chen, F., et al., *B Cells Produce the Tissue-Protective Protein RELMα during Helminth Infection, which Inhibits IL-17 Expression and Limits Emphysema.* Cell Reports, 2018. **25**(10): p. 2775-2783.e3.
143. McConnell, S.A. and A. Casadevall, *New insights into antibody structure with implications for specificity, variable region restriction and isotype choice.* Nat Rev Immunol, 2025.
144. Swanson, E., et al., *Simultaneous trimodal single-cell measurement of transcripts, epitopes, and chromatin accessibility using TEA-seq.* Elife, 2021. **10**.
145. Colino-Sanguino, Y., et al., *Performance comparison of high throughput single-cell RNA-Seq platforms in complex tissues.* Heliyon, 2024. **10**(17): p. e37185.
146. Gurtner, A., et al., *Active eosinophils regulate host defence and immune responses in colitis.* Nature, 2023. **615**(7950): p. 151-157.
147. Catalán, D., et al., *Immunosuppressive Mechanisms of Regulatory B Cells.* Front Immunol, 2021. **12**: p. 611795.
148. Das, A., et al., *IL-10-producing regulatory B cells in the pathogenesis of chronic hepatitis B virus infection.* J Immunol, 2012. **189**(8): p. 3925-35.
149. León, B. and F.E. Lund, *IL-17-producing B cells combat parasites.* Nature Immunology, 2013. **14**(5): p. 419-421.
150. Kudo, M., et al., *IL-17A produced by αβ T cells drives airway hyper-responsiveness in mice and enhances mouse and human airway smooth muscle contraction.* Nature Medicine, 2012. **18**(4): p. 547-554.
151. van der Heijden, P.J., W. Stok, and A.T. Bianchi, *Contribution of immunoglobulin-secreting cells in the murine small intestine to the total 'background' immunoglobulin production.* Immunology, 1987. **62**(4): p. 551-5.
152. Fagarasan, S., et al., *Critical roles of activation-induced cytidine deaminase in the homeostasis of gut flora.* Science, 2002. **298**(5597): p. 1424-7.
153. Suzuki, K., et al., *Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut.* Proc Natl Acad Sci U S A, 2004. **101**(7): p. 1981-6.
154. Wei, M., et al., *Mice carrying a knock-in mutation of Aicda resulting in a defect in somatic hypermutation have impaired gut homeostasis and compromised mucosal defense.* Nat Immunol, 2011. **12**(3): p. 264-70.
155. Shulzhenko, N., et al., *Crosstalk between B lymphocytes, microbiota and the intestinal epithelium governs immunity versus metabolism in the gut.* Nat Med, 2011. **17**(12): p. 1585-93.