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Combating Health Inequities in Pulmonary Inflammation Through Diet and Repair Proteins

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by

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1. Milk Consumption and Respiratory Function in Asthma Patients: NHANES Analysis 2007-2012, March 2021, Nutrients. The co- author Tara M. Nordgren listed in that publication directed and supervised the research which forms the basis for this dissertation. Rachel Bookman assisted in data organization and analysis. Jihyun Ma, Elizabeth Lyden and Corrine Hanson provided technical expertise.

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DEDICATION

I dedicate this work to my family; parents, siblings, grandparents, aunts and uncles, who have nurtured me with laughter, care, and love. To my parents: You have allowed this strong-willed child to grow into a strong-willed adult who fights to carve spaces for all in the world. Thank you for withstanding the growing pains and giving me a landing place if ever I would need. To my five siblings: there is no truer meaning of mutual and unconditional love than we share, and I am lucky to be the eldest of such incredible humans.

This work is dedicated to those that have come before me; women who have made this journey possible for me. I hope that those after me may soar further and higher than I ever will.

I dedicate this work to former professors and coaches who have shown me that there are few limits to my own passion and ability to persevere.

It is my hope that I can make the world a better place for even just a handful of people along my journey, and I dedicate my future to that end.

ABSTRACT OF THE DISSERTATION

Combating Health Inequities in Pulmonary Inflammation Through Diet and Repair Proteins

by

Stefanie Noel Sveiven

Doctor of Philosophy, Graduate Program in Biomedical Sciences
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Dr. Meera G. Nair, Chairperson

The pulmonary system is essential for survival by providing oxygen through its delicate tissue architecture. It is critical for the pulmonary system to maintain tissue homeostasis of a few-cell-layers-thick structure in the lung for efficient clearance of harmful inhaled particulates and effective oxygen delivery. Importantly, deficiency of lung repair following damage can lead to chronic diseases including asthma and chronic obstructive pulmonary disease (COPD). COPD is among the top 10 global killers annually per the World Health Organization, resulting from lung damage by chemicals, pathogens, or unresolved inflammation. Using a model of each of these, this work aims to identify mechanisms of lung repair to help resolve the burden of disease. Since there is currently no therapeutics to promote repair of the lung tissue, this work aims to elucidate the potential for diet and repair proteins to facilitate lung repair and alleviate the global burden of lung disease hallmarked by irreversible damage.

Exposure to agricultural hog barn dust drives neutrophilic and infection with soil-transmitted helminth skews towards eosinophilic lung inflammation, leading to lasting lung damage. These are representative of models of afflictions impacting billions in marginalized communities who may work in agriculture and/or be exposed to helminth infections.

Tackling lung tissue repair using diet and potential therapeutic targets (FFAR4 and RELMα) may prevent the development of chronic lung disease including COPD and improve the quality of life of billions. This work first addresses the capacity for dairy consumption to improve lung function and protect from asthma in those who have and have not been previously diagnosed with asthma. Asthma shares some similarities with COPD as an obstructive disease that impairs airflow and oxygen exchange. Diet provides a tool for individuals to be empowered with their own health, but the limitations are such that these marginalized communities may also have limited access to quality foods. Thus, a second angle of therapeutically targetable proteins is presented: FFAR4 and RELMα can facilitate inflammation resolution and tissue repair. Together, this work highlights a multi-pronged approach as a steppingstone towards the prevention of lung disease and facilitating the repair of lung tissue.

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CHAPTER ONE:
Introduction to pulmonary system and immunity; inequities in lung disease and research models; and paths toward therapeutics

1.1 The Pulmonary System: where epithelial structure meets function in respiration

The pulmonary system is the essential system in allowing the exchange of gases between the internal and external milieu, providing oxygen for cellular processes while ridding the body of waste in the form of carbon dioxide and other gases. The lungs are housed within the chest under the role of the chest wall, pleura, and diaphragm which coordinate pressure gradients between the lung and atmosphere (Figure 1.1). These critical pressure gradients are necessary for the passive flow of air into and out of the lungs during homeostatic breathing. The control of the chest wall and the diaphragm is modulated by the phrenic nerve comprised of cranial nerves 3, 4, 5, which allows respiration to be controlled by neural inputs to meet the body's gas exchange needs. Air is exchanged through cycles of inhalation and exhalation several times per minute. In this process, the lung frequently interacts with immunogenic agents inhaled in each breath, and as such, the lung requires a delicate balance of innate immune responses and resolution of these responses to maintain homeostasis.

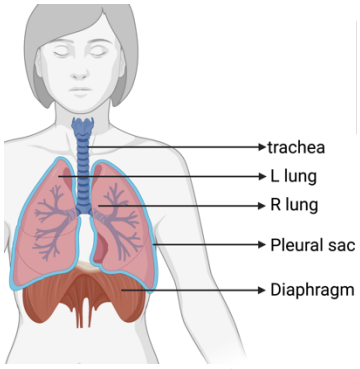


Figure 1.1. Diagram of respiratory system. Created with "biorender.com"

The structure of lung epithelial populations is region- and function-specific, consisting of airway and alveolar compartments (Figure 1.2). Inhaled air is first moved from the mouth through the trachea where it is then transferred to the left and right main bronchi en route to the left and right lungs. Within each lung, bronchi branch into smaller bronchi to conduct air to the three left lung lobes and two right lung lobes. Within each of the lobes, these bronchi branch into small bronchioles moving air deeper into the lungs. This region consists of what is considered 'airway

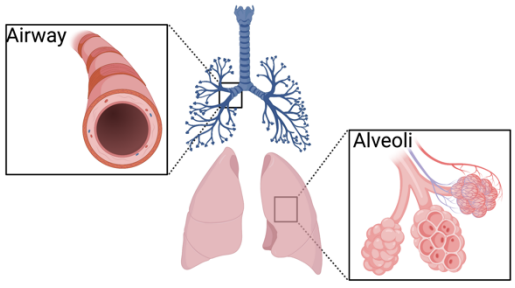


Figure 1.2 Airway and alveolar compartments. Created with "biorender.com"

epithelium' with the primary role of humidifying and filtering inhaled air (done efficiently under homeostasis with 99% of most particulate filtered).

There are a few rarer cell types present such as tuft, ionocyte, and pulmonary neuroendocrine cells in the airway epithelium, though it predominately consists of 4 main cell types: basal, club, goblet, and ciliated pseudostratified columnar (Figure 1.3.). Basal cells in the lung are the tissue stem cells that give rise to the cell types present in the epithelium and represent ~20% of the epithelial cells on average. The airway epithelium has a projected turnover rate of 30-50 days, which is largely facilitated by the basal cell population[1]. A second multipotent stem cell of the airway is the club cell which can replenish goblet and columnar cell populations. Club cells are identifiable by secretaglobin- (CC10) expression as they are a major producer of secretions important to the protective airway surface liquid (ASL) layer covering the lung epithelium. Goblet cells are also important secretory cells in the airway producing mucus that contributes to the airway mucus layer of the ASL, which traps inhaled particulates for air filtration. Once particulates are trapped in the mucus layer, the cilia on the columnar cells beat to move the mucus layer upward, known as the 'mucus escalator,' where it is ultimately coughed out of the airway (expelled or swallowed). The combination of these cells is critical to the role of the airway compartment in filtering immunogenic particulates from the air before the air is transferred to the more physically fragile alveolar compartment for gas exchange.

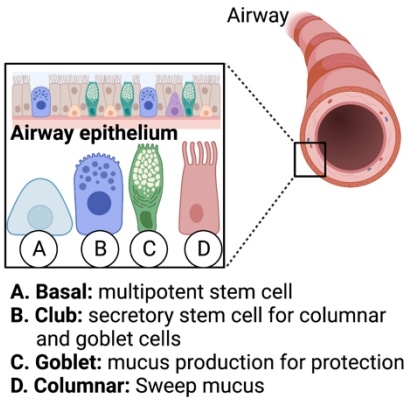
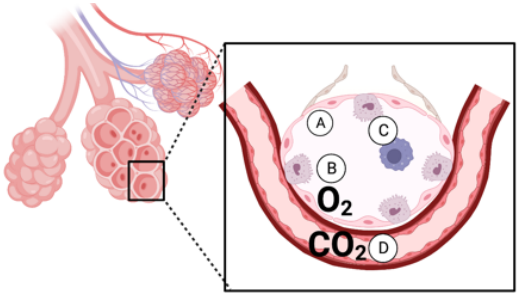


Figure 1.3. Airway epithelial cells types. Created with "biorender.com"

From the smaller bronchioles air is conducted into special structures called alveoli, or airways sacs, that are broken into smaller compartments by septa to increase surface area. The alveolar compartment is where gas exchange occurs, and the structure of the alveolar epithelium

lining this region reflects that function. The alveolar epithelium consists predominantly of alveolar type I (ATI) and type II (ATII) pneumocytes, each cell type with a specific cell shape and function (Figure 1.4.). The ATI cells are squamous which belies their main role in facilitating the rapid exchange of gases between the air in the airway and the blood within the alveolar-affiliated capillaries. Here the oxygen passes through a single ATI cell, a vascular endothelial cell at the capillary, then it can bind heme within the red blood cell.



A. ATI: Site of gas exchange
B. ATII: Stem cell ATI/ATII and secretes surfactant
C. Alveolar macrophage: clears dead cells, role in immunity
D. Capillary: Site of gas exchange

Figure 1.4. Cell types of the alveoli. Created with "biorender.com"

The ATI cells do not divide, and thus the ATII cells are essential in replenishing these ATI cells as well as in self-renewal. The ATII cells are cuboidal in shape, thus secretory cells, and they are critical producers of surfactant which is secreted to counter the force of collapse in the alveoli. There is a resident macrophage population in the alveolar compartment called alveolar macrophages (AMac). These tissue-resident macrophages are less inflammatory to prevent over-activation and function mostly to clear dead cells and debris from the airway under homeostasis. They are recognizable by their high surface expression of the integrin CD11c and lectin SiglecF. These alveolar sacs are surrounded by a dense capillary network that brings deoxygenated blood from the right ventricle. Here the deoxygenated blood exchanges carbon dioxide (CO_2) for oxygen (O_2) with the air within the alveolar space following the pressure gradient. The oxygenated blood is then returned to the left side of the heart to be pumped to the rest of the body completing respiration.

1.2 Pulmonary Immunity: Balancing frequent inhaled immune exposures and homeostasis

The immune system is essential in both mounting a robust response to pathogens to rid the body of non-self-threats and in dampening this damaging response in favor of resolution (Figure 1.5.). The response to pathogens starts with the correct identification of the pathogen, and recruitment of appropriate immune cells for the targeted inflammatory response. When the pathogen has been cleared, the immune response switch from pro-inflammatory to pro-resolution in favor of tissue repair. Finally, repair mechanisms must also be dampened to favor homeostasis as unresolved inflammation and excessive repair both result in organ dysfunction.

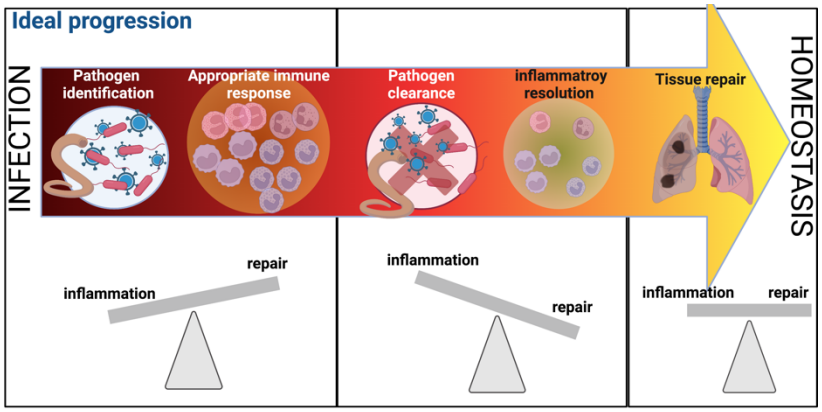


Figure 1.5. Graphic depicting the ideal progression of immune response to resolution following infection. Created with "biorender.com"

The pulmonary epithelium (PE) is the site of immunogenic interaction in every single breath as bacterial, fungal, viral, and inorganic particulate matter permeate the air. Thus, the PE functions in innate immunity as a physical barrier, keeping the internal and external environments divided, and through the expression of pattern recognition receptors (PRR) that identify threats and damage[2]. The epithelium lining the airway and alveolar compartments is selectively permeable, with permeability very tightly regulated. The cells are adjoined by tight junctions that coordinate the passage of some molecules when necessary but remain impermeable to most. Damage to epithelial cells may result in barrier failures due to epithelial cell death or tight junction alterations. For example, cytotoxic chemicals such as those in cigarette smoke can be inhaled, driving epithelial cell death, and resulting in aberrant permeability of harmful inhaled particulates and microbial pathogens. Barrier dysfunction at mucosal barriers, like the lung and

intestines, results in inflammation as the internal immune system responds to invasive pathogens and chemical damage. The inflammation persists until barrier function can be restored and pathogens cleared. Therefore, the maintenance of epithelial barrier integrity is critically important to lung and overall health. This is achieved through efficient re-epithelialization, by epithelial cell proliferation and tight junction formation, to stabilize the epithelial barrier.

Apart from forming a physical barrier, the epithelium participates in pathogen recognition which is an important function of the innate immune response. Innate immunity consists of evolutionarily conserved immune proteins which recognize conserved which do not require previous encounters with pathogens, but rather rely on patterns of pathogenic molecules and damage molecules. Pathogen-associated molecular patterns (PAMP) consist of conserved pathogenic motifs that can be recognized by PRRs. Examples of such include lipopolysaccharide (LPS), glycans, lipoteichoic acid (LTA), viral genetic material, etc which can bind toll-like receptors (like TLR4 in the case of LPS) and initiate innate immune signaling cascades. Damage-associated molecular patterns are molecules that present during cell stress and injury, such as phospholipid membrane changes, and can be recognized by epithelial PRRs like nod-like receptors (NLR). Lung epithelial cells purportedly express all TLRs, underscoring their critical role in the innate immune response beyond serving as a barrier.

Upon activation of lung epithelial PRRs, there is specific activation of pro-inflammatory, pro-resolution, or pro-repair genes depending upon the signaling pathways downstream of the activated PRR. Immunity at mucosal barriers like the lung is well coordinated between resident and peripherally recruited cell populations. In the lung resident populations, include the epithelium, tertiary lymphoid structures like bronchi-associated lymphoid tissue (BALT), as well as a unique population of tissue-resident macrophages called alveolar macrophages which are a self-renewing population of airway macrophages that derive embryonically from the fetal liver

and yolk sac [3]. They are known to be $Mertk^{+}CD11c^{+}SiglecF^{+}$ and have lower MHCII expression than other macrophages. Their primary role is phagocytosing dead cells and debris in the airway and they are less immune-responsive to balance with frequent airway exposures. During exposure to agricultural dust or helminth parasites, the release of cytokines and chemokines allows the epithelium and resident alveolar macrophages to liaise with the peripheral immune system to participate in the activation and recruitment of immune cells.

These communications then extend to myeloid cells such as monocyte-derived alveolar macrophages, neutrophils, and eosinophils, which can be recruited to the lung and airway. During dust exposure, there is a largely neutrophilic response initiated by the airway while helminth infections result in an eosinophil-skewed response. Typically, there is redundancy in the expression of many of these cytokines and chemokines, though some may be specific to epithelial cells or macrophages. Of note, there is a unique protein that is expressed by both epithelial cells and macrophages during helminth infection, called resistin-like molecule α (RELMα) which may play an important role in lung tissue repair. The pulmonary immune responses to dust exposure and helminth infections will be detailed in Chapter Three and Four respectively.

1.3 Agricultural dust exposure and soil-transmitted helminth infections represent health inequities causing lung inflammation and damage: a call for equity

The most up-to-date data from the World Health Organization (WHO) lists three lung-related illnesses among the top 10 list of global causes of death[4]. Per these data, the third highest cause of death globally is chronic obstructive pulmonary disease (COPD) causing the deaths of 3.23 million individuals in 2019. WHO additionally reports that 90% of deaths from COPD occur among those living in impoverished countries. COPD is preventable and treating the related symptoms can prolong life when medical care is accessible and affordable. This

underscores the devastating reality that COPD is a disease of inequity, afflicting and killing those most vulnerable.

The etiology of COPD is multi-factorial, and the risk factors include exposure to air pollution; smoke; work involving inhaled dusts, chemicals, and fumes; and also, previous respiratory infections during young life. Many studies have demonstrated that poor air quality disproportionately impacts low socioeconomic communities and certain racial groups across the globe, and as such it is considered a major health inequity [5, 6]. These inhaled exposures are often chronic, repetitive, immunogenic, and/or they can directly damage the pulmonary barrier contributing to lung diseases like COPD.

COPD consists of two overlaid and irreversible forms of lung damage. The first pathology occurs in the airway and is known as chronic bronchitis. When there is aberrant hyperplasia of goblet cells in the airway compartment, these cells overproduce mucus, and this excess of mucus in the airway leads to airway obstruction. This results in the development of a chronic cough and difficulty moving air efficiently into the lung in chronic bronchitis. In the development of COPD, chronic bronchitis is also overlaid with emphysema. Emphysema is caused by chronic exposure to inhaled particulates that drive the destruction of the alveolar septa. Septal destruction results in the loss of surface area needed for adequate gas exchange and decreased lung elasticity which is required for expelling air from the lungs. A decrease in surface area for gas exchange leads to decreased absorption of oxygen by the blood. Further exacerbating this, when excess air is trapped in the lung, less inhaled air carrying oxygen can fill the lungs. Thus, chronic bronchitis and emphysema together reduce the oxygen taken in by both the lungs and the blood causing hypoxemia and ultimately tissue hypoxia in individuals with COPD.

Agricultural dust exposure is one of the highest-risk occupational exposures and is associated with an increased risk of developing chronic bronchitis and COPD in individuals

exposed. These exposures impact those who work in this industry which is around 2 billion people globally. Other studies have clarified that even those living in close proximity to this industry, more like 3.4 billion people globally, are at increased risk as well due to the air pollution reaching for miles surrounding these farms. Among the types of agriculture, livestock farming is particularly high risk. These individuals can be exposed to aerosolized inorganic chemicals, like methane and ammonia gas, as well as organic particulates. These organic particulates are often immunogenic as they contain bacterial, viral, and fungal components that activate the pulmonary innate immune response. This complex, mixed inhaled exposure type, especially in the chronic and repetitive setting, drives the development of lung diseases like pneumonitis, bronchitis, asthma, and COPD. Despite this robust evidence in human studies, there has been little progress in the way of therapeutics.

Like the broad impact of agricultural exposures, soil-transmitted helminth (STH) infections occur in 1.5 billion people worldwide and disproportionately among impoverished communities[7]. STH infections are the most common infections worldwide and are endemic among rural communities with inadequate sanitary services. The life cycle of common STH infections, such as Ancylostoma duodenale and Strongyloides stercoralis, involves passage through the lung en route to the gastrointestinal tract. Eggs in contaminated soil mature into skin-penetrating larvae that enter the host upon contact of skin with soil. Larvae then enter the vasculature, traveling through the blood to the heart and lungs. Upon reaching the vessels surrounding the lung, the larvae use chemical and mechanical means for transepithelial migration to the airway compartment. This process results in epithelial damage and hemorrhaging acutely in the lung. The parasites are only transiently in the lungs as they are coughed up and swallowed for entry into the GI tract where they complete their life cycle and pass eggs through feces. These STH infections are considered a neglected tropical disease due to their prevalence among

communities that have limited access to clinical care, and as such the availability of clinical data identifying the long-term impacts of the pulmonary changes caused by these parasites is limited. A hallmark paper by Marsland et al., identified the development of emphysema in the lungs of mice infected one time with Nippostrongylus brasiliensis, a commonly used mouse infection model of human helminth infections[8].

With these exposures, there is a clear connection with chronic bronchitis, emphysema, and the development of COPD. When mapped, the increased prevalence of COPD, higher density of impoverished agricultural farmers, and increased prevalence of human helminth infections largely overlap. While the data available are limited, one can hypothesize these phenomena may be associated. Regardless, there is sufficient data to support the need for increased study of these marginalized communities to combat inequities in the burden of diseases like COPD.

1.4 Current therapeutic options for lung repair

The lung under homeostasis is highly regenerative, taking advantage of niche and progenitor cell populations that participate in regenerative healing and maintain the function of the lung. Loss of these healthy healing mechanisms leads to nonregenerative healing which impairs the function of the lungs and causes lung disease. There is currently no therapeutic way to repair, regenerate, or reverse damage to lung tissue repaired through nonregenerative mechanisms. The broad impact of global deaths by pulmonary diseases underscores the need for identifying mechanisms and targets with the goal of promoting lung repair. Available therapeutics aim to relieve symptoms while slowing, hopefully halting, worsening pathology to promote improved quality of life with the disease. Combination therapies are the typical approach to treating emphysema, chronic bronchitis, and COPD emphasizing the importance of health care access in remediating these diseases. Typically, aerosolized medications are given to dilate airways and improve airflow into the lungs while steroids may counter inflammation-driven

symptoms. Depending on the severity of the disease individuals may also need supplemental oxygen to improve oxygenation. Health outcomes are also improved by pulmonary rehabilitation to address modifiable parameters to promote individual well-being and overall lung function. Lastly, the most drastic treatment option is lung transplantation. Apart from the difficulty of finding donor matches for organ transplants, the 5-year survival rate post-lung-transplant is 60%, which is on the lower end of the survival spectrum among other solid-organ donations.

There is a need to identify mechanisms and therapeutics that function to protect the lung from damage, promote tissue resolution, and ultimately better facilitate lung repair. Cell-based therapies utilizing cell transplants and cell-based products are of current interest. One example is the use of mesenchymal stromal cells (MSC), which are self-renewing and multipotent residents of all tissue types surveyed. In general, bone marrow and umbilical cord MSC are the leading choices for clinical studies, as these cell types are more easily accessible to harvest for donation. However, there is data to suggest that tissue-resident MSC may be adequately primed for addressing the needs of the respective damaged tissue and that we should find ways to harness their repair potential from within the organ. Lung-resident mesenchymal stromal cells play diverse roles in lung diseases and represent an important putative cell-based therapy in the treatment of lung disease [9]. Other cell types that have been used in treating lung disease in pre-clinical and clinical trials include endothelial, T- cells.

Cellular products that have potential therapeutically include extracellular vesicles (EV), often derived from MSC cultures. The lack of immunogenic-surface markers improves the uptake of EV and the efficacy of their effects. Originally believed to be cellular waste, EV have since been shown to elicit their effects through content like protein, micro-RNA (miRNA), and even mitochondria. A challenge in EV therapies is that the surface markers are largely under-characterized, which affects the ability to provide specific doses of EV containing desired

content. Further, there is no current method for specifically targeting certain cell types for EV uptake. However, the field is expanding and technology for purifying, identifying, and utilizing EV therapies is improving exponentially.

An additional avenue for prevention and therapeutics is through diet. Omega 3-polyunsaturated fatty acids (ω-3 PUFA), often marketed as fish oil supplements, are lipids with well-characterized benefits in an array of diseases. The ω-3 PUFA family of fatty acids includes α-linoleic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). Diets higher in ω-3 PUFA are associated with better prognoses in cardiovascular disease, diabetes, cancer, Alzheimer's, dementia, depression, and even maternal-child pair health [10-15]. Using mouse studies, DHA protects the lung from inflammation and promotes epithelial repair.

Dietary modulation of disease presents the potential to alleviate the burden of disease, as discussed in Chapter Two, in medically under-served areas where individuals may have limited access to costly therapeutics. Further, preventing disease altogether is the most financially wise path forward for individuals as well as for medical facilities. However, pharmacological intervention can be a method of direct and targeted therapeutics if proteins of interest are known. Dietary factors, although powerful, present their own challenges of accessibility and equity. For this reason, we must acknowledge the need for a multifocal approach to resolving lung disease where diet and pharmaceuticals each play a critical role. The most widely druggable proteins are G-protein coupled receptors (GPCR) which account for nearly 40% of drug targets [16]. Thus, the identification of potential GPCR in the lung that promote repair is a current gap in knowledge that this dissertation aims to address, as these represent targetable transmembrane receptors with an array of diverse functions upon ligation. A GPCR with immunomodulatory and pro-repair potential in the lung is free fatty acid receptor 4 (FFAR4). This receptor is best characterized in its role in metabolism and energy homeostasis particularly as it is lipid-sensing and highly

expressed in the gastrointestinal tract [19, 20]. Here it functions to sense luminal dietary lipids. FFAR4 is also abundantly expressed in the lung however, its role here is less clear since this lipid-sensing function is less relevant in the pulmonary system. We hypothesize that this receptor has important immunomodulatory potential due to its putative role in epithelial homeostasis, which is investigated in Chapter Three [17-20].

Additionally, secreted proteins can serve as biomarkers for disease severity but their role in pathogenesis may be unclear. For example, the RELM family of proteins has been associated with worsened or improved prognoses in an array of human diseases [21, 22]. Of note, RELMα is an immunomodulatory protein that is secreted by myeloid cells and lung epithelial cells alike. This protein is upregulated during type 2 immune responses, such as those initiated by helminth infection, and is a hallmark for alternatively activated macrophages [23-25]. Thus, there is potential for this protein to facilitate tissue repair particularly regarding its expression by alternatively activated macrophages known to promote repair. Chapter Four addresses some potential tissue-reparative roles for RELMα in helminth-induced lung pathology. Clarifying the role of these proteins in the context of a particular disease may help elucidate whether they may be useful therapeutically, either by administering helpful proteins or by inhibiting harmful ones. Factors such as significant changes in a protein level during disease as well as high expression of the protein in the organ of interest are alluring among viable protein targets for pharmacological intervention in lung disease. The overarching goal of this research is to harness the potential of readily available protein receptors or signaling molecules that can be utilized in prevention, treatment, and cure of many diseases that affect the lung.

CHAPTER TWO:
Dairy consumption as a method of prevention and treatment asthma, and promoted overall lung health

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2.1 Abstract

Per the Centers for Disease Control and Prevention, asthma prevalence has steadily risen since the 1980s. Using data from the National Health and Nutrition Examination Survey (NHANES), we investigated associations between milk consumption and pulmonary function (PF). Multivariable analyses were performed, adjusted for a priori potential confounders for lung function, within the eligible total adult population (n = 11,131) and those self-reporting asthma (n = 1,542), included the following variables: milk-consumption, asthma diagnosis, forced vital capacity (FVC), FVC%-predicted (%), forced expiratory volume in one-second (FEV1), FEV1% and FEV1/FVC. Within the total population, FEV1% and FVC% were significantly associated with regular (5+ days weekly) consumption of exclusively 1% milk in the prior 30-days (β:1.81; 95% CI: [0.297, 3.325]; p = 0.020 and β:1.27; [0.16, 3.22]; p = 0.046). Among participants with asthma, varied-regular milk consumption in a lifetime was significantly associated with FVC (β:127.3; 95% CI: [13.1, 241.4]; p = 0.002) and FVC% (β:2.62; 95% CI: [0.44, 4.80]; p = 0.006). No association between milk consumption and FEV1/FVC was found, while milk-type had variable influence and significance. Taken together, we found certain milk consumption tendencies were associated with pulmonary function values among normal and asthmatic populations. These findings propound future investigations into the potential role of dairy consumption in altering lung function and asthma outcomes, with potential impact on the protection and maintenance of pulmonary health.

2.2 Rationale for a role for diet in lung disease

The global burden of pulmonary disease and chronic respiratory diseases (CRD) in particular, are indicated not only by the financial costs of billions of dollars but also in the cost of life by premature mortality of those diagnosed [1,2]. Additionally, as the devastating impacts of

COVID-19 have taken hold this past year, we will likely see increased burden of chronic respiratory disease secondary to irreversible pulmonary fibrosis, for years to come [3]. One such CRD, asthma, is often a challenge to control as environmental triggers are typically unavoidable. Furthermore, airway remodeling which may result from chronic, uncontrolled asthma, increases the risk of emphysema and chronic bronchitis. These pulmonary diseases additionally result in increased risk in developing either chronic obstructive pulmonary disease (COPD), another burdensome CRD that is among the top 5 leading causes of death globally, or asthma-COPD overlap syndrome (ACOS) [4,5]. It is anticipated that the US will spend 300 billion dollars on asthma over the next 20 years, with prevalence likely to keep rising as it has over the past decade [6]. While therapeutic agents for asthma are effective in managing symptoms for most individuals, and are dependent on access to long-term treatment both physically and financially [7–9]. As yet, asthma remains incurable, and individuals with moderate to severe asthma are reliant upon pharmaceutical intervention to preserve a semblance of normalcy until supportive means that are more accessible and/or a cure are identified [10,11].

Diet is an influential and modifiable factor in disease treatment and prevention which allows for autonomous modulation of disease progression and outcomes, to the extent that environmental factors influence disease. Data suggests that asthma can be exacerbated or remediated by dietary factors. A Western diet, as well as obesity, can contribute to a pro-inflammatory physiological state via excess high saturated-fat intake, and thus serve to exacerbate immune-related diseases like asthma [12–14]. Meanwhile, a diet rich in healthy fats, such as the Mediterranean diet, may provide important anti-inflammatory mediators and exert protective effects against inflammatory disease and asthma [15–17]. Dairy products contribute a major share of the food supply, but surprisingly little research has been conducted to evaluate the direct effects of milk consumption and fat content on health outcomes like inflammation. Dairy

consumption may be protective against asthma also has been associated with improvement in lung function and inflammatory biomarkers in the ECLIPSE cohort of individuals with COPD [18,19]. Low fat, but not high fat, dairy intake has also been associated with a decrease in emphysema, improved lung density measured by computed tomography (CT) images, and a potential inverse relationship between high-fat dairy and FEV1/FVC [20].

The National Center for Health Statistics (NCHS), a part of the Centers for Disease Control and Prevention (CDC), has conducted a nationwide snapshot of health and disease across the American population, known as the National Health and Nutrition Examination Surveys (NHANES), since the 1950s [21]. These surveys comprise a publicly available data set, accessible online, which aims to provide researchers with epidemiological data for analyses with a variety of data items such as interviews, laboratory tests, physical examinations and other reports. Among data in NHANES, nutritional assessments in which individuals complete validated assessments about their dietary intake, can be sorted in the context of both self-reported and diagnosed disease. Overall, the aim of this dataset is to associate the impacts of long-term exposures and nutritional patterns on health and disease in the United States.

Using the NHANES 2007–2012 waves, we analyzed milk consumption in participants including those with self-reported asthma to assess for associations between milk consumption and pulmonary function measures among these individuals.

2.3 Materials and Methods

2.3.1. Study Design

Information about the NHANES cross-sectional study design, as well as the methods of participant selection are publicly available through the CDC.gov website. These studies involved NHANES survey years with both lung function data and asthma questionnaires available. The

primary outcomes of this study were self-reported asthma prevalence and lung function measurements. Self-reported asthma was determined using the NHANES questionnaire variables MCQ010 "Ever been told you have asthma: yes/no" and MCQ035 "Still have asthma: yes/no." Throughout the manuscript, those reporting "yes" on MCQ010 are considered to have a history of asthma, while those reporting "yes" to MCQ035 are considered to have current asthma. In addition, NHANES reports SPXNFVC and SPXNFEV1 provided the data for analysis of lung function outcomes. The NCHS acquired these data during home health interviews and mobile examination centers (MEC) to obtain laboratory data. NHANES waves 2007–2012 were used since pre-bronchodilator spirometry was performed these years. The outcomes used to assess lung function included: forced expiratory volume in one second (FEV1), forced vital capacity (FVC), FEV1/FVC ratio, FEV1 percent predicted, and FVC percent predicted. Protocols for these measurements are also summarized within the NHANES resources. Lung function was expressed as a percent predicted based on age, gender, height, and race (white, black or Mexican, Hispanic, or other) from the third NHANES reference values [22].

2.3.2. Participants

Participants aged 19 to 79 with pre-bronchodilator spirometry data (FEV1 and FVC) quality grades A and B were used in the analysis. The NHANES Cohort for waves 2007– 2012 meeting these criteria totaled 30,442. After applying exclusion criteria—pregnant women, and those with energy intake greater than or less than the plausible intake (6000 kcal/day for adult females, 800–8000 kcals/day for adult males)—the remaining eligible participants totaled 11,180. Individuals lacking self-reported asthma status were removed from the dataset, making the final participants for the general population of the study equal 11,131. Of this population, those indicating a history of asthma and/or current asthma totaled 1, 542. These details and a flowchart

following STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) principles are provided in the graphical abstract. NHANES cycles between 2007–2012 were combined with multi-wave weight adjustments. Details for the NHANES study are described within the website reference [21].

2.3.3. Variables

Milk consumption in NHANES includes the following self-reported variables: any type of milk 5x weekly; milk products consumed <30 days; whole (full-fat) milk consumed <30 days; 2% (reduced fat) milk consumed <30 days; 1% (low fat) milk consumed <30 days; skim milk (fat free) consumed <30 days; whole milk only consumed <30 days; 2% milk only consumed <30 days, 1% milk only consumed <30 days, skim milk only consumed <30 days, 2+ types of milk consumed <30 days. The terms of our analyses relating to milk consumption in the NHANES population are defined in Table 3.1.1. Outcome variables assessed included pulmonary function values measured by spirometry (FEV1, FVC, FEV1 percent predicted [FEV1%], FVC percent predicted [FVC%], FEV1/FVC), milk consumption, and self-reported asthma (history of and/or current). Pulmonary function tests are diagnostic for lung diseases including COPD, emphysema, and asthma. The most clinically useful values are FEV1/FVC which may be indicative of obstructive lung disease when <0.70, a category to which some asthma patients may ascribe. However, this ratio is neither sensitive enough nor accurate enough for diagnosis [23]. FEV1% and FVC% values provide clinical information within the context of factors known to impact lung function (e.g., height, age, and gender).

Potential confounding variables were chosen a priori based on previous associations found in literature and included gender, race, education, smoking status, body mass index (BMI)

group, previous work exposure to mineral dusts, previous work exposure to natural dusts, and poverty index [24–29]. Cut-offs for poverty were defined by the NHANES survey variable "INDFMPIR," calculated as a ratio of individual/family income to poverty guidelines determined by the Department of Health and Human Services with a range from 0–5. Values from 0–1.35 were considered "poor", 1.36–1.85 were considered "nearly poor", and values of 1.86+ were considered "not poor" for classification. Mineral dust and natural dust exposure data were determined by the NHANES questionnaire as a response to variable OCQ510, "Ever had work exposure to mineral dusts?", and variable OCQ530, "Ever had work exposure to organic dusts?". Additionally, the use of the term 'gender' here as opposed to 'sex' is in accordance with the NHANES use of 'gender' to describe male or female respondents. The World Health Organization has standardized the following BMI categories which were used in this study: underweight: <18.5, normal range: 18.5–24.9, overweight: 25–29.9, and obese: ≥ 30. The Centers for Disease Control and NCHS define smoking status as follows: Never (has never smoked, or who has smoked less than 100 cigarettes in their lifetime), current smoker (has smoked 100 cigarettes in their lifetime and who currently smokes cigarettes), or former (has smoked at least 100 cigarettes in their lifetime but who had quit smoking at the time of interview).

Table 2.1. Definition of terms used in analyses.

Consumption Frequency Variables	**Definition of Variable**	**Response Categories**
Lifetime regular milk drinker (REGMLK)	Milk consumption, regardless of type, 5+ days per week for a lifetime	Lifetime: most of their life Varied: sometimes a regular milk drinker Never: never a regular milk drinker
Dichotomized lifetime regular milk drinker (REGMLKR)	Milk consumption, regardless of type, 5+ days per week for a lifetime dichotomized as Lifetime/Varied or Never	Yes (Lifetime) No (Varied/Never)
Any type milk consumption within prior 30 days (MLKCONS30)	Frequency of milk consumption, regardless of type, in the 30 days prior to survey date	Daily: 1+ times daily Sometimes: 1+ times a week but not daily Rarely: less than 1 time per week Never: no milk consumption
Dichotomized any type milk consumption within prior 30 days (MLKCONS30R)	Frequency of milk consumption, regardless of type, in the 30 days prior to survey date, dichotomized as 5+ times a week or Sometimes/Rarely/Never	Yes (5+ times a week or more) No (Sometimes/Rarely/ Never)
Type of Milk Consumption Variables	**Definition of Variable**	**Response Categories**
Exclusive milk consumed by type 5+ days per week in prior 30 days (TYPE 30)	Whole (full fat) only, 2% (reduced fat) only, 1% (low fat) only, skim (fat free) only, other type of milk (non-dairy, such as soy) only, 5+ days per week in last 30 days	Yes No

Table 2.1. Variables used throughout the study are separated into two categories (bolded): consumption frequency and consumption type. Each variable, left justified in the first column, is followed by a definition, and then reported with associated response categories.

2.3.4. Statistical Methods

Descriptive statistics included counts and percentages for categorical data and means for continuous data to describe the study population from the NHANES cohort for the years 2007–2012 (Table 3.1.2). Chi-square test for categorical variables and ANOVA test for continuous variables were applied to examine the characteristics of the demographic, socioeconomic, and risk factors related to pulmonary function with participants' current and past asthma. To evaluate associations between milk consumption and pulmonary function and current asthma status, multivariable linear and binomial logistic regression analyses were conducted with other

demographic, socio-economic, and risk factor variables. Diagnostics and fit statistics for the regression analyses were reviewed to assess the validity of our multivariable models. The overall diagnostics were satisfied with regression assumptions and fit statistics significant or acceptable, as appropriate (Wald chi-square <0.0001 for all logistic regressions; diagnostic plots showed no evidence against normality, linearity and equal variances for linear regressions, respectively). A two-sided significance cutoff was set to $p < 0.05$. SAS version 9.4 was used, specifically SAS procedures, "PROC SURVEYFREQ", "PROC SURVEYMEANS", "PROC SURVEYLOGISTIC" and PROC SURVEYREG" were used in computing descriptive and regression analyses as these protocols account for both the weighted data as well as the complexity of sample design.

Table 2.2. Characteristics of NHANES participants and univariable analyses for self-reported asthma status

Characteristic	ALL (n=11,131) % Mean [95% CI]	No History of Asthma (n=9589) % Mean [95% CI]	Yes, Have a History of Asthma (n=717) % Mean [95% CI]	Yes, Still Have Asthma (n=825) % Mean [95% CI]	p-values[1]
Age (year)	44.4 [43.6, 45.2]	44.8 [43.9, 45.6]	40.2 [38.8 41.6]	44.0 [42.6, 45.4]	< 0.001
Gender					< 0.001
Female	51.0% [49.9, 52.0]	49.6% [48.5, 50.8]	53.4% [49.4, 57.3]	64.2% [59.8, 68.6]	< 0.001
Male	49.0% [48.0, 50.1]	50.4% [49.2, 51.5]	46.6% [42.7, 50.6]	35.8% [31.4, 40.2]	-
Race					<0.001
Hispanic	13.6% [10.7, 16.4]	14.2% [11.2, 17.2]	11.1% [8.3, 14.0]	8.7% [6.2, 11.1]	0.001
Non-Hispanic White	69.9% [65.8, 74.1]	69.5% [65.3, 73.8]	70.6% [65.1, 76.1]	74.0% [68.7, 79.2]	-
Non-Hispanic Black	10.3% [8.3, 12.3]	10.0% [8.0, 11.9]	11.9% [9.0, 14.7]	12.8% [9.4, 16.3]	0.088
Other Race	6.2% [5.1, 7.3]	6.3% [5.1, 7.5]	6.4% [4.1, 8.8]	4.5% [3.0, 6.1]	0.113
BMI group					0.007
Underweight/Normal: < 25.0	31.5% [29.5, 33.5]	32.0% [30.0, 34.0]	31.4% [26.2, 36.7]	26.4% [22.0, 30.8]	-
Overweight: 25.0-29.9	33.5% [32.0, 35.0]	34.1% [32.5, 35.7]	29.0% [24.6, 33.3]	30.2% [25.9, 34.5]	0.004
Obesity: > 30	35.0% [33.3, 36.6]	33.9% [32.2, 35.6]	39.6% [33.8, 45.5]	43.4% [38.7, 48.2]	0.438
Smoking					0.391
Non-Smoker	54.8% [52.7, 56.9]	55.2% [53.1, 57.3]	54.9% [50.2, 59.7]	50.4% [44.9, 56.0]	-
Former Smoker	23.8% [22.0, 25.6]	23.7% [21.8, 25.7]	23.7% [19.8, 27.5]	24.5% [20.8, 28.1]	0.555
Smoker	21.4% [20.0, 22.9]	21.1% [19.6, 22.6]	21.4% [17.7, 25.1]	25.1% [20.7, 29.5]	0.151
Poverty					0.019
Poor	21.4% [19.5, 23.4]	20.7% [18.9, 22.6]	22.8% [18.2, 27.3]	28.0% [23.4, 32.7]	0.003
Nearly poor	8.8% [7.9, 9.7]	8.6% [7.7, 9.6]	8.9% [6.2, 11.6]	10.6% [8.0, 13.2]	0.090
Not poor	69.8% [67.3, 72.3]	70.6% [68.2, 73.0]	68.3% [63.2, 73.5]	61.4% [55.3, 67.4]	-
Education					0.143
Less than 12th grade	15.8% [14.0, 17.6]	16.0% [14.3, 17.8]	14.6% [10.5, 18.7]	14.4% [11.2, 17.6]	-
High school/GED	22.0% [20.5, 23.6]	22.0% [20.4, 23.6]	20.7% [17.0, 24.3]	23.5% [19.0, 28.0	0.453
Some college or AA	32.0% [30.5, 33.5]	31.4% [30.0, 32.9]	34.7% [29.4, 39.9]	35.6% [31.7, 39.5]	0.132
College or above	30.2% [27.6, 32.7]	30.5% [27.9, 33.1]	30.1% [25.4, 34.8]	26.5% [21.5, 31.5]	0.833
Mine dust					0.619
No	68.2% [66.6, 70.0]	68.0% [66.2, 69.8]	70.3% [65.2, 75.4]	69.7% [65.9, 73.5]	-
Yes	31.7% [30.0, 33.4]	32.0% [30.2, 33.8]	29.7% [24.6, 34.8]	30.3% [26.5, 34.1]	0.619
Natural dust					0.220
No	76.9% [75.1, 78.7]	79.2% [77.3, 81.1]	81.1% [76.4, 85.9]	75.7% [70.1, 81.3]	-
Yes	23.1% [21.3, 24.9]	20.8% [18.9, 22.7]	18.9% [14.1, 23.6]	24.3% [18.7, 29.9]	0.220
Regular Milk Drinker					0.012
Never been	21.3% [19.9, 22.7]	21.2% [19.8, 22.5]	20.9% [17.0, 24.8]	23.1% [19.8, 26.3]	-
Sometimes/varied	35.8% 34.2, 37.3]	35.8% [34.1, 37.5]	31.6% [26.9, 36.3]	38.8% [34.0, 43.6]	0.683
Lifetime	42.9% [41.3, 44.4]	43.0% [41.4, 44.6]	47.5% [42.5, 52.4]	38.1% [33.5, 42.8]	0.030
Lifetime regular milk drinker (Yes/No)					0.002
Yes	45.2% [43.1, 47.3]	46.0% [43.8, 48.1]	38.5% [29.6, 47.4]	41.4% [35.8, 47.0]	0.002
No	54.8% [52.7, 56.9]	54.0% [51.9, 56.2]	61.5% [52.6, 70.4]	58.6% [53.0, 64.2]	-

Any type milk consumption (30 days)					0.318
Never	15.8% [14.7, 16.9]	15.7% [14.6, 16.8]	14.3% [10.9, 17.7]	18.2% [13.9, 22.4]	-
Rarely: < once a week	15.4% [14.4, 16.5]	15.5% [14.5, 16.5]	13.8% [11.1, 16.6]	15.9% [12.2, 19.6]	0.756
Sometimes: < once a day	29.8% [28.5, 31.0]	30.0% [28.7, 31.2]	28.7% [23.9, 33.5]	28.3% [24.4, 32.1]	0.345
Often: once a day or more	39.0% [37.5, 40.5]	68.8% [37.4, 40.2]	43.2% [38.1, 48.3]	37.6% [32.8, 42.5]	0.201
Any type milk consumption 5X a week or more (30 days)					0.180
Yes	61.0% [59.5, 62.5]	61.2% [59.8, 62.6]	56.8% [61.7, 61.9]	62.4% [57.5, 67.2]	0.180
No	39.0% [37.5, 40.5]	38.8% [37.4, 40.2]	43.2% [38.1, 48.3]	37.6% [32.8, 42.5]	-
Exclusive type milk consumed (30 days)					0.534
Never	15.8% [14.7, 16.9]	15.7% [14.6, 16.8]	14.3% [10.9, 17.7]	18.2% [13.9, 22.4]	-
Regular/whole milk only	17.4% [15.9, 18.9]	17.3% [15.8, 18.9]	19.0% [15.3, 22.7]	16.3% [12.9, 19.8]	0.211
2% milk only	31.5% [29.5, 33.4]	31.4% [29.4, 33.3]	30.9% [26.8, 35.0]	33.1% [26.5, 39.7]	0.741
1% milk only	12.0% [10.6, 13.3]	12.1% [10.8, 13.5]	10.4% [7.5, 13.3]	11.1% [7.9, 14.2]	0.538
Skim milk only	16.5% [14.9, 18.0]	16.4% [14.7, 18.2]	17.5% [13.3, 21.7]	15.7% [11.3, 20.2]	0.364
Other milk only	3.9% [3.2, 4.6]	4.0% [3.2, 4.7]	3.6% [1.8, 5.4]	2.6% [1.5, 3.7]	0.092
2+ types milk combined	3.1%2 [0.7, 3.5]	3.0% [2.6, 3.4]	4.2% [2.5, 5.9]	3.0% [1.4, 4.5]	0.140
Baseline FEV_1 (mL)	3245.6 [3215.5, 3275.7]	3273.9 [3245.3, 3302.6]	3317.1 [3234.5, 3399.8]	2868.2 [2781.8, 2954.5]	< 0.001
Baseline FVC (mL)	4155.4 [4126.0, 4184.8]	4155.4 [4126.0, 4184.8]	4221.7 [4127.8, 4315.6]	3841.3 [3742.9, 3939.6]	< 0.001
FEV_1% predicted	96.3 [95.7, 97.0]	97.1 [96.5, 97.8]	95.3 [93.7, 96.8]	88.7 [87.1, 90.4]	< 0.001
FVC % predicted	99.1 [98.6, 99.7]	99.5 [99.0, 99.9]	98.8 [97.5, 100.1]	96.0 [94.5, 97.4]	< 0.001
FEV_1/FVC	0.78 [0.777, 0.785]	0.78 [0.780, 0.788]	0.78 [0.777 0.791]	0.75 [0.736 0.755]	< 0.001

[1]p-values represent the Wald-type/overall p-values associated with the history of asthma (Yes, have a history and Yes, still have asthma) against no history of asthma. Bolded p-values represent those reaching statistical significance at $p \leq 0.5$. Column headings at the top define the content of each column. Response categories for each characteristic are indented below the corresponding characteristic. Definitions: body mass index (BMI); General educational development (GED) signifying high-school level academic skills; Associate in Arts (AA) degree; forced expiratory volume in one-second (FEV1); forced expiratory volume in one-second (FVC).

2.4. Results

2.4.1. Descriptive Data of Eligible Participants among the NHANES Cohort

For these analyses, the number of total eligible participants was 11,180. Of those participants, 49 were missing self-reported asthma status withdrawn from the eligible participants. Of the remaining 11,131 participants, 49.0% were male and 51.0% female. These eligible participants had a mean age of 44.4 years with 54.8% of participants reporting as non-smokers, 23.8% as former smokers, and 21.4% as current smokers. Participant demographics and milk consumption variables were assessed among the total eligible participants and provided in Table 2.

2.4.2. Milk Consumption Tendencies and Lung Function Measurements in All Eligible Participants

Initial univariable analysis in this total population (n = 11,131) identified several significant associations with asthma diagnosis, including age, gender, race, BMI, poverty status, regular milk-drinker (5+ days per week) status across a lifetime, and measurements of lung function (Baseline FEV1 and FVC, FEV1% predicted and FVC% predicted, as well as FEV1/FVC ratio), as identified in Table 2. In accordance with our hypothesis that milk consumption is associated with better lung function, we explored whether milk consumption was associated with differences in lung function parameters in the total eligible population. As shown in Table 3, multivariable regression models demonstrated that lifetime regular milk consumption was significantly associated with FEV1 (overall $p = 0.004$), where being a lifetime regular milk drinker (β:54.5; $p = 0.001$) or reporting to sometimes be a regular milk drinker throughout life versus never (β:58.4; $p = 0.006$) was associated with significantly higher FEV1 measurements compared to individuals identifying as never being regular milk drinkers. In addition, reporting

milk consumption often in the past 30 days was also associated with increased FEV1 (β:39.6; p = 0.036). An increase in FEV1% was determined in individuals identifying as only regularly drinking 1% milk versus no milk (β:1.81; p = 0.020) in the prior 30 days.

Similar to findings with FEV1, lifetime regular milk consumption was also significantly associated with measured FVC (p = 0.018), where identifying as a lifetime regular milk drinker (β:57.7; p = 0.001) or sometimes being a regular milk drinker across life (β:58.8; p = 0.011) versus never was significantly associated with having higher FVC measurements. While FEV1/FVC was significantly associated with asthma diagnosis in the univariable analysis shown in Table 2, there were no significant associations identified with milk consumption tendencies (all p values > 0.05).

Table 2.3 Multivariable models of associations between milk consumption tendencies and lung function measurements in the total NHANES population.

Milk Consumption Tendencies	FEV_1		FVC		FEV_1%		FVC%		FEV_1 /FVC	
	β[1] [95% CI][2]	p-value[3]	β[1] [95% CI][2]	p-value[3]	β[1] [95%CI][2]	p-value	β[1] [95% CI][2]	p-value[3]	β[1] [95% CI][2]	p-value[3]
Regular Milk Drinker (REGMLK) (vs. Never)		0.004		0.018		0.168		0.248		0.685
Lifetime	54.536 [22.473, 86.600]	0.001	57.714 [14.763,100.665]	0.010	0.587 [-0.290,1.464]	0.185	0.317 [-0.465,1.100]	0.419	0.002 [-.003,0.007]	0.486
Variable/Sometimes	58.418 [18.045, 98.792]	0.006	58.774 [13.879,103.669]	0.011	1.047 [-0.047,2.141]	0.060	0.637 [-0.125, 1.400]	0.099	0.003 [-0.004,0.010]	0.388
30-Day Milk Consumption Frequency (MLKCONS30) (vs. Never)		0.202		0.315		0.347		0.467		0.358
Rarely	26.339 [-19.942,72.621]	0.258	13.877 [-36.301,64.055]	0.581	0.951 [-0.308,2.209]	0.135	0.398 [-0.609, 1.405]	0.431	0.004 [-0,002,0.010]	0.631
Sometimes	30.444 [-17.102,77.990]	0.204	22.105 [-33.276,77.487]	0.426	0.972 [-0.244, 2.187]	0.111	0.643 [-0.370, 1.656]	0.208	0.004 [-0.004,0.007]	0.151
Often	39.551 [2.730, 76.372]	0.036	40.924 [-3.997, 85.844]	0.073	0.917 [-0.219, 2.053]	0.115	0.733 [-0.239, 1.704]	0.136	0.001 [-0.003,0.010]	0.246
Regular 30-day Exclusive Type Milk Consumption (TYPE30) (vs. No per Type)		0.140		0.301		0.165		0.074		0.110
Whole	-1.101 [-44.644, 42.442]	0.960	-5.931 [-54.304, 42.441]	0.806	0.290 [-0.873, 1.453]	0.619	0.124 [-0.820,1.068]	0.793	0.000 [-0.006,0.007]	0.324
2%	36.344 [-3.529, 76.216]	0.073	37.017 [-12.103, 86.136]	0.136	0.919 [-0.203, 2.041]	0.106	0.807 [-0.093, 1.707]	0.078	0.002 [-0.004,0.008]	0.570
1%	59.500 [-6.273, 125.269]	0.075	52.020 [-26.657, 130.696]	0.190	1.811 [0.297, 3.325]	0.020	1.270 [0.024, 2.516]	0.046	0.005 [-0.001,0.012]	0.114
Skim	50.411 [-0.836, 101.658]	0.053	45.959 [-13.092, 105.010]	0.124	1.283 [-0.194, 2.760]	0.087	0.828 [-0.381,2.037]	0.175	0.004 [-0.004,0.011]	0.324
Other	30.504 [-41.082, 102.090]	0.396	12.634 [-77.477, 102.745]	0.779	0.649 [-1.335, 2.632]	0.514	-0.034 [-1.928,1.861]	0.972	0.006 [-0.000,0.014]	0.080
2+ types	32.723 [-53.052, 118.497]	0.447	6.153 [-91.164, 103.469]	0.899	0.091 [-2.061, 2.244]	0.932	-0.589 [-2.413, 1.245]	0.519	0.006 [-0.004,0.016]	0.209

[1] Regression coefficient (β); Each regression model shows milk consumption adjusted for the following confounding factors: age, gender, race, education, poverty level, BMI group, smoking status, mineral dust exposure in the past, and natural dust exposure in the past. [2] Standard Error (SE); Due to small values of regression coefficient and standard error, values were displayed a after multiplying by 10^3.[3]p-values ≤ 0.05 are bolded and have been rounded to the nearest hundredth with ≤ 0.01for any values less than 0.01; data rounded to the nearest hundredth (greater than or equal to 0.005 rounded up) with ≤0.01 for any values less than 0.01; Non-italicized p-values (top p value for each variable) represent Wald-type/overall p-values. Column headings at the top define the content of each column. Response categories for each characteristic are indented below the corresponding characteristic. Definitions: forced expiratory volume in one-second (FEV_1); forced expiratory volume in one-second (FVC).

2.4.3. Milk Consumption Tendencies and Current Asthma Report in All Eligible Participants

When considering regular, 5+ days per week, milk consumption, multivariable analysis between regular milk consumption across a lifetime and likelihood of reporting current asthma (responding 'yes' to "still have asthma"), no significant association was identified (Figure 1A). However, when dichotomized (Yes/No) for being a lifetime regular milk drinker, there was a significant association (OR: 0.81; p = 0.026) between identifying as a lifetime regular milk consumer and decreased likelihood of having current asthma. Regular milk consumption in the past 30 days (regardless of type) did not have any significant association with current asthma (Figure 1B). Although, reporting regular milk consumption of exclusively 'other' milk (e.g., soy) was significantly associated with reduced current asthma (OR: 0.51 [0.28, 0.93]; p = 0.028; Figure 1C) which may be in part due to the lack of cholesterol and decreased saturated fats in soy milk, as well as its ability to reduce LDL compared to cow's milk. It has been shown that serum cholesterol and dyslipidemia are associated with asthma exacerbations [62].

Figure 2.1

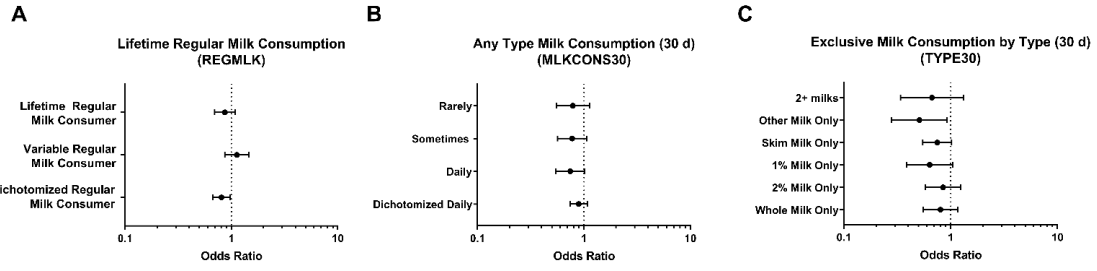

Figure 2.1. Associations between milk consumption tendencies and reporting current asthma in the NHANES total population. Odds ratios for reporting current asthma compared to the total NHANES population based on identifying as (A) a lifetime regular milk drinker, or sometimes being a regular milk drinker across life as compared to identifying as never being a regular milk drinker; (B) based on any type milk consumption tendencies in the previous 30 days; (C) based on exclusive regular milk consumption by type.

2.4.4. Milk Consumption Tendencies and Current Asthma Report in Participants Reporting Asthma (History or Current)

Limiting our analysis to individuals specifically reporting a history of asthma or current asthma, Table 4 outlines the characteristics of these asthmatic participants. The total number of participants self-reporting either a history of previous asthma or current asthma was 1542. In this subset of individuals, multivariable analyses identified significant associations between individuals answering 'yes' to "still have asthma" versus answering 'no', including age, gender, poverty status, and/or lifetime regular milk consumption status. Amongst patients self-reporting a history of asthma, we identified a significant association between lifetime regular milk consumption and current asthma status (overall $p = 0.006$). As shown in Figure 2A, those who reported being a lifetime regular milk consumer had a decreased likelihood of reporting current asthma compared to those who reported never being a regular milk drinker (OR: 0.75; 95% Confidence Interval [0.56, 1.01]), while individuals reporting variable regular milk consumption did not exhibit the same effect (OR: 1.13; 95% CI: [0.76, 1.69]). When this variable was dichotomized, we similarly identified a significant association ($p = 0.001$), where individuals that were identified as regular milk drinkers were significantly less likely to report current asthma compared to individuals not identified as being regular milk drinkers (OR: 0.70; 95% CI: [0.56, 0.86]).

Table 2.4. Characteristics of milk drinkers and association to symptoms among participants reporting a history of asthma (n = 1542).

Characteristic	Current Asthma No (n=717) % Mean	Current Asthma Yes (n=825) % Mean	Current Asthma Yes vs. No Univariable p value[1] OR[2] [95% CI] [3]	Current Asthma Yes vs. No Multivariable p value[1] OR[2] [95% CI] [3]
Age (year)	40.21	44.03	p<0.001 1.02 [1.01, 1.02]	p=0.001 1.02 [1.01, 1.03]
Gender			p=0.001	p=0.029
Female	53.4%	64.2%	1.56 [1.21, 2.02]	1.43 [1.04, 1.96]
Male	46.6%	35.8%	1	1
Race			p=0 .107	p=0.319
Hispanic	11.1%	8.7%	0.74 [0.52, 1.07]	0.74 [0.51, 1.08]
Non-Hispanic White	70.6%	74.1%	1	1
Non-Hispanic Black	11.8%	12.7%	1.03 [0.76, 1.40]	0.96 [0.68, 1.37]
Other race	6.4%	4.5%	0.67 [0.42, 1.06]	0.71 [0.40, 1.25]
BMI group			p=0.229	p=0.893
Underweight/Normal: > 25.0	31.4%	26.4%	1	1
Overweight: 25.0-29.9	29.0%	30.2%	1.25 [0.87, 1.78]	1.10 [0.75, 1.61]
Obesity: > 30	39.6%	43.4%	1.30 [0.96, 1.77]	1.08 [0.73, 1.60]
Smoking			p=0.257	p=0.600
Non-smoker				
Former smoker	54.9%	50.4%	1	1
Smoker	23.7%	24.5%	1.13 [0.83, 1.54]	0.99 [0.69, 1.41]
	21.4%	25.1%	1.28 [0.96, 1.70]	1.17 [0.85, 1.62]
Poverty			p=0.120	p=0.044
Poor	22.8%	28.0%	1.37 [1.00, 1.87]	1.47 [1.04, 2.08]
Nearly poor	8.9%	10.6%	1.32 [0.84, 2.10]	1.41 [0.92, 2.14]
Not poor	68.3%	61.4%	1	1
Education			p=0.652	p=0.637
Less than 12th grade	14.6%	14.4%	1	1
High school/GED	20.6%	23.5%	1.15 [0.76, 1.75]	1.34 [0.82, 2.18]
Some college or AA	34.7%	35.6%	1.04 [0.71, 1.52]	1.29 [0.83, 2.00]
College or above	30.1%	26.5%	0.89 [0.60, 1.33]	1.13 [0.71, 1.81]
Mine dust			p=0.791	p=0.085
No	70.3%	69.7%	1	1
Yes	29.7%	30.3%	1.03 [0.83, 1.28]	1.27 [0.97, 1.67]
Natural dust			p=0.083	0.051
No	75.4%	78.8%	1	1
Yes	24.6%	21.2%	0.83 [0.66, 1.03]	0.77 [0.59, 1.00]
Regular milk drinker			p=0.002	p=0.006
Lifetime	47.5%	38.1%	0.73 [0.57, 0.93]	0.75 [0.56, 1.01]
Sometimes/Varied	31.6%	38.8%	1.12 [0.78, 1.60]	1.13 [0.76, 1.69]
Never been	20.9%	23.1%	1	1

[1] Note: Wald-type/overall p-values ≤ 0.05 are bolded; 2 Odds ratio (OR); 3 95% Confidence Interval (95% CI); percents rounded to the nearest tenth; OR, CI, have been rounded to the nearest hundredth with ≤0.01 for any values less than 0.01. Column headings at the top define the content of each column. Response categories for each characteristic are indented below the corresponding characteristic.

When considering milk consumption (of any milk type) over the 30 days prior to survey participation, we identified no significant association between milk consumption and reporting current asthma (overall p = 0.100). Shown in Figure 2B, as compared to individuals never drinking milk in the past 30 days, consumers reporting once daily or more milk consumption had reduced odds of reporting current asthma (OR: 0.62 [0.40, 0.95]; p = 0.028). Those reporting

sometimes or rarely did not exhibit a similar association. When this variable was dichotomized to those reporting daily milk consumption in the past 30 days versus those not reporting daily milk consumption, there was no significance (OR: 0.76; 96% CI: [0.56, 1.02]; p = 0.063). Furthermore, as shown in Figure 2C, when assessing regular, exclusive consumption of a specific milk type in the prior 30 days, individuals reporting exclusive consumption of whole milk only versus no milk had a significantly decreased likelihood of reporting current asthma (OR:0.60; 95% CI: [0.38, 0.96]; p = 0.032).

Figure 2.2

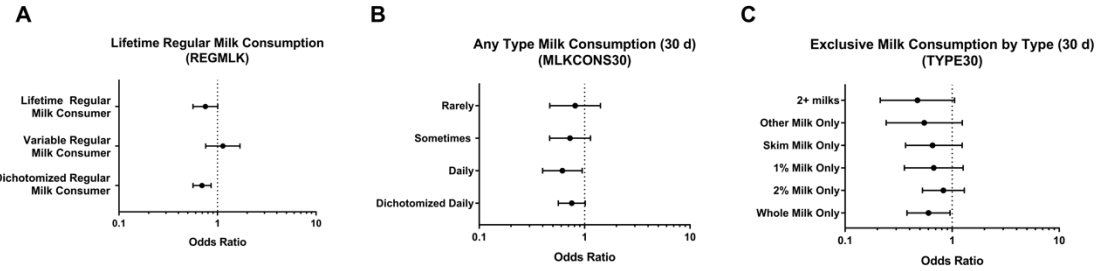

Figure 2.2. Associations between milk consumption tendencies and reporting current asthma in participants reporting asthma (history or current). Odds ratios for reporting current asthma amongst individuals reporting asthma based on identifying as (A) a lifetime regular milk drinker, or sometimes being a regular milk drinker across life as compared to identifying as never being a regular milk drinker; (B) based on any type of milk consumption tendencies in the previous 30 days; (C) based on regular (5+ days per week) exclusive type of milk consumption.

2.4.5. Milk Consumption Tendencies and Lung Function Parameters in Participants Reporting Asthma (History or Current)

As in our assessment of the general population, we also evaluated for relationships between lung function and milk consumption in the cohort reporting current/history of asthma (Table 5). Here, we found no significant association between identifying as a regular milk consumer across lifetime and any differences in FEV1 or FEV1% measures. When looking at FVC, lifetime regular milk consumption tendencies and FVC measurements did not reach significance (overall p = 0.058). Although, individuals who reported sometimes being a regular

milk drinker across their life had significantly higher FVC measurements compared to participants who did not identify as ever being a regular milk drinker (β:127.28; p = 0.030). This finding is paralleled by the FVC% findings of a significant association between lifetime regular milk consumption tendencies and FVC% (overall p = 0.045) with those reporting as variably being regular milk drinkers having significantly higher FVC% (β:2.62; p = 0.020) vs. those identifying as never being a regular milk consumer. Meanwhile, exclusive consumption of specific types of milk in the prior 30 days was also associated with significant differences in FVC outcomes (overall p = 0.0084) and FVC% outcomes (overall p = 0.009). Here, consumption of only skim milk the 30 days prior to completion of the questionnaire (β:2.76; p = 0.045) was associated with higher FVC% measurements compared to those consuming no milk in the prior 30 days.

Table 2.5 Associations between milk consumption tendencies and lung function measurements in NHANES participants reporting past or current asthma.

Milk Consumption Tendencies	FEV_1		FVC		FEV_1%		FVC%		FEV_1 /FVC	
	β[1] [95% CI][2]	p-value[3]	β[1] [95% CI][2]	p-value[3]	β[1] [95% CI][2]	p-value[3]	β[1] [95% CI][2]	p-value[3]	β[1] [95% CI][2]	p-value[3]
Regular Milk Drinker (REGMLK)		0.378		0.058		0.380		0.045		0.461
(vs. Never)										
Lifetime	21.485 [-81.007, 123.978]	0.675	67.810 [-56.787, 192.407]	0.280	0.392 [-2.805, 3.588	0.807	1.344 [-1.343, 4.031]	0.320	-0.010 [-0.0257,0.006]	0.229
Variable/Sometimes	60.609 [-43.290, 164.510]	0.247	127.282 [13.134, 241.430]	0.030	1.644 [-1.124, -4.413]	0.238	2.617 [0.438, 4.796]	0.020	-0.008 [-0.023,0.008]	0.310
30-Day Milk Consumption Frequency (MLKCONS30)		0.260		0.450		0.251		0.556		0.467
(vs. Never)										
Rarely	106.540 [-22.946, 236.027]	0.104	116.104 [-35.197, 267.405]	0.130	2.932 [-0.332, 6.187]	0.077	2.232 [-0.887, 5.350]	0.158	-0.009 [-0.022,0.005]	0.198
Sometimes	44.763 [-83.866,173.392]	0.488	69.626 [-66.649, 205.901]	0.310	0.935 [-2.273, 4.143]	0.561	1.310 [-1.201, 3.821]	0.300	0.002 [-0.016,0.021]	0.810
Often	21.084 [-101.213,143.380]	0.731	60.320 [-79.273,199.913]	0.389	0.484 [-3.037, 4.006]	0.783	1.167 [-1.743, 4.077]	0.424	-0.002 [-0.017,0.013]	0.813
Regular 30-day Exclusive Type Milk Consumption (TYPE30)		0.078		0.008		0.2233		0.009		0.539
(vs. No per Type)							-1.217 [-3.764,1.331]			
Whole	-78.398 [-182.009, 25.212]	0.135	-76.798 [-203.185, 49.789]	0.229	-1.706 [-4.653,1.241]	0.250	1.436 [-1.160, 4.033]	0.342	-0.010 [-0.027,0.008]	0.291
2%	67.544 [-46.365, 181.453]	0.239	108.135 [-22.100, 238.370]	0.102	1.025 [-1.889, 3.938]	0.483	4.197 [-0.111, 8.505]	0.272	-0.004 [-0.015,0.007]	0.521
1%	114.602 [-43.446, 272.649]	0.152	144.527 [-37.854, 326.909]	0.118	4.244 [-0.498, 8.987]	0.078	2.759 [0.070, 5.448]	0.056	0.000 [-0.019,0.020]	0.962
Skim	86.530 [-58.639, 231.700]	0.237	138.043 [-22.424, 298.510]	0.090	2.073 [-1.436, 5.584]	0.241	-1.214 [-5.731,	0.045	-0.007 [-0.023,0.010]	0.401
Other	83.761 [-101.368, 268.890]	0.368	51.153 [-137.083, 239.388]	0.588	0.261 [-4.776, 5.398]	0.918	3.302]	0.591	0.009 [-0.018,0.036]	0.504
2+ types	15.822 [-182.614, 214.259]	0.873	21.343 [-219.196, 261.882]	0.859	2.005 [-4.235, 8.244]	0.4831	1.727 [-3.950, 7.404]	0.544	0.001 [-0.020,0.023]	0.899

[1] Regression coefficient; Each regression model shows milk consumption adjusted for the following confounding factors: gender, race, age, education, poverty level, BMI group, smoking status, mineral dust exposure in the past, and natural dust exposure in the past. 2 Standard Error; Due to small numbers of regression coefficient and standard error, values were displayed after multiplying by 103.[3] p-values ≤ 0.05 are bolded and have been rounded to the nearest hundredth with ≤0.01 for any values less than 0.01; data rounded to the nearest hundredth (greater than or equal to 0.005 rounded up) with ≤0.01 for any values less than 0.01; non-italicized p-values (top p value for each variable) represent Wald-type/overall p-values. Column headings at the top define the content of each column. Response categories for each characteristic are indented below the corresponding characteristic. Definitions: forced expiratory volume in one-second (FEV_1); forced expiratory volume in one-second (FVC).

2.5. Discussion

Asthma has been on the rise across the world [30], and dietary modification is a viable option for reducing incidence of respiratory diseases like asthma. Lately, there is growing awareness about the role of dairy products in health, with studies identifying that intake of dairy products does not increase the risk for disease and may be protective [31–34]. Our findings suggest that milk consumption may be an autonomous factor that individuals can modify to benefit lung health outcomes with consumption reducing instances of current asthma reports in those with and without a history of asthma. In these investigations, we have utilized the NHANES 2007–2012 dataset to assess relationships between milk consumption tendencies and asthma-related outcomes, including reporting current asthma as well as lung function measurements. Since these data are generated from a cross- sectional study, the FEV1% and FVC% are more informative to pulmonary performance since they are inherently compared to expected values. Among the total population, we found FEV1% and FVC% to be significantly associated with regular consumption of exclusively low-fat 1% in the prior 30-days (β:1.81; $p = 0.020$ and β:1.27; $p = 0.046$). Among the total population, we also identified significant associations with FEV1 ($p = 0.0040$), FVC ($p = 0.018$), and regular milk consumption. There was a significant association between FEV1 and FVC in participants describing regular milk consumption as lifetime regular consumption (FEV1 β: 54.5; $p = 0.0013$ and FVC β:57.7; $p = 0.010$) or varied regular consumption ({FEV1, β:58.4; $p = 0.006$ and FVC, β:58.8; $p = 0.011$).

Among participants with asthma, varied-regular milk consumption in a lifetime was significantly associated with FVC ($p = 0.002$) and FVC% ($p = 0.006$). These paralleled findings may be indicative of the importance of timing in milk consumption as we explore below, in which regular milk consumption earlier in life is most strongly associated with changes in lung

function [35–37]. Among these participants, individuals who were regular milk drinkers during childhood, as many parents transition children to cow's milk in the earlier years, may have ceased frequent consumption in adulthood. This is an example warranting a "varied/sometimes" response to lifetime regular milk consumption, where the beneficial effects of cow's milk during earlier years could provide protection to pulmonary function into adulthood. In our investigations, FVC and FVC% of participants with asthma were additionally associated with regular single-type milk consumption in the prior 30-days ($p = 0.008$ and $p = 0.009$, respectively) with regular consumption of exclusively low-fat 1% in the prior 30-days associating significantly with FVC% (β:2.76; $p = 0.045$). These data have revealed that individuals identifying as lifetime regular milk consumers are less likely to answer yes when asked if they have had or still have asthma and also had higher FEV1 and FVC measurements compared to individuals not identifying as lifetime regular milk drinker. Meanwhile, individuals reporting a history of asthma did not have any associations with lifetime milk consumption and FEV1. We identified a potential protective association for exclusive consumption of low fat (2%, 1%, or skim) milks including reduced likelihood of current asthma or higher lung function measurements in both the general and asthmatic populations. Together, these data from NHANES support additional findings in the literature on the effects of dairy consumption and lung health, particularly in the case of low-fat milks. We cannot make causative claims based on these results, but in summation with similar findings in the literature, we can appreciate the potential impact of dairy consumption on pulmonary health.

Varied results in our findings may be the consequence of the inability to assess milk quality and content in these individuals. Many studies have clarified the impact animal well-being has on milk components. Heat-stress resulting from challenging temperature conditions exhibits detrimental effects to the animals' physiological state, altering the quantity and quality of milk

products. It has been demonstrated that heat stress results in a decrease in yield, as well as fat and protein milk-content, and likely aberrant nutritional value [38,39]. Furthermore, significant changes to the triacylglyceride composition (decreased SCFA, MCFA, lipid polar classes and increased LCFA) of milk due to acute heat stress presumably alters the biological properties of milk [40]. Alternatively, transportation stress demonstrated an increase in fat content, when comparing milk from transported to non-transported cows, and there were significant decreases in pH, yield, as well as lactose and solid non-fat content associated with transportation [41]. Transportation stress was also significantly associated with increased leukocyte, neutrophil, eosinophil, and monocyte count, although cytokines were not measured. Additionally, breed and genetics of cows play an important role in the quality of milk produced, particularly as this field is heavily reliant upon SNP analysis and sequencing screening [42–44]. The properties of milk are likely to differ between dairy farms, as well as the brands that are available at participants' grocery stores. Thus, we anticipate these factors to be influential in our reported results and we suggest further studies investigating how they alter the biological impacts of dairy consumption on human lung physiology and asthma.

Finally, protocols for milk processing (pasteurization, homogenization, milk-fat removal) modify the properties of milk. Most commonly, milk consumed in the United States is treated with ultra-high temperatures for pasteurization and homogenization, and therefore the impact of milk consumption is dependent on milk-processing. The literature on the benefits of raw milk in human physiology, immunology, and protection against asthma, is abundant. Since raw milk is not heat-processed, the protein structures remain intact as well as the microbial diversity. The GABRIEL Advanced study demonstrated, using a questionnaire and blood samples when available, that among 79,888 school-aged children living in rural Austria, Germany and Switzerland, both raw and pasteurized milk consumption may half the risk of developing asthma

[45]. A literature review by Barbara Sozánska M.D. summarized that consumption of raw cow's milk is protective against allergies and asthma among children and adults [31]. These studies demonstrate the influence that dairy consumption may have specifically on lung health, via a variety of mechanisms, including dietary absorption of lipids, macronutrients, or milk-derived exosomes containing small regulatory RNAs and immunoglobulins. The fat-soluble vita- mins in milk, D and E, have promising if not well-established associations with improved inflammatory processes, and specifically in the airways [46]. Vitamin D deficiency, for example, results in aberrant lung structure and function in a mouse model [47]. Vitamin D deficient mice have significantly less thoracic gas volume, greater airway resistance, and lower alveolar air volume and less alveoli. In human, insufficient serum vitamin D is associated with asthma severity markers such as elevated IgE, eosinophilia, and increased hospitalizations [48]. A meta-analysis identified a direct relationship between vitamin D levels and FEV1, FEV1%, and FEV1/FVC [49]. Additionally, vitamin D plays an important role in inhibiting human airway smooth muscle cells, which is an important measure of asthma severity [50].

Dairy fat is comprised mainly of long, medium, and short-chain saturated fatty acids (LCFA, MCFA, SCFA). Certain long-chain fatty acids have been associated with increased serum lipid levels, and corresponding risk for cardiovascular disease, however milk has a much higher proportion of short- and medium chain saturated fatty acids. These varying chain lengths impact blood lipid levels and inflammation differently and may be more important than previously recognized. Evidence suggests these short-chain fatty acids have potential anti-inflammatory effects due to their influence on the regulation of various molecular signaling pathways [51], and around 11% of the fat content of milk is SCFA [52]. Studies suggest that intake of dairy products, high fat or raw, lowers inflammatory markers in adults and increases the intake of the anti-inflammatory ω-3 polyunsaturated fatty acid in children, respectively [53,54].

These findings, along with findings defining alterations to milk properties, demonstrate the impact milk quality may have on lung health and support observations found in the current study.

When considering the potential mechanisms by which milk elicits its effects on human lung health, it is important to consider the many homologous components of bovine milk such as immunoglobulins, lactoferrin, lactadherin, and various cytokines that may enhance the immune activity upon consumption and absorption [54]. Low-fat dairy intake also has implications in positive clinical outcomes of moderately improved lung density, measured by CT scan, the mechanisms of which were not fully elucidated [20]. Furthermore, current consumption of farm milk leads to a significant increase in regulatory T cell counts and potential increase in activated T cells [55]. Furthermore, we have previously shown that mice fed a diet containing bovine milk exhibited altered immune responses to an environmental dust challenge, compared to mice fed the same diet where the milk was first sonicated to disrupt milk exosomes. Here, mice fed the unsonicated milk diet exhibited a skewing towards an M1-type inflammatory response, while mice fed the sonicated milk diet exhibited a polarization towards an M2-like response that is classically seen in asthma and allergic disease settings [56]. Diet-derived small RNAs are also implicated in gene expression regulation at the level of the consumer. The analogous nature of many miRNAs, from bovine to human, implicates the role of milk-derived non-coding RNAs in targeting genes related to inflammation including in disease settings such as asthma [54,56,57]. Therefore, lung function and immunity may be modulated by dairy consumption.

In a cohort of men and women, aged 45–84 years, the MESA-Lung Study questionnaire demonstrated the significance of higher low-fat dairy consumption on improved CT-measured lung density [20]. The implications of this study are that dairy consumption increases tissue-to-air ratio as a measure of improvement of the expanded airways and alveolar tissue destruction as

found in emphysema. Additionally, pulmonary function testing is a clinical standard for diagnosing and managing obstructive lung diseases like asthma [58]. While no CT data were available for this NHANES cohort, spirometry can be used to ascertain improvements in lung health. In this cohort, milk consumption overall was associated with higher pulmonary function, measured by spirometry, in asthmatics. Among those reporting asthma, those that reported sometimes or regularly consumed dairy products across life and/or within the prior 30 days had overall better FEV1/FVC measurements, with the exception of individuals reporting regular whole milk consumption in the prior 30 days (as mentioned above).

It is worth mentioning that people with asthma can be avoidant of dairy products because of what is known as the "milk-mucus theory." This theory suggests that consumption of dairy products exacerbates asthma symptoms, but the summation of data from a wealth of studies does not directly link milk consumption to asthma, and overall there is no evidence of cause and effect [59]. There are studies in which people with asthma reported improved respiratory outcomes when eliminating dairy consumption, however a number of studies demonstrated that placebo control also reported improved lung function [60,61]. While the evidence does not support the milk-mucus theory, it is important to be aware of this when educating patients/individuals with asthma about any recommendations of incorporating dairy products.

The results reported through our investigations and derived from a cross-sectional study provide an interesting, although limited, perspective on the impact of dairy con- sumption and pulmonary health outcomes. For example, there are likely unmodelled confounding variables and interactions that we have not assessed. Additionally, NHANES relies on participants self-reporting asthma, thus this work lacks quantitative data of asthma diagnoses, symptomology, and potential ongoing therapeutics usage. Due to the exploratory nature of these studies in assessing

for significant associations between milk consumption and pulmonary function/disease outcomes, we did not apply any multiplicity corrections. In addition, though frequency of milk consumption was described, there were no data available regarding the quantity of milk consumption, thus quantity is not effectively modeled, which is likely to impact these outcomes. Beyond milk consumption, it would be beneficial to study if dairy consumption in forms of cheese and yogurt are associated with lung function and/or asthma in the NHANES dataset, or if participants differentiated between these products. These limitations could be addressed through further studies focused on elucidating how frequency, quantity, and type of dairy consump- tion alter pulmonary outcomes across a period of time, to better understand the role of milk in improving lung health outcomes. In particular, identifying how soon after milk consumption habits might elicit alterations in pulmonary outcomes would be important if dairy products are to be recommended for lung health. To address the shortcomings of this cross-sectional study design, further investigation using reversibility testing, highly sensitive for asthma, could also be used. a challenge of these recommendations are that 45% of children with asthma also have food allergies, some to dairy products, so these are considerations for future studies and recommendation [63, 64]. All together, these data in combination with previously published data, highlight the importance of further studies on dairy and its potential to modify lung disease outcomes, prevent disease, and use in disease treatment where current access to therapeutics falls short.

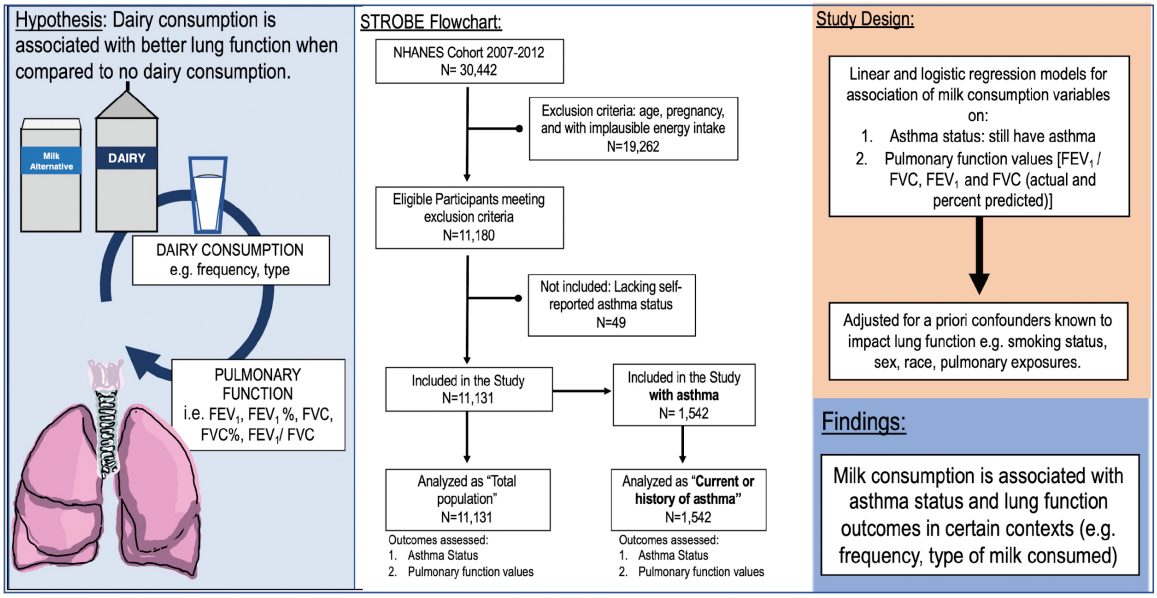

Figure 2.3 Summary Figure. Created with "biorender.com"

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CHAPTER THREE:

Free fatty acid receptor 4 is a targetable regulatory protein in pulmonary epithelial homeostasis and response to immunogenic exposures

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3.1 Abstract

The role of pulmonary free fatty acid receptor 4 (FFAR4) is not fully elucidated and we aimed to clarify the impact of FFAR4 on the pulmonary immune response and return to homeostasis. We employed a known high-risk human pulmonary immunogenic exposure to extracts of dust from swine confinement facilities (DE). WT and Ffar4-null mice were repetitively exposed to DE via intranasal instillation and supplemented with docosahexaenoic acid (DHA) by oral gavage. We sought to understand if previous findings of DHA-mediated attenuation of the DE-induced inflammatory response are FFAR4-dependent. We identified that DHA mediates anti-inflammatory effects independent of FFAR4 expression, and that DE-exposed mouse lacking FFAR4 had reduced immune cells in the airways, epithelial dysplasia, and impaired pulmonary barrier integrity. Analysis of transcripts using an immunology gene expression panel revealed a role for FFAR4 in lungs related to innate immune initiation of inflammation, cytoprotection, and immune cell migration. Ultimately, the presence of FFAR4 in the lung may regulate cell survival and repair following immune injury, suggestive of potential therapeutic directions for pulmonary disease.

3.2 Rationale for investigating FFAR4 in lung inflammation, injury, and repair

Lipids play a critical role in metabolic and immune homeostasis, which underscores their potential as diet-based therapeutics in the treatment of human disease. One such family of lipids with well-characterized anti-inflammatory and pro-resolving effects includes omega-3-polyunsaturated fatty acids (ω-3-PUFA) such as docosahexaenoic acid (DHA)[1-3]. Dietary supplementation with PUFA introduces these lipids into lipoproteins which can be cleaved by lipoprotein lipase in the vasculature and target tissues. Use of ω-3-PUFA, experimentally and clinically, demonstrates improvements in: the resolution of inflammation; tissue repair;

cardiovascular health; metabolic homeostasis and dyslipidemia; chronic diseases like chronic obstructive pulmonary disease (COPD); among others[2, 4-11]. Among these ω-3-PUFAs, studies identify that DHA in particular exhibits the broadest anti-inflammatory response via numerous modes of action and thus has strong therapeutic potential in diseases such as those of the lung [12-14]. DHA is critical to cell membrane integrity and participates in anti-inflammatory signaling through generating lipid mediators and through ligation of lipid-sensing free fatty acid receptor 4 (FFAR4) [15-18].

FFAR4, previously GPR120, is highly expressed in the gut where it is activated by an ingested luminal long-chain fatty acid, resulting in energy homeostasis regulation. FFAR4 is targeted therapeutically in clinical trials for the treatment of metabolic disease (clinicaltrials.gov identifier: NCT03285750, NCT02444910, NCT03062592, NCT05068557). However, this lipid-sensing role of FFAR4 is less intuitive in the pulmonary system, which exhibits some of the highest Ffar4 expression [19, 20]. Publications outlining the functionality of FFAR4-signaling in the pulmonary context are limited to a handful of papers, but importantly a role in improved airway repair in a murine model of naphthalene-induced airway injury has been elucidated by Lee et. al. [21]. We have previously reported the benefits of DHA administration on inflammatory outcomes, both in vitro and in vivo, using an organic swine dust exposure model which causes pulmonary inflammation[1, 2]. These data suggest potential crosstalk between DHA and FFAR4 in protecting the lung which may be important as therapeutic targets in lung tissue repair [22].

This model is of clinical importance due to the Centers for Disease Control's classification of agricultural dust inhalation as one of the highest-risk occupational exposures as it contains organic and inorganic immunogenic particulates (bacteria, viruses, fungi, and chemicals) that drive lung inflammation [23-26]. The long-term implications of these exposures, which are

typically frequent and repetitive, are an increased risk of pulmonary inflammatory diseases, like COPD, the third leading global cause of death per the World Health Organization [27, 28]. COPD is marked by two overlaying, irreversible pathologies: chronic bronchitis and emphysema, which often result from inhaled exposures and dysregulated repair. Without established therapeutics to reverse lung damage, exploring DHA and FFAR4 as potential targets in lung repair is enticing, especially as GPCRs comprise over 40% of current therapeutic targets and data demonstrates the positive role of DHA on COPD morbity[29, 30].

Due to the numerous ways DHA functions in vivo, these anti-inflammatory and pro-repair effects are not always FFAR4 dependent [31, 32]. Thus, this current work aims to identify the role of FFAR4 in our previous elucidation of the DHA-mediated resolution of pulmonary inflammation. To accomplish this aim we employed a well-established mouse model of agricultural dust-induced lung inflammation and complemented these studies using additional exposure models known to facilitate lung damage through different mechanisms. Our mouse model of this dust exposure allows for the study of pulmonary inflammation and resolution as these exposure models are self-limiting [33, 34]. This study clarifies the impact of FFAR4 on the inflammatory process through repair with the long-term objective of ultimately identifying potential therapeutics and interventions of chronic lung diseases, such as DHA and FFAR4, for which few current therapeutics are available.

3.3 Results

3.3.1. Ffar4 deficiency leads to dampened inflammatory cell influx to the airways independent of DHA

Ffar4 wild type (WT), heterozygous (HET), and knockout (KO) mice were supplemented with DHA or mineral oil vehicle by oral gavage and repetitively exposed to hog barn dust extract (DE)

or saline vehicle (Fig. 3.3.1a). Total immune cell influx to the airways was quantified from bronchoalveolar lavage fluid (BALF). As previously published, exposure to DE significantly induced the recruitment of cells into the airway compartment in WT mice, hallmarked by neutrophilia in BALF (Fig. 3.3.1b-e). Notably, cell influx following DE exposure was significantly diminished in Ffar4 KO mice compared to WT, independent of DHA administration (Fig. 3.3.1c; $P < 0.0001$). Analysis by 3-way ANOVA revealed significant main effects of genotype, treatment, and exposure. Further, Tukey's multiple comparisons calculated a significantly higher total cell count in WT than in KO in the vehicle supplement group (Fig 3.1c-d). Though total cell counts were altered, there was no significant change to cell differentials apart from significant induction of neutrophils in dust-exposed airways compared to genotype-matched saline controls (Fig. 3.3.1e; main effect of infection: $P <0.0001$). To address differences in airway compartment inflammation, perivascular inflammation was scored from hemotoxylin and eosin (H&E)-stained formalin-fixed, paraffin-embedded (FFPE) tissues using our previously published histopathology scoring techniques [35]. Dust exposure significantly induced perivascular inflammation with immune cell infiltrate in both WT and KO mice when compared to their genotype-matched saline controls, while DE-exposed KO when compared to DE-exposed WT mice had significantly higher perivascular inflammation scores (Fig. 3.3.1f; Adj. $P = 0.0127$ and <0.0001 respectively).

We additionally challenged these mice with an exposure eliciting a differently skewed inflammatory response of helminth infection with the rodent hookworm Nippostrongylus brasiliensis (Nb), which infects the lung and small intestine (Fig. 3.3.2a). The subsequent type-2 skewed immune response and lung epithelial damage causes M2-like (alternatively activated) macrophage polarization which contrasts with the M1-like macrophage polarization following DE exposure [36]. Like the DE model, Ffar4-deficient mice had significantly less immune cell

recruitment to the airway following helminth infection (Fig. 3.3.2b; Adj. P = 0.0294). Similarly, as well to the BAL data in the DE model, the infection promoted eosinophilia as expected in both genotypes (main effect of infection: P = < 0.0001), while the proportions of immune cells implicated in helminth infections (alveolar macrophages and eosinophils) were equivalent (Fig. 3.3.2d-e). Despite decreased airway cell recruitment, Ffar4-deficient mice exhibited improved anti- changes in present populations during immunogenic exposures, regardless of whether the stimulus induces a neutrophilic or eosinophilic response. helminthic immunity, with significantly reduced total worms counted in the intestines (Fig. 3.3.2f; P = 0.0006). Together these data reveal an important role of FFAR4 in airway total immune cell counts with no identified changes

present populations during immunogenic exposures, regardless of whether the stimulus induces a neutrophilic or eosinophilic response.

Figure 3.1

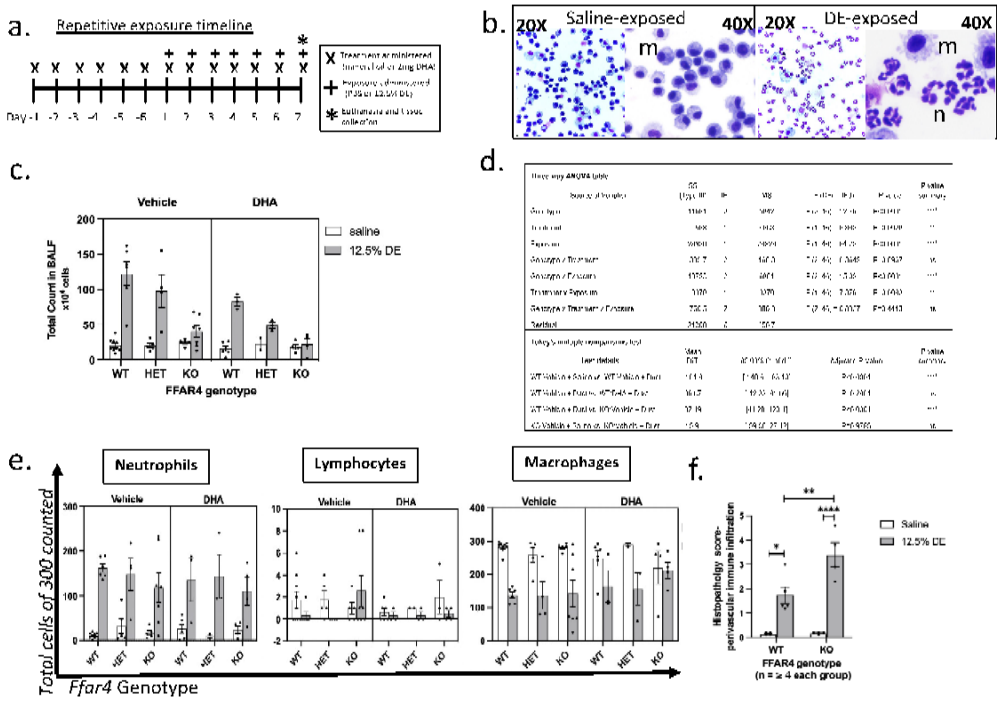

Fig 3.1. Significantly dampened immune recruitment in Ffar4-/- following repetitive pulmonary dust exposure with no changes to cell populations. a. A schematic of the experimental timeline of repetitive exposure with treatment. Mice were given 6-day pre-treatment of mineral oil or DHA by gavage, followed by 7-day concurrent treatment with exposures to saline or DE. Mice were euthanized 5 hrs after the final doses on day 7 of the exposure period. b. Representative DiffQuick-stained cytospins of BAL cells for saline and DE-exposed mice show macrophages (m) and neutrophils (n) in the airways (20X and 40X). Repetitive-DE exposure over 7 days significantly (three-way ANOVA followed by Tukey's post hoc test) induces BALF cell influx in an exposure and genotype-dependent manner (c. and d.). This influx is marked by neutrophilia in DiffQuick-stained-cytospins detrmined by cell differential analysis from masked samples (e). There were no significant changes in cell differentials between genotypes, though exposure induced neutrophilia (main effect of exposure: $P < 0.0001$). f. H&E stained FFPE lung sections were masked and scored for vascular inflammation. Inflammation was induced by DE exposure in both WT and KO compared to their genotype-matched controls (Adj. $P = 0.0127$ and <0.0001 respectively). Ffar4 KO mice had significantly higher inflammatory scores than WT (Adj. $P = 0.0095$). Experiments were run in triplicate (at minimum) with all groups represented in each separate experiment.

Figure 3.2

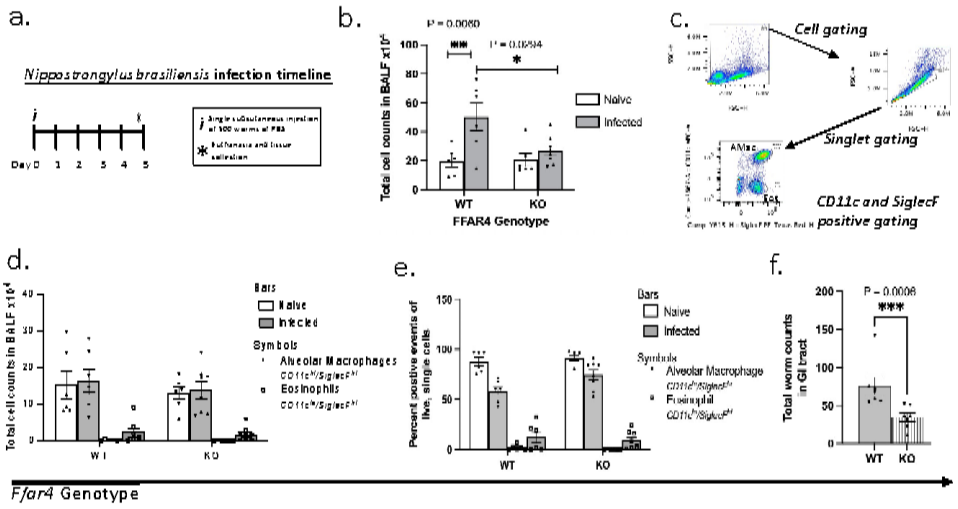

Fig 3.2 *Ffar4*-/- phenotype of dampened immune influx without changes to cell populations is confirmed in another model: *Nippostrongylus brasiliensis* (Nb) infection. a. A schematic of the helminth infection timeline. Mice were subcutaneously injected with 500 *Nippostrongylus brasiliensis* L3 larvae in 200 µL PBS or PBS only on day 0. Mice were euthanized on day 5 post-infection. b. Helminth-infected WT mice had significantly elevated cell counts in the airway compared to their naïve controls measured by total cell counts in the BALF, this effect was significantly dampened by Ffar4-deficiency which was comparable to naïve controls. c-e. BAL cell differentials, determined by flow cytometry, were not significantly altered by Ffar4 presence, though the infection significantly impacted cell differentials by promoting eosinophilia in the airways (two-way ANOVA, main effect $P < 0.0001$). Expressing as totals or percents did not alter this outcome. These data were generated from two separate experiments with representative groups included in each experiment. f. *Ffar4*-deficiency dampened the presence of worms in the GI tract, with significantly fewer worms counted from the jejunum of Ffar4-null mice.

3.3.2 Dysregulated epithelial homeostasis is exacerbated by DE exposure in Ffar4-null mice

To identify if DE-exposed mice had epithelial pathology, histopathological scoring was conducted on H&E stained-lung tissue from mice that were repetitively exposed to DE. Overall, Ffar4-deficient lung tissue had significantly dysplastic epithelium compared to WT mice (Fig. 3.3.3b-c; Adj. $P < 0.0001$). The average thickness of the epithelium of respiratory airways was significantly increased in DE-exposed Ffar4-deficient lung tissue compared to DE-exposed WT and naïve KO (Fig. 3.3.3d; Adj. $P = 0.0001$ and 0.0002 respectively). DE exposure significantly altered the airway epithelial area in WT and KO mice compared to their genotype-matched saline controls, with no significant differences determined between genotypes (Fig. 3.3.3e; Adj. $P = 0.0285$ and 0.004 respectively). To assess if the self-limiting nature of this known epithelial

disruptor is FFAR4-dependent, mice repetitively exposed to DE were given a 3-day-exposure-free recovery period before lung barrier integrity was assayed using a commonly employed epithelial permeability assessment with 70,000 KDa Rhodamine-B-conjugated dextran (RhoB), which was administered intranasally to assess pulmonary permeability [37]. WT mice had no difference in plasma RhoB levels between DE-exposed and saline control mice, while DE-exposed Ffar4-null had significantly elevated plasma fluorescence compared to DE-exposed WT mice (Fig. 3.3.3f; Adj. P = 0.0081). To supplement this functional data, we used a model of acute lung injury to directly challenge the epithelial response to chemical injury. Porcine pancreatic elastase (PPE) or vehicle saline was administered intranasally at 0.9-1.2 U. Ffar4-KO mice were significantly less likely to survive this acute epithelial injury model (Fig. 3.3.3h, Log-rank test: P = 0.0224).

Figure 3.3

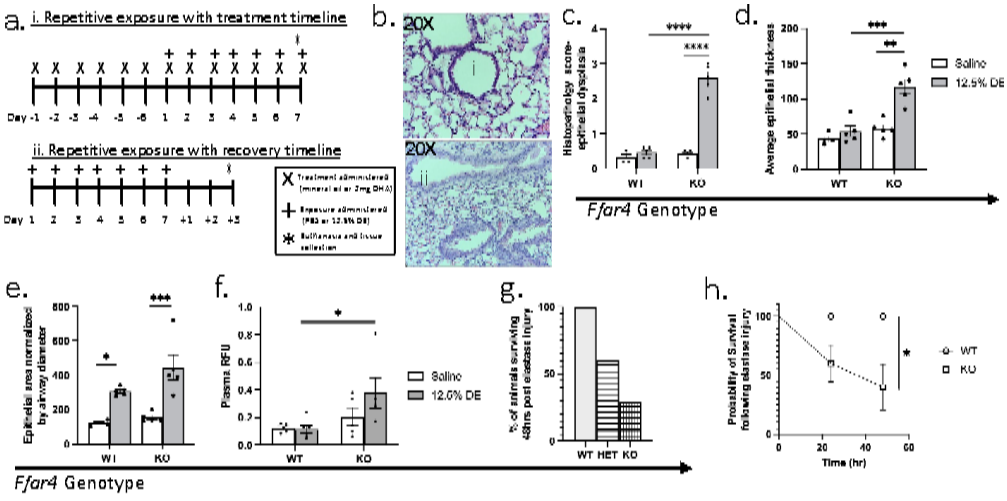

Fig 3.3. Lack of Ffar4 impairs airway epithelial barrier homeostasis. a. Schematic of repetitive exposure timeline without (i) and with (ii) the recovery period. The recovery studies did not include treatments, and mice were left to recover without exposure for three days. On the third day of recovery, 1 hour before euthanizing, mice were given intranasal RhoB-dextran in PBS. X's represent gavage treatments of mineral oil (vehicle control) or 2 mg DHA, plus-signs (+) represent the administration of intranasal exposure (PBS or 12.5% DE), and the asterisk (*) signifies euthanasia. If euthanasia falls on the day of an exposure animals were euthanized 5hrs after administration. b. H&E stained FFPE lung sections reveal dysplastic epithelial appearance (representative images of i. normal and ii. dysplastic epithelium, at 20X). Ffar4-deficient mice exposed to DE had significantly higher airway epithelial dysplasia scores compared to DE-exposed WT and Naïve KO (c., Adj. $P < 0.0001$ for each) and similar findings in quantified epithelial thickness (d., Adj. $P = 0.0001, 0.0002$ respectively). Airway area normalized to diameter was only significant in both dust-exposed WT and KO mice compared to respective genotype-matched saline controls (d., Adj. $P = 0.0285$ and 0.004 respectively). f. Using intranasal Rhodamine B dextran delivery, Ffar4-deficient mice had significantly greater plasma fluorescence following repetitive-DE plus three days for repair (Adj. $P = 0.0081$). g-h. Ffar4-deficient mice had significantly reduced survival in an acute epithelial injury model of a single dose of intranasal porcine pancreatic elastase (h., Log-rank test, $P = 0.0224$).

3.3.3 Hippo pathway as a potential mechanism of dysregulated immune recruitment and epithelial homeostasis

Among a multitude of diverse functions, the Hippo pathway regulates the stability of transcriptional co-activators yes-associated protein 1 (YAP) and transcriptional coactivator with PDZ-binding motif (WWTR1/TAZ). This well-conserved pathway is an important and well-established regulator of both lung epithelial homeostasis and the immune system [38]. In mammals, YAP signaling downstream of Hippo promotes inflammatory responses, including the bacterial response which dominates the swine dust exposure [39]. Further, GPCRs which signal through Gαq11 and tight junction proteins are established regulators of this pathway. Indeed, FFAR4 has been implicated as a regulator of the Hippo pathway in some cancers but there is little in the

literature regarding this synergism in the lung [39, 40]. Given the importance of YAP/TAZ signaling in lung epithelial homeostasis, the significant epithelial dysplasia with barrier and immune-response deficits in Ffar4-deficient mice led to the hypothesis of YAP/TAZ dysregulation in mice lacking Ffar4 [41, 42]. To investigate this, lung sections were taken from FFAR4-/- mice and wild-type littermates after exposure to 12.5% DE or saline control as described above. YAP and TAZ staining intensity was assessed semi-quantitatively across complete sections (Fig. 3.3.4), revealing a significant reduction of YAP intensity in the lungs of Ffar4-/- mice (2-way ANOVA; main effect of genotype, $P = 0.0424$). These findings are consistent with the impaired immune response, epithelial dysplasia, and barrier dysfunction suggesting a potential role of the Hippo pathway and/or the effector YAP in FFAR4-mediated epithelial homeostasis.

Figure 3.4

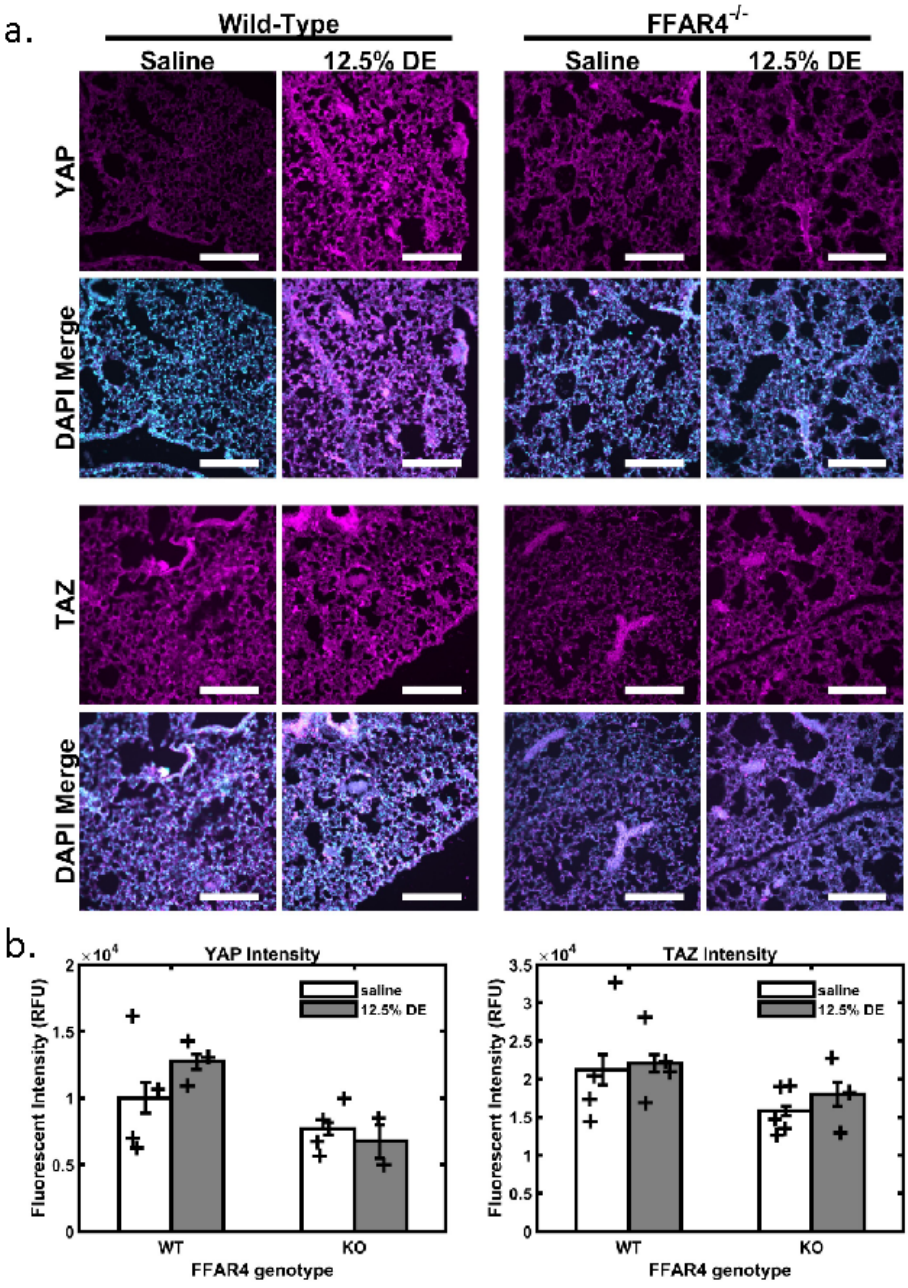

Fig 3.4. Lack of Ffar4 and reduced YAP signal in lung immunofluorescence. Immunofluorescence was performed on FFPE lung sections from mice repetitively exposed to 12.5% DE or saline (5µm sections imaged at 20X; scale bars are 200µm). a. Anti-murine YAP or TAZ antibodies were used followed by secondary antibody incubation, then counter-stained with DAPI. b. YAP intensity showed a significant main-effect of genotype (2-way ANOVA, $P = 0.0424$) while TAZ was not significant in these comparisons.

3.3.4 Genes related to the innate immune response and apoptosis are downregulated in Ffar4-deficient mice

We sought to examine gene expression changes that could corroborate the DHA-independent phenotypes (i.e. epithelial dysfunction, dampened immune infiltrate, and reduced YAP intensity by IF) from repetitive DE exposures in Ffar4 WT and KO mice using total lung RNA from vehicle-treated WT and KO mice repetitively studies exposed to saline or DE. Both wild-type and Ffar4-null mice exhibited upregulation of numerous immune-related genes following repetitive DE exposures as expected in the inflammatory model (Fig. 5a-b). DE-exposure significantly induced 51 differentially expressed genes (DEG) in the WT comparison and 34 in the KO comparison (Fig. 3.3.5 a. and b. respectively; Adj. $P < 0.05$).

Figure 3.5

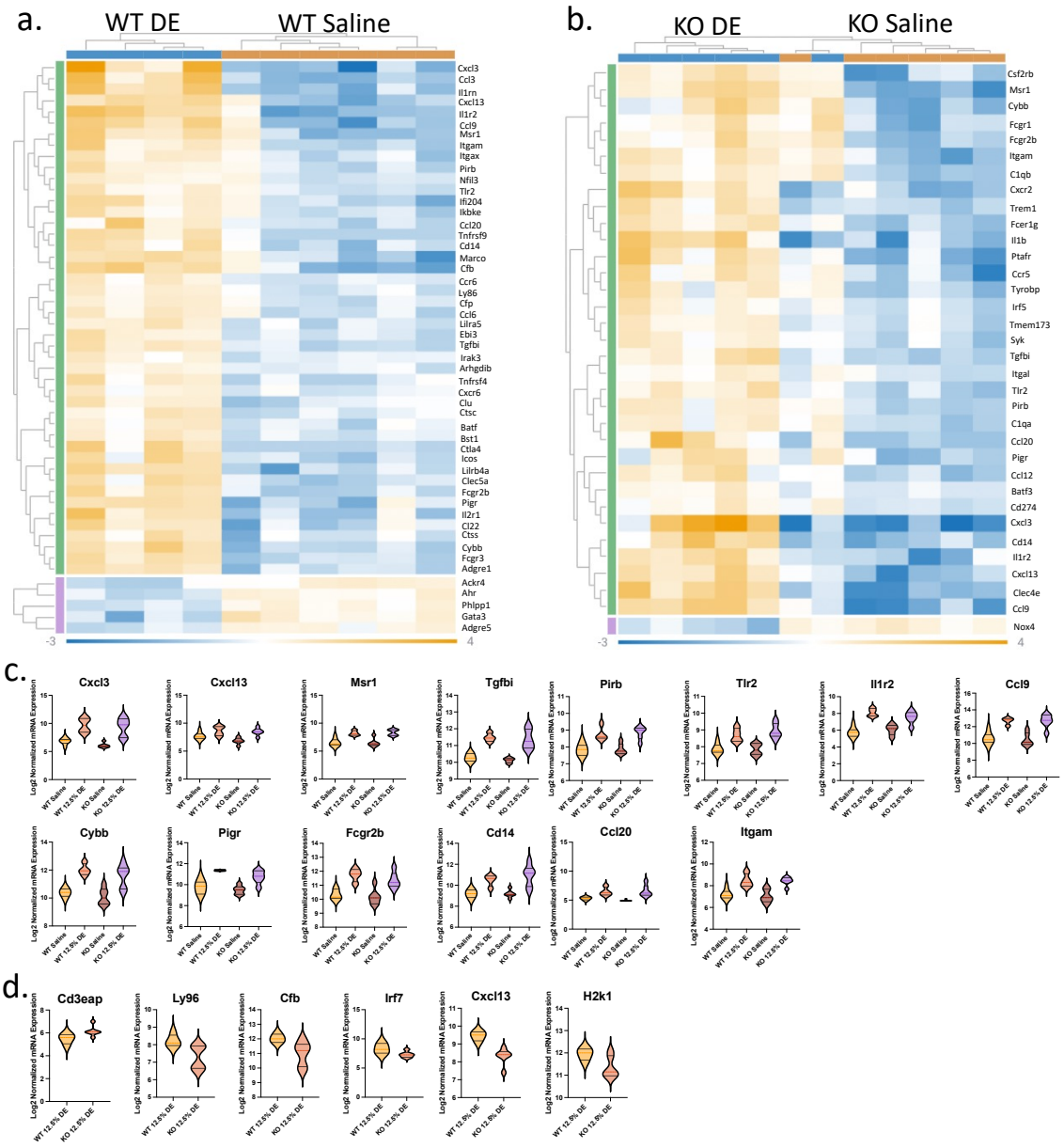

Fig 3.5. repetitively exposed mice. Total lung bulk RNA from mice repetitively exposed to 12.5% DE or saline was analyzed by the mouse immunology NanoString nCounter panel. Significant genes (Adj. $P \leq 0.05$) with a fold change of +/- 1.5x are reported as heat maps of differentially expressed genes (DEG) in DE-exposed WT mice compared to WT saline controls (a., 51 genes) or DE-exposed KO mice compared to KO saline controls (b., 34 genes). One of the KO DE samples clusters with the saline group which could be an artifact of poor intranasal delivery. Violin plots of the c. 14 DEGs overlapping in both DE-exposed WT and KO samples compared to their genotype-matched saline controls (Adj. $P < 0.05$, FC: +/- 1.5X) and d. 5 DEG between DE-exposed KO compared to WT ($P < 0.05$, FC: +/- 1.5X) are reported.

Most genes were upregulated following DE exposure, though there were a few genes downregulated in DE compared to saline groups (5 genes in WT and 1 in KO). The 5 DEGs which were downregulated in WT following DE-exposure include immunomodulatory genes (Ackr4, Ahr, Gata3), while Adgre5 affects cell-cell adhesions and Phlpp1 regulates mucous production. The down-regulated gene from the KO comparison group is Nox4 which plays a critical role in the generation of reactive oxygen species (ROS) from polymorphonuclear neutrophils, important in the progression of inflammation, particularly in this neutrophilic-dust-exposure response.

Of these DEG derived from each genotype comparison of DE-exposed to genotype-matched saline controls, 14 upregulated DEG were common between WT and KO comparisons (Fig. 3.3.5c). These 14 common DE-induced DEGs exhibit functions such as chemokine and interleukin signaling (e.g. Ccl20, Cxcl3, Cxcl13, Il1r2, Tlr2); LPS detection and signaling cascades (e.g. Cd14, Tlr2); and TLR4 signaling (e.g. Tlr2, Cd14, Itgam), among others. Comparing the genotype-specific responses in DE-exposure, Ffar4-deficient mice had 5 downregulated DEG compared to WT and 1 upregulated gene (Fig 3.5. d.). Uniquely expressed DEG by genotype were grouped according to pathway analyses to determine genotype-driven functional differences in the responses to dust (Table 3.1 a.-b.). DE-exposure in KO mice alters pathways involved in pyroptosis and necrosis which could implicate a role for FFAR4 in cell survival (Table 3.1b.). The pathway analysis from the genotype-specific responses reveals effectors of toll-like receptor (TLR) signaling cascades among the genes downregulated in KO lungs. Overall, the gene expression differences in dust-exposed WT and Ffar4-/- mice allude to a deficiency in key players of the innate immune response and differential responses to DE exposure.

Table 3.1	
a. Pathway Analysis of Unique DEGs from WT DE-exposed v. WT saline comparison	
Reactome Pathway Name	P-value
RUNX1 and FOXP3 control the development of Tregs	5.13E-05
Chemokine receptors bind chemokine	6.03E-05
Interleukin-10 signaling	5.40E-04
Alternative complement activation	2.24E-03
Activation of C3 and C5	2.24E-03
Interleukin-4 and interleukin-13 signaling	3.38E-03
b. Pathway Analysis of Unique DEGs from KO DE-exposed v. KO saline comparison	
Reactome Pathway Name	P-value
CLEC7A/inflammasome pathways	2.55E-06
Interleukin-10 signaling	2.74E-05
Interleukin-1 processing	3.06E-05
Pyroptosis	4.78E-04
C-type lectin receptors	1.04E-03
RUNX3 Regulates Immune Response and Cell Migration	2.12E-03
Regulated necrosis	4.76E-03
Dectin-2 family	7.14E-03
c. Pathway Analysis of DEGs in DE-exposed KO vs. WT mice	
Reactome Pathway Name	P-value
Activation of IRF3/IRF7 mediated TBK1/IKK epsilon	4.66E-04
TRAF6 mediated IRF7 activation TLR7/8 or 9 signaling	2.21E-03
TICAM1-dependent activation of IRF3/IRF7	4.42E-03
TRAF6 mediated IRF7 activation	8.81E-03
TRAF3-dependent IRF activation pathway	8.81E-03
Alternative Complement Activation	1.32E-02
Activation of C3 and C5	1.32E-02
DEx/H-box helicases activate type I IFN and inflammatory cytokines production	1.53E-02
MyD88 dependent cascade initiated on endoscope	1.94E-02
Toll Like receptor 7/8 (TLR7/8) Cascade	1.98E-02
TRIF-mediated programmed cell death	2.19E-02
Toll-like receptor 9 cascade	2.25E-02
MyD88-independent TLR4 cascade	2.34E-02
TRIF(TICAM1)-mediated TLR4 signaling	2.34E-02
TRIF-mediated programmed cell death	2.41E-02
Toll-like receptor 4 cascade	3.24E-02
Activation of IRF3/IRF7 mediated by TBK1/IKK epsilon	3.35E-02
Caspase activation via Death Receptors in the presence of ligand	3.48E-02
activation of TAK1 complex upon TLR7/8 or 9 stimulation	3.82E-02
TRAF6-mediated induction of TAK1 complex within TLR4 complex	4.00E-02
IRAK deficiency (TLR2/4)	4.17E-02
IKK complex recruitment mediated by RIP1	4.47E-02
Caspase activation via extrinsic apoptotic signaling pathway	4.69E-02
Heme signaling	4.91E-02

Table 3.1. Summary of pathway analysis findings using significant DEGs. Differentially expressed genes induced by DE-exposure which were unique in WT (a.) or KO (b.) compared to their genotype controls were input into REACTOME, an open-source pathway database. Only genes that were unique to WT or KO with an Adj. $P < 0.05$ and fold change of +/- 1.5X were used. c. DEGs in DE-exposed KO compared to WT samples with $P < 0.05$ and a fold-change of +/- 1.5X were input into REACTOME. Only significant pathways with a P-value < 0.05 and FDR < 0.1 are reported in this table.

3.4. Discussion

Lung tissue is a top expresser of the lipid-sensing GPCR, FFAR4, canonically involved in metabolic regulation and insulin sensitivity, but it also plays a role in PUFA-mediated anti-inflammation[3]. PUFAs, like the omega-3 fatty acid DHA, are well-established molecules in attenuating inflammation, tipping the balance towards the resolution of the inflammatory process. Our previous data have revealed that DHA dampens the inflammatory response to swine dust exposure, lessening the release of protein inflammatory mediators as well as the recruitment of inflammatory cells, namely neutrophils, to the lung [1, 2, 43]. The high expression of FFAR4 in the lung, and the fact that dietary lipids are first pumped through the pulmonary system before traveling to the liver, led us to hypothesize that DHA ligation of pulmonary FFAR4 is important in the observed dietary-DHA-attenuated pulmonary inflammation.

As previously characterized, DE exposure induced BAL neutrophilia independent of FFAR4 status, and there were no significant differences in recruited cell types determined from cytospins (Fig. 3.3.1c-e)[44-48]. While DHA did significantly dampens the dust-induced airway immune cell recruitment in wild-type mice within these current studies ($P<0.0001$), Ffar4-deficient mice had reduced total immune recruitment, independent of DHA administration, and which were like saline controls (Fig. 3.3.1b, Fig. 3.3.1d). It has been discussed in the literature that DHA can mediate anti-inflammatory effects independent of FFAR4-signaling, the means of which include: bioavailability of immune-related substrates; lipid raft modifications which interrupt immune signaling pathways; and DHA-derived mediators called specialized pro-resolving mediators (SPMs) like resolvins[32, 49-51].

These studies are in line with what we have previously published as treatment with DHA alleviated the inflammatory cell influx. Surprisingly, FFAR4 had a significant and independent effect on cell influx (Fig. 3.3.1d)[1, 2]. Entrapment of immune cells in the mesenchyme has been

demonstrated in Myd88-null mice repetitively exposed to DE [33, 43]. These published studies revealed no significant changes to cells in the BAL with significant histopathologic changes in the Myd88-deficient mice including immune cell entrapment and epithelial dysplasia. Similar outcomes were significantly altered in Ffar4-null mice in our studies, which also had significantly increased perivascular inflammation scores using blinded histopathology scoring (Fig. 3.3.1f). FFAR4 may regulate the migration of immune cells out of the vasculature and into the airway. Thus, we complemented these findings using an eosinophilic model of inflammation to evaluate the consistency of these data across different immune responses[52, 53]. Here, Nippostrongylus brasiliensis (Nb) infection was utilized to identify if the observed deficiency in FFAR4 with DE exposure was inclusive of other exposures promoting airway immune infiltration. Similarly, this eosinophil-skewed parasitic response, which significantly elevated BAL cell counts in wild-type mice ($P = 0.0060$), was also dampened by the loss of Ffar4 without changes to recruited cell populations (Fig. 3.3.2a, Fig. 3.3.2b-c respectively).

These findings corroborated the dysregulated immune cell presence in the airway as seen in the DE exposure model, regardless of the type of immune response elicited. What these data suggest is that a lack of Ffar4 plays a role in the migration of immune cells into the airway space. The expectation of anti-helminthic immunity, if simply considering the impaired immune recruitment, would be increased parasite survival in mice lacking Ffar4. Interestingly, the parasite burden was significantly lower in Ffar4-deficient mice (Fig. 3.3.2f), however, the lack of Ffar4 in the gut may differentially impact parasite survival in the jejunum, from which the worm burdens are quantified [54]. This is a limitation of studies utilizing global gene knockout strategies, however removing Ffar4 from the lung specifically would be challenging since its expression is across a number of lung-resident cells (i.e. pneumocytes, endotheliocytes, macrophages etc.) and

would require a number of cell-specific knockout mouse strains to identify which cells contribute to each phenotype.

From blinded histopathological scoring of lung epithelium, we identified significantly increased dysplastic appearance and airway epithelial thickening, resembling hyperplasia, in DE-exposed Ffar4-null airways compared to wild-type (Fig. 3.3b-d; $P = 0.0079$ and $P = 0.0079$, respectively). It has been demonstrated that silencing FFAR4 in intestinal cell lines resulted in increased cell proliferation [55]. The epithelial dysplasia phenotype from the histopathological analyses in our Ffar4-deficient mice could similarly be the result of increased proliferation of lung epithelial cells upon immunogenic activation, and more studies to examine this potential are warranted. The literature dictates that FFAR4-signaling dampens cell proliferation and maintains mucosal barriers, thus mice deficient in Ffar4 exhibit lung epithelial dysplasia possibly through unregulated cell proliferation, which could further translate to impaired barrier integrity [55, 56]. Of note, epithelial dysplasia (usually hyperplasia of certain cell populations) and increased permeability are identified in epithelial pathologic remodeling among individuals with COPD, a disease for which hog barn dust exposure is a risk factor [24, 57-59].

We sought to identify if the FFAR4-dependent epithelial dysplasia had functional consequences for barrier integrity. Exposures to airway immunogens like diesel exhaust, allergens, and environmental dust result in mucosal epithelial barrier dysfunction marked by increased permeability but are reversible upon successful epithelial repair mechanisms [60, 61]. Further, in vitro studies using human bronchial epithelial cells, grown in air-liquid interface, demonstrate increased dextran permeability following repetitive swine dust extract exposure [62]. Unlike in human exposures, which are linked to chronic pulmonary deficits, the DE dose used in our mouse exposure model is self-limiting by design and the lungs can recover under homeostatic conditions. Thus, we sought to clarify if this return to homeostasis is Ffar4-dependent,

particularly in the epithelial restoration of barrier function. In a pilot in vivo study, we identified that repetitive DE-exposure increased pulmonary epithelial permeability to intranasally delivered 70,000 KDa Rhodamine-B-conjugated dextran (RhoB70) (Supplementary S1). Similarly, previous data using intestinal epithelium have demonstrated that Villin-cre Ffar4-floxed mice had increased intestinal permeability and decreased expression of basement membrane-affiliated genes, suggestive of the role of FFAR4 in mucosal barrier integrity [55]. After implementing a three-day recovery period following repetitive DE exposures, we identified persistent barrier deficits among Ffar4-null mice that were not identified in the wild-type mice which restored barrier integrity by this time. Mice lacking Ffar4 had increased leakiness to RhoB70 measured as plasma fluorescence (Fig. 3.3f; $P = 0.0368$). Like data reported in the literature, Ffar4-expression in these mice impacts the efficient reestablishment of epithelial barrier integrity during this early time point of repair in our DE model. Further, tight junction proteins are known to mediate the transmigration of immune cells, and dysplastic epithelium with poorly assembled tight junctions could prevent the transepithelial migration to the airway compartment following immunogenic challenge [63]. This could be a contributing factor to the decreased cell counts in BAL contrasted to the histopathologic findings of increased immune infiltrate in the mesenchymal lung compartments for mice lacking Ffar4 (Fig. 3.3c-d).

We then assessed the functionality of the epithelium in an acute setting using porcine pancreatic elastase (PPE)-induced acute lung injury model. Mice partially or completely deficient in Ffar4 were less likely to survival during a 48 h timepoint while WT mice had 100% survival (Fig. 3.3 g-h). This data evidence the hypothesis of insufficient inflammatory activation and/or impaired epithelial integrity in the absence of Ffar4 which in the case of PPE injury, resulted in death[64]. Imperative to proper epithelial receptor signaling of membrane-associated receptors, such as through GPCRs like FFAR4, is the luminal and basilar polarization of lung epithelium.

The significant epithelial dysplasia and subsequent impaired polarization of these cells could contribute to inadequate membrane-receptor-mediated responses to stimuli and impaired immune cell interactions during migration to the airway. To explore a potential link to these mechanisms, we turned to the Hippo pathway. This pathway regulates cell proliferation and survival and is important in modulating pulmonary epithelial homeostasis. GPCRs, and particularly those signaling via Gαq11 like FFAR4, are known regulators of the Hippo pathway [65, 66]. Relevant to the DE-exposure model, TLR4 activation is upstream of the Hippo pathway and LPS has been shown to activate actuators of the Hippo pathway. Thus, the Hippo pathway plays a role in mediating TLR4-dependent immune response through lymphocyte trafficking, antigen recognition, as well as immune tolerance[39]. With these considerations in mind, we performed immunofluorescence labeling of hippo actuators YAP and TAZ on FFPE lung sections from repetitive dust exposures. A Ffar4-dependent decrease in YAP activity was quantified, which is compliments the epithelial deficits in this work due to the published role of decreased YAP leading to impaired epithelial repair ($p = 0.0427$, Fig. 5) [42, 67]. The decreased expression of YAP may be linked to the observed epithelial dysregulation, or the dampened immune response measured as recruited cells to the airway. Given the apparent role of YAP in lung injury response, decreased YAP expression may also contribute to the epithelial healing defects observed in Ffar4$^{-/-}$ mice; further work is needed to elucidate these mechanisms.

We next aimed to clarify potential gene signatures underpinning the immune and epithelial deficits identified in the absence of FFAR4 using an RNA panel tailored to immunological responses. These data provided insight into DE exposure- or genotype-related transcript alterations. Total lung tissue RNA from repetitively exposed wild-type and Ffar4-null mice revealed that DE exposure when compared to genotype-matched saline controls, resulted in the upregulation of several genes assayed and far fewer downregulated genes. This is in line with

expectations that an immunogenic stimulus, like swine dust extract, would promote the upregulation of inflammation and immune-related genes [68]. DE exposure in WT mice resulted in 46 and KO in 33 upregulated differentially expressed genes (DEGs). 14 DEG are shared among DE-exposed KO and WT samples, with upregulation of genes important in innate-immune recognition of DE components (Cd14, Tlr2, Pigr) and immune cell chemoattraction (Cxcl13, Ccl9, Ccl20, Il1r2, Itgam).

Significant pathways associated with these upregulated genes include arms of the innate and adaptive immune response, which occurs as the inflammatory response to repetitive exposures connects innate and adaptive immunity. The innate immunity pathways in DE-exposed WT mice include complement activation while the adaptive pathways include the regulation of control Treg development and IL-4/IL-13/IL-10 signaling. These phenomena in repetitive swine dust exposure are discussed in the literature from both in vitro and in vivo studies [34, 69-72]. The only overlapping pathway term in DE-exposed KO data is IL-10 signaling suggesting the transition from the acute response to chronic/adaptive response to DE mediating immune-regulatory signaling to control inflammation. The KO DE-response is marked by pathways involving inflammasome, pyroptosis, immune cell migration, necrosis, and IL-1 signaling. Inflammasome pathways, pyroptosis and necrosis are all related to cell death suggesting a cytoprotective role of Ffar4 in the inflamed lung, a role that is not well-characterized in publications. However, DHA-mediated cytoprotection and redox regulation has been explored and determined to have some dependence on FFAR4 [73-76]. These pathways may provide context for the lack of cell infiltration in the BAL, barrier deficits, and poor survival to acute lung injury among Ffar4-null mice in this work.

Another major difference in DE-exposed outcomes is complement activation pathways in WT but not KO. Complement activation is a characteristic response to swine dust exposure in

humans, both in vivo and in vitro [77]. Complement is an essential part of pathogen recognition and plays an important role in both induction of sufficient immune responses and pathogen clearance for adequate resolution [78, 79]. Of the 5 DEG comparing KO to WT DE-exposed, an important complement effector gene, Cfb, was downregulated in mice lacking Ffar4. This may contribute to a failure to sufficiently promote the alternative complement pathway and initiate a sufficient inflammatory response, seen as dampened immune infiltrate in the BAL. In addition, lower expression of Irf7, H2k1, Ly96, and Cxcl13 will also dampen the initial and sustained immune response following DE-exposure. It has been shown that LPS-challenged mice exhibit interferon response in an IRF7-TLR4 dependent manner with deficiency leading to dampened cytokine release [80]. H2k1 is critical in antigen processing and presentation via major histocompatibility receptor I (MHCI) in the progression of the inflammatory response. In addition, epithelial progenitor populations in the lung also express H2k1 and drive barrier restoration[81]. Lymphocyte antigen 96 (Ly96), also called myeloid differentiation factor 2 (Md2), dimerizes with TLR4 to recognize LPS and initiate MyD88 signaling cascades driving pro-inflammatory gene expression in response to swine dust exposure [33, 82, 83]. MyD88 KO mice repetitively exposed to DE exhibited significantly reduced chemokine and cytokine release, dampened BAL cell influx, and epithelial dysplasia. Though not as pathological a phenotype as in the MyD88 KO, this reduced Md2 and Irf7 expression could contribute to MyD88-dependent dampened BAL infiltrate and epithelial dysplasia in the Ffar4-null mice from our studies. Taken together these data reveal phenotypic findings which are corroborated by gene expression data in mice lacking Ffar4, such as immune response deficits, failure to survive, and impaired epithelial homeostasis.

These results broaden the scope for FFAR4-signaling and pose new questions as to its role in lung homeostasis. Chronic inflammatory diseases, like COPD and diabetes mellitus, are

hallmarked by altered lipoprotein profiles and lipid metabolism disorders causing cardiovascular comorbidities [84, 85]. High-fat diet and diabetic dyslipidemia induces atherosclerosis and lipoprotein imbalances in human and non-human studies [86, 87]. DHA is recommended clinically for dyslipidemia because it attenuates atherogenic lipoproteins and FFAR4 senses excess lipids in high-fat diet [5, 88]. Studies have shown a decrease in FFAR4 during heart failure with Ffar4-null mice exhibiting aberrant oxylipin transcriptomics during cardiac pressure overload. FFAR4-signaling was responsible for the maintenance of oxylipins in lipoproteins and may be a novel therapeutic strategy in the treatment of atherosclerosis [89]. Clinical trials targeting FFAR4 are abundant in the treatment of diabetes-related metabolic disorders highlighting the potential diverse utility of this receptor in the treatment of lipid- and inflammatory-related disease [90, 91]. The results of these studies must be taken within the context of global Ffar4 knockout mice such that it cannot be ignored that many of these effects may be the result of systemic metabolic changes in these mice. DHA as well as FFAR4 signaling regulate the availability of metabolites and lipid mediators that could impact the balance in the immune system and on the cellular level. This work underscores the need to study non-canonical roles of FFAR4-signaling on innate immune responses at mucosal barriers where Ffar4 is highly expressed. Future directions for this work include determining the pathway effectors for this FFAR4-mediated regulation of pulmonary immunity and epithelial homeostasis in order to identify the potential of FFAR4 therapeutic in the treatment of lung disease.

3.5. Materials and Methods

3.5.1 Ffar4 knockout mouse model

The knockout mouse model used in these studies was generated by Bjursell at al.[31]. As described in their publication, using mice of C57BL/6NCrl (Charles River), the Ffar4 gene was

removed and replaced by a LacZ reporter sequence. Animals were housed in a specific pathogen-free facility (12 h dark, 12 h light) with ad libitum access to standard chow. Heterozygous mice were used as breeders allowing for the comparison of littermates in these studies. Mice were used between the ages of 8-12 weeks of age and experiments were conducted with age, sex, and littermate-matched controls.

3.5.2 3R Statement

Mice were bred for use in specific experiments and multiple tissues were collected from experimental mice to reduce the number of animals used in these studies. All mice euthanized were used for experimental or training purposes to ensure efficient and limited use of mice. All experiments were performed with approval from the University of California (Riverside, CA) Animal Care and Use Committee (A-20210017; and A-20200014), in compliance with the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals.

3.5.3 Preparation of hog-barn dust extract (DE)

Settled dust was collected, at a height of approximately 1 meter, from within confined animal feeding facilities housing 500-700 swine, as previously described[92, 93]. Collected dust samples were stored at -20 °C, and extracts were periodically generated and stored as 100% stock. To prepare the 100% extracts, 5 g of settled dust was stirred into 50 mL of sterile Hank's balanced salt solution for 1 h at room temperature in a fume hood. The stirred solution was then transferred to a polypropylene 50 mL conical tube, centrifuged at 2500 rpm for 20 min and 4 °C. This step was performed twice, collecting only supernatant fractions after each centrifugation step. The supernatant fraction was then sterile filtered using 0.22 μm syringe filters and aliquoted in 1.5 mL microcentrifuge tubes. For mouse experiments, 12.5% DE was prepared from the 100% solution by dilution into sterile phosphate-buffered saline (PBS) and stored at -20 °C and thawed just

before use. All dust extract was generated from the same settled dust collection, therefore no batch effects are present.

3.5.4 Dust exposure model with exogenous DHA administration

This model has been utilized and previously published to study the impacts of inhaled exposure on murine respiratory responses[93]. DHA was administered as previously published; DHA (Cayman Chemical, Ann Arbor, MI) stock solution was prepared in mineral oil at 20 mg/mL[1]. Each mouse received a 2 mg dose of DHA delivered in a 100 μL bolus by oral gavage, daily for 12 consecutive days (6 days prior to DE exposure and 7 days concurrently). Following oral gavage on days 7+, mice were anesthetized under isoflurane using a small-animal anesthesia vaporizer set to 1.5-2% (v/v). Mice were determined to be anesthetized when there was a noticeable slowing of their breathing rate and a negative hind pedal reflex. Using a micropipette, 50 μL of well-mixed 12.5% DE or PBS was administered over both nares until fully inspired. Animals were held supine and angled upright for a few seconds and replaced in the cage as they began to awaken. The DE was administered once daily for 7 consecutive days, and animals were sacrificed 5 hours following the final DE dose. For the recovery studies, mice were not administered DHA or vehicle and were instead given a 3-day recovery period following the final dose of the 7-day DE exposure period. Mice in the recovery group were intranasally administered fluorescence-conjugated dextran, FITC 4 KDa (Sigma Aldrich, cat no. 46944), and Rhodamine B 70 KDa (Sigma Aldrich, cat no. R9379), at a concentration of 8 mg/kg of each conjugate, q.s. to 50 μL in sterile PBS. The dextran was prepared individually for each animal right before instillation and the animals were euthanized 1 hour after dextran administration. To achieve 85% power in detecting a 20% difference in histopathological changes, the least sensitive parameter in quantifying DE-induced outcomes, our power analyses determined 8 mice per group were needed

in these studies. Experiments were repeated 3-4 times with at least 3 animals from each group represented in every experiment, apart from elastase studies which were repeated twice.

3.5.5. Acute lung injury model

Intranasal delivery of porcine pancreatic elastase (PPE) has been used to model acute lung injury and emphysema[94, 95]. For these studies, mice were given a single instillation of 0.9 or 1.2 U PPE (Sigma cat. No. E7885) in PBS at 50 μL total volume.

3.5.6. Parasitic worm infection model

Nippostrongylus brasiliensis life cycle was maintained via rat infection and collected feces were cultured in vermiculture medium on humidified petri dishes. L3 larvae were gravity-extracted in saline from these plates on the day of infection. Mice were anesthetized by isoflurane and subcutaneously injected with 500-600 L3 worms in 200 μL PBS or PBS alone using a 22G needle (considered infection day 0), as previously published[53, 96]. Mice were euthanized for tissue collection on day 7 post infection.

3.5.7. In vivo outcomes

For in vivo outcomes, the experimentalist was masked to the genotype and treatment of the mice.

3.5.7.1. Plasma: Upon euthanasia of the mice, 250-500 μL of blood was collected from the renal artery using a 25-gauge needle and transferred to a lavender-capped blood collection tube. Blood was centrifuged for 15 minutes at 2,000 x g and plasma was transferred to a microcentrifuge tube and stored at -80 C.

3.5.7.2. Bronchoalveolar lavage (BAL): A cannula was placed into the trachea, near the hyoid, and tied off with suture string to avoid movement (BD, cat no. 381434). For BAL washes, 1 mL

of ice-cold PBS was slowly administered through the cannula and slowly withdrawn. The first wash was reserved for cytokine analyses by enzyme-linked immunoassays (ELISA), while the remaining two washes were stored together in a separate tube. All tubes were centrifuged at 1200 rpm for 5 min at 4 °C to pellet the airway cells, and the supernatant fraction of the first tube was aliquoted to store at -80 °C for use in ELISA. All three cell pellets were combined and treated with red blood cell lysis buffer. A total cell count was generated manually by hemocytometer using masked sample IDs, counted twice, and averaged on the day of euthanasia. Cytospins were prepared from 100,000 cells for each BAL sample and were stained by Diff-Quick and coverslipped with a toluene-based mounting medium. The cytospins were imaged (5 images per slide), and a square box of the exact same size was placed centered on each image. 300 cells were counted from within the boxes and marked as either macrophages, neutrophils, eosinophils, or lymphocytes to determine cell differentials of airway cells.

3.5.7.3. RNA: Following BAL fluid collection, the left main bronchus was tied off with a suture and the left lung was cut away, placed in a 1.5 mL microcentrifuge tube and flash frozen in liquid nitrogen. Frozen lung was crushed into a powder using a liquid nitrogen-cooled mortar and pestle (BelArt Cat. no. H37260-0100). 100 μg of tissue powder was used for RNA isolation with commercial RNA isolation kits (Invitrogen Purelink).

3.5.7.4. Lung histology: The right lung and trachea were excised, inflated with 400 μL of 10% buffered formalin, and transferred to an apparatus to finalize the inflation of the lungs at constant pressure (20 cm). The lungs were trimmed and transferred to cassettes stored in EtOH. The cassettes were shipped to the University of California Irvine (UCI) Experimental Tissue Resource facility where they were paraffin-embedded. We obtained sections that were H&E-stained by UCI for histopathological analyses.

3.5.7.5. NanoString nCounter transcript analyses

RNA was prepared from flash-frozen, powdered left lung using commercial RNA kits as described above. Initial concentration and purity were determined using a Nanodrop. RNA was cleaned up with additional on-column washes when contamination persisted. A second concentration was determined using a Qubit and then samples were delivered to the Genomic Core Facility at UC Riverside for bioanalyzer analyses for sample QC prior to nCounter→ analysis. Qubit values for concentration were used to determine nCounter→ panel load volumes, adjusting for poorer quality RNA determined by bioanalyzer. Samples were prepared with the Mouse Immunology V2 code set from NanoString per the manufacturer's protocols, hybridized for 16 hours, and left in the final 4 C cycle for no longer than 30 minutes. The nCounter→ runs were completed per manufacturer instructions using an nCounter→ SPRINT Profiler, with an N of at least 3 per group and as previously published [97]. Normalization was completed using nSolver 4.0 Data Analysis software with default settings, except that the background thresholding was set to 30 for these analyses. Heatmaps were generated using the ROSALIND™ online analysis platform (www.rosalind.bio) with gene cutoffs set to $P < 0.05$ and fold-change of + or – 1.5 times. Differentially regulated genes that were statistically significant were input into REACTOME (www.reactome.org), an open-source pathway database, for gene pathway analyses[98, 99]}. Only pathways with a $P < 0.05$ and FDR < 0.1 were reported.

3.5.8. Histopathological scoring of FFPE lungs

3.5.8.1. Airway dysplasia scoring: Hematoxylin and eosin-stained slides were blinded at the start of the scoring period. Slides were first scanned on 4X and 20X and notes were taken on each of the lung compartments (pleura, conducting airway, alveolar, and vascular compartments). Prominent changes to airways were noted and airways with complete ring-cross-sectional

structures were analyzed to avoid artifact of sagittal airway cuts and branching. Published examples of airway dysplasia were referenced and images were taken from the sample slides to establish an in-house reference of scores from 0-5[100]. Slides were then scanned after establishing the scoring criteria, and a score was given to 5-7 individual airways per slide and averaged for the final dysplasia score.

3.5.8.2. Airway area calculations: Blinded images were taken at 20X of airways that were complete (forming a circle) to be used in these analyses. The basement membrane border was traced in ImageJ to represent the outer airway circumference[101]. The total area measurement was taken, and the luminal circumference was selected giving luminal area measurements. Subtracting the luminal area from the total area gave the representative epithelial area. Using the outer area, we calculated the predicted diameter to normalize the epithelial area to airway diameter.

3.5.8.3. Airway thickness calculations: From the same blinded images, the thickness was measured as a linear length in ImageJ at three randomly selected points of each airway. These points were averaged across 5-7 airways to generate an average airway thickness for each sample.

3.5.8.4. Perivascular Inflammation scoring: Blinded slides were scanned for vascular inflammation and the presence of immune cell aggregates and diffusion surrounding medium to large vessels. The mice were not perfused, so vessels were easily identified by the presence of erythrocytes and surrounding smooth muscle cells. As before, images were taken to establish a scoring reference from 0-5 based on the thickness of immune cells surrounding the airway and the occlusion of vessels with immune cells. 10 medium to large vessels were scored per sample and averaged to generate a score per animal.

3.5.9. Immunofluorescence & Microscopy

FFPE slides were deparaffinized by xylene, rehydrated in decreasing percent ethanol solutions, followed by heat-induced epitope retrieval with a tris-EDTA solution for 20 minutes (10mM Tris, 1mM EDTA, 0.5% Tween-20, pH 9.0, heated at 100C for 20 minutes). Slides were then treated with blocking buffer for 1hr and stained with anti-YAP (1:500, rabbit polyclonal, 13584-1-AP, Proteintech Group, Rosemont, IL) or anti-TAZ (1:500, rabbit polyclonal, 23306-1-AP, Proteintech Group) antibodies diluted in blocking buffer overnight at 4 °C. Blocking buffer consisted of 1% bovine serum albumin (ThermoFisher Scientific, Waltham, MA), 0.2% cold-water fish gelatin (Sigma-Aldrich, St. Louis, MO), 0.1% Tween-20 (ThermoFisher Scientific), and 0.1% sodium azide (Sigma-Aldrich) in PBS. Slides were washed three times in PBS and stained with Cy5-conjugated goat anti-rabbit (1:500, 072021506, KPL) diluted in blocking buffer for 2hrs and counterstained with DAPI in mounting media (Vector Laboratories VECTASHIELD Antifade Mounting Medium with DAPI, UX-93952-24). Lung sections were imaged on a Leica DMI8 automated microscope using a DFC9000 sCMOS camera and a 20X Plan Apochromat objective (Leica, Buffalo Grove, IL). For each tissue, a total area of approximately 5.4 mm 2 was imaged as a tilescan. Each tilescan was stitched into a single image using a custom MATLAB (MATLAB 2021a, Mathworks, Natick, MA) implementation of the Phase Correlation Method previously described[102]. Images were analyzed for YAP and TAZ expression using a semi-automated segmentation. Briefly, a masked observer manually annotated images to remove large artifacts or non-lung tissue. Following this the DAPI channel was filtered using a 3×3 median filter (1.95 μm ×1.95 μm) and a 9.75 μm radius rolling ball filter. A nuclear mask was then segmented from the background using global binary thresholding. Each image and segmentation were manually checked by a masked observer. The fluorescent staining intensity of YAP and

TAZ was averaged over the nuclear mask. To remove variable background from the imaging, background subtraction was performed with a 650 μm radius rolling ball filter.

3.5.10. Statistical analyses

Statistical analyses were performed using Graphpad Prism Version 9.4.1. For bar graphs with error bars, the standard error of the mean (SEM) was used. Ordinary two- and three-way ANOVA analyses were fit to a full model, followed by Tukey's post hoc test, and reported with multiplicity-adjusted P-values. Mann-Whitney analyses were used for intestinal worm burden data. The α-cutoff was set at 0.05 for all analyses.

SUMMARY FIGURE 3.6

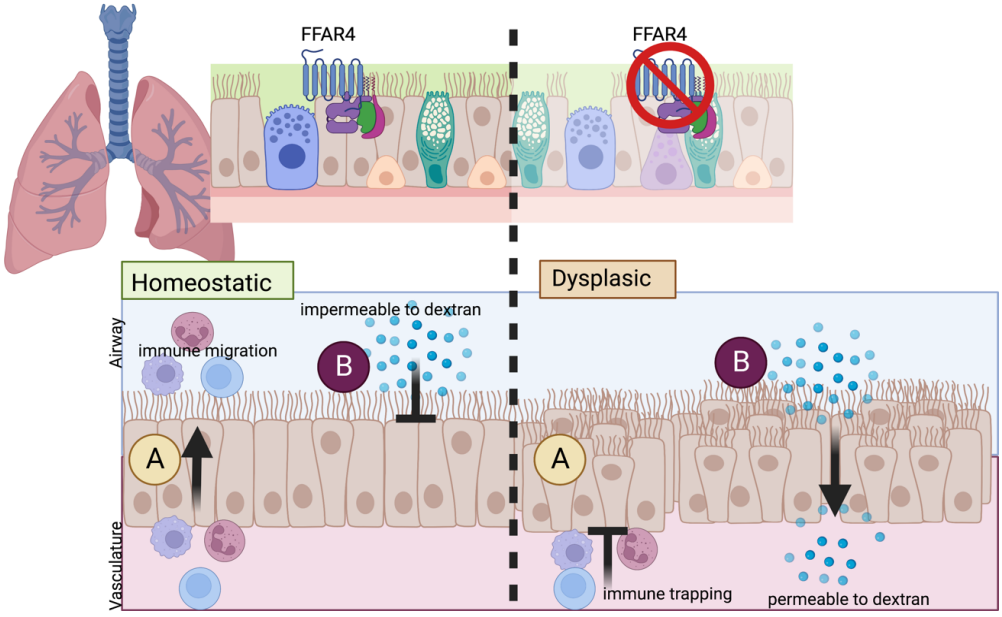

Figure 3.6 In the presence of FFAR4 expression, epithelium is homeostatic and allows transepithelial migration of immune cells while maintaining impermeable barrier. Without FFAR4, dust exposure causes dysplasia. The dysplastic epithelium does not allow immune cells to traffic and becomes permeable to dextran. Created with "biorender.com"

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CHAPTER FOUR

Delineating the effect of cell-specific RELMα expression in shaping the chronic immune response to lung helminth infection and promoting lung tissue repair

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A version of this chapter will be submitted for publication.

4.1. Abstract

Soil-transmitted helminth (STH) infections impact billions of individuals globally, however, there is a need for clarifying the long-term impacts of these infections on pulmonary health due to their transient migration through and subsequent damage to the lungs. In mouse models of these infections using Nippostrongylus brasiliensis, lung pathology progressively worsens at later time points post-single infection. These studies also indicate persistent transcriptional expression of resistin-like molecule α (RELMα), an immunomodulatory protein characteristic of type 2 immunity and alternatively activated macrophages. Using complete and cell-specific gene knockout mouse strains, we identified RELMα protein remains elevated at day 30 post-infection and alters the immune cell signature in lung compartments. Further, histopathological assessment of alveolar damage paired with acellular scaffold in vitro studies reveal a role for RELMα in tissue repair, highlighting the importance of persistently elevated RELMα both systemically and within the lung. These findings underscore a need to identify the long-term impacts of helminth infection on human pulmonary disease, particularly as alveolar destruction can develop into chronic obstructive pulmonary disease (COPD), which remains among the top global causes of death. Translation of these findings to analogous human protein resistin presents therapeutic opportunities in lung repair.

4.2. Rationale

Soil-transmitted- helminth (STH) infections impact 24% of the world's population, particularly among the poorest and most vulnerable communities, and are considered a neglected tropical disease (NTD) [7{Brooker, 2006 #159]}. The host life cycle of these STH begins with infection by larvae in feces-contaminated soil, and the larvae of human hookworms *Necator americanus* and *Ancylostoma duodenale* migrate through the lung to be coughed up and

swallowed into the gastrointestinal tract [26]. The transient migration of these hookworms through the lung results in chemical and mechanical injury to the vasculature and pulmonary epithelium, the long-term implications of which are not well characterized in human infection. However, mouse models of these infections, using the hookworm *Nippostrongylus brasiliensis*, have indicated progressive long-term lung pathology, resembling emphysema, following a single infection *with N. brasiliensis*[8].

N. brasiliensis transiently migrates through the lungs days 2-4 post-infection. We and others have reported infection-induced hemorrhaging in the lungs, eosinophilia, alveolar destruction, and upregulation of the immunomodulatory protein resistin-like molecule α (RELMα) during these early time points which may parallel lung damage to that of human infection [25]. RELMα is a major type 2 effector protein and marker of alternatively activated macrophages and is upregulated in the airway, measured by bronchoalveolar lavage, and systemically in the serum within the first week of infection.The host immune response to these parasites is dominated by Type 2 immunity, airway eosinophilia, and alternatively, activated macrophage populations that clear the parasites and promote tissue repair. Papers delineating the progressive development of emphysema post-infection also demonstrate continuous expression of *Retnla* transcript at these timepoints [8]. Studies suggest a role for RELMα in tissue repair (promoting angiogenesis, proliferation, fibroblast differentiation and collagen crosslinking), though the function of RELMα during the later phases post-infection needs further clarification[24, 27].

Based on these prior studies, the focus of this project is to elucidate the cellular source of persistently elevated RELMα and the impact on emphysematous lung pathology using three *Retnla* targeted gene knockout mouse models: whole-body *Retnla* knockout, as well as CD11c+ and CC10+ cell-specific cre-recombinase knockout systems, which target macrophage and lung

epithelial contributors of RELMα respectively [28-30]. At day 30 post single infection with *N. brasiliensis*, we analyzed the RELMα-dependent responses contributed by peripheral and resident cellular populations with the goal of identifying if RELMα has a tissue-protective role in progressive helminth-induced alveolar destruction. We employed an ex vivo repair model using acellular scaffolds from infected and naïve lungs of both WT and KO mice to assess if RELMα is altering epithelial repair via the ECM. These studies revealed cell-intrinsic effects of RELMα expression of peripheral and resident macrophage populations as well as impacts on lung histopathological outcomes and extracellular matrix-epithelial interactions. These studies revealed cell-intrinsic effects of RELMα expression of peripheral and resident macrophage populations as well as impacts on lung histopathological outcomes.

4.3. Results

4.3.1. Cell-specific deletion of RELMα from myeloid cells versus epithelial cells differentially impacts RELMα protein secretion in the alveolar spaces and the blood.

RELMα is expressed by macrophages and lung epithelial cells at day 7 post-infection shown by immunofluorescent staining in the airway and mesenchyme of lung sections (Fig 4.1a). We validated the effectiveness of our complete, myeloid ($CD11c^{Cre}$), and epithelial ($CC10^{Cre}$) knockout systems using a variety of methods (i.e. immunofluorescence, intracellular staining flow cytometry, and ELISA; Fig 4.1b). $CC10^{Cre}/Retnla^{fl/fl}$ were validated by immunofluorescent staining of the airways with fluorescent quantification revealing a significant reduction in RELMα fluorescent intensity at day 7 of infected $Cre^{+/-}$ mice compared to $Cre^{-/-}$ (Fig. 4.1c). $CD11c^{Cre}/Retnla^{fl/fl}$ were validated for RELMα-deficiency by intracellular flow cytometry which demonstrated a reduction in the frequency of $RELM\alpha^{+}CD11c^{+}$ cells which was mirrored by a quantified reduction in mean fluorescent intensity (MFI) for RELMα. We generated constitutive knockouts, $Retnla^{Td/Td}$, as we have previously published which were validated for these studies by undetectable limits in RELMα sandwich enzyme-linked immunosorbent assay (ELISA, data not

shown)[28]. The cell-specific knockouts allowed us to clarify if it is epithelial or myeloid cells responsible for maintaining RELMα levels in the peripheral and lung-specific compartments at the later timepoints post infection (Fig 4.1d). We found $CC10^{Cre+}/Retnla^{fl/fl}$ mice demonstrated reduced RELMα concentration in bronchoalveolar lavage fluid (BALF), with unaffected serum levels. Conversely, $CD11c^{+}$ cells are responsible for maintaining serum RELMα levels, with $CD11c^{Cre+}/Retnla^{fl/fl}$ mice demonstrating significant reductions in RELMα levels within serum but not BALF. Further, in addition to RELMα expression being maintained in the lung, as previously published by Marsland et al., we also identified RELMα protein concentration remains elevated in the blood [8]. Thus, RELMα concentration is maintained by specific cell populations in a compartment-dependent manner, with lung localized RELMα largely mediated by $CC10^{+}$ lung epithelial cells both at day 7 and day 30.

Figure 4.1

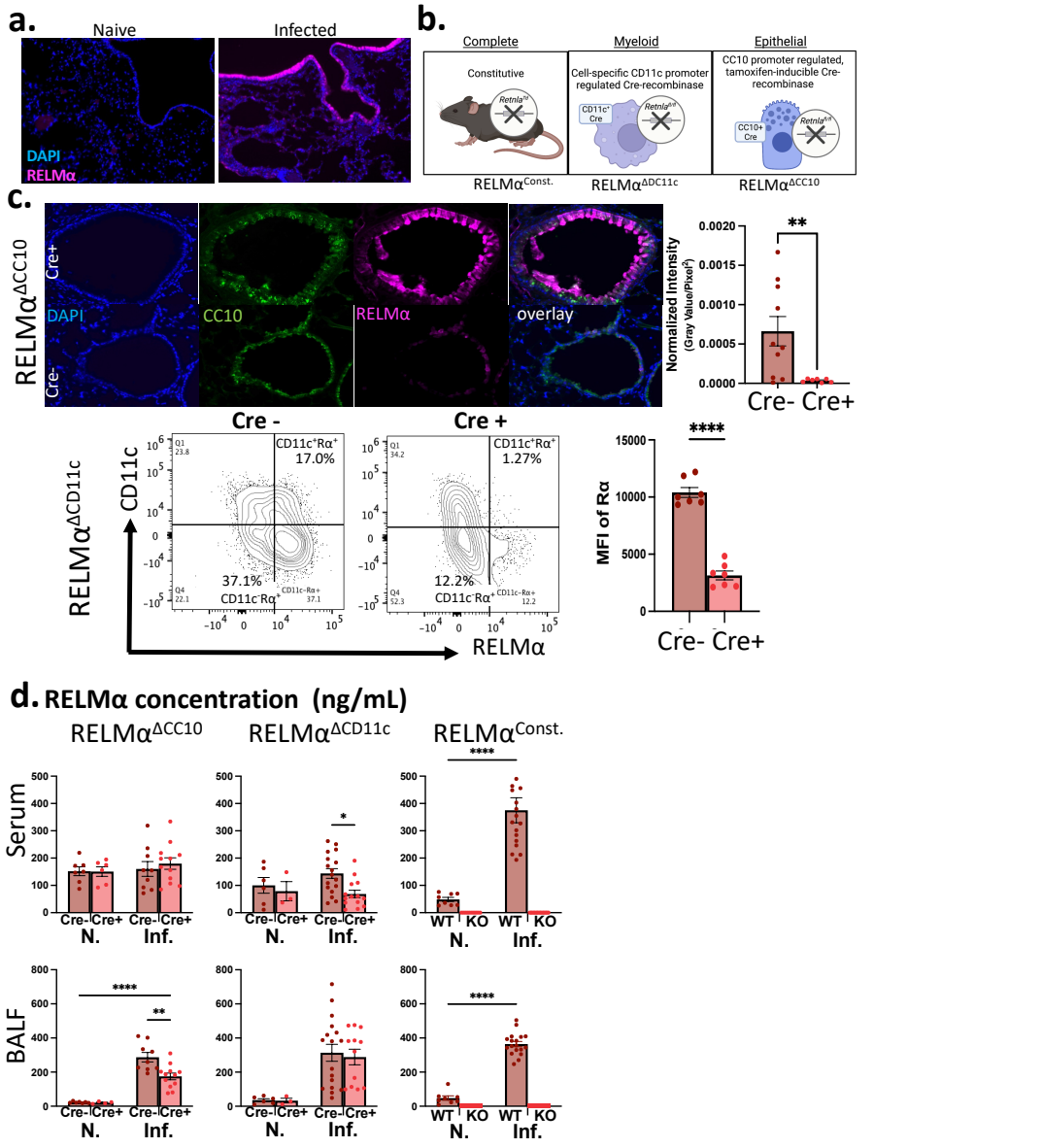

Figure 4.1. a) Immunofluorescent images of cryo-sectioned lung, labelled with anti-murine RELMα and counter-stained with DAPI, demonstrated expression of RELMα in the epithelium and mesenchyme of the lung at day 7 post-infection (5µm, 10Xobj.). **b**) Schematic of knockout strategies for the transgenic mice used in this study (created with biorender.com). **c**) Immunofluorescent labelling for CC10 and RELMα was performed on cryo-preserved lung and airways were quantified to reveal a significant decrease in RELMα intensity (5µm, 10X obj., quantified on ImageJ). Extracellular and intracellular flow staining was performed on lung cell homogenate to demonstrate a reduced frequency of $CD11c^{+}RELM\alpha^{+}$ populations and a significant reduction in RELMα expression among $CD11c^{+}$ myeloid cells. **d**)Sandwich ELISA for murine RELMα was performed on BALF and serum which reveled compartment-specific contributions of myeloid and epithelial RELMα secretion.

4.3.2. Distinct effects of complete versus cell-specific deletion of RELMα on peripheral and resident cell populations in the pulmonary compartment

Alveolar macrophage (AM) populations consist of a tissue-resident (TR-AM) and myeloid-derived (mono-AM) repertoire, with tissue-resident deriving from fetal liver and yolk sac while the myeloid derived are recruited from the blood (Fig 4.2a). AM are $CD11c^{hi}SiglecF^{hi}Mertk^{hi}$ while expression of MHCII distinguishes TR- from mono-AM ($MHCII^{lo}$ and $MHCII^{hi}$, respectively, Fig 4.2a-b). Reduction analysis using tSNE plots of representative experiments reveals subtle differences visually (Fig. 4.2c). The frequency of AM and mono-AM as a percent of live cells was quantified for each transgenic system to clarify RELMα-dependent maintenance of AM populations (Fig 4.2d). In $RELM\alpha^{\Delta CC10}$ and $RELM\alpha^{\Delta const}$, where lung RELMα is decreased, there were reduced frequencies of AM and mono-AM in the lung of infected mice compared to their naïve controls, suggesting deficits in regaining AM homeostasis at day 30 post infection. Constituitive deletion of RELMα resulted in reduced mono-AM in naïve mice which was exacerbated by infection. No differences were detected between infected RELMα-deficient mice or their respective wild-type counterparts.

Figure 4.2

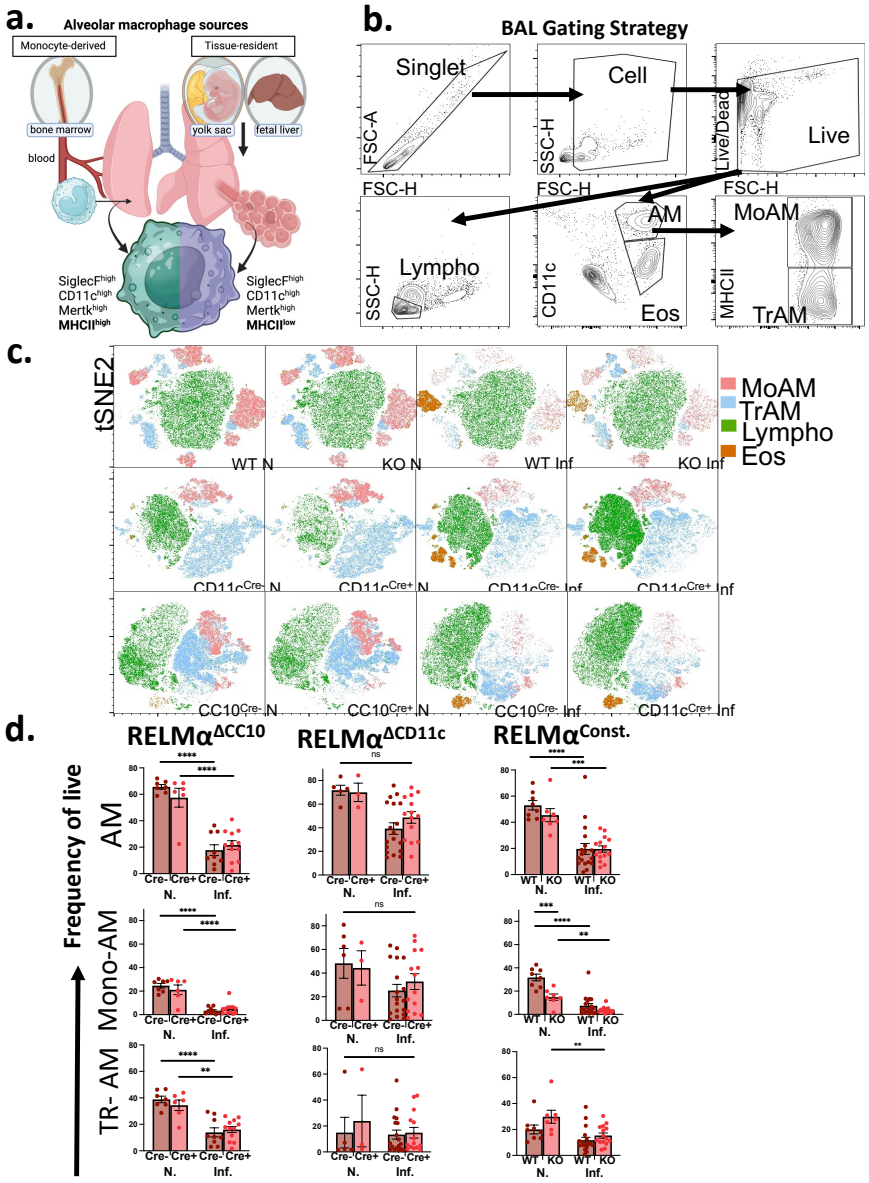

Figure 4.2. a) Schematic outlines the distinct sources of alveolar macrophages and their distinguishing surface marker profiles. (created with biorender.com) **b**) Gating strategy for BALF and subsequent tSNE (**c.**) and frequency (**d.**) analysis of BALF populations. Data is plotted as mean with error bars as standard error mean. Analyzed by two-way ANOVA with Tukey's post hoc. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, ****$P \leq 0.0001$. Experiments run in duplicate or more, with at least three biological replicates representing each group in every experiment.

4. 3.3. Both epithelial and myeloid-derived RELMα are sufficient to promote lung tissue repair following helminth infection

Lungs were inflated with formalin at constant pressure to ensure physiological airspaces for comparison across mice of variable lung volumes prior to paraffin embedding (Fig 4.3a). Histopathological scoring of hematoxylin and eosin (H&E) stained lungs was performed on blinded slides to assess the role of RELMα expression on alveolar destruction following helminth infection (Fig 4.3b). When compared to naïve controls, infection resulted in higher scores for alveolar destruction regardless of RELMα status. Comparing the RELMα-deficient mice to their respective controls, we identified a RELMα-protective role in this destruction. While knocking out RELMα from either $CC10^{+}$ or $CD11c^{+}$ cells alone was insufficient to result in statistically significant changes, complete deletion of RELMα resulted in worse alveolar destruction at day 30 post-infection, in line with previous studies conducted at earlier time points which suggest a tissue-protective role for RELMα in helminth-induced lung damage [27].

Figure 4.3

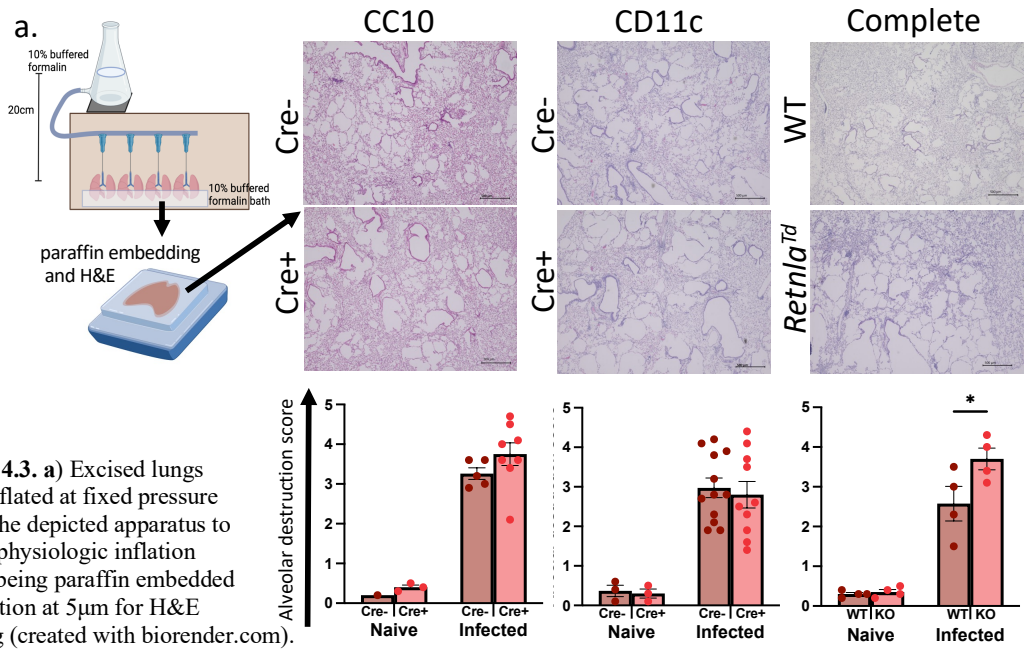

Figure 4.3. a) Excised lungs were inflated at fixed pressure using the depicted apparatus to ensure physiologic inflation before being paraffin embedded and section at 5µm for H&E staining (created with biorender.com). **b**) Representative brightfield images at 4X of infected lung sections from each genotype are shown here. Below each figure, data is plotted from the blinded histopathology analysis of alveolar destruction. The whole lobe was scored in 5 sections and an average of these scores is reported per animal. Graphs are plotted as mean and error bars at the standard error mean. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, ****$P \leq 0.0001$. Experiments run in duplicate or more, with at least three biological replicates representing each group in every experiment.

4.3.4. Infection changes extracellular matrix interaction with epithelial cell line using ex vivo wound repair assay and acellular scaffolds

At later timepoints post-single infection with *N. brasilinesis*, there are gross alterations to the lung appearance, with infected lungs marked by enlarged, nodular lobes (Fig 4.4a). Stitched images qualify the diffuse alveolar destruction of H&E stained FFPE lung sections. Using 20X images taken of the alveolar spaces, mean linear intercept was determined by semi-automated quantification methods using an ImageJ plugin devised by Nolan et al[31] . An increasing chord length suggest further distances between tissue-intersection, demonstrating a loss of alveolar surface area. Using these methods, infected mice had significantly longer average chord lengths than naïve, which suggests an emphasematous-like pathology post-infection as previously described by others.

We sought to determine if RELMα deficiency during the infection recovery period affects the extracellular matrix (ECM) either directly through its role on collagen cross-linking, or indirectly via RELMα-responsive cells with ECM remodeling roles, which could contribute to the lung repair process via epithelial-ECM interactions[24, 32]. Decellularized lungs from $Retnla^{WT}$ and $Retnla^{Td}$ mice, both naïve and infected, were inflated with agarose for vibratome sectioning at 300μm thick for in vitro re-epithelialization (Fig. 4.4b). A mouse lung epithelial cell line (MLE-12-CRL2110) was plated onto each scaffold to assess effectiveness of re-epithelialization over a period of 6 days. Hoescht staining of scaffolds revealed epithelial integration onto each scaffold type, and alveolar destruction on the infected scaffolds was also apparent (Fig 4.4c). Nuclei counts were performed on 4X images of the scaffolds and WT and KO infected compared to WT naïve showed significantly reduced average nuclei counts, demonstrating an infection-associated decrease in scaffold-associated cell adherence to and/or proliferation on the scaffolds. There also seems to be a potential nonsignificant deficit in the KO naïve which may result in no significance with the infected groups. Cellular ATP was assayed and revealed a significant reduction in ATP from KO infected scaffolds when compared to both WT and KO naïve. This reveals that infection in the presence of RELMα deficit exacerbates cell viability and/or metabolic activity estimated by ATP. To normalize for potential differences in scaffold protein content between the groups, protein was extracted from each scaffold following lysis of cells during the ATP assay, and using these methods there were no significant differences comparing the scaffolds used in these studies (Fig 4.4d). Further, acellular lung was assessed for soluble collagen content which also did not reveal any significant differences among groups.

Figure 4.4

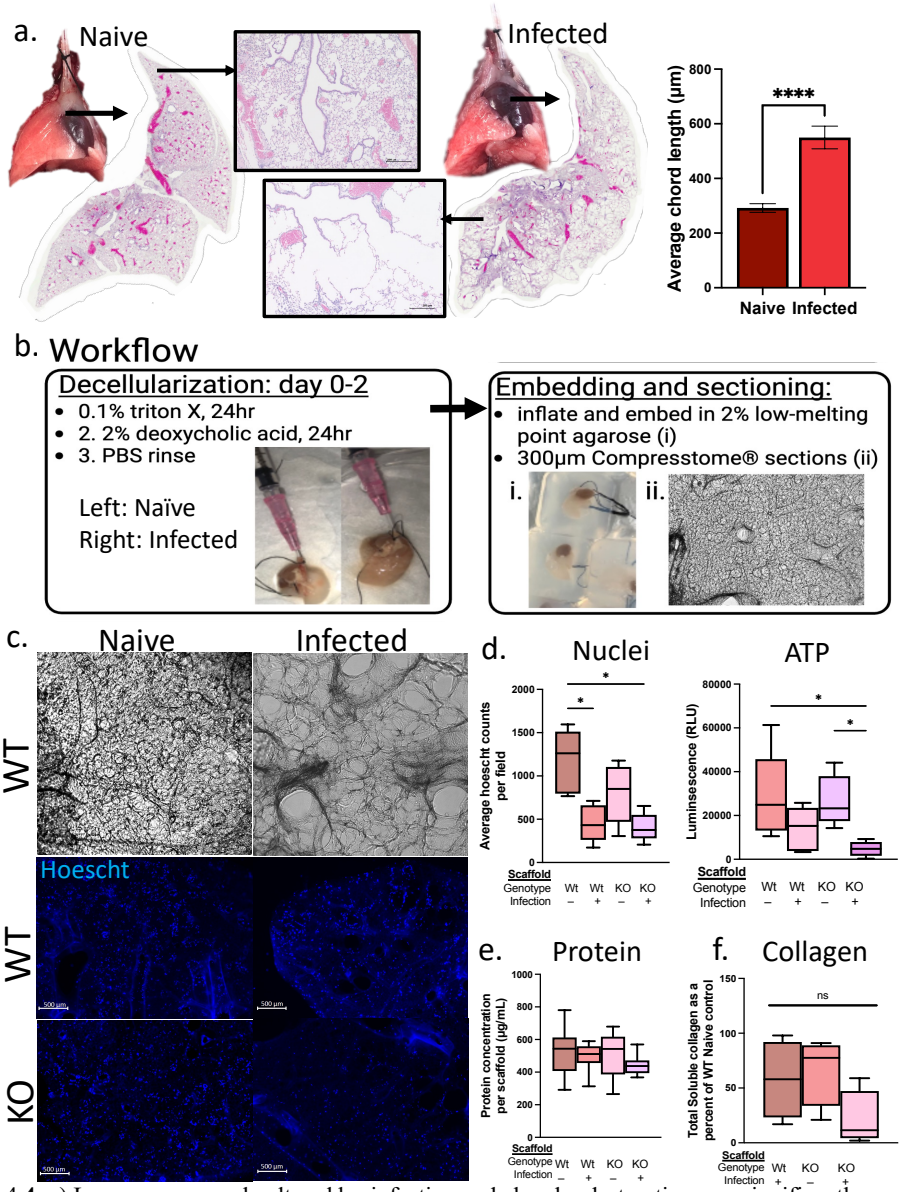

Figure 4.4. **a**) Lungs were grossly altered by infection and alveolar destruction was significantly increased as assessed by mean linear intercept quantification (ImageJ analysis package). **b**) Excised lungs were inflated with solutions to remove cells, embedded in agarose (i) and vibratome sectioned at 300µm thick(ii). **c**) Hoescht stained MLE-12 on scaffolds were imaged at 4X. Cellular ATP was then assayed and nuclei quantified by ImageJ package. **d**) Each scaffold was then processed for protein content to control for infection-induced changes and total soluble collaged was determined from an additional piece of acellular lung. Scaffold experiments were repeated 5 times, 5 biological replicates per group and three technical replicates per experiment. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, ****$P \leq 0.0001$. Box and whisker plots are reported using the average of the technical replicates for each condition. Collagen assay was performed on an n or 4-7 per group.

4. 4 Discussion

Human soil-transmitted helminth infections and associated morbidities afflict billions of people each year, however little is known of the long-term impacts of these infections on lung health. With staggering numbers of individuals affected among poor communities, it is imperative that we examine the potential impacts of these infections on the lungs especially since lung diseases resulting from damage, like COPD, are highly prevalent in these regions [33, 34]. Using the rodent parasite, *Nippostrongylus brasiliensis*, that models these human infections we and others seek to address the gaps in our knowledge surrounding the persisting pulmonary impacts of these infections.

Marsland et al. demonstrate progressive emphysematous-like pathologies in mice at days 30-200 post-single infection with N. brasiliensis. Since, others have also corroborated these findings, filling in some critical information that even while these parasites have left the lungs, there may be long-term consequences of their transient passage. We seek to identify protein regulators of this pathology so that we may better understand this progression towards worsening emphysema which may be relevant in treating other methods of alveolar damage. Of note, Marsland et al. identified that early-responder-RELMα mRNA remains upregulated in the lung at day 40 post infection. This murine protein shares functional and structural homology with human resistin, which has broad clinical impacts [21]. The function of RELMα as protective or pathogenic in these late post-infection time points is not well elucidated. We have previously shown that RELMα is expressed by both respiratory epithelium and parenchymal cells (such as macrophages) at day 9 post-infection (shown here at day 7, Fig. 4.1 a) [25]. We, therefore, sought to elucidate the role of RELMα in emphysematous lung pathology as well as the cellular source of this persistent expression at day 30 post-infection.

At day 7 post infection with *N. brasilinesis*, the expression of RELMα is induced among lung epithelial and mesenchymal cells, which is detectable by immunofluorescence of lung tissue (Fig 4.1a). This expression is a hallmark of the type 2 immune response instigated by parasitic infection like those of helminths. This acute activation of RELMα is well documented in this model and it is increasingly demonstrated that RELMα remains transcriptionally upregulated even at later stages post infection [8]. This led us to using three transgenic mouse model system to better elucidate the cellular sources, macrophage or epithelial, of this persistent and long-term RELMα expression (Fig 4.1b).

We were able validate our transgenic systems and demonstrate that RELMα mains elevated at the protein level in both the airway and blood of mice even 30 days after infection with *N. brasiliensis* (Fig 4.1c-d). The successful deletion of RELMα from $CC10^{+}$ club cell progenitors was sufficient to significantly reduce RELMα concentration in the bronchoalveolar lavage fluid (BALF), suggesting that epithelial cells are prominent secretors of this airway RELMα, in line with our previous findings at day 7. While serum RELMα levels were unaffected by the epithelial deletion, we found significant reductions in serum RELMα concentrations among $CD11c^{Cre+}$ and $Retnla^{Td}$ animals. Thus, airway and blood RELMα derives from distinct cellular source, and since neither cell-specific knockout system fully depleted airway or blood RELMα, this highlights the redundancy in expression of RELMα from varying cell types and leads to questions as to the importance of its expression in mice recovering from helminth infection.

To better understand the impact of RELMα expression on lung immune cell populations at this recovery time point, we performed flow cytometry on cells isolated from BALF. We focused on distinguishing tissue-resident (TR-) and monocyte-derived (mono-) AM due to their differing functions in lung repair and that monocyte-derived AM secrete RELMα (Fig 4.2a-b).

Remarkably, there is a predominant impact of infection on depleting BAL alveolar macrophage (AM) populations even at day 30 post infection (Fig. 4.2d). Infection, but not RELMα expression, resulted in significantly decreased TR-AM frequencies in infected CC10 cre+ and cre- mice compared to their naïve controls. This pattern also held for the constituitive knockouts and wild type. There was no significant difference in the $CD11c^{Cre+}$ frequencies which may be the result of insufficient power to detect this difference. Overall, the depletion of TR-AM reserves is driven by infection and is not directly affected by RELMα deletion at this time point.

However, the BAL mono-AM populations are affected by cell-intrinsic RELMα expression in myeloid cells. While all genotypes saw significantly reduced frequencies of mono-AM in the BAL, only whole knockout had RELMα-dependent differences. Comparing naïve $Retnla^{+/+}$ to $Retnla^{-/Td}$ demonstrated reduced mono-AM frequencies under homeostatic conditions suggesting a deficit in the mono-AM recruitment to the lung. It was recently published by Sanin et al., using these same Retnla whole knockouts, that cell-intrinsic expression of RELMα is critical in monocyte transition to macrophages of all types[35]. Thus, these above data support those findings as monocytes rely on RELMα expression to facilitate their migration and inhabitation of tissues like the lung.

To address the question of tissue repair, we collected lungs from day 30 post infection for paraffin embedding and blinded histopathology scoring (Fig 4.3a). The lungs post-infection had significantly higher alveolar destruction scores compared to their respective naïve controls (Fig 4.3b). Partial depletion of RELMα in the cell-specific knockouts was insufficient to yield detectable differences in alveolar destruction by scoring. However, complete deletion of RELMα resulted in significantly higher alveolar destruction scores, underscoring the tissue protective role of RELMα in lung recovery from damage. These data support assertions that RELMα plays an important role in dampening the progressive alveolar destruction that follows helminth infection.

Krljanac et al. demonstrated this using *Nippostrongylus brasiliensis*, in which deletion of RELMα increased fatality in infected mice linking the importance of RELMα expression to lung homeostasis and thus survival[24].

The long-term alteration in lung histology and structure is observable grossly, with significantly increased diffuse alveolar destruction (Fig 4.4a). By modulating target cells RELMα can impact extracellular matrix composition, and others have shown RELMα can directly impact collagen cross-linking, which may be another mechanism by which RELMα facilitates lung repair. We set out to identify is RELMα may facilitate some of this tissue repair via alterations of ECM. We generated acellular lung scaffolds from naïve, infected and WT, KO mice which allows us to address this question ex vivo (Fig 4.4b). The structural damage to alveolar septa is visible on acellular scaffolds from infected mice, but all scaffolds were successfully inhabited by the murine epithelial cells that were plated (Fig 4.4c). After 6 days, an ATP assay on only cells adhered to the scaffolds revealed an important role of RELMα on re-epithelialization as an assessment of repair. There was significantly less cellular ATP in cells plated on scaffolds from $Retnla^{Td}$ compared to naive scaffolds from $Retnla^{Wt}$ and $Retnla^{Td}$. This demonstrates both a combined infection and RELMα-dependent role of ECM-epithelial interactions guiding repair. The KO infected scaffold may be reducing the proliferative capacity or activity of epithelial cells. We complemented this ATP assay with nuclei counts which revealed a significant reduction in nuclei adhered to scaffolds of WT and KO infected compared to WT Naïve. This data clarifies that RELMα may be altering the activity or proliferation of epithelial cells via its effect on the ECM. We assessed the protein content of each individual scaffold used in these studies and determined no significant difference in protein concentration on a per scaffold basis. Due to the role of RELMα on collagen, we also used acellular lung tissue to clarify that there were no significant differences in the amounts of acid soluble collagen relative to WT naïve as a baseline.

Taken together these studies suggest that infection and RELMα expression both modulate the ECM, such that epithelial cells may have reduced proliferation, attachments and/or activity. This is an important perspective in our understanding of how a single infection with helminths results in persistent alveolar damage and identifies specific proteins that may be involved such as RELMα, which could be targeted for improved tissue repair.

4.5 Materials and Methods

4.5.1 Transgenic mice

Retnla^{Td} were generated by genOway as previously published. These mice were back-crossed with C57BL/6 to generate knockout and wild-type controls, bred in-house. Retnla^{flox} mice were also generated by genOway in a similar fashion to the Retnla^{Td} mice, and bred in-house to generate Retnla$^{fl/fl}$ mice. B6.Cg-Tg(Itgax-cre)1-1Reiz/J (strain #: 008068; common name: CD11c-Cre) and B6N.129S6(Cg)-Scgb1a1tm1(cre/ERT)Blh/J (strain #: 016225; common name: Scgb1a1-CreER™ or CC10-Cre) were purchased from the Jackson Laboratory. Cre heterozygous CD11c-Cre and CC10-Cre mice were bred in-house with Retnla$^{fl/fl}$ mice to generate homozygous Retnla-floxed Cre$^{+/-}$ and Cre$^{-/-}$ mice. CC10-Cre mice are tamoxifen-inducible and were administered 5 doses of 100μL, 20mg/mL tamoxifen (Sigma) in corn oil intraperitoneally every other day. Following the 5th does, mice were given 7 days to recover prior to the start of helminth infection. Mice were sex- and age-matched (6-13 weeks) and housed in a specific pathogen-free vivarium with standard 12hr light cycles. Mice had ad libitum access to standard chow and water. Sex- and age-matched mice with littermate controls were used in these studies. Animal experiments were conducted in accordance with National Institutes of Health guidelines, the Animal Welfare Act, and the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Protocols for the use of these animals were approved and followed in

accordance with the University of California Riverside Institutional Animal Care and Use Committee (IACUC, protocol number A-20210017).

4.5.2. *Nippostrongylus brasiliensis* infection

Mice were infected subcutaneously with L3 larvae from *Nippostrongylus brasiliensis* at 600 worms in 200µL saline, as previously published [31]. Mice were given 30 days to recover from this single infection and were euthanized on day 30 following infection day 0. For acellular scaffold studies, mice were euthanized on day 80 post-infection. Weight was monitored to prevent egregious weight loss in these animals in the few days after the initial infection.

4.5.3. Tissue harvest

After euthanasia, blood was collected from the renal arteries which was allowed to coagulate and then processed for serum by centrifugation. A small incision was made on the diaphragm, and a pleural lavage was conducted with the pleural cavity washed twice with 500µL of cold phosphate-buffered saline (PBS) and combined. Revealing the trachea, another small incision was made to fit the cannula, a luer-lock shield from an 18-gauge shielded IV Catheter (BD Insyte Autoguard, cat # 381447). This was tied to the trachea with nylon suture wire and used with a 1mL syringe to perform the bronchoalveolar lavage. 800µL of cold saline was inserted and removed from the lung twice, each collected in separate flow tubes for processing. Supernatant from the pleural and first BAL wash was used for protein analyses, while the cell pellets were used for flow cytometry. Prior to the excision of the heart and lungs en bloc, vascular immune cells were cleared from the lungs by cardiac perfusion with PBS. Nylon suture was tied around the right main bronchus, and the right lung lobes were removed and processed by mechanical (mincing with scalpels) and chemical digestion (Collagenase/DNase) to create a single-cell suspension for flow cytometry. The remaining left lobe was partially inflated with 300µL of 10% buffered formalin via the tied cannula and placed on an apparatus used to inflate

the lungs under constant pressure. This apparatus uses the pressure of 10% buffered formalin at 22cm above the lungs for inflation, with the lungs surrounded by 10% buffered formalin, using reagent reservoirs, and were sent to Sanford Burnham Prebys or UCLA histology cores for paraffin embedding. Hematoxylin and eosin (H&E) staining was also performed by these histology cores on 5μm FFPE lung sections.

4.5.4. Acellular scaffold assays

Mice were euthanized on day 80 post-infection and perfused by cardiac perfusion with PBS until they turned white. Acellular scaffolds were prepared using an adapted protocol for the decellularization of human lungs from our previous publication [17]. A cannula was placed in the trachea, like BAL washes above, and tied tightly with nylon suture wire. Lungs were gently excised, inflated with 1mL 0.1% Triton X in ddH20, and the syringe was left attached to maintain the volume in the lungs. The lungs were then placed in a plastic container and covered with ddH2O for overnight incubation at 4C. The next day lungs were deflated and reinflated with 2% deoxycholic acid (Spectrum) and placed back in the container with fresh ddH2O for overnight incubation at 4C. The final day lungs were deflated and rinsed with 2% p/s PBS (inflated deflated). Prior to vibratome sectioning, lungs were inflated with 2% low melting point agarose in PBS and chilled on ice to set the agarose. Lungs were sectioned with the manufacturer's tungsten carbide blades at 300μm thick and placed in 100% ethanol to sterilize and for cold storage (Precisionary Instruments Compresstome; speed setting: 1, oscillation setting: 10). To standardize lung size, sections were identified by cut number and only cuts 6-10 were used in these studies. Hoescht staining of representative scaffolds revealed successful decellularization.

Prior to assay, scaffolds were incubated in 2% penicillin-streptomycin in PBS overnight at 37.5C to clear the agarose. The following day, the 2% p/s in PBS was replaced for two more 20-minute washes at 37.5C. The murine lung epithelial cell line MLE 12 – CRL2110™ (ATCC,

passages 6-8) was plated at 200,000 cells per scaffold in 48-well plates to assay for ex vivo lung repair. Cells were plated directly onto the scaffolds in 100μL of ATCC-recommended HITES media for overnight incubation at 37.5C in a humidified cell culture incubator at 5% CO_2. The following day, an additional 200μL of HITES media was added to each scaffold. On day 3, the media was replaced. On day 6, the scaffolds were stained with Hoechst (1:2500 of 20mM, cat #) and transferred to a new 24-well plate for imaging at 4X objective on the Keyence BZ-X810.

4.5.5. Quantification of cell viability

After imaging, scaffolds were gently transferred to new 24-well opaque plates for analysis of cellular ATP using the Promega CellTiter-Glo™ 3D Cell Viability Assay per manufacturers protocol (cat # G9681). This allows quantification of ATP from only those cells associated with the scaffold. The protein content of each scaffold was determined by bicinchoninic acid (BCA) assay (Pierce). Cellular ATP was normalized to the respective scaffold protein content to adjust for differences in available surface for cell adherence. Nuclei count analysis was performed using an automated protocol on ImageJ by Christine Labno at the University of Chicago.

4.5.6. Flow Cytometry

BAL and pleural cells were pelleted by centrifugation at 800xg for 5min. The excised right lobes were minced using a scapula then incubated in a digestion solution for 1hr while shaking at 37.5C. 15 minutes prior to the final 1 hr mark, lungs were homogenized using an 18 gauge needle (pulled 10 times) and returned for the 15min 37.5C incubation. The incubated sample was then poured over 70μm cell strainers and pelleted. For all pleural and lung pellets, RBC lysis was performed per the manufacturer protocol (Biolegend 10X) Cells were resuspended in PBS and cell counts were quantified using an automated cell counter (Denovix CellDrop™). Up to 1 million cells of each sample were prepared for flow cytometry using v-bottom 96-well

plates. The panel of Biolegend antibodies used for flow analysis are listed here: The panel of Biolegend antibodies used for flow analysis is listed here used at 1:400; FITC anti-mouse MERTK (Mer) Antibody (clone: 2B10C42, Biolegend), Alexa Fluor® 700 anti-mouse MHCII (I-A/I-E) Antibody (clone: M5/114.15.2, Biolegend) APC/Cy7 anti-mouse CD11b Antibody (clone: M1/70, Biolegend, Brilliant Violet 510™ anti-mouse Ly-6G Antibody (clone 1A8, Biolegend), Zombie Aqua™ Fixable Viability Kit, Brilliant Violet 605™ anti-mouse CD11c Antibody (clone: N418, Biolegend), Brilliant Violet 650™ anti-mouse F4/80 Antibody (clone: BM8, Biolegend), PE-CF594 Rat Anti-Mouse Siglec-F (clone: E50-2440, BD Biosciences), PE/Cyanine7 anti-mouse Ly-6C Antibody (clone: Hk1.4, Biolegend). Samples were analyzed using the Agilent Novocyte Quanteon and flow data was performed using FlowJo™ version 10.

4.5.7. Sandwich Enzyme-Linked Immunosorbent Assay (ELISA)

Recombinant murine RELMα protein, rabbit-anti-murine RELMα and biotinylated-rabbit-anti-murine RELMα were purchased from Peprotech (cat # 450-26, 500-P214 and 500-P214BT, respectively). Plates were coated overnight at 4C with rabbit anti-murine RELMα at a concentration of 0.5μg/mL, and the ELISA was completed on the following day. Detection antibody was also used at a concentration of 0.5μg/mL.

4.5.8. Histopathology assessment

H&E slides were blinded and scored from 1 to 5 on alveolar destruction and vascular inflammation across the lung which was divided into 5 equally spaced sections: apex to base. Each of the 5 spaces was assessed for percent of alveolar damage, scored, and the scores were reported as an average for each mouse. Scoring parameters: 0 for no detectable damage, 0.5 for 5% damage, 1.0 for 10%, 1.5 for 20%, 2.0 for 30%, 2.5 for 40%, 3.0 for 50%, 3.5 for 60%, 4.0 for 70%, 4.5 for 80%, and 5 for 90% or more.

4.5.9. Statistics

Statistical analysis were performed using Graphpad Software Prism 9. Experiments were repeated at least twice with an n of 3 or more per group.

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CHAPTER FIVE:

Conclusions and future directions

5.1 Conclusions

Many of the conclusions of this work are confirmatory of what has been recently published in the literature. What is apparent is that dietary modifiers of inflammation and immunity can be utilized among individuals when possible to contribute to overall better health outcomes. Supplementation with PUFAs like omega 3 fatty acids and DHA is a well-studied in its role to improve outcomes of a number of disease states. What is shown here is how dairy, which does have a percentage of healthy fats, can improve lung function and decrease the symptoms associated with asthma for individuals who have never had an asthma diagnosis as well as for those with asthma diagnoses. Further, mouse models reveal that DHA supplementation can dampen pulmonary inflammation which may be upstream of lung function and damage, suggestive of tissue protection and resolution potential. Diet presents an opportunity for lifestyle changes to improve lung health in communities with limited access to medical providers, while access to these diets may yet present a financial or availability challenge. Thus, a multi-pronged approach is still more likely to improve overall outcomes and address the needs of all individuals.

In addition to diet, receptor proteins and adipokines, such as FFAR4 and RELMα, provide the opportunity for targeted therapeutics. FFAR4 as a GPCR is a strong candidate for drug design and the presence of this receptor on the airway makes it accessible by the bloodstream and as an aerosolized inhalant. This receptor is already the subject of numerous clinical trials, therefore transitioning this drug to other functions in lung-specific clinical trials would be feasible. The evidence of FFAR4 playing a role in epithelial repair, shown in chapter 3 and as published by Lee et al [20], suggests that additional research may yield clinical potential in

the treatment of lung disease and airway damage. FFAR4 expression among epithelial progenitor cells, club cells, may underscore the potential for this receptor in mechanisms of epithelial homeostasis and with further studies we may identify a potential clinical use. Perhaps FFAR4 could be beneficial in airway epithelial diseases like chronic bronchitis and goblet cell hyperplasia which contributes to COPD.

We also identified RELMα as a candidate secreted protein that promotes tissue repair. Through the use of transgenic mice where RELMα was completely deleted, or deleted specifically in epithelial cells or myeloid cells, we demonstrate that either cellular source is sufficient to promote tissue recovery following lung helminth infection. RELMα deficiency also had long term effects on the lung architecture, given that lung scaffolds from RELMα deficient mice were less able to support epithelial cell growth compared to lung scaffolds from WT mice. Together, this work highlights a few future directions in the quest for pulmonary repair and in treating lung diseases which afflict billions of people globally.

5.2 Future directions

One of the fields of research that is paramount in our understanding of tissue repair is embryological development. Harnessing the power of stem cell regenerative capacity and mechanisms driving development could provide the tools necessary to facilitate repair in the lung. As mentioned previously, there are not yet available therapeutics that effectively promote repair in the lung. Most currently available treatments address symptoms and may slow the progression of the disease, but neither reverse damage. The overarching future direction for researchers in the field of lung health and disease would be to identify mechanisms involved in tissue repair. The more specific directions supported by this work involve a better understanding of the downstream

effectors involved in FFAR4-mediated epithelial phenotypes so that using FFAR4 therapeutically progresses toward a clinical possibility. With regard to RELMα, the priority should be in clarifying the receptor and downstream players involved in this adipokines effects. The analogous human resistin is also used as a clinical biomarker for numerous diseases, but so far no therapeutic directions have been presented and this is a current gap in knowledge that could be filled with additional research.

Lastly, we need access to clinical data summarizing the lung health of individuals afflicted by helminth infection. A major roadblock to this future direction is that communities afflicted are often impoverished and lacking clinical care. However, the widespread impact of helminthiases as well as the global burden of pathological lung diseases certainly underscores the significance of such work, which could improve lives on the order of billions. Medical expenses associated with chronic disease are an enormous financial burden for individuals as well as for societies, therefore it is financially beneficial to all to support these endeavors. With more clinical data, we can better understand if the long timepoints of STH infections in mouse models are appropriately modeling human disease, and if so, we can provide better care for those who are impacted.

5.3 Call to action

The problem of equity is pervasive across disciplines with the common outcome of impacting the health of marginalized individuals. Inequitable access to healthy foods, unpolluted air, chemical-free houseware, and medical care are major global crises that need our immediate attention. Study after study has delineated the impact of these intersectional exposures on poorer health outcomes across the US and the globe which disproportionately impact Black, Brown, and

Indigenous communities and those of lower socioeconomic status. This call to action is for scientists to consider the individuals represented in their research models and to highlight areas of need so we can advocate for change. Ensuring that research models are representative of all individuals or that models used address the conditions faced by marginalized communities is an important next step for many scientists. I hope to encourage scientists to make these considerations in their experimental design and in the interpretations of their data. Further I hope through this lens that science policy and community engagement can provide avenues for individual advocacy to promote better access to healthy foods, medical care, and reconciliation of the increased risk of exposure to harmful chemicals that disproportionality impact marginalized communities. We can all contribute to the betterment of lives in our communities and globally, so long as we are aware and make the effort.

5.4 Citations for Chapter One and Chapter Five: Introduction and Discussion

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