THESIS

MEDIATORS OF MUCOSAL INTEGRITY IN THE CONTEXT OF AGRICULTURE DUST EXPOSURE

Submitted by

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ABSTRACT

MEDIATORS OF MUCOSAL INTEGRITY IN THE CONTEXT
OF AGRICULTURE DUST EXPOSURE

Inhalation of particulate matter, such as agricultural dust, can lead to lung inflammation and increased risk for chronic respiratory diseases. Agricultural workers (e.g., hog farmers) are constantly exposed to organic dusts from the workplace. Chronic pulmonary obstructive disease (COPD) is a type of lower respiratory disease that results in an excessive inflammation cycle that leads to disease progression. Currently, available treatments merely treat the patient's symptoms with no effect on the prevention of disease progression. Metabolites of omega-3 fatty acids, called specialized pro-resolving mediators (SPM), can aid in inflammation resolution and promote immunity in the context of respiratory infection. IL-22 promotes mucosal immunity by regulating inflammation, inducing the production of antimicrobial peptides (AMP), and promoting epithelial repair. Mucosal surfaces in the airways are lined with epithelial cells, a mucus layer, and immune cells that act as the first line of defense against inhaled pathogens. The respiratory epithelium cells express antimicrobial peptides like beta-defensin-2 and Regenerating islet-derived protein 3 gamma (Reg3g); this expression can be stimulated by IL-22. In this study, we explored the effects of omega-3 fatty acids after organic dust exposure using a fat-1 transgenic mouse model, which represents the ideal ratio between omega-3 and omega-6 fatty acids. To further explore the impacts of IL-22 on AMP expression after organic dust exposure, a whole-body IL-22 knockout mouse model also was used in this study. Since omega-3 fatty acids and IL-22 promote inflammation resolution, we studied their impact on mucosal immunity and

epithelial repair following repetitive challenges with extracts of organic dust *in vivo*. Wildtype, IL-22 knockout, and fat-1 mice were exposed by intranasal installations five times a week for 3 weeks. Lung tissue from mice exposed to either organic dust or saline were obtained and evaluated for AMP and wound repair markers. To evaluate the impact of IL-22 on AMP expression in the context of organic dust exposure, immunofluorescence (IF) staining, enzyme-linked immunosorbent assays (ELISAs), and RT-qPCR arrays were used. IF staining was done to assess beta-defensin-2 expression within the bronchial epithelial cells in IL-22 knockout and wildtype mice. While trends of positive staining for beta-defensin-2 were observed, no statistical significance was found. ELISAs were performed to assess for Reg3g expression in mice lung tissue; concentrations were found to be present in both the wildtype and IL-22 knockout models after saline and dust exposure. To assess for markers of wound repair in IL-22 knockout and wildtype mice models, a custom RT^2 profiler PCR array was ordered to detect gene expression of different antimicrobial peptides, anti- and pro-inflammatory markers, and wound repair markers. The findings were as follows. Expression of AMPs—specifically S100A8 and S100A9—indicates a decrease among organic dust-exposed groups. The expression of wound repair markers CCL7 and ITGA3 exhibited decreases in the context of organic dust exposure. Expression of the anti-inflammatory marker MIF also exhibited a decrease among dust-exposed groups. To evaluate the impact of omega-3 fatty acids on AMP expression in the context of organic dust exposure, lung tissue from fat-1 and wildtype mice were stained for Reg3g expression. While trends of positive staining for Reg3g were observed, no statistical significance was found. IL-22 signaling exhibited trends of increased expression of AMP and pro-resolution mediators of mucosal integrity in the context of chronic dust exposure. Further studies should be conducted to determine the effects of omega-3 fatty acids on AMP expression in the context of

organic dust exposure. These findings can be utilized to develop new treatment strategies for lung disease that focus on pro-resolution rather than solely anti-inflammatory methods.

Keywords: chronic pulmonary obstructive disease (COPD), agricultural dust exposure, specialized pro-resolving mediators, antimicrobial peptides, IL-22 knockout, Reg3g, Beta-defensin-2

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INTRODUCTION

1.1 Background on Chronic Pulmonary Diseases

Inhalation of particulate matter, such as agricultural dust, can put agriculture workers at elevated risk for the development of chronic pulmonary diseases (Crawford et al. 2021). Per the Centers for Disease Control and Prevention ([CDC] 2020), almost 16 million Americans were diagnosed with chronic obstructive pulmonary disease (COPD) and nearly half of agricultural workers reported lower respiratory symptoms (National Center for Health Statistics [NCHS] 2023; Xu et al. 2022). As a demographic, agricultural workers are at an increased risk for developing respiratory diseases ranging from chronic bronchitis, asthma, and COPD due to dust exposure in animal confinement facilities (Crawford et al. 2021; Rumchev et al. 2019). Within animal confinement facilities, agricultural workers are continuously exposed to organic dusts that contain bacterial products like peptidoglycan, fungal spores, and particulate matter, which can all lead to airway irritation and inflammation (Nordgren and Bailey 2016). The inhalation of dust can lead to chronic inflammation and alter mucosal integrity thus leaving agricultural workers at a heightened risk for infection. In 2021, the sixth leading cause of death in the United States was chronic lower respiratory diseases, for which few to no therapies are available (Xu et al. 2022).

Smoking is the largest factor in COPD, but nearly 30% of COPD cases are caused by occupational exposure (Nordgren and Bailey 2016). There is evidence that occupational exposure correlates with a high prevalence of the respiratory symptoms related to COPD, especially in agricultural workers exposed to farm animals (Nordgren and Bailey 2016). In a study that compared dairy farmers to office workers, researchers found rates of COPD were

higher in dairy farmers, with 10.7% of the latter reporting COPD versus a rate of 2.7% found in office workers (Nordgren and Bailey 2016). COPD is often associated with high mortality rates since there is less oxygen diffusing into the blood as the disease progresses and breathing becomes more difficult.

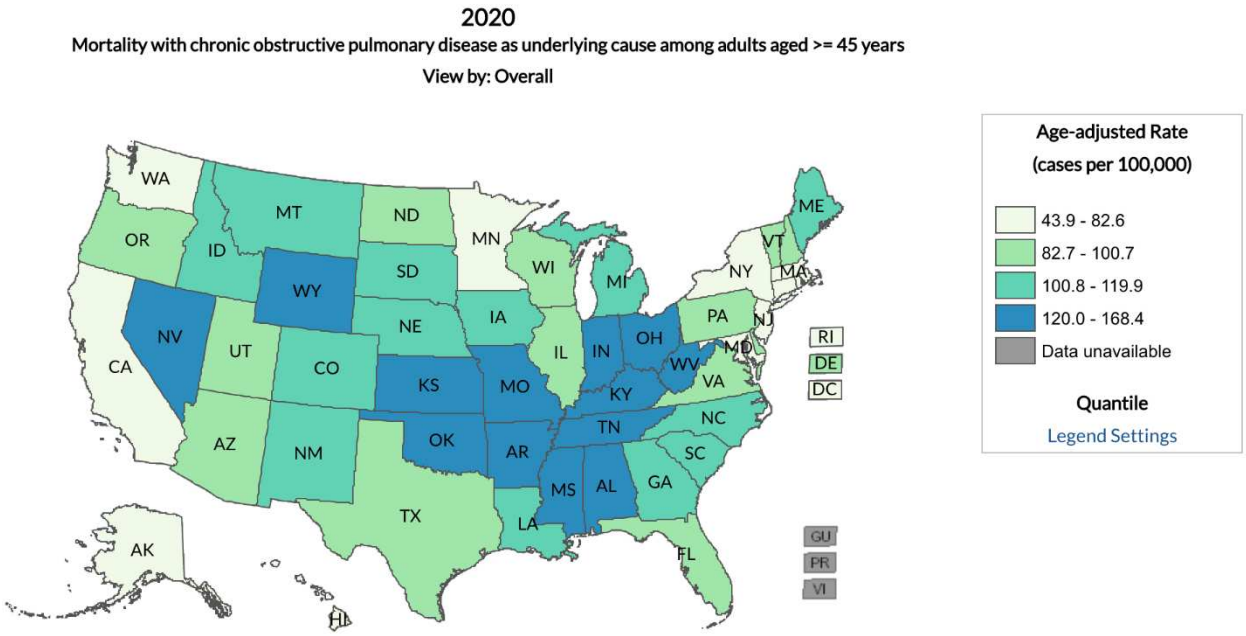

Figure 1. *COPD Mortality Rates Per State for 2020*

Note: From the Centers for Disease Control and Prevention (2020) with data from the National Center for Chronic Disease Prevention and Health Promotion's National Vital Statistics System. Dark blue indicates states with the highest mortality rates from COPD and these correlate with the states with high agricultural production.

The American Academy of Family Physicians describes COPD as a lower respiratory disease that "is characterized by the gradual progression of irreversible airflow obstruction and increased inflammation in the airways and lung parenchyma" (Dewar and Curry 2006, p. 669). The condition is initiated by the inhalation of smoke, dust, or pollutants that cause the immune system to trigger a persistent inflammatory response (Baggio et al., 2020). Chronic inflammation leads to increased disease progression as it causes the lung tissue to thicken and obstruct airways (Crawford et al. 2021). Macrophages, epithelial cells, and other immune cells recruited to the alveolar spaces promote inflammation and cause lung function to decline (van Eeden and Hogg 2020). Once at the site of injury, these cells release proteases, which causes elastin degradation and emphysema, leading to the destruction of the alveoli. This results in an excessive inflammatory cycle with no therapies available to stop disease progression and only limited therapies available to help treat the symptoms of COPD (Aghasafari et al. 2019).

Current therapies available to treat symptoms of COPD include bronchodilators, corticosteroids, macrolides, and phosphodiesterase inhibitors (van Eeden and Hogg 2020). However, these treatments can merely increase patient quality of life, they have no effect on the disease itself. Bronchodilators are a Beta-agonist and antimuscarinic therapy used to control symptoms while increasing airflow to lungs (van Eeden and Hogg 2020). Corticosteroids are an anti-inflammatory treatment that have been shown to reduce membrane thickening and inflammatory cell infiltration in airways (van Eeden and Hogg 2020). Macrolides help to clear bacteria colonization out of the airways to help mitigate inflammation and reduce COPD exacerbations (van Eeden and Hogg 2020). Corticosteroids and macrolides can be used in combination to enhance the anti-inflammatory effect while clearing out pathogens. Although phosphodiesterase inhibitors are an older treatment type, they are still used because they have

both bronchodilation and anti-inflammatory effects (van Eeden and Hogg 2020). The limited treatment regimens available indicate the need for a therapy that allows for control of the inflammatory response with a focus on actual resolution of inflammation (pro-resolving mediators) rather than simply lessening or temporarily reducing inflammation (anti-inflammatory).

1.2 Omega-3 Fatty Acids

Omega-3 fatty acids can be beneficial in inflammation control and repair, due to their ability to produce specialized pro-resolving mediators (SPM; Mendivil 2021). Omega-6 fatty acids, which tend to be more prominent in the typical western diet, have both positive and negative traits. This is primarily because of the omega-6 fatty acid linolenic acid, which the human body can convert into arachidonic acid. The latter, in turn, can be used by the body in the production of "molecules that calm inflammation and fight blood clots," but can also produce inflammation promotion factors (Harvard Health Publishing 2019). SPMs derived from Omega-3 fatty acids comprise the following classes: resolvins, protectins, and maresins (Bannenberg and Serhan 2010). These SPMs are known for being anti-inflammatory as well as possessing tissue protecting and repair functions (Bannenberg and Serhan 2010; Ulu Velazquez et al. 2022). During an inflammation response, SPMs are produced as part of the “resolution” phase, actively aiding in the resolution of inflammation. SPMs are crucial to stopping the recruitment of granulocytes, for the activation of macrophages, and in helping repair damaged tissue (Sandhaus and Swick 2021). In order to explore the effects of Omega-3 fatty acids, we utilized a transgenic

fat-1 mouse model that uses the *Caenorhabditis elegans* (*C. elegans*) fatty acid desaturase gene to create the ideal ratio of omega-3 fatty acid to omega-6 fatty acid (Ula Velazquez et al. 2022).

1.3 Mucosal Immunity

Mucosal immunity is the body's first line of defense against pathogens and fighting infection (Aliouche 2023). Agricultural workers with impaired or damaged mucosal immunity from dust inhalation have a higher chance of developing chronic respiratory diseases such as emphysema and chronic bronchitis. Mucosal surfaces, which are thin and permeable barriers in the interior of the lungs that aid in gas exchange, are also vulnerable to infection (Aliouche 2023). Mucosal immunity plays a big role in protecting these mucous membranes as well as in the prevention of harmful immune responses (Bojanowski et al. 2021). Within the airway, the mucosal surface includes epithelial cells, mucus, and resident and recruited immune cells that aid in the clearance of pathogens and particulates (McKelvey et al. 2021). Epithelial cells within the bronchioles line the airways and play a role in the detection of pathogens and the secretion of antimicrobial peptides (AMP; McKelvey et al. 2021).

1.4 Interleukin-22

Interleukin (IL)-22 is part of the IL-10 family of cytokines; it has been shown to regulate inflammation through tissue repair and promoting host defenses, with production being shown by both innate and adaptive immune cells (Ula Sveiven et al. 2022). IL-22 is traditionally thought to be restricted to lymphocytes and is known to have roles in both anti- and pro-inflammatory responses. In a previous study, it was found that macrophages also express IL-22 (Ula Sveiven et al. 2022). Within the epithelial tissue of the alveolar, when dust exposure occurs, inflammation arises. The macrophages secrete the IL-22 which in turn signals to the other cells to promote inflammation resolution and epithelial repair (see Fig. 2; Ula Sveiven et al. 2022).

Ula Sveiven et al. (2022) utilized IL-22 knockout and wildtype mice and employed a 3-week repetitive organic dust extracts (DE) model. The researchers found IL-22 is expressed within the airway epithelial cells and macrophages (Ula Sveiven et al. 2022). The respiratory epithelial cells express antimicrobial peptides like B-defensin-2 and regenerating islet-derived protein 3-gamma (Reg3g), which recruit inflammatory cells and can be stimulated by IL-22 (Ahlfors et al. 2014). IL-22 plays an important role in mucosal immunity by maintaining epithelial barrier integrity and promoting the production of antimicrobial peptides (Ahlfors et al. 2014).

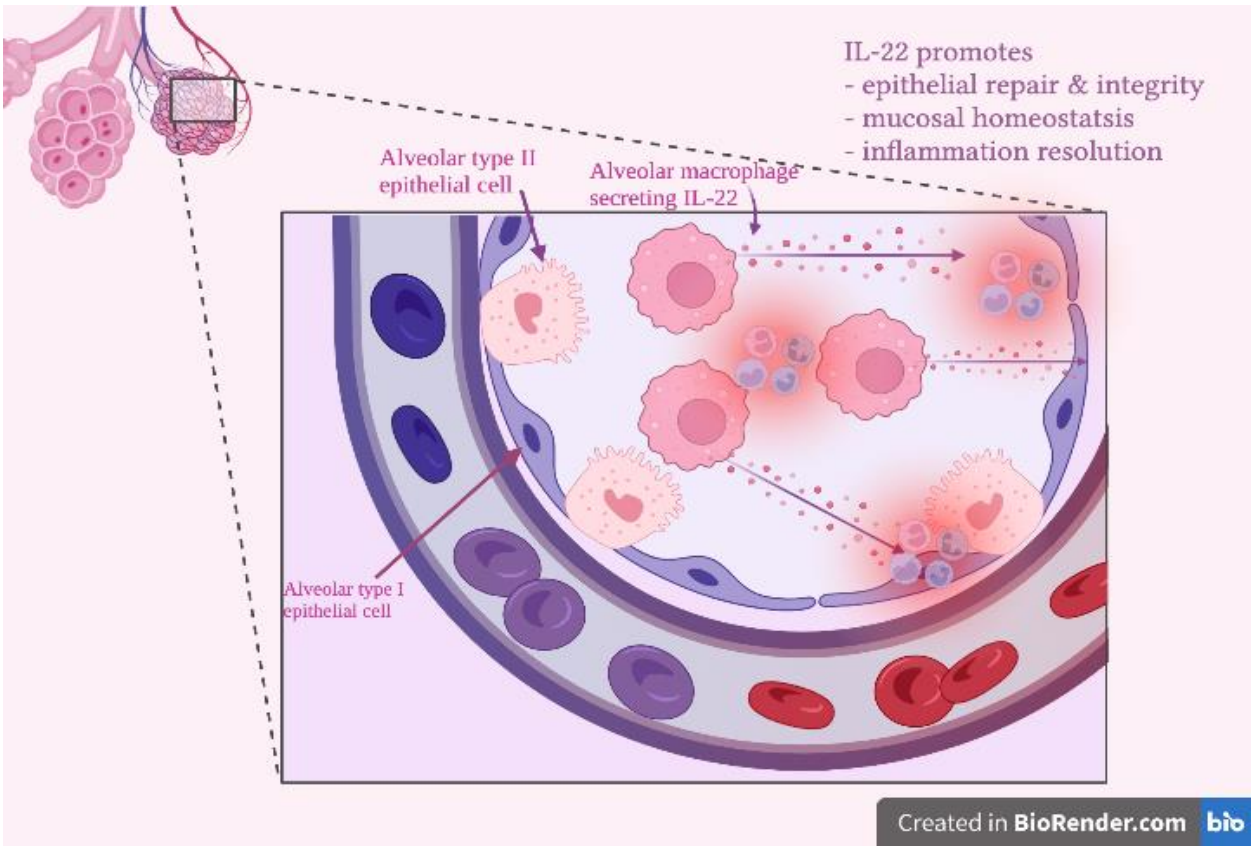

Figure 2. *Visual Cue of IL-22 Signaling Within the Alveolar Epithelium*

Note: Image represents an interior perspective within the alveoli after dust exposure. Macrophages are recruited and secrete IL-22, which signals to promote pro-resolution and epithelial repair. Image created by author using BioRender and consulted Ulu Sveiven et al. (2022).

In a previous study done to explore the role of IL-22 during an inflammatory response after chronic dust exposure, demonstrated a protective role for IL-22 (Ula Sveiven et al. 2022). Mice were exposed to DE for 3 weeks, 5 times a week, and bronchial epithelial tissue was assessed for IL-22 expression (Ula Sveiven et al. 2022). The data indicate that IL-22 was significantly increased in DE exposed groups compared to their saline counterparts (see Fig. 3; Ula Sveiven et al. 2022). IL-22 has a protective effect during an inflammatory response by

providing communication between specialized tissue cell types and the immune system (Fang et al. 2022).

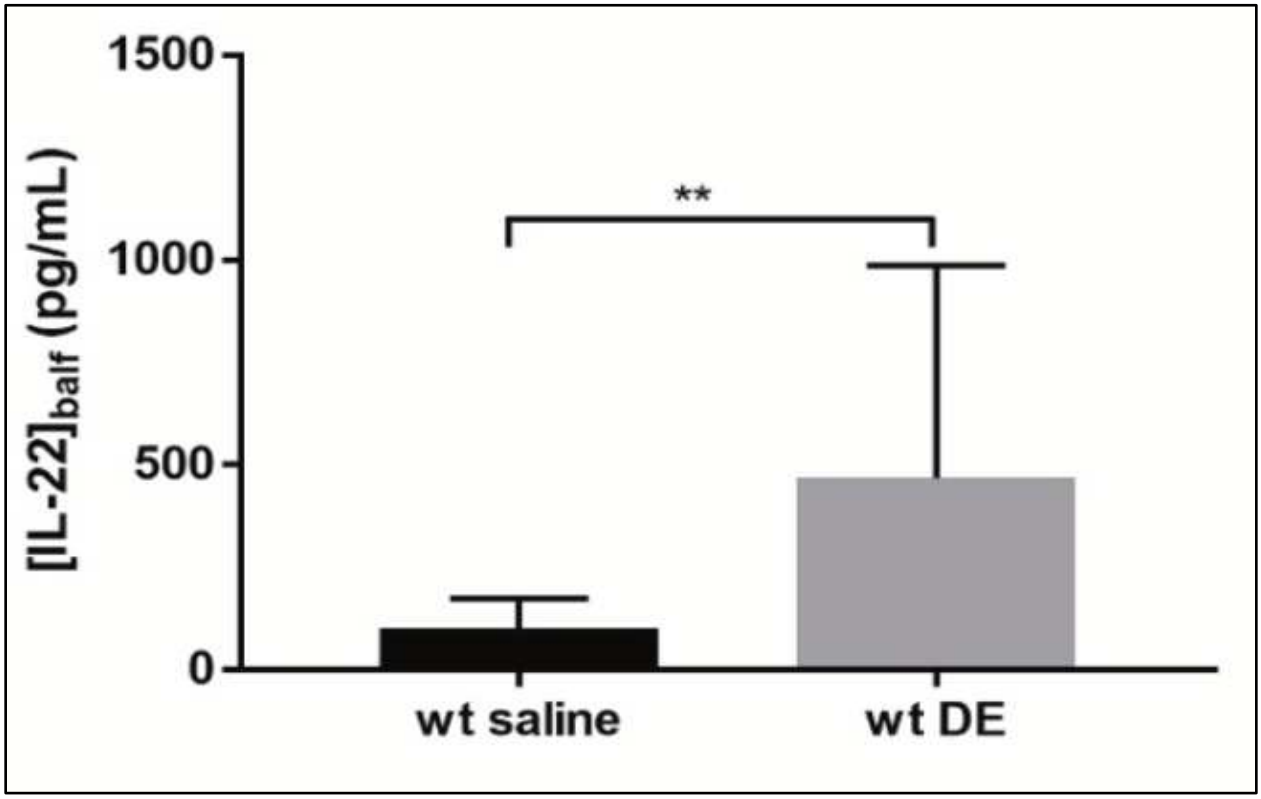

Figure 3. *IL-22 Expression in BALF*

Note. Data from Ula Sveiven et al. (2022) showing that IL-22 expression is increased in the BALF after DE exposure.

1.5 Antimicrobial Peptides

Antimicrobial peptides are effectors of the innate immune system and help aid in elimination of pathogens and have been recognized for their roles in homeostasis of pulmonary health (Diamond et al. 2009). They are constitutively expressed within the respiratory tract and are mainly produced by phagocytic cells and mucosal epithelial cells (Meyerholz and Ackermann 2005). The expression of AMP can be induced by pathogens or cytokines as part of the host defense response (Aghamiri et al. 2021; Diamond et al. 2009). It is thought that by enhancing the expression of AMP, there may be a decrease in disease susceptibility. A study done with cystic fibrosis patients, suggested that they are at higher risk for pulmonary infections due to the inactivation of antimicrobial activity of beta defensins (Bojanowski et al. 2021).

1.5.1 Beta-Defensins

Beta-defensins have broad antimicrobial effects against bacteria, fungi, and viruses and are expressed in the epithelial cells of mucosal surfaces such as respiratory, urinary, and gastrointestinal tracts (Meyerholz and Ackermann 2005). There are various types of beta-defensins, each exhibiting a specific antimicrobial activity (Dalcin and Ulanova 2013). Specifically looking at beta-defensin-2, expression can be increased following an inflammatory response (Dalcin and Ulanova 2013). While beta-defensin-2 can be expressed in unstimulated cells in basal amounts, cytokines such as, IL-22, can stimulate secretion (Dalcin and Ulanova 2013). Beta-defensin-2 is found to be most concentrated within lung, tonsils, and trachea and for that reason is critical to the resolution of pulmonary diseases (Dalcin and Ulanova 2013. Beta-defensin-2 will interact with the invading microorganism through electrostatic amphipathic attraction between the AMP and the negatively charged phospholipid bilayer, thus exerting antimicrobial effect by rupturing the membrane causing death of the microorganism (Dalcin and

Ulanova 2013). In a study done on patients with COPD, those with low levels of beta-defensin-2 were more found to be more likely to experience exacerbations (see Fig. 4; Feng et al. 2023). The study included patients with varying degrees of COPD progression (using GOLD 0-4 criteria) as well as healthy individuals as the control and analyzed the sputum for beta-defensin-2 levels (Feng et al. 2023).

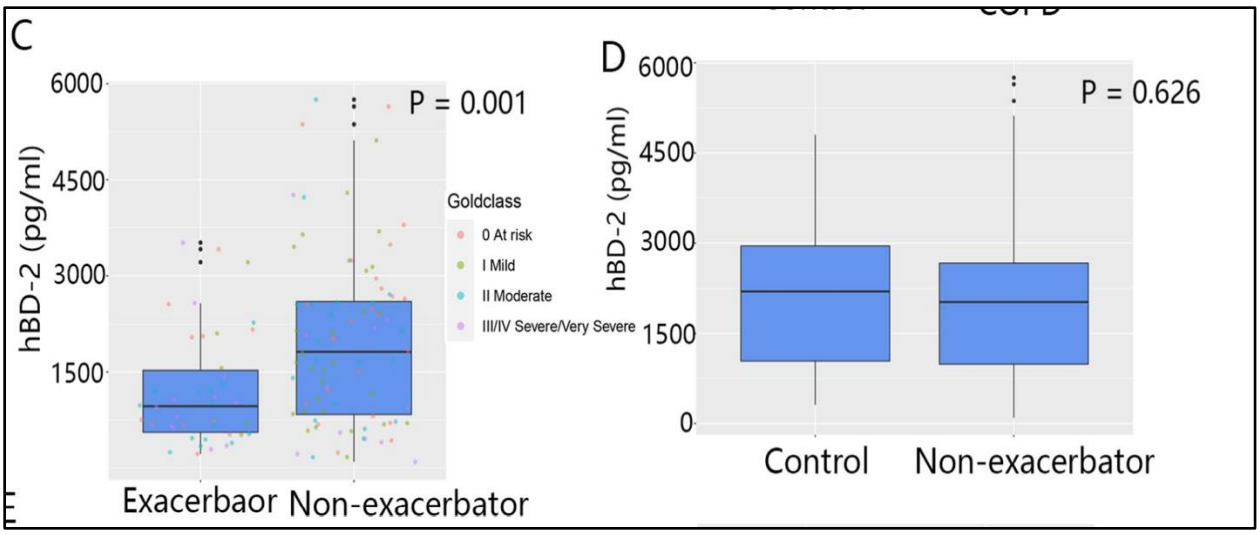

Figure 4. *Sputum Beta-Defensin-2 Levels Between COPD Patients and Healthy Individuals*

Note. Panel (C): The hBD-2 levels of patients who never experienced exacerbation were compared with those of patients who experienced exacerbation at least once. *x*-axis = amount of HbD2 present in sputum; *y*-axis = patients who have experienced exacerbations and patients who have not experienced exacerbations. Panel (D): The sputum hBD-2 levels of patients without any exacerbation were compared to those of controls. *x*-axis = amount of HbD2 present in sputum; *y*-axis = no exacerbation and control group. This study utilized the Goldclass criteria to determine COPD severity. Figure from Feng et al. (2023).

1.5.2 Regenerating Islet-Derived Protein 3-Gamma

Reg3g proteins are C-type lectins traditionally thought to be found in the mucosa of the gastrointestinal tract (Shin and Seeley 2019). However, recent investigations have identified roles for Reg3 in the lung, liver, pancreas, and skin (Shin and Seeley 2019). Reg3 proteins can be categorized into four different subtypes, which are α, β, γ, and δ. Herein, we specifically look at Reg3γ (Reg3g), which like many other antimicrobial peptides, is found within mucosal surfaces and is expressed by epithelial cells (Choi et al. 2013; Shin and Seeley 2019). In a study utilizing a house-dust asthma model, Ito et al. (2017) found that Reg3g was induced by IL-22 from lung epithelial cells. This research also determined that, in the lungs, Reg3g was the most highly-

expressed of all the Reg3 proteins (Ito et al. 2017). While there is a well-defined role for Reg3g in the gastrointestinal tract, little known about its role in the lung epithelium. Therefore, this research aimed to further define the role Reg3g plays in pulmonary inflammation after chronic dust exposure.

1.6 Purpose of the Study and Hypotheses

The overall objective of the research was to support the development of new treatment strategies for chronic respiratory disease through the exploration of AMPs and mediators of wound repair, mediated by IL-22, and the role omega-3 fatty acids play in the promotion of Reg3g. To achieve this goal, certain expectations were established prior to the start of this study. First, we hypothesized that when IL-22 signaling is present, an increase in the expression of AMP and pro-epithelial repair pathways will be observed after chronic dust exposure. In addition, it was hypothesized that IL-22 signaling would increase pro-resolution mediators of mucosal immunity and wound healing in the context of dust exposure. Next, in the context of omega-3 fatty acids, it was expected that an increase in AMP expression after chronic dust exposure would be seen.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 In vivo *Organic Dust Extract Exposure Model*

All mouse studies were completed at the University of California Riverside and approved by the Institutional Animal Care and Use committee. All animal studies and organic dust extract preparation was completed as described in Ula Sveiven et al. (2022) and Ula Velazquez et al. (2022). Male C57BL/6J (WT), whole-body IL-22 knockout (IL-22 KO) mice, and transgenic FAT-1 mice that were 6–12 weeks of age were obtained from Jackson Laboratories (Bar Harbor, ME, USA). They were housed in pathogen-free conditions and given free access to standard mouse chow and water. Intranasal (IN) instillations of either 12.5% DE or sterile saline were administered five times a week for 3 consecutive weeks (Ula Sveiven et al. 2022; Ula Velazquez et al. 2022).

2.1.2 Agriculture Dust Extract

Dust was collected one meter above the ground in closed swine confinement facilities in Nebraska. The dust was then suspended in Hank's Buffered Salt Solution at 1 g of dust per 10 mL of saline solution, and incubated at room temperature for 1 hr. The aqueous extract was then centrifuged twice at 4250 xg for 20 min each. The resulting supernatant was sterile filtered through 0.22 μm syringe filters. Then the aqueous dust extracts were frozen into aliquots and stored at -20 ℃ for later use.

2.2 Methods

2.2.1 Immunofluorescence

To determine antimicrobial peptide expression, mouse lung tissue sections from the left lobe were stained for B-defensin-2 and Reg3g. Lung tissue sections were deparaffinized and rehydrated with serial ethanol washes and PBS after which they were incubated in 2N HCl for antigen retrieval. Tissues were then blocked in 5% PBST + milk for 1 hr at room temperature. Then, tissues were incubated overnight at 4 ºC with primary antibody against mouse B-defensin-2 (ab 203077) or Reg3g (PA5- 102588) at a 1/50 concentration. The secondary antibody, Alexa Fluor™ 647 goat anti-Rabbit (Thermo Fisher Scientific), was incubated at room temperature for 2 hr and covered using VECTASHIELD® PLUS Antifade Mounting Medium for fluorescence with DAPI (Vector Laboratories). Tissue sections were imaged with an Olympus Microscope BX63 Fluorescence using Olympus cellSens™ Microscope Imaging Software. Image analysis was also performed using cellSens™. The quantification of B-defensin and Reg3g was performed in two images obtained from each mouse, regions of interest (ROI) were then selected around the bronchiole epithelial to quantify the amount of positive staining per ROI. Data points were graphed as an average of each image ($n = 2$) as well as the average of each group (i.e., $n =$ 2–4 mice/group).

2.2.2 Enzyme-Linked Immunosorbent Assays

Reg3g concentrations in IL-22 KO and WT mice were determined using sandwich enzyme-linked immunosorbent assay (ELISA), which uses two antibodies to sandwich the antigen. A capture antibody is coated on the plate after which the sample is added and the protein of interest binds. Then a detection antibody is added and binds the additional epitope onto the target protein. The substrate is added to produce a signal that is proportional to the amount of

protein present in the sample. For this study, ELISA kit for Reg3g (SEE676Mu) was purchased from Cloud-Clone Corp. (Katy, TX). The 96-well plate was pre-coated with an antibody specific to Reg3g. Lung homogenate protein samples were removed from −20 ºC storage and placed in ice to thaw. Then the standards and samples were added to the appropriate wells with a biotin-conjugated antibody specific to Reg3g. All samples were done in duplicate. Next, Avidin conjugated to Horseradish Peroxidase (HRP) was added to each well and incubated. The TMB substrate solution was then added and after 20 min, the reaction was ended by the sulfuric acid solution. The color change was measured on a FLUOstar® Omega spectrophotometer (BMG Labtech) at wavelength 450 nm using Omega software (Version 5.7).

2.2.3 Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

To access for markers of wound repair, a custom RT^2 Profiler PCR Array was configured using 24 genes and allowing for four samples per plate to be run. Mouse lung tissues were frozen in −80 ºC for storage and put on ice to thaw. RNA was extracted from IL-22 KO and WT mice lung tissues. Tissue samples were prepped first by weighing 30 mg of tissue, each of which were then placed in a homogenizer tube with RLT buffer and then placed in homogenizer. After homogenization, sample was added to QIAshredder column and centrifuged at max speed for 3 min. To begin extraction, 75% ethanol was then added to the homogenate and transferred to an RNeasy spin column to be centrifuged. Then, RW1 buffer was added directly to the membrane and centrifuged again. Next, the Dnase solution was prepped before by adding Dnase with RDD buffer to each sample and mixed by gentle inversion, following the direction that the DNase solution should be added directly to the membrane and incubated. RPE buffer was added and centrifuged, after which flow-through was discarded. This step was then repeated, and the spin column was placed in a new collection tube and centrifuged. Following this step, the spin

column was placed into a new collection tube and nuclease-free water was added directly to the membrane and centrifuged. The nuclease-free water was then re-pipetted directly on the column membrane and centrifuged again. Concentrations were read on Nanodrop and stored at −20 ºC for future use. Purified RNA was then used to prepare cDNA using the iScript™ cDNA synthesis kit (Bio-Rad); the protocol provided was followed with no modifications. Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) was performed using the custom RT^2 Profiler PCR Array obtained from Qiagen as shown in Figure 5. This plate includes: (a) housekeeping genes and controls (PPC, MGDC, RTC, Gapdh, Actb), (b) antimicrobial peptides (Defb, Reg3g, CAMP, S100A8, S100A9), (c) pro-inflammatory markers (IL1b, MIF, Ptger2), (d) anti-inflammatory markers (IL-10, IL-22, IL-22ra1, Fpr2), and (e) wound repair markers (Mmp9, Plat, Acta2, Ccl7, Ifng, Itga3, B2m).

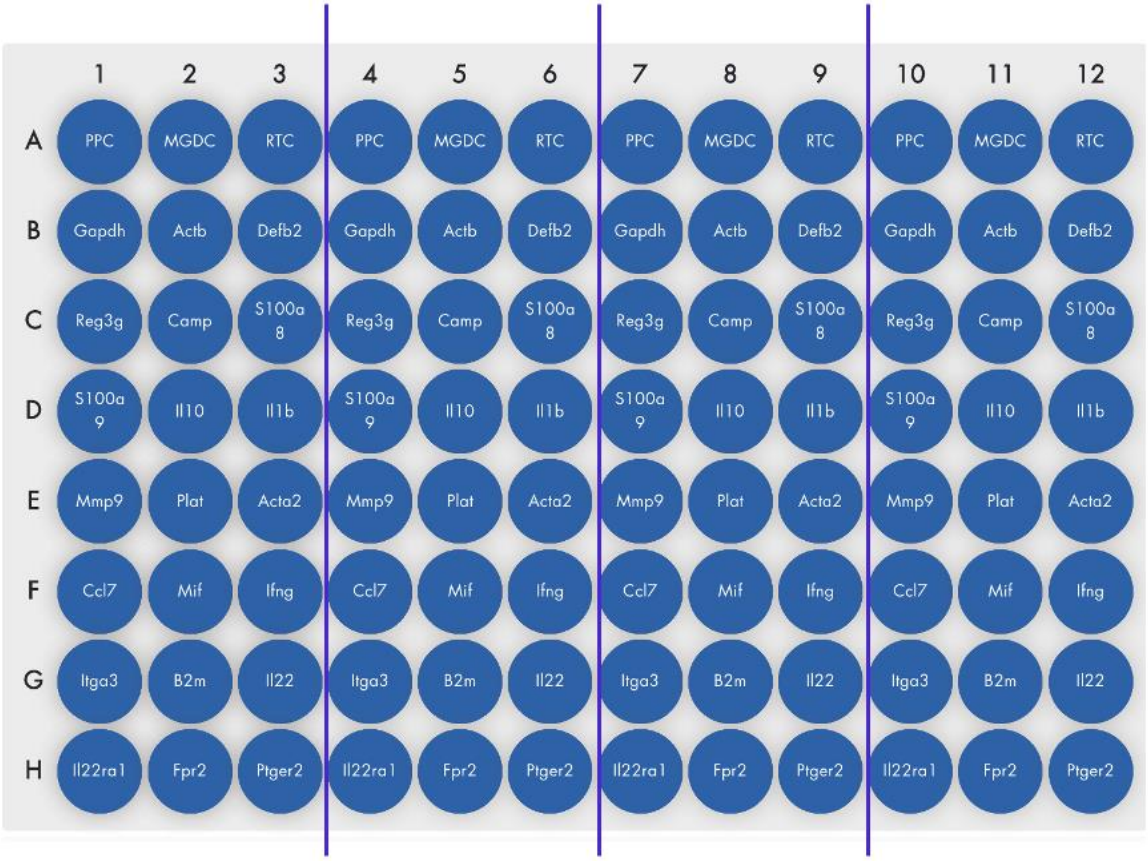

Figure 5. *Custom RT² Profiler PCR Array Set-Up*

Note: Array was arranged to allow for four samples per plate with 19 genes being tested and 5 housekeeping genes.

2.2.4 Statistical Analysis

To determine differences between treatments groups, two-way ANOVA followed by Tukey post-hoc multiple comparison tests were conducted. GraphPad Prism software (Version 9) was used to generate all data figures, and p value < 0.05 was considered statistically significant. Omega MARS software (Version 4.0) was used to analyze the ELISA data and to calculate the concentration of Reg3g based on the standard concentration curve. The average of each sample's replicate was taken for statistical analysis. Bio-Rad CFX96 software was used to analyze RT-

qPCR data and calculate the expression of each protein being analyzed based on the housekeeping genes. The Cq (threshold cycle) was collected in GraphPad Prism and used to generate all data figures and *p* values.

CHAPTER 3

RESULTS

3.1 *In vivo* Interleukin-22 Knockout Repetitive DE Exposure Model

3.1.1 Effects of Dust Exposure on Beta-Defensin-2 Expression

To evaluate the role IL-22 plays in beta-defensin-2 production, we assessed whether beta-defensin-2 expression is altered between wildtype and IL-22 knockout mice lung tissue. Beta-defensin-2 exhibited trends of similar punctate IF staining but in variable locations throughout exposure groups. While there were no statistical differences between exposure groups, genotype groups, and genotype x exposure interactions, trends of varying amounts of beta-defensin-2 were observed to be present within the epithelial cells. Both groups treated with saline seemed to show trends of similar amounts of B-defensin-2. The wildtype mice that were exposed to organic dust extracts were shown to have more B-defensin-2 production than their knockout counterpart, which is consistent with our hypothesis. However, these showed no significant trends. We also found trends of varying levels of beta-defensin-2 production between the exposure and genotype groups. The data from IF staining exhibited no significant differences. ANOVA results indicated $p = 0.5720$ for exposure groups, $p = 0.4746$ for genotype groups, and $p = 0.5978$ for interactions.

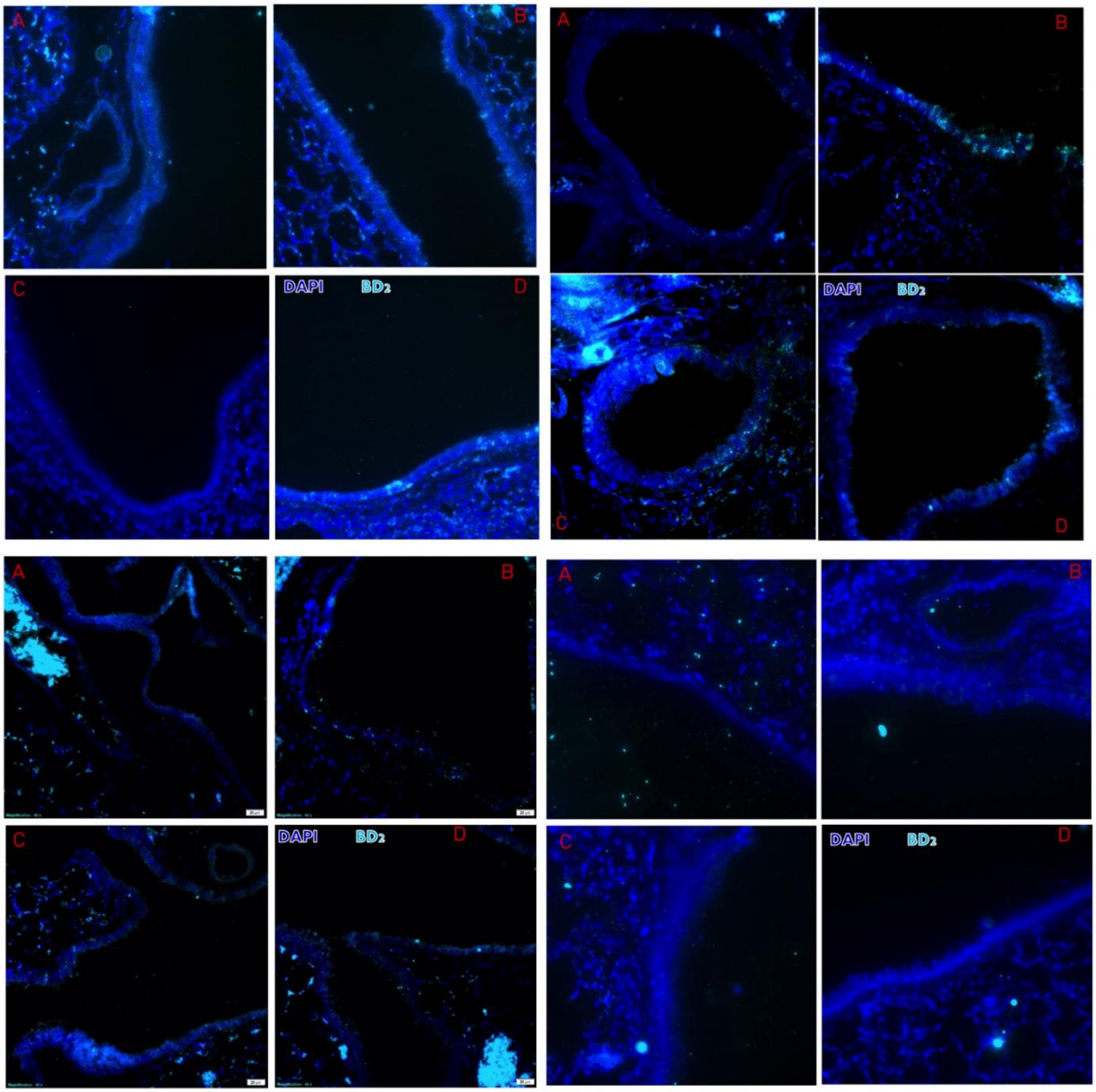

Figure 6. *Effects of IL-22 on Beta-Defensin-2 Production in DE-Exposed and Saline-Exposed Wildtype and IL-22 Knockout Mouse Lung Tissue*

Note: Images show airway, epithelium, and alveolar space. The mice were not perfused so there is background present. Panel (A): Wildtype Saline; Panel (B): IL-22 Knockout DE; Panel (C): Wildtype DE; Panel (D): IL-22 Knockout Saline. Dark Blue Stain = DAPI, Cyan Stain = beta-defensin-2 (BD2). $n = 4$.

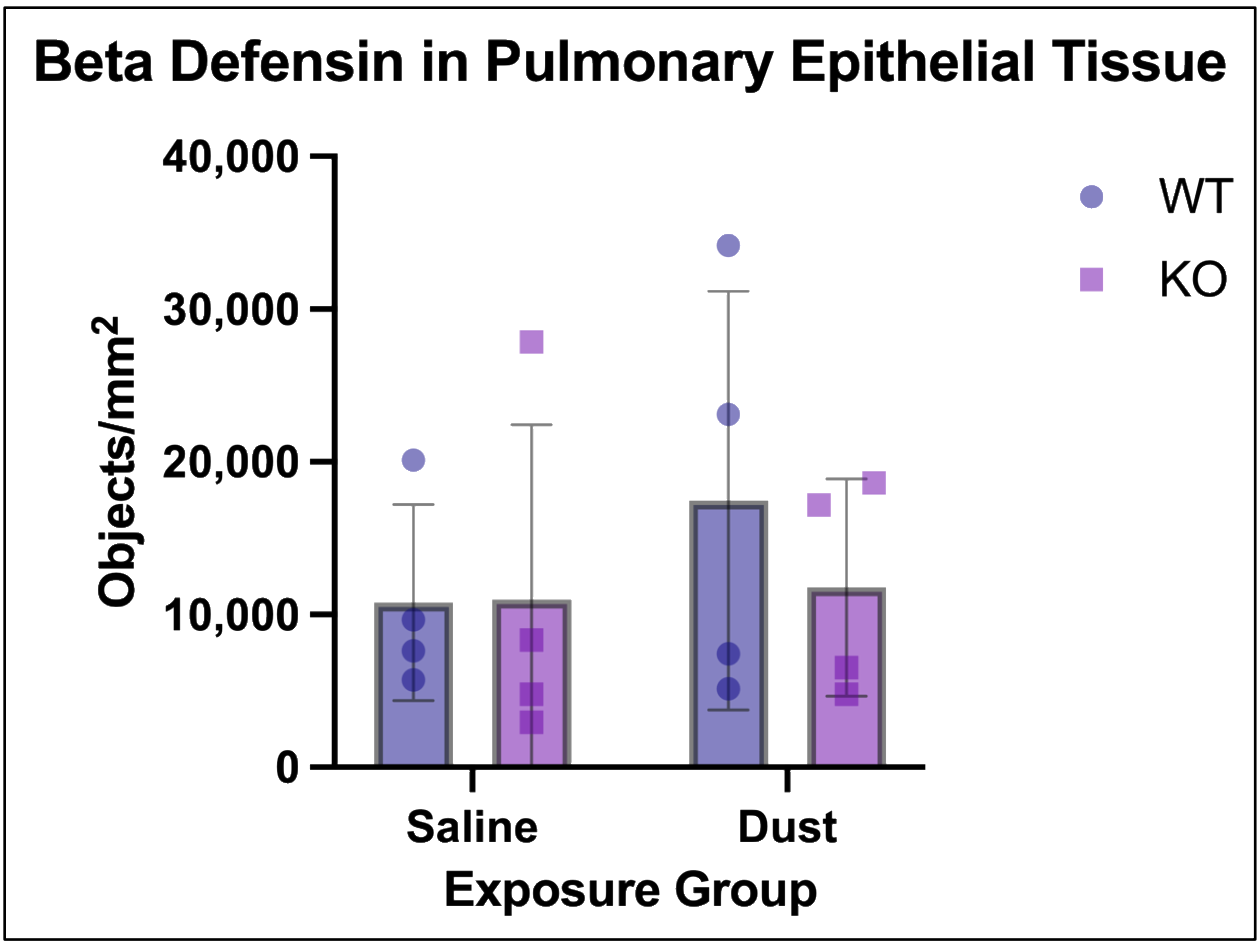

Figure 7. *Quantification of Beta-Defensin-2 in Pulmonary Epithelial Tissue From IF Images*

Note: Results are presented as the amount of positive stain per mm^2 within the epithelium. $n = 4$.

3.1.2 Effects of Dust Exposure on Reg3g Expression

To explore the role IL-22 plays in the expression of AMP and Reg3g, we assessed pulmonary tissues to determine the concentration of Reg3g protein present. Both wildtype and IL-22 knockout showed trends of similar concentrations of Reg3g when exposed to saline. The data from the ELISA exhibited a decreasing trend in genotype. Trends exhibited by the wildtype exposure groups stayed relatively the same, but the IL-22 knockout dust-exposed group had a decrease in Reg3g concentration compared to saline. This suggests that an interaction could be occurring between the genotype and exposure. However, a larger sample size is needed to determine significance.

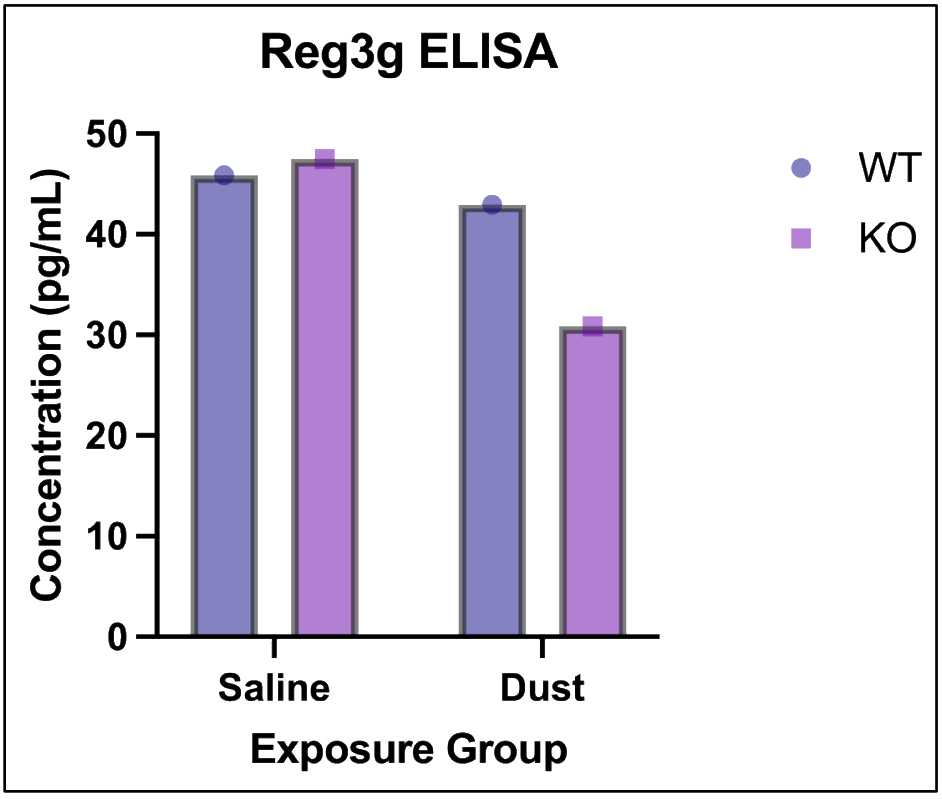

Figure 8. *Concentration of Reg3g in Pulmonary Epithelial Tissue From ELISA*

Note: Results are presented as the amount of protein present in each sample. $n = 1$.

3.1.3 Effects of Dust Exposure on Wound Healing

In order to explore the impacts of IL-22 on mediators of wound repair, we assessed pulmonary tissue mRNA for antimicrobial peptides, beta-defensin-2 (Defb2), regenerating islet-derived 3 γ (Reg3g), cathelicidin antimicrobial peptide (CAMP), S100 calcium-binding protein A8 (S100A8), and S100 calcium-binding protein A9 (S100A9). Defb2 exhibited no statistical significance for genotype groups, exposure groups, and genotype x exposure interactions. No clear trends are demonstrated for Defb2 expression. Reg3g exhibited no statistical significance for genotype groups, exposure groups, and genotype x exposure interactions. Reg3g demonstrated trends of expression in all mice regardless of genotype or exposure groups.

The antimicrobial peptide CAMP exhibited trends of increased expression in wildtype mice and decreased expression in IL-22 knockout mice in both saline- and DE-exposed groups. CAMP also exhibited trends of increased expression in DE-exposed IL-22 knockout mice compared to their saline-exposed counterparts. However, there was no statistical significance for genotype groups, exposure groups, and genotype x exposure interactions for CAMP. The expression of S100A8 and S100A9 exhibited similar decreasing expression when exposed to DE. ANOVA results demonstrated significance for IL-22 knockout saline-exposed and DE-exposed groups ($p = 0.0473$) for S100A8. ANOVA results demonstrated significance for IL-22 knockout saline-exposed groups and wildtype DE-exposed groups ($p = 0.0096$) and significance for IL-22 knockout saline- and DE-exposed groups ($p = 0.0290$) for S100A9.

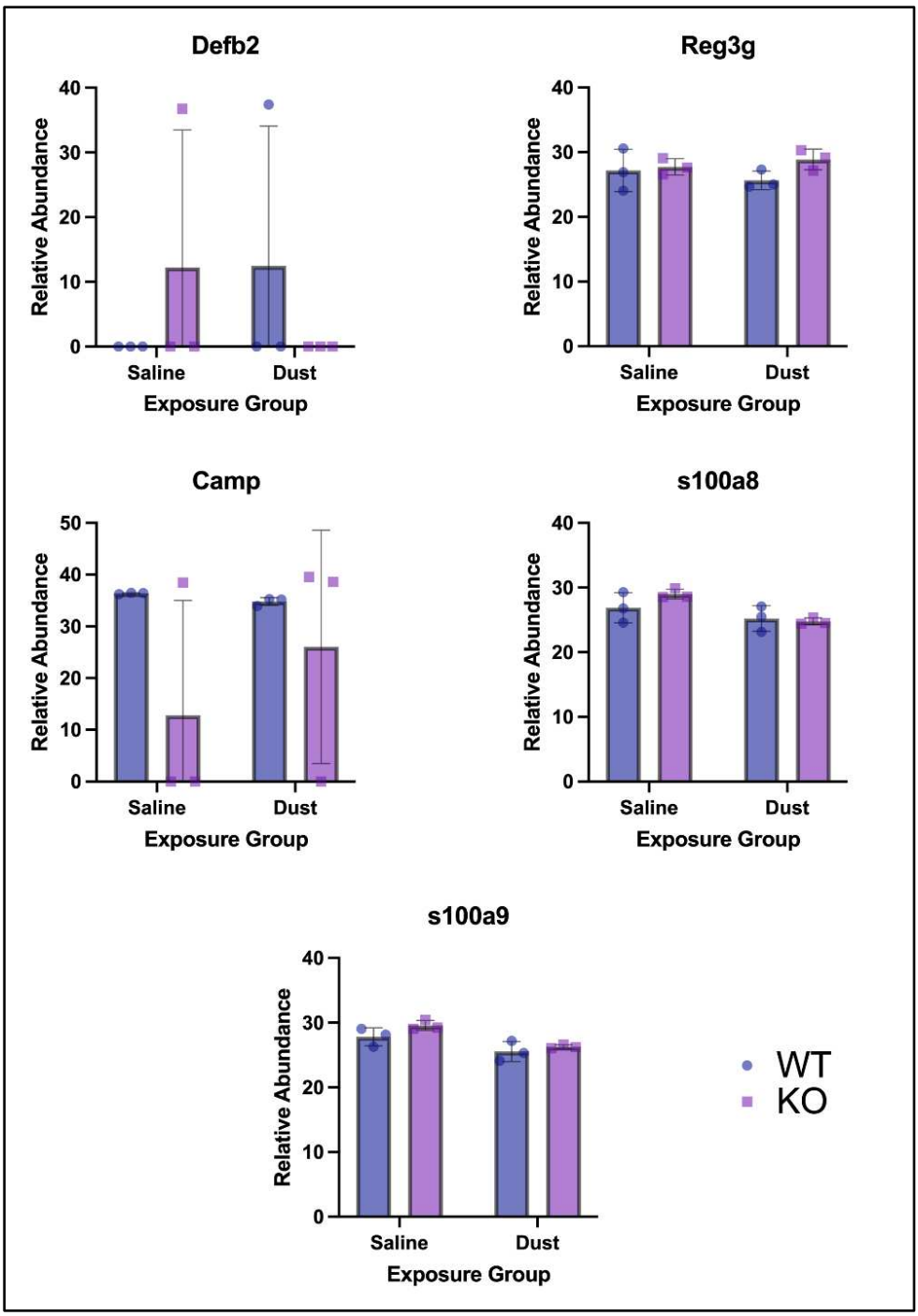

Figure 9. *Relative Abundance of Antimicrobial Peptides in Pulmonary Epithelial Tissues*

Note: Results are presented as the relative abundance, which is the number of cycles in the PCR amplification to see a positive signal present in each sample. $n = 3$. The significant findings were: S100A8: IL-22 KO saline-exposed vs. IL-22 KO DE-exposed: $p = 0.0473$. S100a9: IL-22 KO saline-exposed vs. WT DE-exposed: $p = 0.0096$. IL-22 KO saline-exposed vs. IL-22 KO DE-exposed: $p = 0.029$.

In order to explore the impacts of IL-22 on mediators of wound repair, we assessed pulmonary tissue mRNA for mediators of the anti-inflammatory response, interleukin 10 (il10), interleukin 22 (il22), interleukin 22 receptor, alpha 1 (il22ra1), and formyl peptide receptor 2 (Fpr2). Il10 exhibited no statistical significance for genotype groups, exposure groups, and genotype x exposure interactions. There were trends of decreased exposure between IL-22 knockout and wildtype groups in the context of both saline and DE. IL22 exhibited no statistical significance for genotype groups, exposure groups, and genotype x exposure interactions. IL22ra1 exhibited no statistical significance for genotype groups, exposure groups, and genotype x exposure interactions. There were trends of increased expression shown in wildtype DE-exposed mice compared to their saline counterparts as well as trends of decreased expression in IL-22 knockout saline-exposed mice compared to their DE-exposed counterparts. Frp2 exhibited no statistical significance for genotype groups, exposure groups, and genotype x exposure interactions. Trends exhibited expression in wildtype DE mice was increased compared to saline exposed counterparts and expression in IL-22 knockout saline exposed mouse was decreased compared to the DE-exposed counterpart.

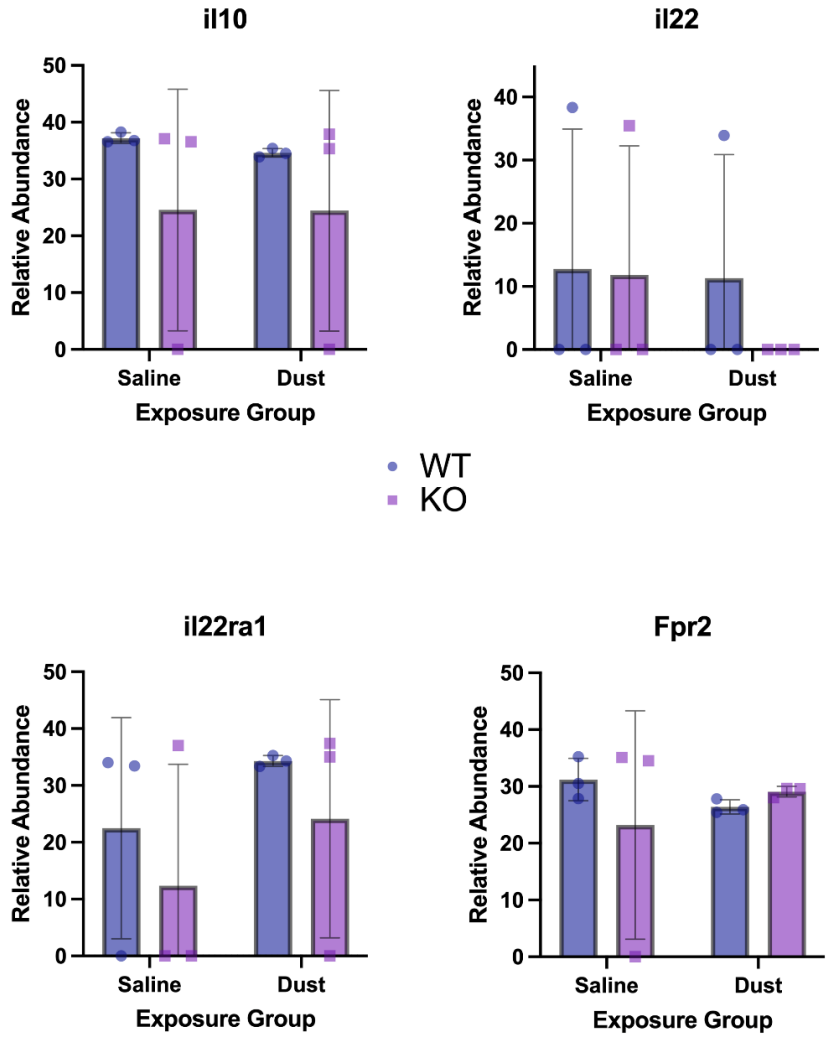

Figure 10. *Relative Abundance of Anti-Inflammatory Markers in Pulmonary Epithelial Tissues*

Note: Results are presented as the relative abundance, which is the number of cycles in the PCR amplification to see a positive signal present in each sample. $n = 3$.

In order to explore the impacts of IL-22 on mediators of wound repair, we assessed pulmonary tissue mRNA for mediators of the pro-inflammatory response, interleukin 1 beta (il1b), macrophage migration inhibitory factor (MIF), and prostaglandin E receptor 2 (Ptger2). IL1b exhibited no statistical significance for genotype groups, exposure groups, and genotype x

exposure interactions. The trends regarding exhibited expression of Il1b were increased in the context of DE-exposed groups for both wildtype and IL-22 knockout. Expression of MIF shows little difference in expression occurring between genotype and exposure groups. ANOVA results exhibited statistical significance for expression of MIF in IL-22 knockout saline-exposed vs. wildtype DE-exposed ($p = 0.0199$). Ptger2 exhibited no statistical significance for genotype groups, exposure groups, and genotype x exposure interactions. Expression of ptger2 demonstrated trends of increased expression in DE-exposed mice as compared to saline-exposed groups.

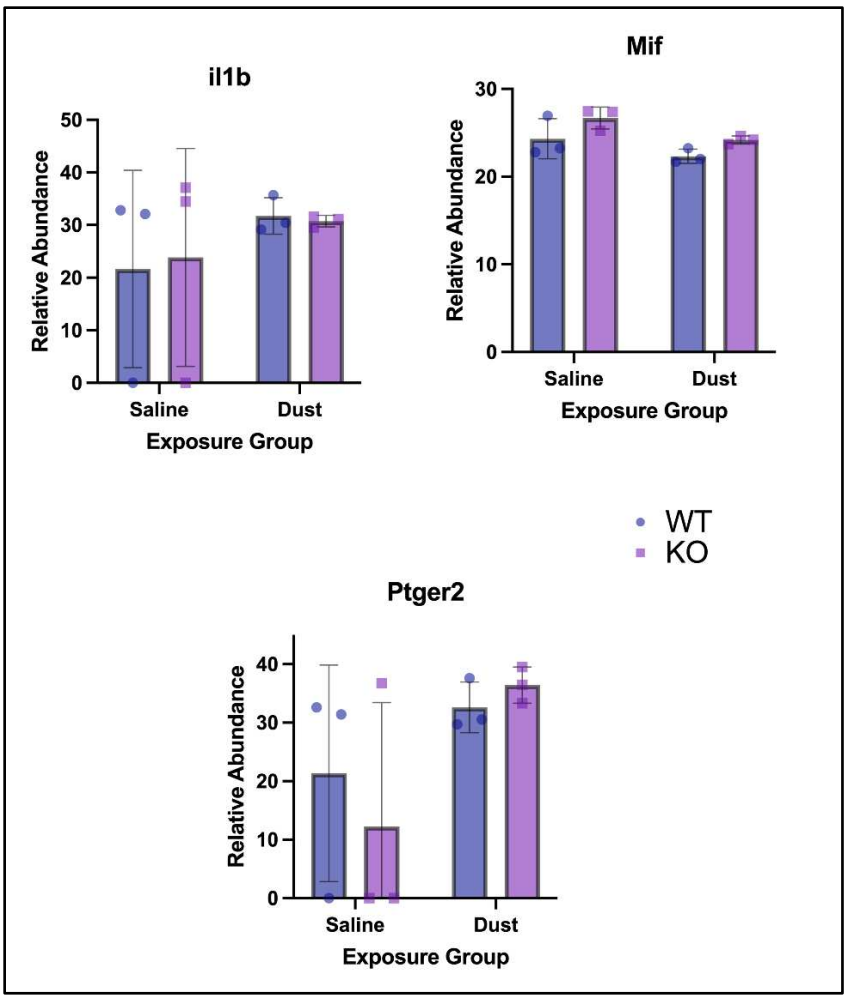

Figure 11. *Relative Abundance of Pro-Inflammatory Markers in Pulmonary Epithelial Tissues*

Note: Results are presented as the relative abundance, which is the number of cycles required in the PCR amplification to see a positive signal present in each sample. $n = 3$. MIF for IL-22 knockout saline-exposed vs. wildtype dust-exposed was significant at $p = 0.0199$.

To explore the impacts of IL-22 on mediators of wound repair, we assessed pulmonary tissue mRNA for markers of wound repair mechanisms, matrix metallopeptidase 9 (Mmp9), plasminogen activator, tissue (Plat), actin alpha 2, (Acta2), chemokine ligand (Ccl7), interferon gamma (Ifng), integrin alpha 3 (Itga3), and beta-2 microglobulin (B2m). Mmp9 exhibited no statistical significance for genotype groups, exposure groups, and genotype x exposure

interactions. Trends of expression exhibited that mmp9 was increased in IL-22 knockout DE-exposed groups as compared to their saline counterparts; no change was found in both saline- and DE-exposed wildtype mice. Acta2 exhibited no statistical significance for genotype groups, exposure groups, and genotype x exposure interactions. Trends exhibited that acta2 was decreased in DE-exposed mice compared to their saline counterparts for both IL-22 knockout and wildtype groups. IFN-gamma exhibited no statistical significance for genotype groups, exposure groups, and genotype x exposure interactions. Trends exhibited that ifng was increased in DE-exposed mice compared to their saline counterparts for both IL-22 knockout and wildtype groups. Plat exhibited no statistical significance for genotype groups, exposure groups, and genotype x exposure interactions. Trends demonstrated that expression was increased in IL-22 knockout DE mice but decreased in their saline counterparts, whereas wildtype mice exhibited no clear trends. Ccl7 was found to be decreased in IL-22 knockout DE-exposed mice compared to their saline-exposed counterparts. ANOVA results demonstrated significance for IL-22 knockout saline-exposed vs. wildtype DE-exposed ($p = 0.0193$) and IL-22 knockout saline-exposed vs. IL-22 knockout DE-exposed ($p = 0.0169$). Itga3 exhibited a decreased expression in DE-exposed mice compared to their saline counterparts. ANOVA results demonstrated significance for IL-22 saline-exposed vs. wildtype DE-exposed ($p = 0.0334$). B2m exhibited no statistical significance for genotype groups, exposure groups, and genotype x exposure interactions. Trends demonstrated that expression was decreased in the context of DE-exposed groups as compared to saline counterparts.

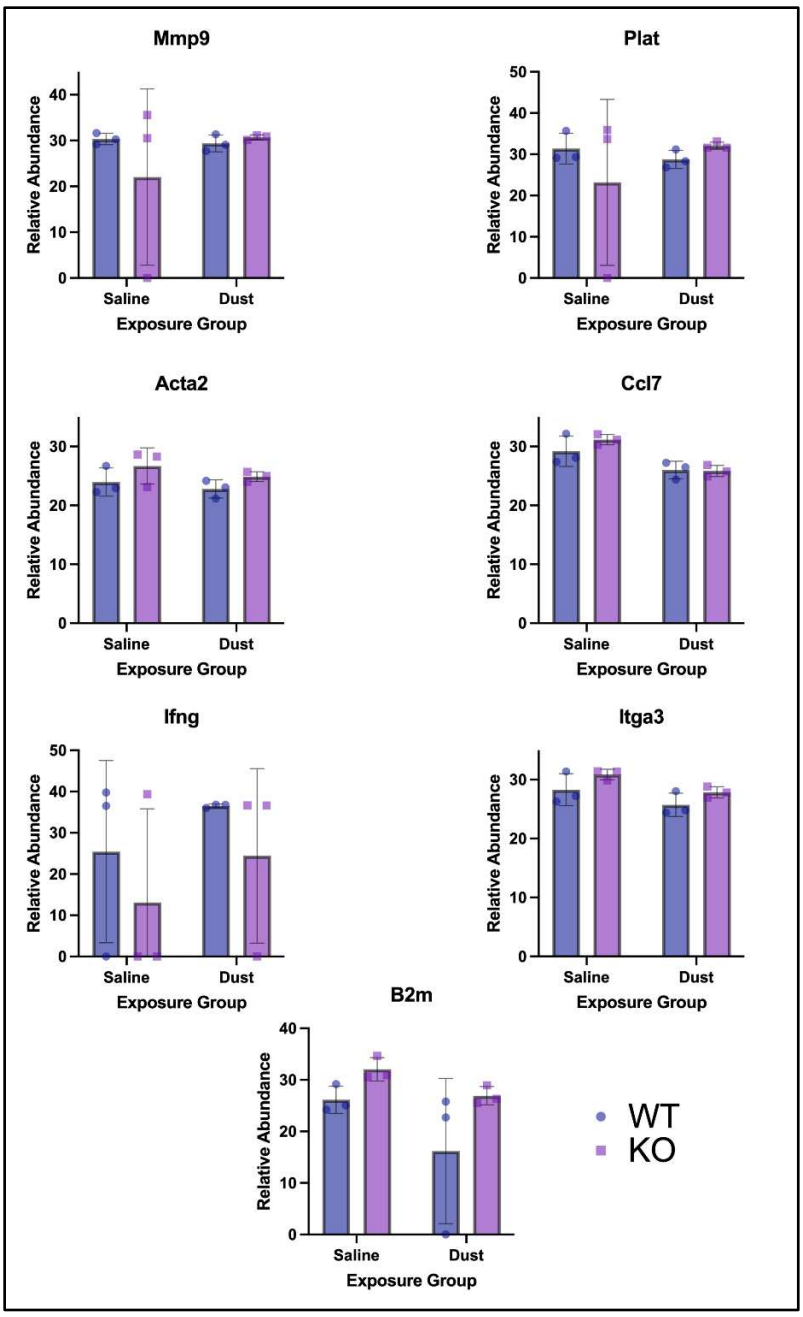

Figure 12. *Relative Abundance of Wound Repair Markers in Pulmonary Epithelial Tissues*

Note: Results are presented as the relative abundance, which is the number of cycles in the PCR amplification to see a positive signal present in each sample. $n = 3$. The following were found to have significant p-values. For Ccl7: IL-22 KO saline-exposed vs. WT DE-exposed, $p = 0.0193$. For IL-22 KO saline-exposed vs. IL-22 KO DE-exposed, $p = 0.0169$. For Itga3: IL-22 KO saline-exposed vs. WT DE-exposed, $p = 0.0334$.

3.2 *In vivo* Fat-1 Repetitive DE Exposure Model: Effects of Dust Exposure on Reg3g

To evaluate the role omega-3 fatty acids play in the expression of Reg3g, we stained pulmonary epithelial tissues in fat-1 and wildtype mice. The IF staining, as shown in the images in Figure 13, showed trends of variable staining through genotype and exposure groups. The data exhibited trends that Reg3g expression was increased in wildtype DE exposed groups. While there was no significance between genotype and exposure groups, trends exhibit that there may be an interaction occurring that caused an increased expression in the wildtype DE-exposed model.

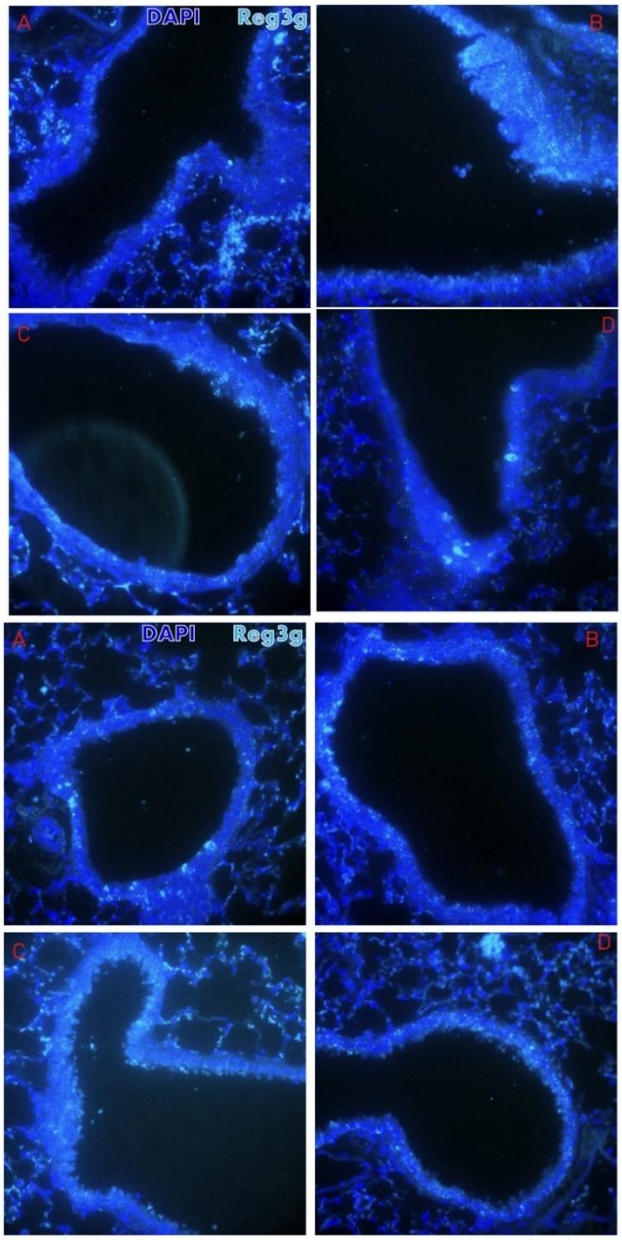

Figure 13. *Effects of Omega-3 Fatty Acids on Reg3g Production in DE-Exposed and Saline-Exposed Wildtype and in Fat-1 Mouse Lung Tissue*

Note: Images show airway, epithelium, and alveolar space. Mice were not perfused so there is background present. Panel (A): WT Saline; Panel (B): Fat-1 DE; Panel (C): WT DE; and Panel (D): Fat-1 Saline. Dark Blue Stain = DAPI, Cyan Stain = Reg3g. $n = 2$.

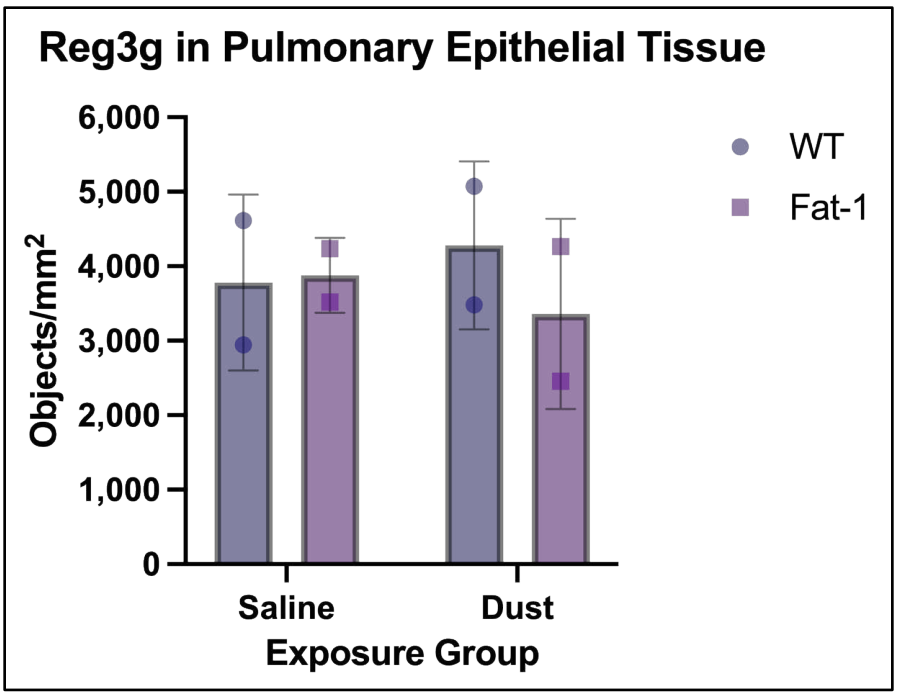

Figure 14. *Quantification of Reg3g in Pulmonary Epithelial Tissue from IF Images*

Note: Results are presented as the amount of positive stain per mm^2 within the epithelium. $n = 2$.

CHAPTER 4

SUMMARY OF FINDINGS

4.1 Discussion

This study aimed to explore the impacts that IL-22 and omega-3 fatty acids have on mediators of mucosal integrity following chronic dust exposure. Special attention was paid to the expression of AMPs, beta-defensin-2 and Reg3g. In addition, we looked for markers of wound repair in mouse lung tissues. We expected to see an increase in AMP expression and wound repair mechanisms when IL-22 was present in tissue. We also expected that omega-3 fatty acids would enhance the expression of Reg3g in the context of DE exposure.

In the investigation of the expression of beta-defensin-2 in bronchial epithelial tissue, the data exhibit variable punctate staining. While there were no statistical differences between exposure groups, trends were observed of varying amounts of beta-defensin-2 being present within the epithelium. As previously stated, it is well known that AMP can be secreted or induced by cytokines. Therefore, it is possible this punctate staining is due to beta-defensin-2 being expressed in unstimulated cells in basal amounts (Dalcin and Ulanova 2013; Diamond et al. 2009). The variable staining shows that beta-defensin-2 is not being expressed by all epithelial cell types, but rather only certain epithelial cells. Goblet and club cells are epithelial cells that are known for their roles in inflammation resolution and homeostasis (Crystal et al. 2008; Liu et al. 2019). These cells could be crucial in the expression of beta-defensin-2 and further studies into the role they play on beta-defensin-2 secretion should be conducted. In the IF staining, we also began to see trends of highly positive staining associated in the branchpoints of airways. This might be related to the presence of microfold—or M—cells, which aid in mucosal

integrity and are typically found in the epithelium overlying organized mucosal lymphoid tissues (Dillon and Lo 2019). Further exploration on the accumulation of M cells and their role in the promotion of beta-defensin-2 could aid in identifying the cause of variable beta-defensin-2 staining. Looking at the data from quantifying the IF staining of beta-defensin-2, we find an increasing trend in beta-defensin-2 expression in wildtype dust-exposed mice. This indicates that with IL-22 present, beta-defensin-2 expression is upregulated in the context of dust exposure, which is also consistent with our hypothesis.

The study also examined the expression of Reg3g protein in the pulmonary tissues to better understand the role of IL-22 in the promotion of AMP. Reg3g showed trends that decreased in the context of dust exposure when IL-22 was not present. There is little known about Reg3g's role in pulmonary infection. However, a previous study conducted by Ito et al. (2017) explored the roles of IL-22 and Reg3g in the context of dust-mite induced asthma. The authors found that Reg3g production was enhanced by IL-22 induced from House Dust Mite (HDM) stimulation in lung epithelial cells in STAT3 dependent manner (Ito et al. 2017). STAT3 activation has been shown to promote IL-22–dependent mucosal wound healing and when stat3 was inhibited, Reg3g induced by HDM was decreased. Another study that explored the impacts of IL-22 on STAT3 activation found that IL 22 knockout mice exhibited a complete lack of STAT3 activity (Pickert et al. 2009; Ratsimandresy et al. 2017). This indicates that it could be hypothesized that the downward trend seen in the IL-22 knockout DE is associated with activation of STAT3. Further studies into the role STAT3 plays on Reg3g expression in the context of organic dust exposure are needed.

When assessing the role IL-22 plays in wound repair mechanisms, we utilized a RT^2 custom profiler array to analyze antimicrobial peptides, wound repair genes, pro-inflammatory

genes, and anti-inflammatory genes. To investigate the interaction for the effect of IL-22 on antimicrobial peptides in the context of dust exposure, we analyzed AMP genes in wildtype and IL-22 knockout mice. We found a decrease in the DE exposure groups for the expression of AMP, S100A8, and s100a9 in both IL-22 knockout and wildtype mice. It is possible that S100A8 and S100A9 had interactions between the genotype and exposure groups that caused a decrease in their expression. S100A8 and S100A9 have been shown to have both pro and anti-inflammatory properties and can be expressed or induced depending on the cell type and stimuli in the environment (El-Gazzar 2015). The decrease in the context of organic dust could be due to excessive inflammation occurring, which results in these AMPs being decreased due to their role in inflammation in the context of dust exposure. By exploring expression in the bronchoalveolar fluid, we can obtain a better understanding of the secretion patterns exhibited by these AMPs and hopefully learn more about their role in inflammation and resolution. Trends also a showed a difference between the genotype groups in the expression of CAMP. CAMP exhibited a trend of decreased expression in IL-22 knockout mice in both dust and saline groups. Previous studies have found that IL-22 and IL-17A, which is a pro-inflammatory cytokine, will stimulate the expression of CAMP (Kanda et al. 2012; Sakabe et al. 2014). It is likely that the lack of expression seen is due to the lack of IL-22 to aid in the promotion of the antimicrobial peptide CAMP (Kanda et al. 2012; Sakabe et al. 2014).

We also analyzed anti-inflammatory genes in wildtype and IL-22 knockout mice to investigate how IL-22 affects anti-inflammatory markers in the context of dust exposure. Our research found there were trends of low IL-22 expression seen in both genotype and exposure groups. These trends could be seen due to IL-22 having pro-resolution properties and the cell secreting pro-inflammatory proteins at this point. Previous studies conducted in the Colorado

State University Nordgren lab have shown IL-22 to be upregulated in the BALF after chronic dust exposure. Trends show small amounts of IL-22 is present in the RNA at the 5-hr time point, but further exploration of IL-22 expression at different time points could provide greater insights into its role (Ulu Sveiven et al. 2022). We found there were trends of increasing expression of the IL22ra1 gene in DE-exposed groups in both wildtype and IL-22 knockout mice. Moreover, wildtype mice exhibited trends of increased expression compared to IL-22 knockout mice. IL22ra1 is a receptor that mediates IL-22 as well as other cytokines and has roles in pathogenesis (Ahlfors et al. 2014; Carrión et al. 2013) Previous research has shown that IL22ra1 expression is increased in the lungs in the context of dust exposure (Ulu Sveiven et al. 2022). We also saw trends showing Frp2 expression is decreased in wildtype DE mice. Fpr2 plays a crucial role in the resolution of inflammation by its interaction with the polyunsaturated fatty acid Resolvin D1. This decrease in the context of dust exposure is likely due to excessive inflammation occurring and the lack of Resolvin D1 treatments (Li et al. 2020). To further explore the role of Frp2 on inflammation resolution, we should further investigate its expression in the context of omega-3 fatty acids and Resolvin D1.

In this study, we also investigated the expression of pro-inflammatory genes to better understand how IL-22 helps to regulate inflammation and promote resolution. The expression of IL-1b exhibited an increasing trend in the context of dust-exposed groups in both IL-22 knockout and wildtype mice. This implies there could be an interaction occurring between genotype and exposure groups. A larger sample size will help us to determine significant differences and clearer trends of the role of IL1b in wound repair after chronic dust exposure. IL-1b is a pro-inflammatory cytokine and participates in the inflammation process through activation from inflammasomes. Overactivation of Il-1b has been found to increase inflammation-associated

tumor invasiveness (Garon et al. 2020). In the context of dust exposure, inflammation is occurring, hence the trends exhibiting increased expression of IL-1b. MIF expression exhibited a decrease in dust-exposed groups in both wildtype and IL-22 knockout mice. MIF is an inflammatory mediator and expression is not mediated by IL-22, which is why expression is seen in both genotype groups (Florez-Sampedro et al. 2020). Ptger2 exhibited trends of increased expression in dust-exposed groups in both wildtype and IL-22 knockout mice. Ptger2 is a receptor for prostaglandin E_2 (PGE_2) which is involved in the pathogenesis of chronic inflammatory diseases. It is produced during an inflammatory response and is reported to be increased in patients with COPD (Mani et al. 2021).

Markers of wound repair in the context of dust exposure were also studied in this research. Specifically, we analyzed genes associated with wound repair in wildtype and IL-22 knockout mouse models. Trends of expression show Plat and Acta2 stay relatively the same throughout exposure and genotype groups. Wound repair genes are involved in homeostasis, so we would expect to see them in the context of inflammation and resolution. These wound repair markers show trends of not being mediated through IL-22, as they have no change in expression when IL-22 is not present. B2m exhibits a decreasing trend in the dust-exposed groups in both wildtype and IL-22 knockout mice as compared to saline, with trends of increased expression in IL-22 knockout mice as compared to wildtype mice. A previous study that looked at the correlation between IL-22 and B2m in the context of multiple myeloma, found a positive correlation between IL-22 levels in serum and B2m. The data obtained here could be due to interactions happening in the context of dust exposure (Tsirakis et al. 2015). Both Ccl7 and Itga3 were shown to decrease in the context of dust exposure. Itga3 is widely expressed in the lung epithelium and has roles in cell survival, cell proliferation, and Itga3 functions within tissues.

Studies have shown that loss of function of Itga3 leads to increased disease progression (Yalcin et al. 2015). Ccl7 is a chemokine that aids in the wound-healing process through regulation of angiogenesis and recruitment of immune cells. Ccl7 has been found to be involved in the inflammatory step of wound healing by attracting macrophages and aiding in angiogenesis (Ridiandries et al. 2018).

To obtain insights into the impacts of omega-3 fatty acids on expression of Reg3g, we looked at expression in pulmonary epithelial tissues in fat-1 and wildtype mice. There was a trend of increases in expression in wildtype DE mice. Not much is known about the role omega-3 fatty acids play in the expression of AMP. However, previous studies on omega-3 fatty acids have shown that they possess their own antimicrobial activity and may not need the secretion of AMP to aid in resolution (Chanda et al. 2018).

4.2 Limitations

In this research, *in vivo* studies were limited to male mice due to the availability of lung tissue provided. Previous studies have shown that in the fat-1 mouse model, females have different conversion rates of fatty acids than males (Ulu Velazquez et al. 2022). This could lead to changes in gene expression of cytokines and alter the secretion of AMP found in the lung epithelial (Ulu Velazquez et al. 2022). Another limitation to our study is the use of the transgenic fat-1 model. This model achieves the ideal 1:1 ratio of omega-3 to omega-6 fatty acids in tissues, which is not translational to the human condition. Human omega-3 intake varies among individuals, which means the effects of omega-3 fatty acid will vary in that population. The lack of investigation for beta-defensin production in the fat-1 mouse model is another limitation. Our model also used intranasal instillation, which is not the same way that agricultural workers inhale these organic dusts. Therefore, this model lacks the real-world implication of a human breathing

in organic dusts accompanied by certain of the surrounding inhalants one subject population of interest—agricultural workers—might be exposed to (e.g., chemicals, animal by-product, air pollution). Inclusion of such might result in slightly different results as inhaled particulate matter may have greater effect on the mucosal layers in comparison to intranasal instillation.

The *in vivo* IL-22 knockout studies showed no significant data in IF staining for beta-defensin-2. ELISA studies were limited to the expression of Reg3g and lacked exploration of beta-defensin-2. All animal studies were limited to lung tissue due to availability of samples. AMP are known to be produced and secreted, so exploring other components of the respiratory system, such as lavage, might obtain greater insights into the variable expression we observed in the IF staining. We could also look into the expression and transcription levels of AMP at different time points after dust exposure. Our studies looked at 5-hr post-DE exposed but looking at other time points—like 18 hr or 3 days—could give us further insights regarding when these AMPs are being secreted and how they are participating in the resolution process.

4.3 Future Directions

Beta-defensin-2 showed variable expression in IF staining in IL-22 knockout and wildtype mice models after dust exposure. Expanding studies beyond lung tissues to include lavage samples would allow us to quantify AMPs being secreted out of the epithelial cells. AMPs are shown to be associated with pulmonary inflammation and disease severity (Chen et al. 2004; Feng et al. 2023). Chen et al. (2004) found that beta-defensin-2 is present in the BALF, so being able to compare amounts shown in lung tissue to amounts in lavage would be beneficial to study the promotion and secretion of AMP. Furthermore, our *in vivo* IL-22 knockout dust exposure studies exhibited no significant differences between exposure groups. This indicates

that a power analysis should be done to estimate the proper sample size required to see statistical significance in the data (Brownlee 2020).

Another future direction would be to explore the impacts of omega-3 on AMP expression, mediated through IL-22. Studies were done looking at the effects of omega-3 fatty acids and IL-22 on AMP production independently after dust exposure. To evaluate the impacts of omega-3 fatty acids on AMP after dust exposure, as mediated through IL-22, we could utilize a fat-1^{tg}il$22^{-/-}$ model, which would express the *C. elegans* fat-1 gene while also having a whole body IL-22 knockout. This would mean that we could explore the role of IL-22 in AMP expression with omega-3 fatty acids present after chronic dust exposure. Expanding further on this model, we could introduce resolvin, protectin, and maresin treatments to evaluate the substrates of omega-3 fatty acids and the role they play in the resolution of chronic inflammation.

4.4 Conclusions

Immunofluorescence staining of beta-defensin-2 in IL-22 knockout models exhibited similar trends in staining throughout exposure groups but in variable locations. We can confirm that beta-defensin-2 is present in bronchial tissue following chronic dust exposure. We did not expect to see beta-defensin-2 in the IL-22 knockout models; however, AMPs are known to reside in mucosal surfaces in unstimulated basal amounts. Reg3g concentrations were present in both wildtype and IL-22 knockout tissues after saline and dust exposure. CAMP showed trends of low expression in IL-22 knockout models. This demonstrates that CAMP could rely on IL-22 to aid in its expression. Expressions of S100A8 and S100A9 were shown to decrease among dust-exposed groups. This could be due to the function of these AMPs in the context of organic dust. Reg3g was shown to be decreased in fat-1 dust-exposed models, which could be due to the

antimicrobial nature of omega-3 fatty acids. Further studies that include exploring the lavage for secreted AMP should be conducted to better understand the expression and release of AMPs. Ultimately, we hope that our studies will offer new avenues for therapy options for agricultural workers who suffer from lower respiratory diseases such as COPD.

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