THESIS

RESOLVIN D1 MODULATES THE PULMONARY IMMUNE RESPONSE TO AGRICULTURE DUST EXPOSURE

Submitted by

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ABSTRACT

RESOLVIN D1 MODULATES THE PULMONARY IMMUNE RESPONSE

TO AGRICULTURE DUST EXPOSURE

Occupational exposure to agriculture dust causes a variety of acute and chronic pulmonary diseases including allergies, asthma, chronic obstructive pulmonary disease (COPD) and organic dust toxic syndrome (ODTS). These diseases have high impact on the healthcare system and limited treatments with variable efficacy. In addition, workers often display low compliance with required workplace personal protective equipment (PPE), increasing their risk for developing these diseases. Therefore, the development of new pharmacological interventions is critical to alleviate the burden on the healthcare system and improve the quality of life for patients who will inevitably develop occupational-related pulmonary diseases. Interleukin-22 (IL-22) is a cytokine in the anti-inflammatory interleukin-10 (IL-10) family of cytokines that has demonstrated a protective role in murine models of acute and chronic lung injury. It has been described as being exclusively produced by lymphocytes, however methodological limitations of the primary cited study restricted the exploration of other cell types as producers of IL-22. Upregulation of this cytokine by pharmacological means could prove beneficial for delaying the progression of occupational chronic pulmonary diseases. Omega-3 fatty acids and their metabolites have well-documented anti-inflammatory and pro-resolution functions in chronic pulmonary diseases and have been implicated in the induction of IL-22. Omega-3 fatty acids have shown overwhelming evidence in being anti-inflammatory by their function as substrates

for the production of specialized pro-resolving mediators (SPMs), lipid metabolites that signal immune cells to transition to a resolution and repair state following inflammation. Resolvin D1 (RvD1), a metabolite of the omega-3 fatty acid docosahexaenoic acid (DHA), has shown to have anti-inflammatory and protective functions in a murine acute lung injury model.

To evaluate the source of IL-22 in the pulmonary response to agricultural dust, mouse alveolar macrophages were co-exposed to 1% hog dust extract (DE) collected from swine confinement facilities in the Midwest US and treated with either 10 nM or 100 nM RvD1. Cells were incubated for up to 24 hours, supernate was collected at the desired timepoint, and enzyme-linked immunosorbent assays (ELISAs) were performed to assess protein expression. Cells were also lysed to determine intracellular IL-22 protein concentrations. Cells exposed to DE exhibited increased pro-inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) as well as increased IL-10 and IL-22 production, demonstrating macrophages as a source of IL-22 in the immune response to organic dust. Cells exposed to DE and treated with RvD1 demonstrated significant decreases in IL-6 and TNF-α and increases in IL-10.

To determine the efficacy of RvD1 as an inducer of IL-22, and as a potential treatment for organic dust-induced lung injury, C57BL/6 (WT) and full-body IL-22 knock-out (KO) mice were intranasally instilled (IN) with 12.5% DE 5 days/week for 3 weeks and injected intraperitoneally (IP) with 250 ng RvD1 once per week. Animals were allowed to recover for 5 hours or 3 days before sacrifice where bronchoalveolar lavage fluid (BALF) was collected for cytokine and cellular infiltrate evaluation to determine the role of RvD1 in the reduction of the immune response to organic dust exposure. BALF cytokines exhibited significant increases in the production of IL-10 in KO mice exposed to DE and treated with RvD1 with a 3-day recovery. Cellular infiltrates demonstrated decreased neutrophil infiltration and increased

lymphocyte recruitment in KO mice exposed to DE after a 3-day recovery and further significant decreases in mice treated with RvD1 with a 3 day recovery.

The data support the production of IL-22 by alveolar macrophages and its induction by RvD1. They also demonstrate the effects of RvD1 on the pulmonary immune response to agriculture dust and as a potential therapeutic for organic dust-induced chronic pulmonary diseases.

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CHAPTER 1: BACKGROUND

1.1 Introduction

Agricultural workers report higher incidence of respiratory diseases such as allergies, asthma, chronic obstructive pulmonary disease (COPD), and organic dust toxic syndrome (ODTS)[5,34,45,99,148,165]. There are currently limited treatments for these diseases, which have undesirable immunological side effects, so development of improved pharmacological interventions is of increasing concern in public health[44,80,81,136,137]. Effective treatments exist for many asthma sufferers, but often with immunological side effects[20,86]. COPD patients in particular have limited options for symptom management, no course to slow the progression of the disease, and most inevitably die as a result of the disease[23,142]. Populations living around agriculture facilities are also at increased risk of these diseases[5]. To exacerbate matters, many agriculture workers do not comply with personal protective equipment (PPE) requirements, further necessitating the development of pharmacological interventions for the resulting chronic inflammatory diseases[68].

Inflammation is a desirable process. Many people would be surprised by that statement when medical professionals often prescribe anti-inflammatory drugs. The inflammatory response is beneficial to protect the body from foreign material and pathogens, and restriction of this process would allow for opportunistic infections[54]. Current treatments for many chronic inflammatory diseases focus on reducing inflammation, often resulting in blockage of resolution, as both responses are dependent on each other, and the inflammatory signals are critical to transitioning to a resolution response and restoring tissue homeostasis after injury[47,97].

The goal in the development of new treatments for chronic inflammatory diseases is to regulate the inflammatory response so that it does not become harmful while allowing for the resolution and repair processes to progress unhindered. The inflammatory response is initiated by the release of exosomes and other damage associated molecular patters (DAMPs) from injured epithelial cells[77]. It is then progressed and primarily coordinated by tissue-resident macrophages that produce pro-inflammatory cytokines such as interlukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) to influence and recruit other immune cells to clear the inflammatory trigger[55,67,104,108,148,161]. Once the trigger has been eliminated, anti-inflammatory and pro-resolution cytokines are produced, such as interleukin-10 (IL-10) by macrophages and interleukin-22 (IL-22) by lymphocytes[36,104].

IL-22 is a member of the IL-10 superfamily, a family of anti-inflammatory cytokines that are up-regulated in inflammation and assist in the resolution response and tissue repair after inflammation[104,155]. We have previously shown that increasing the production of IL-22 may play a role in shifting the immune response from pro-inflammatory to pro-resolution and could be an effective treatment to reduce the chronic inflammation that causes the progression of these diseases and increase epithelial repair and barrier integrity after organic dust exposure[108,148].

Omega-3 (ω-3) fatty acids are metabolized to specialized pro-resolving mediators (SPMs) during the inflammatory response and function to transition inflammation to resolution and repair processes[76,101,121]. The docosahexaenoic acid (DHA) derivative Resolvin D1 (RvD1) in particular has previously demonstrated anti-inflammatory and pro-resolution properties in many murine models of acute lung injury, sepsis, and chronic organic dust exposure[34,38,75,148]. We have also previously shown that RvD1 assists in the production of IL-22 and modulates the pulmonary immune response to organic dust[148].

1.2 Agriculture Dust-Induced Chronic Pulmonary Diseases

Agriculture workers who directly work on farms account for approximately $164.7 billion, or 0.7% of the United States' (US) Gross Domestic Product (GDP) as shown in **Figures 1 and 2**. This provides 2.6 million jobs, or 1.3% of the US workforce[149].

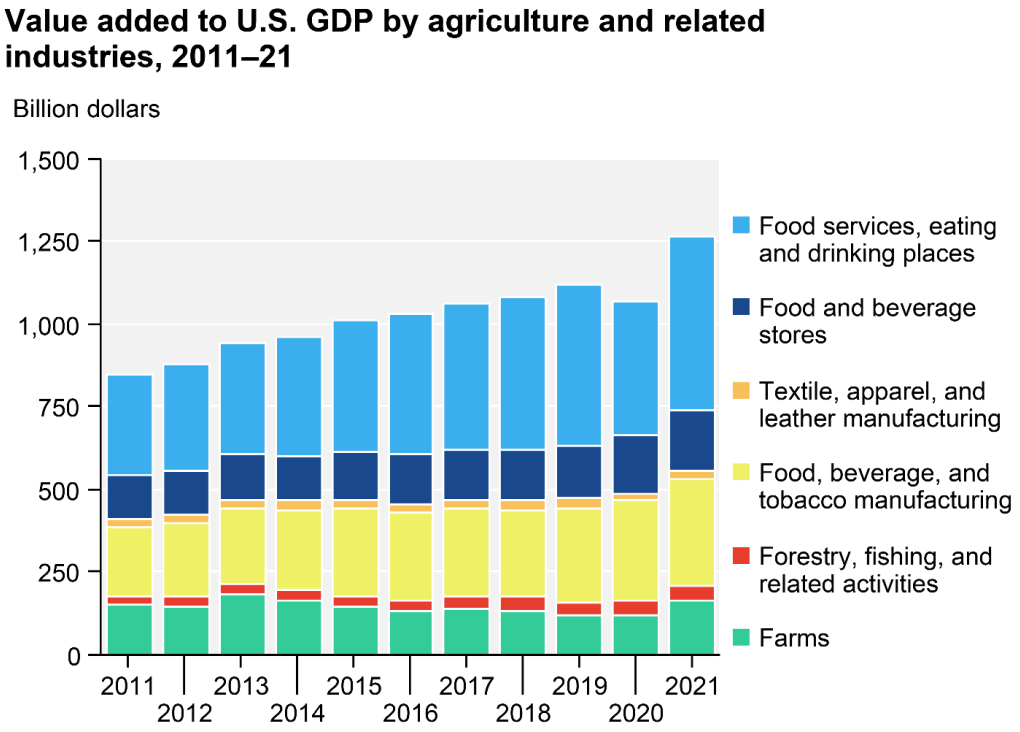

Figure 1. Agriculture and Food Industry Contribution to US Gross Domestic Product[149]

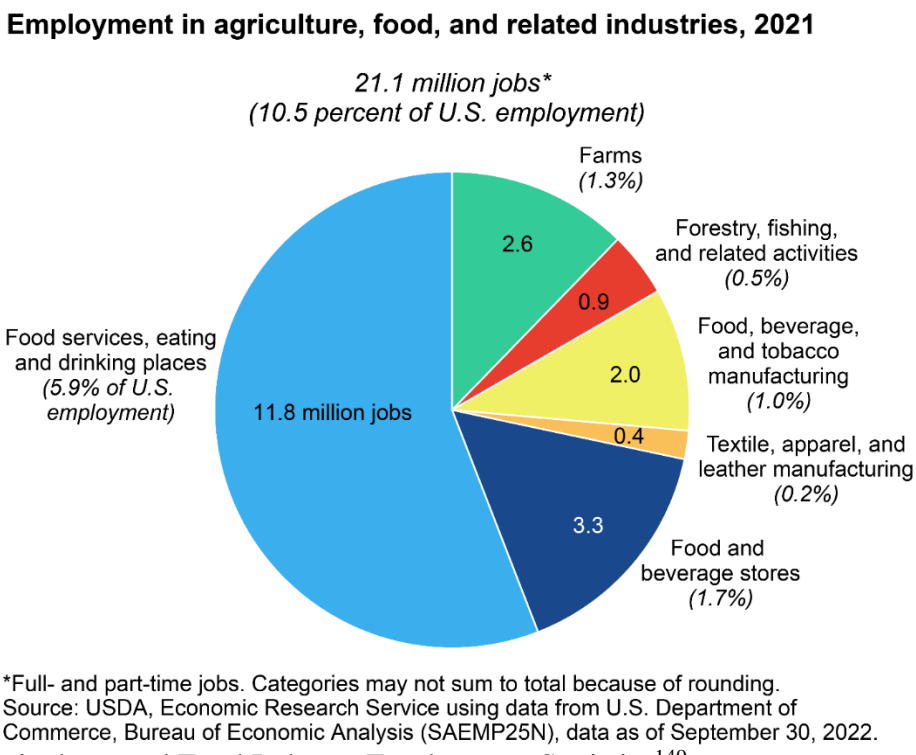

Figure 2. Agriculture and Food Industry Employment Statistics[149]

Agricultural workers are at elevated risk for chronic pulmonary diseases such as allergies, asthma, and COPD due to high exposure to organic dusts[42,100]. Agriculture dusts contain a wide variety of bacteria, fungi, and viruses, as well as endotoxins and lipopolysaccharides (LPS) that can contribute to the immune response[19].

26-53% of COPD cases in patients who have never smoked are attributed to occupational exposures, including dusts, with organic dusts significantly increasing the risk for chronic pulmonary diseases[141]. In addition, chronic lower respiratory tract diseases such as COPD are the 6th leading cause of death in the US[46]. Respiratory diseases associated with agricultural occupations pose a significant burden to the healthcare system and development of treatments is essential to relieve that burden[156].

Risk for organic dust-induced respiratory diseases can be reduced in workers by using personal protective equipment (PPE) designed to limit respiratory injury including but not limited to, disposable dust masks, respirators with cartridges or canisters, and face shields [4,12,141].

In a publication by Kearney et al., out of 129 workers surveyed, 46% reported never using dust masks, 15% reported using masks more than half of the time, and only 40% reported consistent use. For face shields, 69% of workers reported no use, 9% reported use over half of the time, and 23% reported consistent use. Cartridge respirator users reported no use in 69% of workers, use more than half the time in 11% of workers, and consistent use in 19% of workers. Canister respirator users reported no use in 77% of workers, 6% reported use more than half time, 17% reported consistent use. Workers reported the highest influence for wearing PPE was desire to avoid injury and warning stickers on equipment[68].

Despite this, a large population of workers do not comply with PPE requirements and receive the full potential dose of organic dusts, causing significant risk for respiratory diseases. 48% reported ease of using PPE had some influence over compliance while 36% reported strong influence. 34% reported cost of PPE as having some influence with 22% reporting strong influence. 37% reported the time required to put on PPE as having some influence and 20% reported it having strong influence. 16% reported personal appearance having some influence and 15% reported it having strong influence[68]. Time and effort required to don PPE combined with the cost makes workers less likely to use equipment intended to prevent respiratory and other diseases, leading to their inevitable development.

As compliance with PPE requirements is variable, therapeutics are needed to combat inevitable disease development. Therefore, a comprehensive approach of campaigns for increasing PPE use in workers and the development of pharmacological therapies for organic

dust-induced chronic respiratory diseases is required to reduce the number of patients who progress to severe respiratory disease due to their occupations.

1.2.1 Allergies

Other particulates are inhaled with organic dusts during agricultural work and can cause inflammatory responses in workers[19]. General allergies are caused by the adaptive hypersensitivity response to fungal and bacterial spores[131]. Agricultural workers are also highly susceptible to a specific form of allergies known as farmer's hypersensitivity pneumonitis (FHP), caused by inhalation of mold spores from animal feed[45]. Dendritic cells and eosinophils respond to the allergen to cause an immune response and tissue damage[28,130]. Eosinophils degranulate and release tissue-damaging substances while dendritic cells prime T cells to continue the immune response and cause further tissue damage and severe symptoms such as difficulty breathing[28,130].

Allergic reactions are characterized by an early and late phase. The early phase begins within minutes of exposure and involves cross-linking of IgE, and degranulation of mast cells and basophils to release histamines, leukotrienes, and prostaglandins and can last up to 3 hours[95,131,145]. The early phase is the main contributor to rhinorrhea symptoms[145]. The late phase occurs within 4 hours of exposure and can last up to 12 hours, It is characterized by the increase of leukocyte adhesion molecules to promote recruitment of monocytes, eosinophils, T cells, and basophils to the pulmonary mucosa and increased production of TNF-α by mast cells[95,132,138].

Allergic reactions to the spores in organic dust can be acute or chronic and may be associated with higher risk of asthma[45,111]. Acute symptoms typically manifest within 4 hours of exposure, peaking at 12 hours, and lasting up to 48 hours; they include fever, chills, frequent productive cough, shortness of breath, and chest discomfort. Chronic FHP can last significantly

longer and presents as a long chest cold. These symptoms can decrease a patient's quality of life and reduce their ability to perform necessary tasks[45].

There are limited treatments for allergies, with most research focusing on food sensitivities[136]. Antihistamines are the recommended treatment for exposure allergies, but often do not adequately control symptoms and may have adverse effects[84,136,166]. Immunotherapy is being explored for allergic rhinitis, where patients are inoculated sublingually with their sensitized agent to promote increased tolerance. However, it is only effective for IgE-mediated allergies, so is a viable option for workers with IgE-mediated allergies caused by organic dust exposure [86]. However, in workers that develop FHP, these treatments are not effective, so exploration of other therapies is needed[86,134]. In workers with fungal sensitivities, immunotherapy has low efficacy is controlling rhinitis symptoms, requiring alternative treatments[20]. RvD1 has demonstrated efficacy in reducing gut mucosal hypersensitivity through reduction of histamine release [107]. It has also shown to alter B cell class switching to inhibit IgE production while maintaining IgM, IgG, or IgA concentrations, protecting against allergic reactions while maintaining mucosal barrier integrity, indicating it may be an effective treatment for respiratory allergies[70].

1.2.2 Asthma

Asthma is a disease characterized by coughing, wheezing, shortness of breath, and difficulty breathing[4]. It can be exacerbated by environmental triggers and cause significant detriment to patients' quality of life[4,50,160]. Asthma classically presents as eosinophil-mediated, with some dendritic and T cell involvement[59,80]. Some patients may also present with

neutrophilic asthma, often during an attack and is associated with reduced efficacy of steroid treatments and higher mortality[112].

As of 2020, there are currently 21 million adults in the US living with asthma, which makes up 8.4% of the population[4,50]. Females are more likely to be diagnosed with asthma than males, a statistic that is consistent with females experiencing higher prevalence and severity of chronic pulmonary diseases[4,12,16].

Work-related asthma (WRA) includes the exacerbation of pre-existing asthma or the new onset of asthma in patients who have never experienced it before, due to one's occupation[159]. The American Thoracic Society estimates that 15% of American adults with asthma can attribute their disease to their occupation[159]. Populations repetitively exposed to organic dust, whether from their occupation or residence, exhibited a positive correlation with increased severity of pre-existing asthma and increased development of new asthma patients[165]. In the US, adult asthma patients account for over 1 million emergency room visits per year, with over 100,000 patients requiring in-patient hospital stays to manage their symptoms, and approximately 4,000 patients dying from asthma each year[94]. Asthma treatment and related hospital stays are estimated to cost the US healthcare industry approximately $50 billion each year[25].

Development of new therapies to treat WRA is paramount to decreasing the monetary and labor burden on the healthcare system and reduce side effects of current therapies. There are many current treatments for asthma, all carrying side effects and risks, and some are only effective against some subtypes[49,112]. Corticosteroids are the most commonly prescribed treatment[50]. They reduce the Th2 inflammatory response and modulate the immune system to reduce inflammation from asthmatic triggers[49,50]. Long-acting β-agonists (LABAs) are often prescribed in combination with corticosteroids to reduce their side effects and reduce the risk of

death associated with LABAs[50,87]. Leukotriene modifiers are prescribed to prevent the Th2 and Th17 responses, which can interfere with resolution actions of the Th17 pathway[50,153]. Theophyllines reduce symptoms through promotion of anti-inflammatory pathways through reduction of neutrophil recruitment and pro-inflammatory cytokine production[50].

Current treatments focus on anti-inflammatory therapies, which subsequently block the resolution response and return of tissue homeostasis[47,97]. IL-22 has been implicated as a regulator of asthma, and its production is thought to be driven by SPMs, therefore SPMs as a treatment would be effective to mediate the immune response to transition to a pro-resolution state, reducing symptoms and repairing tissues[13,64].

1.2.3 Chronic Obstructive Pulmonary Disease

COPD is a class of chronic irreversible lower respiratory diseases that include emphysema and chronic bronchitis. It is characterized by coughing and wheezing with increased pulmonary mucus production, eventually leading to shortness of breath, and difficulty taking deep breaths[12].

COPD patients experience reduced quality of life, with difficulties performing normal activities[78], often not engaging in social activities, and experience additional comorbidities including cardiovascular diseases, asthma, and cognitive difficulties[51,156]. COPD has also been linked to increased risk of developing severe COVID-19 infections by 47.2%[113]. The cost of COPD medical bills increased from $32.1 billion to $49 billion in 2020. 51% of COPD medical bills were paid by Medicare, 25% by Medicaid, and 18% by private medical insurance, placing a significant burden on the state-funded medical system[29].

Organic dust-induced COPD is associated with high mortality rates[142]. As the disease progresses, patients experience reduced oxygen availability[12]. Mortality of COPD patients in agriculture occupations account for 11.3% of the chronic pulmonary disease-related deaths in the US[142]. COPD can also be associated with cardiovascular disease, heart failure, arrhythmia, and pulmonary embolism[16]. Women are affected more severely than men and have a higher mortality rate, and as of 2018, it is the 4th leading cause of death among adult women in the US[12,24].

The primary cause of COPD is smoking, but occupational pollutants such as agricultural dust, are also a significant contributing factor[12,142]. COPD is caused and perpetuated by chronic immune activation from foreign particles such as smoke or organic dusts[1]. The inflammation in COPD is initiated by macrophages and epithelial cells reacting to foreign material in the alveolar spaces[1]. Macrophages are the primary coordinators of the inflammatory response in COPD, but other cells such as neutrophils, eosinophils, and lymphocytes also contribute to the chronic inflammation and tissue damage[37,157,162].

Neutrophils are recruited as part of the immune response but cause tissue damage through release of granules and neutrophil extracellular traps (NETs) when chronically activated[22].

There is conflicting evidence for the association of eosinophils and COPD. High eosinophil levels in the lung have previously been associated with exacerbated disease states and increased inflammation, contributing to eosinophilic COPD[37]. Eosinophilic COPD endotypes are associated with higher healthcare costs[103]. Recent studies have shown lung-resident eosinophils contribute to resolution of inflammation and the restoration of tissue homeostasis[89,90].

COPD histology is associated with increased lymphocyte infiltration and aggregation, tissue destruction, and signs of significant remodeling[41]. Patients exhibit elevated production of IL-6, TNF-α, and IL-10[150]. As the disease progresses, serum IL-10 levels decrease while IL-6

levels increase[66,135]. Chronic inflammation in COPD leads to fibroblasts activating the remodeling process in excess, causing tissue thickening in the airways and obstructed breathing[1].

Due to its negative effects on patient quality of life, exploring effective therapies to slow the progression of the disease and prolong the patient's quality of life is of increasing interest in biomedical and pharmacological research groups. Current treatments aim to control the symptoms through bronchodilators such as β-agonists, and anti-inflammatory drugs including corticosteroids, phosphodiesterase inhibitors, and macrolides[37]. However, these therapies have not been shown to reduce the progression of the disease or impact mortality rates[37]. β-agonists often have severe side effects and risk of death, requiring combinational therapy with corticosteroids[50,87]. Corticosteroids and other anti-inflammatory drugs block the inflammatory response, which leads to a reduction of the resolution response and prevents tissue repair[47,97].

Control of the inflammatory response to prevent chronic activation and promote resolution is preferred to prevent progression of the disease[63]. SPMs have been implicated in aiding the transition of the inflammatory response to resolution, increasing tissue repair, and reducing chronic inflammation-caused pathology[63,129]. Resolvins have demonstrated pro-resolution properties in a wide range pulmonary injury models, suggesting they could be a viable treatment for COPD[34,38,128].

1.2.4 Organic Dust Toxic Syndrome

Organic dust toxic syndrome (ODTS) is an acute respiratory disease mediated by a severe immune reaction to organic dust[44]. Symptoms appear within 4-6 hours of exposure and include flu-like symptoms: fever, chills, cough, muscle aches, weakness and fatigue, and loss of appetite[45].

The disease is primarily facilitated by the innate immune response to fungal spores and endotoxins in organic dust[8,85,114]. Pathophysiological effects of ODTS are primarily facilitated by the release of chemotactic molecules from alveolar macrophages to initiate neutrophil recruitment to the lungs from the vasculature[43]. Febrile symptoms are the result of production of pyrogenic cytokines such as IL-6 by pulmonary epithelial cells and alveolar macrophages[116]. Bronchoalveolar lavage fluid (BALF) from patients with ODTS revealed increased neutrophil counts, IL-6, and TNF-α[43,158].

Patients are often misdiagnosed with infectious diseases due to the presenting symptoms and laboratory tests[96]. They often recover without medical attention but are left with increased risk of chronic bronchitis[44,73]. Current routine treatment for ODTS is acetaminophen for febrile symptoms and generic non-steroidal anti-inflammatory drugs (NSAIDs) to treat the other symptoms, which reduce the immune response[115]. Attenuation of the immune response is not desired as its processes are critical to clear the foreign substances causing tissue damage[47,97].

Use of PPE such as respirators was associated with reduced neutrophil recruitment, IL-6 nasal lavage levels and, reduced endotoxin levels[35,106]. Supplemental DHA has been associated with reduced neutrophil recruitment to injured tissues within 4 hours by blocking chemotaxis[53,139]. Resolution of the immune response and transition to repair processes has previously been demonstrated to be mediated and enhanced by SPMs, including RvD1, indicating it could be a viable treatment for ODTS[38,126].

1.3 The Pulmonary Immune System and Response to Organic Dusts

The pulmonary system is constantly assaulted by microorganisms and toxins in the air we breathe, and maintaining the integrity of the mucosal barrier and immune response is essential to prevent chronic infections and diseases. It is critical to maintain tissue integrity in the pulmonary system due to its close interface with the circulatory system to prevent transfer of foreign particles or pathogens into systemic circulation[3]. The pulmonary system is comprised of mucosal tissue with resident immune cells positioned to patrol for foreign particles and pathogens[9]. Cells involved in the immune response to particulate exposure such as agriculture dust include macrophages, neutrophils, eosinophils, and lymphocytes[90,92,110,152].

Macrophages are the primary coordinators of the pulmonary immune system, producing IL-6 and TNF-α in response to foreign particles[60,119]. After resolution of inflammation, they will also produce IL-10 to initiate tissue repair[118,133]. Recruited lymphocytes produce IL-22 to aid in the resolution of inflammation and tissue repair processes[109,110].

1.3.1 Signaling Molecules in the Pulmonary Immune Response

Pro-inflammatory cytokines such as IL-6 and TNF-α are common indicators of acute inflammation which, after repeated exposure to toxins or particles, can progress to chronic inflammatory diseases [38]. Pro-inflammatory cytokines are produced by epithelial cells, monocytes, and macrophages during an inflammatory response initiated by pathogen associated molecular patterns (PAMPs) and/or damage associated molecular patterns (DAMPs)[74,144].

In the case of organic dust exposure, damage to the epithelial cells from the dust particles causes apoptosis and the release of cellular contents as DAMPs, such as mitochondrial DNA

(mtDNA) and cellular proteins that are recognized by toll-like receptors on macrophages, which induces IL-6 and TNF-α production[164]. Organic dusts may also contain microorganisms such as bacteria, viruses, and fungal spores which activate PAMP pathways to initiate an immune response with the release of IL-6 and TNF-α[74].

IL-6 can be produced by monocytes, macrophages, T cells, B cells, and epithelial cells[71,163]. IL-6 is produced by macrophages and monocytes at the initiation of the immune response to signal the maturation of B cells into antibody-producing plasma cells[56,143]. It functions to increase pulmonary vascular permeability to aid the recruitment of other immune cells[52].

TNF-α is produced by monocytes and macrophages in response to infection or injury to activate neutrophils and natural killer (NK) cells and increase macrophage phaocytosis[48,60]. Excessive production can result in apoptosis or necrosis, damaging tissues and furthering the immune response to a chronic state[18].

The cytokines IL-10 and IL-22 are regarded as anti-inflammatory and pro-resolution, promoting epithelial repair and homeostasis after inflamamtion[104]. IL-10 is produced by monocytes and macrophages during the immune response to promote the transition to resolution and aid in tissue repair[133]. IL-22 has been implicated in promoting tissue repair and epithelial barrier integrity[109]. Studies indicate that attenuation of IL-22 is associated with worse disease state outcomes[148]. It is widely regarded as being exclusively produced by lymphocytes, but macrophages have been implicated as a possible source in our previous studies[36,148].

1.3.2 Pulmonary Immune Cells

Alveolar macrophages are located in the alveoli and airways and function to patrol for foreign materials and pathogens, acting as the primary initiators and coordinators of the pulmonary immune response[33]. Macrophages, including alveolar macrophages, exist in an anti-inflammatory basal state to protect the delicate tissues from immune-modulated injury, but like all macrophages, exist on a spectrum which allows for the production of both pro-inflammatory and anti-inflammatory mediators as needed[91].

Neutrophils are circulating granulocytes that are recruited from the vasculature by chemokines secreted from macrophages and damaged epithelial cells during an immune response[22,117]. They produce and release cytotoxic compounds to assist in the elimination of pathogens and foreign material, but often cause tissue collateral damage in the process[152]. They also assist in phagocytosis of foreign particles and pathogens, as well as produce NETs to trap foreign materials for phagocytosis and elimination by macrophages and neutrophils[22]. Recent evidence has implicated them in resolution and repair processes, indicating they may not only serve a damaging role[152].

Eosinophils are granulocytes involved in many inflammatory processes, most notably in parasitic infections and allergies, but are also elevated in COPD and asthma[10,28]. They can be tissue-resident or recruited from the vasculature during inflammation and are involved in the complement cascade to assist in pathogen clearance as well as secrete cytokines and chemokines to coordinate immune functions[28,90,154]. Recruitment of eosinophils into the pulmonary tissues depends on IL-22 signaling from T lymphocytes[17]. They have also been implicated in the repair process after allergic reactions[28].

Lymphocytes are recruited from the blood during inflammation by chemotactic molecules[15]. αβ $CD4^+$ T cells are upregulated in dust exposure mouse models and are primarily polarized to Th1 and Th17 responses[110]. In organic dust-exposed COPD patients, NK cells and $CD8^+$ T cells are significantly decreased compared to non-COPD patients[69]. COPD patients in general have elevated lymphocyte populations compared to no-COPD patients, which contributes to the chronic inflammation and tissue damage[69].

1.3.3 IL-22 In the Pulmonary Immune Response to Organic Dust

IL-22 is a cytokine in the IL-10 superfamily, which are generally described as being anti-inflammatory[36,104]. Previous evidence has indicated that increased expression of IL-22 it may aid in reduction of inflammation[64]. Its role in controlling inflammation is demonstrated by its correlation with reduced neutrophil infiltration in a murine *Pseudomonas aeruginosa* pneumonia model[21]. Therapies that increase this cytokine could aid in preventing the inflammatory response from progressing to chronic pulmonary disease[34,58].

Previous studies have indicated that IL-22 is produced exclusively by lymphocytes, however, the investigators gated only on the lymphoid lineage, excluding myeloid populations in their exploration of IL-22 expression[2,36]. In the pulmonary system, IL-22 is produced by innate lymphoid cells during the innate immune response and Th17 αβ T cells, γδ T cells, and NK cells during the adaptive immune response[90,92,109,110,152].

The IL-22 receptor IL22Ra1 is present on pulmonary epithelial cells and acts to direct repair and maintain mucosal homeostasis[98,109]. The IL-22 receptor is comprised of two subunits, the IL10R2 subunit and the IL22R1 subunit, suggesting it would have similar downstream intracellular functions as IL-10[93]. IL-22 acts on receptors on ciliated airway epithelial cells to

promote epithelial repair and inflammatory resolution[6,109]. During inflammation, IL22Ra1 is upregulated on parenchymal epithelial cells, indicating IL-22 is critical to restoring and maintaining epithelial homeostasis[109].

Studies have demonstrated neutralization of IL-22 increases mucosal susceptibility to microbial challenge by reducing neutrophil recruitment and infiltration into pulmonary tissues, indicating its role in mucosal barrier maintenance and microbial resistance[21]. IL-22 also has pro-inflammatory roles in the presence of IL-17, recruiting eosinophils in asthma models of mice sensitized to ovalbumin[17].

1.4 Lipids in the Pulmonary Inflammatory Response

Lipids are critical in activating, modulating, and resolving inflammation. Polyunsaturated fatty acids (PUFAs) perform many functions in the immune response as mediators for both inflammation and resolution and repair. During inflammation, omega-6 (ω-6) PUFAs are cleaved from immune and epithelial cell membranes and metabolized to pro-inflammatory mediators, and both ω-6 and ω-3 PUFAs are cleaved to be metabolized to SPMs, which initiate repair and resolution (**Figure 3**)[6,61,122].

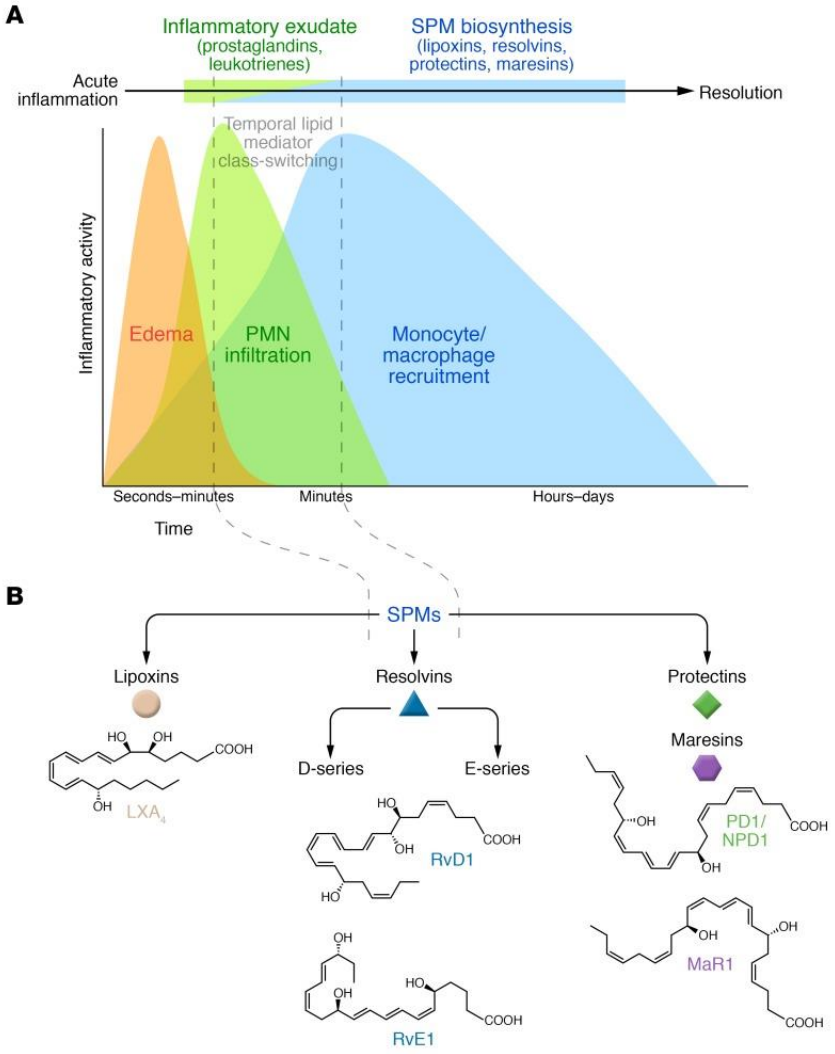

Figure 3. Inflammation-Resolution Relationship Mediated by SPMs[127]

1.4.1 Omega-6 Fatty Acids in Inflammation

Omega-6 (ω-6) fatty acids are metabolized by leukocytes during inflammation to produce pro-inflammatory lipid mediators that act on other immune cells to induce the production of pro-inflammatory cytokines[83,151].

The primary substrate is arachidonic acid (AA), which is cleaved from the cell's plasma membrane and acted on by cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P450 (CYP) enzymes. COX reactions produce prostaglandins, LOX reactions produce

leukotrienes, and P450 reactions produce hydroxyeicosatetraenoic acids (HETEs)[83,146,151]. Prostaglandins and HETEs are chemotactic for circulating immune cells, driving leukocyte infiltration into tissues[83]. Leukotrienes enhance leukocyte adhesion to vessels, aids infiltration into tissues during inflammation[83]. HETEs aid in vasodilation, an important factor in facilitating immune cell infiltration into tissues[146].

1.4.2 Omega-3 Fatty Acids in Inflammation

ω-3 fatty acids are known to control inflammation and aid in reducing symptoms of chronic diseases[88]. Diets high in the ω-3 PUFA, DHA have been shown to be protective in a murine model of chronic organic dust exposure and other chronic inflammatory diseases by increasing the concentration of substrates necessary to transition the immune response to a pro-resolution state[88,147].

ω-3 fatty acids are cleaved from immune cell membranes during the resolution phase of inflammation to be metabolized into SPMs, which act on macrophages and lymphocytes to induce the production of anti-inflammatory and pro-resolution cytokines and increase phagocytosis and other antimicrobial functions[26,31,125]. They also act on receptors on epithelial cells to initiate tissue repair processes[109]. SPMs function during the peak of the inflammatory response to control and mitigate tissue damage and restore homeostasis[127].

1.4.3 Specialized Pro-Resolving Mediators

The resolution of inflammation has traditionally been considered a passive process but has since been demonstrated to be highly bioreactive[121]. SPMs are produced from

polyunsaturated ω-3 fatty acids in response to inflammation by cleavage from epithelial, macrophage, and other immune cell membranes[124].

Inflammation is protective but can become damaging if left unresolved[127]. Resolution of inflammation is critical to restoring tissue homeostasis and preventing further tissue damage[127]. SPMs are considered anti-inflammatory and pro-resolution because they control the severity of the response by restricting immune cell recruitment and cytokine production to only what is necessary to clear the trigger while limiting tissue damage[30,76,82,127]. SPMs decrease the time interval of inflammation by limiting immune cell infiltration while enhancing efferocytosis, phagocytosis, and microbial elimination (**Figure 4**)[26,32]. In contrast, administration of NSAIDs and other anti-inflammatory drugs can cause immunosuppression, reducing the effectiveness of the immune response during pathogen or particle challenge[127]. SPMs drive the polarization of immune cells to a pro-resolution state, inhibiting the production of pro-inflammatory cytokines and upregulating the production of pro-resolution cytokines to promote tissue repair[7,26]. Cytokine production alteration by SPMs acts on innate lymphocytes, NK cells, T cells, and B cells[11,70,72].

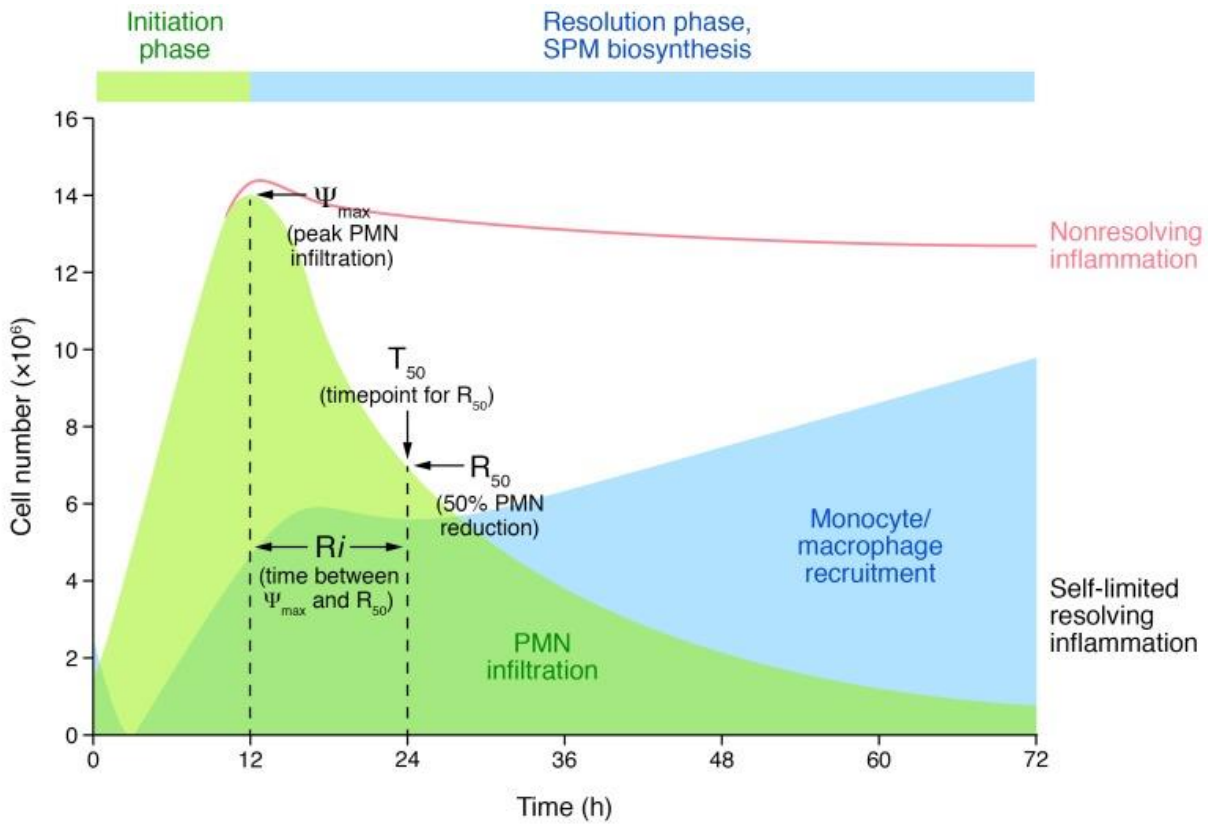

Figure 4. Resolution of Inflammation vs Nonrevolving Inflammatory Response[127]

1.4.4 Resolvin D1 and Aspirin-Triggered Resolvin D1

RvD1 is an SPM produced by macrophages and neutrophils in response to inflammation[31,124]. RvD1 has been demonstrated to peak 5 hours post-DE exposure and continue into the resolution phase in a murine model or agricultural dust exposure[31,147]. RvD1 is produced by pulmonary mucosal tissues in response to acute lung injury following hydrochloric acid challenge and reduces pro-inflammatory cytokine production[38]. It is produced *in vivo* and rapidly inactivated by eicosanoid oxidoreductase to significantly less bioactive metabolites 8-oxo-RvD1 and 17-oxo-RvD1[140].

Aspirin-Triggered RvD1 is an epimer of RvD1 (AT-RvD1) produced through acetylation of the cyclooxygenase-2 (COX-2) enzyme by aspirin, causing a conformational change in the

enzyme and altering the metabolism of docosahexaenoic acid (DHA) to form 17(R)-RvD1 instead of RvD1[120,140]. AT-RvD1 is converted to its 8-oxo-RvD1 and 17-oxo-RvD1 metabolites at a reduced rate, so has longer-lasting, more potent pharmacological activity[1,140]. RvD1 is converted to these metabolites within 25 minutes, whereas AT-RvD1 was shown to be resistant to this degradation pathway[140]. Stereochemistry is critical to biological activity and potency, with the 17(R) conformation being favored biologically and 17(R)-AT-RvD1 being favored in studies[34,38,57,123].

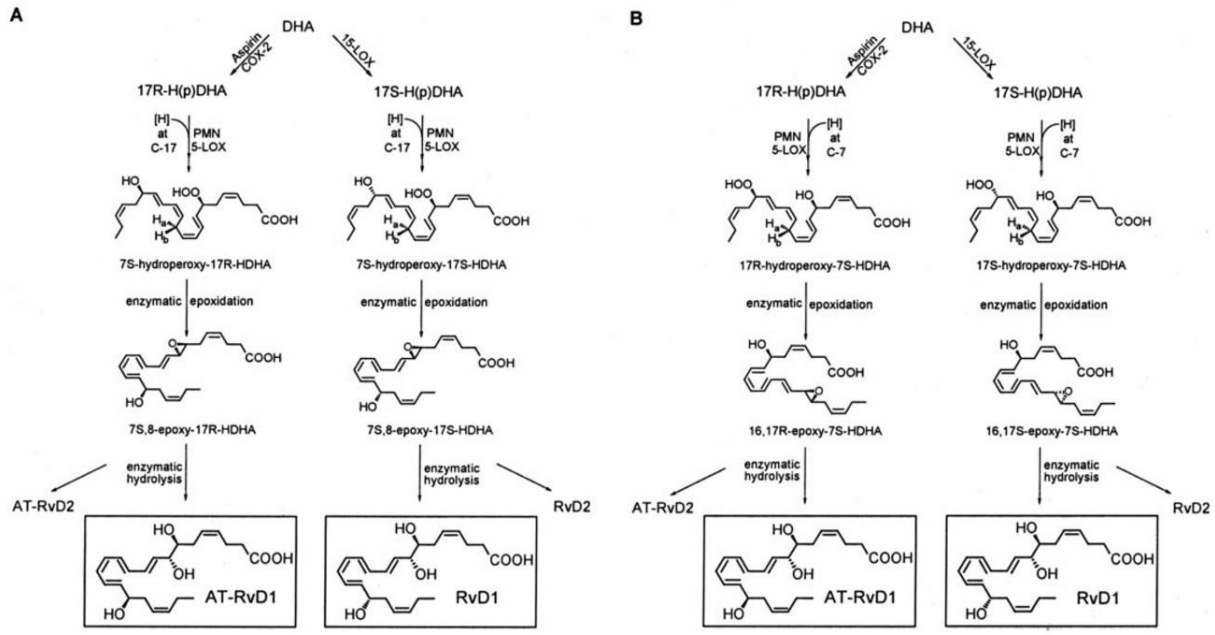

Figure 5. Metabolic pathway for formation of RvD1 and AT-RvD1[140]
(A) Formation of 7(S),17(R) AT-RvD1 by acetylation of COX-2 by aspirin and 7(S),17(S) RvD1 by 15-LOX.; **(B)** Formation of 17(R),7(S) AT-RvD1 by acetylation of COX-2 by aspirin and 17(S),7(S) RvD1 by 15-LOX.

RvD1 production and tissue concentrations are correlated with disease outcomes in acute and chronic inflammatory disease as well as autoimmune diseases[105,127]. In an acute lung injury murine model, AT-RvD1 reduced BALF IL-6 and TNF-α concentrations and histology displayed higher epithelial integrity compared to those without RvD1 treatment[38]. It has also demonstrated

significantly reduced infiltration of macrophages, neutrophils, eosinophils, and lymphocytes[34]. In a murine cancer model of chronic dust exposure, AT-RvD1 administration was correlated with reduced neutrophil and lymphocyte infiltration and increased IL-10 concentrations in BALF[34]. RvD1 has also exhibited a protective role in neutrophil-mediated reperfusion injury[140]. These studies indicate it may assist in maintaining homeostasis between inflammation and resolution.

1.5 Justification and Specific Aims

Organic dust exposure in agriculture workers is a leading cause of COPD and other acute and chronic respiratory diseases, of which there are limited treatments with many side effects[20,62,96,99,137]. Studies have shown low compliance with PPE in agricultural workers, leading to higher prevalence of chronic inflammatory diseases, and highlighting the need for improved treatments for organic dust-induced chronic inflammatory diseases[23,40,68,150]. The cytokine IL-22 functions to maintain the mucosal barrier and restore tissue homeostasis after an inflammatory response and the literature primarily accepts it is produced by lymphocytes[36,64]. RvD1 is an SPM derived from the ω-3 PUFA, DHA that has exhibited a protective role in a murine acute lung injury model and a murine chronic dust exposure cancer model[34,38].

Our previous finding indicate attenuated IL-22 production is associated with worse disease outcomes in a repetitive organic dust exposure model with IL-22 knock out mice[148]. We have also demonstrated ω-3 fatty acid metabolites may contribute to increased IL-22 production by macrophages, as have other researchers of acute and chronic lung injury[38,148]. Disruptions in SPM production are associated with attenuated IL-22 production and correlate with worse outcomes in a murine sepsis model[27]. SPMs, specifically RvD1 have been previously shown to

induce a pro-resolution response after initiation of inflammation and play a protective role in tissue repair[38].

This project aims to determine the source and production of IL-22 in the context of organic dust exposure and RvD1 treatment and evaluate the efficacy of RvD1 as a treatment for repetitive organic dust exposure via induction of IL-22. Based on previous data, we hypothesize *in vitro* alveolar macrophages exposed to DE and treated with RvD1 will have increased IL-22 and IL-10 production and decreased IL-6 and TNF-α production. We also hypothesize that *in vivo* murine model of repetitive DE exposure with RvD1 treatment will demonstrate increased IL-22 and IL-10 BALF concentrations, decreased IL-6 and TNF-α BALF concentrations, as well as decreased immune cell infiltration into the lungs, especially neutrophils. In addition, we predict in an *in vitro* murine IL-22 knock-out model of repetitive DE exposure with RvD1 treatment, will exhibit increased IL-6, TNF-α, IL-10, and cell infiltrate counts in BALF.

CHAPTER 2: METHODS

2.1 *In Vitro* Mouse Alveolar Macrophage Model

2.1.1 Mouse Alveolar Macrophage Culture and Co-Exposure Model

A mouse alveolar macrophage immortalized cell line (MH-S ATCC CRL-2019) was cultured in RPMI-1640 medium (Mediatech Inc.; Virginia, USA) with 10% fetal bovine serum (FBS) (Mediatech Inc.; Virginia, USA), 1% penicillin-streptomycin (Life Technologies Corporation, New York, USA), and 1% L-glutamine solution (HyClone Laboratories; Logan, Utah, USA) at 37 °C with 5% CO_2. Medium was refreshed every 2-3 days.

Cells were harvested from culture flasks with 0.25% trypsin, 0.53 mM ethylenediaminetetraacetic acid (EDTA) solution (HyClone Laboratories; Logan, Utah, USA) and counted via hemocytometer to seed 24-well plates (Fisher Brand) at a density of $1x10^6$ cells per well. Plates were incubated overnight in growth medium and no more than 24 hours before treatment. Solutions for treatments with hog dust extract (DE) and 17(R)-AT-RvD1 (RvD1) (Caymen Labs) were prepared: medium with no additives, medium with 1% DE, medium at 10 nM RvD1, medium at 100 nM RvD1, medium at 10 nM RvD1 with 1% DE co-exposure, and medium at 100 nM RvD1 with 1% DE co-exposure were prepped. Overnight growth media was aspirated, and the treatments were added with four replicates each and incubated for 1, 2, 6, 18, or 24 hours. All studies were repeated twice for a total number of 8 wells per treatment. A schematic of the 24-well plate set-up is detailed in **Figure 6**.

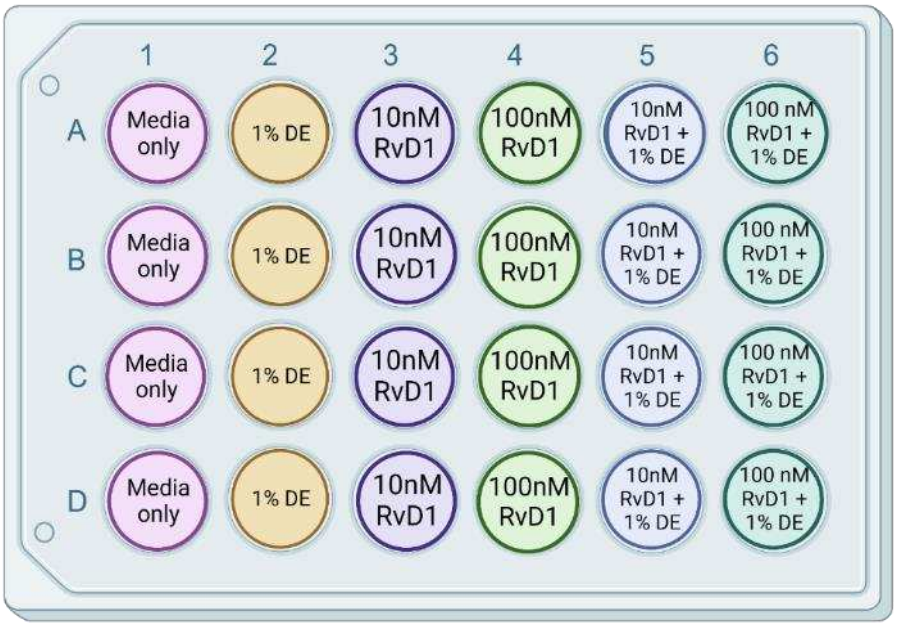

Figure 6. MH-S Experimental Plate Set-Up

2.1.2 Supernatant Collection

Supernate was aspirated at 1, 2, 6, 18, and 24 hours post co-exposure, placed in 1.5 mL microcentrifuge tubes (VWR), centrifuged at 5000 rpm at 4 °C for 10 minutes, and stored at -20 °C for future assays.

2.1.3 Cell Lysate Collection

Cell lysate was collected for analysis of intracellular IL-22 production. Supernatant was aspirated as previously discussed and cells were washed twice with 500 µl ice-cold PBS. 150 µl of ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Fisher) with 100 µl/ml protease inhibitor (Thermo Fisher) was added to each well. The plate was rocked gently, placed on ice for 30 minutes, then rocked gently every 10 minutes during incubation. Lysate was aspirated, centrifuged at 5000 rpm at 4 °C for 10 minutes, and stored at -20 °C for later use[65].

2.2 Enzyme-Linked Immunosorbent Assays

Cytokine protein concentrations in culture media and bronchoalveolar lavage fluids (BALF) were quantified using sandwich enzyme-linked immunosorbent assays (ELISAs). Kits were purchased from DuoSet (R&D Systems) with the modification of capture antibody diluted in BupH Carbonate-Bicarbonate Buffer (Voller's Coating Buffer, Thermo Scientific). High-binding half-well 96-well plates (VWR) were coated with capture antibody and incubated for 24 hours before progression of assay. Samples were removed from -20 °C storage and placed on ice for 1 hour to thaw before centrifugation at 5000 rpm at 4 °C for 10 minutes prior to addition to the plates in duplicate. Protocol was completed as outlined by DuoSet with no other modifications. Peroxidase substrate with peroxide (Thermo Scientific) was used as the indicator and 2N sulfuric acid (Sigma-Aldrich) was used as stop solution. Plates were read on a FLUOstar Omega spectrophotometer at 450 nm using Omega software version 5.7.

2.3 *In Vivo* Repetitive Organic Dust Extract Exposure Model

In vivo studies were conducted at the Colorado State University Laboratory Animal Resources Painter Facility and approved by the Institutional Animal Care and Use Committee (IACUC, Protocol Number 2887). C57BL/6 (WT) and whole-body IL-22 knock-out (IL22KO) mice 8-12 weeks of age were housed in the Painter Facility in a pathogen free environment with free access to standard mouse feed and water. Treatment groups included animals that received intranasal (IN) instillation of sterile saline or 12.5% hog dust extract (DE) with tail vein intravenous (IV) or intraperitoneal (IP) administration of 95 µl sterile saline with 5 µl ethanol, and animals that received sterile saline or 12.5% DE with 500 ng of AT-RvD1 IV in the tail vein or 250 ng of AT-RvD1 IP.

2.3.1 Hog Dust Extract Preparation

Dust was collected at least one meter above the ground from enclosed swine confinement facilities in the Midwest US and stored at -20 °C for later use. 10 g of collected dust was added to 100 mL of Hank's Balanced Salt Solution (HBSS) (HyClone Laboratoies; Logan, Utah, USA) and stirred for one hour on a magnetic stir plate at room temperature. The solution was transferred to 50 mL conical tubes and centrifuged at 2500 rpm at 4 °C for 20 minutes. Supernatant was transferred to new 50 mL conical tubes, and the pellets were discarded. The solution was centrifuged again at 2500 rpm at 4 °C for 20 minutes. The supernatant was filtered through 0.22 µm syringe filters into new 50 mL conical tubes and aliquoted into microcentrifuge tubes at 1 mL 100% DE per tube and stored at -20 °C.

2.3.2 Saline and Hog Dust Extract Instillation

Mice were intranasally (IN) instilled with 50 µl 12.5% DE or sterile saline 5 days/week for 3 weeks under light isoflurane sedation using SomnoSuite Small Animal Anesthesia System (Kent Scientific Cooperation). Animals were placed into anesthesia receptacle and parameters were set at a flow rate of 100 mL/minute at 2.8-3.6% anesthesia depending on the size and tolerance of the animal. Animals were removed for instillation once breathing appeared slowed and even. 50 µl of the appropriate exposure was loaded into a pipette and deposited at the tip of the animal's nose to allow the animal to naturally inhale the liquid. After instillation, DE animals were marked on their tails with sharpie for later identification and tracking of exposure groups. Animals were returned to their enclosures on their backs to encourage recovery from the anesthesia and monitored to ensure return of normal behaviors.

2.3.3 Saline and AT-RvD1 Administration

Injections were prepared with 5 µl 17(R) AT-RvD1 or ethanol in 95 µl PBS per mouse to produce solutions of 500 ng/100 µl of AT-RvD1 or 5% ethanol. Animals were instilled, then placed in a catch cage under a heat lamp to assist in dilating the tail vessels for IV administration. They were then placed in a holder for tail vein injections and administered 100 µl of AT-RvD1 (500 ng) or saline with 5% ethanol intravenously (IV). Animals that received treatment intraperitoneally (IP) received 50 µl of AT-RvD1 (250 ng) or saline with 5% ethanol.

2.3.4 Animal Sacrifice

Animals were euthanized by isoflurane followed by cervical dislocation. Dissection of the chest and neck was performed to expose the lungs and trachea. Blood was collected via the right ventricle. Serum was obtained by centrifuging the blood at 5000 rpm for 15 minutes and stored at -20 °C for later cytokine analysis. Bronchoalveolar lavage fluid (BALF) was collected by inserting a 25 g catheter into the trachea and securing it with suture followed by lavage of the lung three times with 1 mL sterile saline for each wash. The first wash was collected in one tube while washes 2 and 3 were pooled in the same tube. The left lung lobe was tied off with suture, extracted, place in a 1.5 mL microcentrifuge tube (VWR) with 400 µl RNAlater solution, and stored at -20 °C for later use. The right lobes, trachea, and heart were removed from the animals and inflated with 0.7 mL 10% formalin (Fisher Scientific), hung submerged in 10% formalin overnight at 20 cm H_2O pressure, then placed in tissue cassettes and stored in 10% formalin for further histological analysis.

2.3.5 Bronchoalveolar Lavage Cellular Infiltrate Analysis

BALF was centrifuged at 1200 rpm at 4 °C for 5 minutes. Supernatant of the first wash was retained and aliquoted at 105 µl in 1.5 ml microcentrifuge tubes (VWR) for cytokine analysis by ELISA. Supernate of pooled washes two and three was discarded. Cell pellets of all washes were combined with 400 µl of red blood cell lysis buffer (Life Technologies Corporation, New York, USA) and placed on ice to incubate. After 5 minutes, washes were checked for red color and the process was repeated if necessary. After incubation, washes were centrifuged at 1200 rpm at 4 °C for 5 minutes. Supernatant was discarded and cell pellets were reconstituted in 200 µl PBS and counted on a hemocytometer to determine the total number of cells present and

calculate the additional volume of PBS necessary to bring the concentration to $1x10^6$ cells/mL. 200 µl were then added to a cytocentrifuge (Thermo Cytospin 4, Thermo Scientific) and spun at 600 rpm for 5 minutes. Slides were dried overnight and differentially stained using Volu-Sol dip-stain kit for microscopic analysis (Volu-Sol; Salt Lake City, Utah, USA). Differential counts were performed by counting 300 cells on each slide to assess the types of immune cell infiltrations present. Macrophages, lymphocytes, and polymorphonuclear cells were reported. Counts were assisted by Fiji Cell Counter version 1.54b.

2.3.6 Lung Pathology

Fixed tissues were embedded in paraffin, sliced 4-5µm thick, and mounted on slides for histological analysis. Slices were stained using hematoxylin and eosin for structural visualization (Sigma-Aldrich). Images were taken at 4x magnification using an Olympus BX35 microscope and cellSense software version 4.1 to evaluate immune cellularity, peribronchiolar/perivascular inflammation, bronchiolar epithelial hyperplasia, alveolar inflammation, and lymphoid aggregates. Immune cellularity was determined by the number of immune nuclei in the parenchyma. Peribronchial and perivascular inflammation was evaluated by increased cellularity and decreased lumen area in the bronchi and vasculature. Bronchiolar epithelial hyperplasia was determined by the number of epithelial nuclei surrounding the airways. Lymphoid aggregates were determined as 20 or more lymphoid cells aggregated with limited spaces between.

2.3.7 *In Vivo* DE and RvD1 Murine Pilot Study

A pilot study was conducted on WT mice to determine appropriate timepoints and AT-RvD1 administration routes for observation of AT-RvD1 on inflammation, resolution, and repair before proceeding with additional studies. Animals were instilled IN with either sterile saline or DE 5 days/week for 3 weeks. Once per week the animals were injected intravenously (IV) in the tail vein with 500 ng AT-RvD1/100 µl PBS or intraperitoneally (IP) with 250 ng AT-RvD1/50 µl PBS for the treatment group, and 100 µl 5% (EtOH) in PBS IV or 50 µl 5% EtOH in PBS IP for the control groups.

A table displaying the number of animals per timepoint and administration route is displayed below, as well as a schematic of the study timeline.

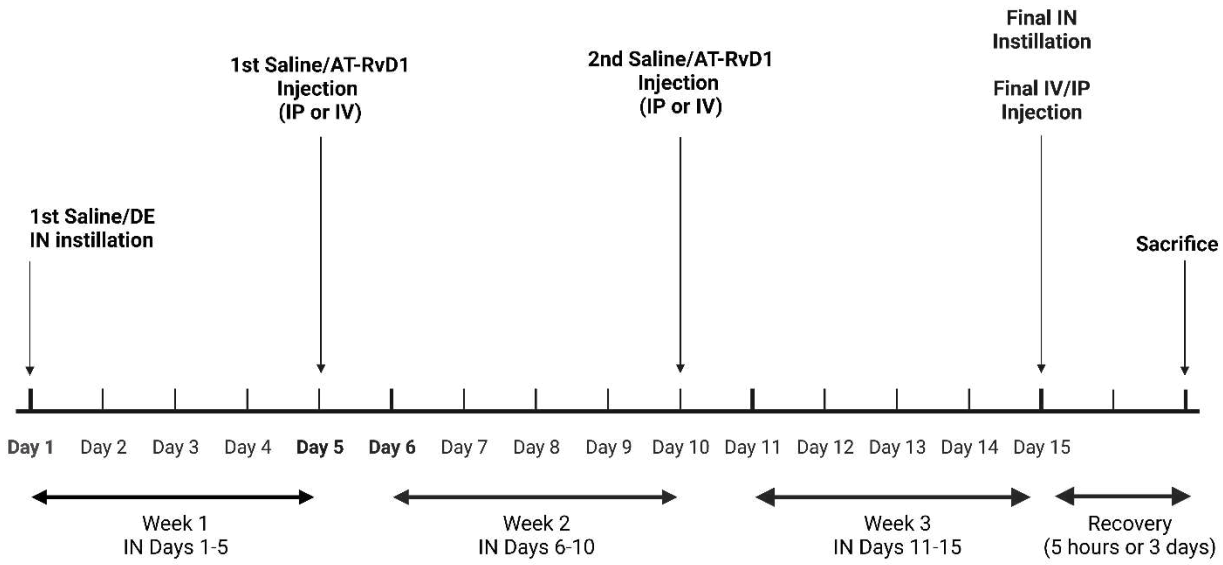

Figure 7. Murine Pilot Study Timeline

Table 1. Animals to be sacrificed 5 hours post-instillation

Number of animals to be sacrificed 5 hours post-instillation

Saline IN + Saline IV		DE IN + Saline IV		Saline IN + AT-RvD1 IV		DE IN + AT-RvD1 IV	
Male	Female	Male	Female	Male	Female	Male	Female
1	1	0	1	0	1	1	1
Saline IN+ Saline IP		DE IN + Saline IP		Saline IN + AT-RvD1 IP		DE IN + AT-RvD1 IP	
Male	Female	Male	Female	Male	Female	Male	Female
1	0	1	1	1	0	0	1
Total: 11 animals							

Table 2. Animals to be sacrificed 3 days post-instillation

Number of animals to be sacrificed 3 days post-instillation

Saline IN + Saline IV		DE IN + Saline IV		Saline IN + AT-RvD1 IV		DE IN + AT-RvD1 IV	
Male	Female	Male	Female	Male	Female	Male	Female
1	1	1	1	1	0	1	1
Saline IN+ Saline IP		DE IN + Saline IP		Saline IN + AT-RvD1 IP		DE IN + AT-RvD1 IP	
Male	Female	Male	Female	Male	Female	Male	Female
0	1	1	1	2	2	2	2
Total: 18 animals							

2.3.8 *In Vivo* DE and RvD1 IL-22 Knock-Out Murine Study

After determining the appropriate RvD1 dosage and route of administration, 48 total animals including IL22KO animals were instilled, injected, and sacrificed as outlined below.

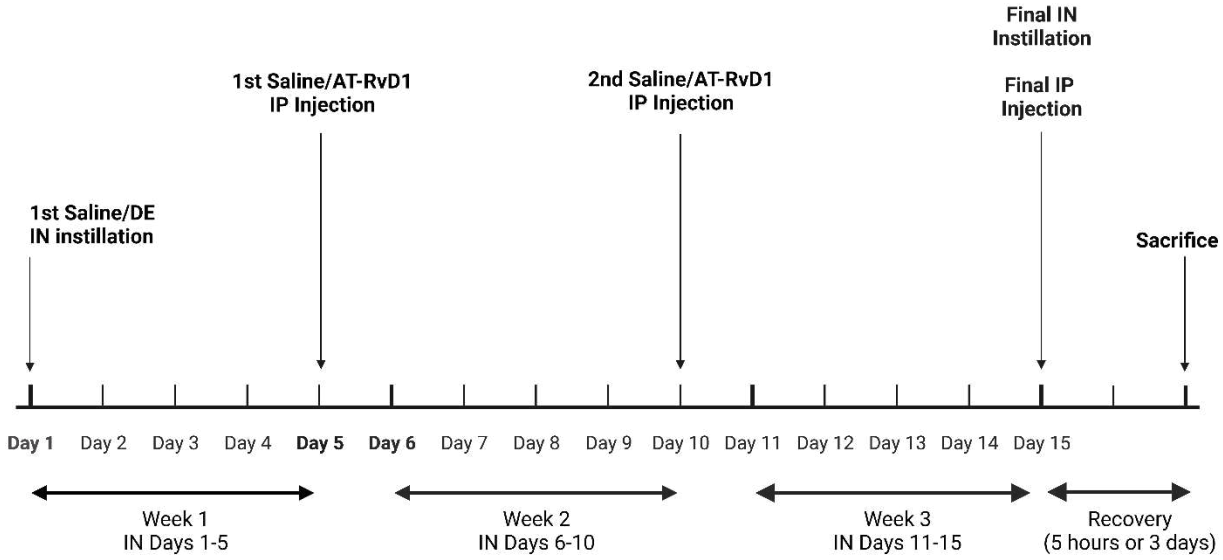

Figure 8. Murine AT-RvD1 IL22KO Study Timeline

Animals were divided evenly by sex into treatment groups and recovery timepoint as outlined below. Animals were staggered in groups of 32 animals total, with 16 animals per sacrifice timepoint with an even number of animals per group. Each group is identical, containing one animal of each sex per treatment, per genotype.

Table 3. Wild-type animals to be sacrificed 5 hours post-instillation

Number of WT animals to be sacrificed 5 hours post-instillation							
Saline IN + Saline IP		DE IN + Saline IP		Saline IN + AT-RvD1 IP		DE IN + AT-RvD1 IP	
Male	Female	Male	Female	Male	Female	Male	Female
2	2	2	2	2	2	2	2
Total: 24 animals							

Table 4. IL-22 knock-out animals to be sacrificed 5 hours post-instillation

Number of IL22KO animals to be sacrificed 5 hours post-instillation							
Saline IN + Saline IP		DE IN + Saline IP		Saline IN + AT-RvD1 IP		DE IN + AT-RvD1 IP	
Male	Female	Male	Female	Male	Female	Male	Female
2	2	2	2	2	2	2	2
Total: 24 animals							

Table 5. Wild-type animals to be sacrificed 3 days post-instillation

Number of WT animals to be sacrificed 3 days post-instillation							
Saline IN + Saline IP		DE IN + Saline IP		Saline IN + AT-RvD1 IP		DE IN + AT-RvD1 IP	
Male	Female	Male	Female	Male	Female	Male	Female
2	2	2	2	2	2	2	2
Total: 24 animals							

Table 6. IL-22 knock-out animals to be sacrificed 3 days post-instillation

Number of IL22KO animals to be sacrificed 3 days post-instillation							
Saline IN + Saline IP		DE IN + Saline IP		Saline IN + AT-RvD1 IP		DE IN + AT-RvD1 IP	
Male	Female	Male	Female	Male	Female	Male	Female
2	2	2	2	2	2	2	2
Total: 24 animals							

2.4 Statistical Analysis

ELISA data were analyzed using Omega MARS software version 4.0 to calculate concentration based on 4-parameter calculations of a standard concentration curve. Averages of technical replicates were used for statistical analysis. Concentration readings below the level of detection were set to 0.0001 for statistical analysis and graphing to indicate it may be present but is not detectable by the assay.

Significance was determined with two-way ANOVA for *in vitro* experiments and three-way ANOVA for *in vivo* experiments with post-hoc Tukey tests using GraphPad Prism Version 9. Temporal relationships of cytokine concentrations were determined by mixed effects analysis and graphed as averages of all replicates.

For all *in vivo* study data, males and females were combined and differentiated on graphs to show sex differences but were calculated together for statistical analysis. Males were defined as filled shapes and females were defined as open shapes on graphs. Significance was determined by a p-value < 0.05.

GraphPad Prism Version 9 was used for production of all data figures. On graphs, significance is denoted by, * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$; **** = $p \leq 0.0001$.

CHAPTER 3: RESULTS

3.1 *In Vitro* Cytokine Protein Expression

3.1.1 Effects of RvD1 on IL-6 Production in DE-Exposed Alveolar Macrophages

IL-6 is widely considered to be a marker for inflammation produced by macrophages in the immune response[119]. In an alveolar macrophage model, we aimed to determine how treatment of RvD1 in concentrations of 10 nM and 100 nM would alter the production of this cytokine. The results of the Tukey post-hoc test revealed that exposure to 1% DE and addition of 10 nM ($p<0.0001$) and 100 nM ($p<0.0001$) RvD1 treatment significantly reduced production at 6 hours post co-exposure. All statistically significant comparisons are provided in **Table 7**.

Table 7. Statistical Comparisons of *In Vitro* IL-6 Production

Timepoint	Comparisons	P-value
2hr	Medium vs. DE	<0.0001
	Medium vs. 10 nM RvD1 + DE	<0.0001
	Medium vs. 100 nM RvD1 + DE	<0.0001
	10 nM RvD1 vs. DE	<0.0001
	10 nM RvD1 vs. 10 nM RvD1 + DE	<0.0001
	10 nM RvD1 vs. 100 nM RvD1 + DE	<0.0001
	100 nM vs. DE	<0.0001
	100 nM vs. 10 nM RvD1 + DE	<0.0001
	100 nM vs. 100 nM RvD1 + DE	<0.0001
6hr	Medium vs. DE	<0.0001
	Medium vs. 10 nM RvD1 + DE	0.0009
	Medium vs. 100 nM RvD1 + DE	0.0096
	10 nM RvD1 vs. DE	<0.0001
	10 nM RvD1 vs. 10 nM RvD1 + DE	0.0008
	10 nM RvD1 vs. 100 nM RvD1 + DE	0.0093
	100 nM vs. DE	<0.0001
	100 nM vs. 10 nM RvD1 + DE	0.0008
	100 nM vs. 100 nM RvD1 + DE	0.0093
	DE vs. 10 nM RvD1 + DE	<0.0001
	DE vs. 100 nM RvD1 + DE	<0.0001
18hr	Medium vs. DE	<0.0001
	Medium vs. 10 nM RvD1 + DE	<0.0001

	Medium vs. 100 nM RvD1 + DE	<0.0001
	10 nM RvD1 vs. DE	<0.0001
	10 nM RvD1 vs. 10 nM RvD1 + DE	<0.0001
	10 nM RvD1 vs. 100 nM RvD1 + DE	<0.0001
	100 nM vs. DE	<0.0001
	100 nM vs. 10 nM RvD1 + DE	<0.0001
	100 nM vs. 100 nM RvD1 + DE	<0.0001
24hr	Medium vs. DE	<0.0001
	Medium vs. 10 nM RvD1 + DE	<0.0001
	Medium vs. 100 nM RvD1 + DE	<0.0001
	10 nM RvD1 vs. DE	<0.0001
	10 nM RvD1 vs. 10 nM RvD1 + DE	<0.0001
	10 nM RvD1 vs. 100 nM RvD1 + DE	<0.0001
	100 nM vs. DE	<0.0001
	100 nM vs. 10 nM RvD1 + DE	<0.0001
	100 nM vs. 100 nM RvD1 + DE	<0.0001

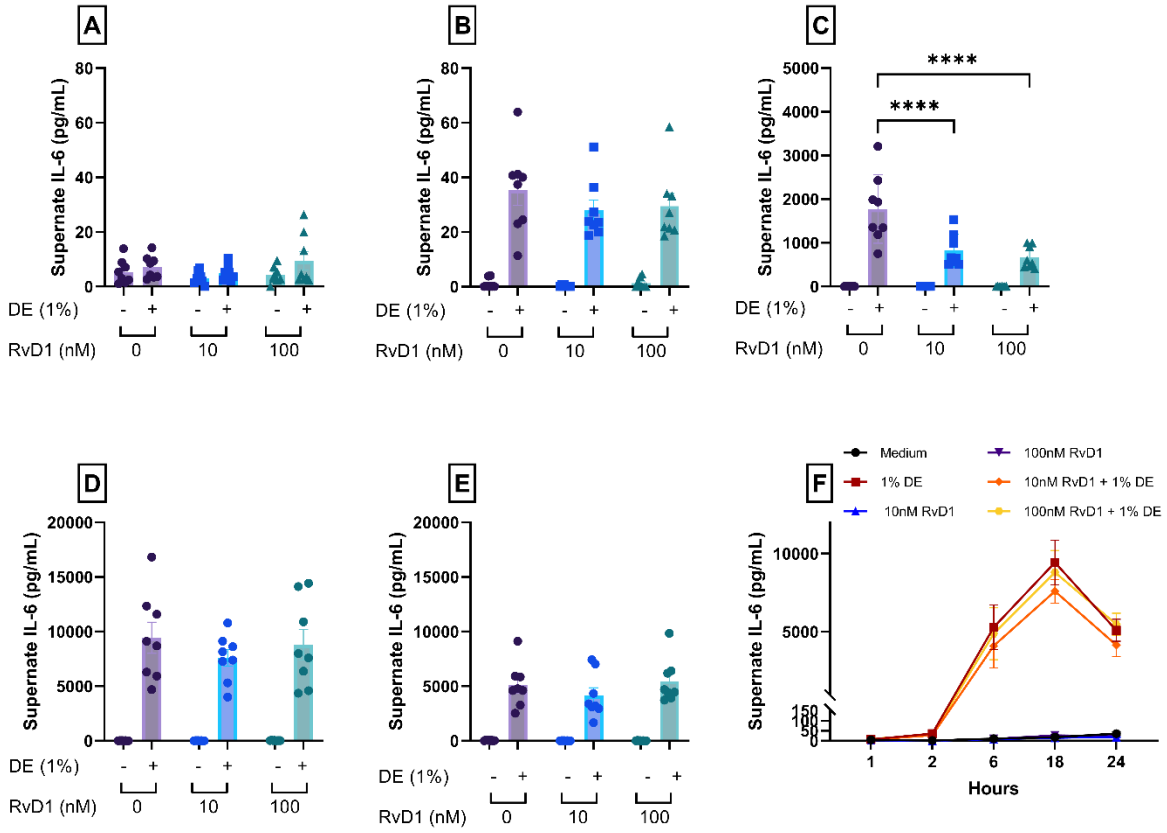

Figure 9. Effects of DE exposure and RvD1 treatment on IL-6 induction in alveolar macrophages over 24 hours

(A) Concentrations 1 hour post co-exposure; **(B)** concentrations 2 hours post co-exposure; **(C)** concentrations 6 hours post co-exposure; **(D)** concentrations 18 hours post co-exposure; **(E)** concentrations 24 hours post co-exposure; **(F)** temporal relationships of treatment groups over 24 hours.

3.1.2 Effects of RvD1 on TNF-α Production in DE-Exposed Alveolar Macrophages

TNF-α is another pro-inflammatory cytokine produced by macrophages in response to organic dust exposure[161]. We aimed to determine the effects of RvD1 on TNF-α production by alveolar macrophages *in vitro*. Statistical significance was observed between DE-exposed cells and DE-exposed cells treated with 100 nM RvD1 at 6 hours. All other statistically significant comparisons are shown in **Table 8**.

Table 8. Statistical Comparisons of *In Vitro* TNF-α Production

Timepoint	Comparisons	P-value
1hr	Medium vs. DE	0.0001
	Medium vs. 10 nM RvD1 + DE	0.0162
	Medium vs. 100 nM RvD1 + DE	<0.0001
	10 nM RvD1 vs. DE	<0.0001
	10 nM RvD1 vs. 10 nM RvD1 + DE	0.0003
	10 nM RvD1 vs. 100 nM RvD1 + DE	<0.0001
	100 nM vs. DE	<0.0001
	100 nM vs. 10 nM RvD1 + DE	<0.0001
	100 nM vs. 100 nM RvD1 + DE	<0.0001
2hr	Medium vs. DE	<0.0001
	Medium vs. 10 nM RvD1 + DE	<0.0001
	Medium vs. 100 nM RvD1 + DE	<0.0001
	10 nM RvD1 vs. DE	<0.0001
	10 nM RvD1 vs. 10 nM RvD1 + DE	<0.0001
	10 nM RvD1 vs. 100 nM RvD1 + DE	<0.0001
	100 nM vs. DE	<0.0001
	100 nM vs. 10 nM RvD1 + DE	<0.0001
	100 nM vs. 100 nM RvD1 + DE	<0.0001
	DE vs. 10 nM RvD1 + DE	<0.0001
	DE vs. 100 nM RvD1 + DE	<0.0001
6hr	DE vs. 100 nM RvD1 + DE	0.0142
18hr	Medium vs. DE	<0.0001
	Medium vs. 10 nM RvD1 + DE	<0.0001
	Medium vs. 100 nM RvD1 + DE	<0.0001
	10 nM RvD1 vs. DE	<0.0001
	10 nM RvD1 vs. 10 nM RvD1 + DE	<0.0001
	10 nM RvD1 vs. 100 nM RvD1 + DE	<0.0001
	100 nM vs. DE	<0.0001
	100 nM vs. 10 nM RvD1 + DE	<0.0001
	100 nM vs. 100 nM RvD1 + DE	<0.0001
24hr	Medium vs. DE	<0.0001

	Medium vs. 10 nM RvD1 + DE	<0.0001
	Medium vs. 100 nM RvD1 + DE	<0.0001
	10 nM RvD1 vs. DE	<0.0001
	10 nM RvD1 vs. 10 nM RvD1 + DE	<0.0001
	10 nM RvD1 vs. 100 nM RvD1 + DE	<0.0001
	100 nM vs. DE	<0.0001
	100 nM vs. 10 nM RvD1 + DE	<0.0001
	100 nM vs. 100 nM RvD1 + DE	<0.0001

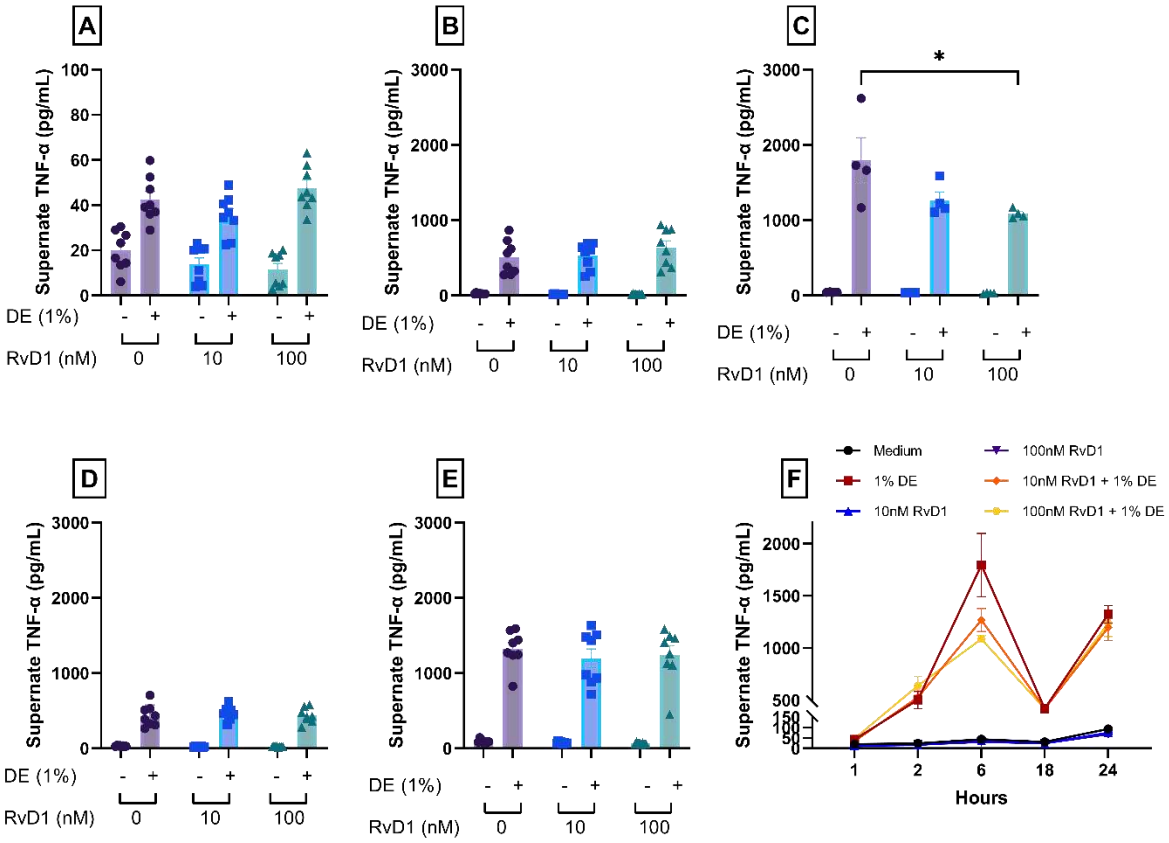

Figure 10. Effects of DE exposure and RvD1 treatment on TNF-α induction in alveolar macrophages over 24 hours
(A) Concentrations 1 hour post co-exposure; **(B)** concentrations 2 hours post co-exposure; **(C)** concentrations 6 hours post co-exposure; **(D)** concentrations 18 hours post co-exposure; **(E)** concentrations 24 hours post co-exposure; **(F)** temporal relationships of treatment groups over 24 hours.

3.1.3 Effects of RvD1 on IL-10 Production in DE-Exposed Alveolar Macrophages

IL-10 was evaluated as a marker for resolution of inflammation and the anti-inflammatory effects of RvD1 in alveolar macrophages[104]. Treatment of RvD1 in DE-exposed cells did not significantly alter the production of IL-10. All statistically significant comparisons are given in **Table 9**.

Table 9. Statistical Comparisons of *In Vitro* IL-10 Production

Timepoint	Comparisons	P-value
1hr	Medium vs. DE	0.0112
	Medium vs. 10 nM RvD1 + DE	0.0384
	10 nM RvD1 vs. DE	0.0061
	10 nM RvD1 vs. 10 nM RvD1 + DE	0.0221
	100 nM vs. DE	0.0095
	100 nM vs. 10 nM RvD1 + DE	0.0332
2hr	Medium vs. DE	<0.0001
	Medium vs. 10 nM RvD1 + DE	0.0039
	Medium vs. 100 nM RvD1 + DE	0.0007
	10 nM RvD1 vs. DE	<0.0001
	10 nM RvD1 vs. 10 nM RvD1 + DE	0.0044
	10 nM RvD1 vs. 100 nM RvD1 + DE	0.0008
	100 nM vs. DE	<0.0001
	100 nM vs. 10 nM RvD1 + DE	0.0078
	100 nM vs. 100 nM RvD1 + DE	0.0015
6hr	Medium vs. 10 nM RvD1 + DE	0.0387
	Medium vs. 100 nM RvD1 + DE	<0.0001
	10 nM RvD1 vs. DE	0.0304
	10 nM RvD1 vs. 10 nM RvD1 + DE	0.0102
	10 nM RvD1 vs. 100 nM RvD1 + DE	<0.0001
	100 nM vs. DE	0.0448
	100 nM vs. 10 nM RvD1 + DE	0.0156
	100 nM vs. 100 nM RvD1 + DE	<0.0001
18hr	Medium vs. 10 nM RvD1 + DE	0.0122
24hr	Medium vs. 10 nM RvD1	0.0101
	10 nM RvD1 vs. 100 nM RvD1 + DE	0.0011

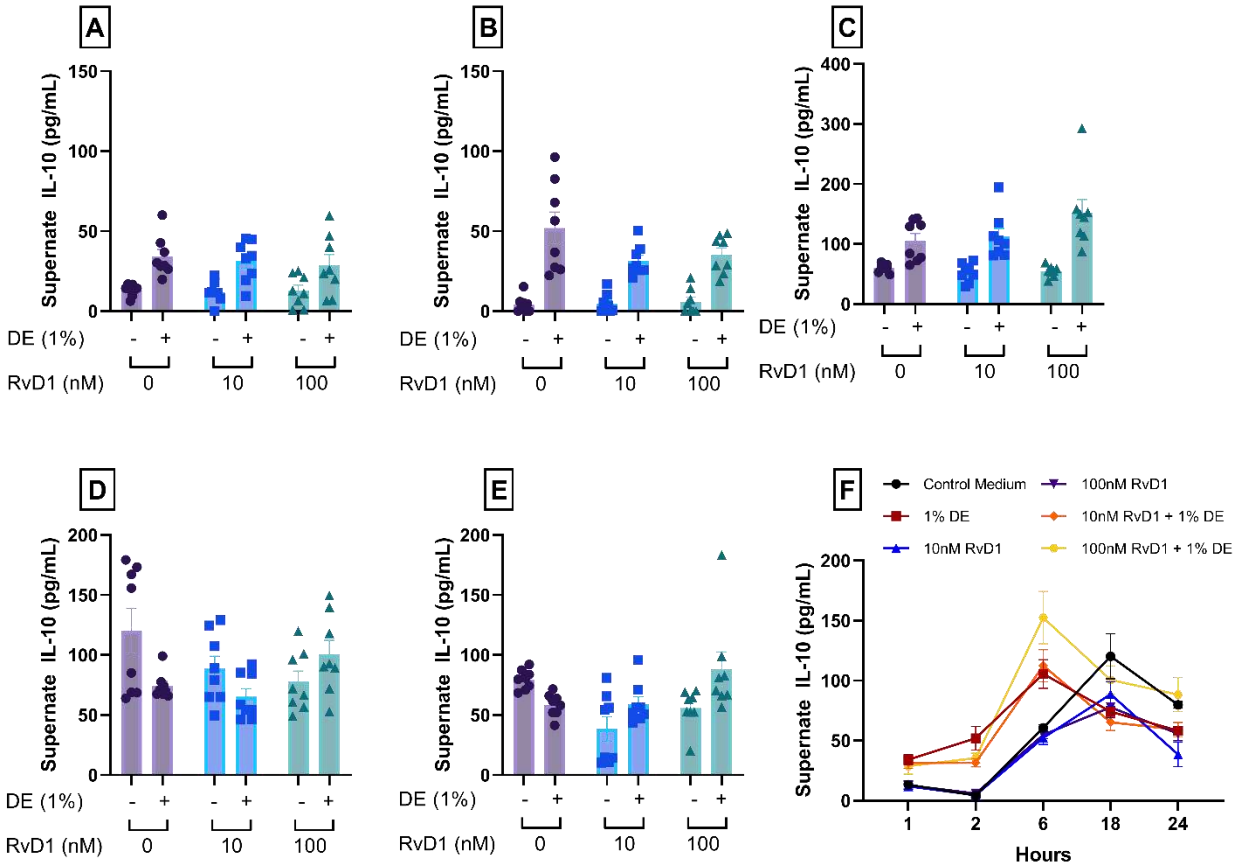

Figure 11. Effects of DE exposure and RvD1 treatment on IL-10 induction in alveolar macrophages over 24 hours
(A) Concentrations 1 hour post co-exposure; **(B)** concentrations 2 hours post co-exposure; **(C)** concentrations 6 hours post co-exposure; **(D)** concentrations 18 hours post co-exposure; **(E)** concentrations 24 hours post co-exposure; **(F)** temporal relationships of treatment groups over 24 hours.

3.1.4 Effects of RvD1 on IL-22 Production in DE-Exposed Alveolar Macrophages

Our recent studies have shown alveolar macrophages produce IL-22 in response to organic dust and that it was Golgi-localized[148]. We tested induction of IL-22 in mouse alveolar macrophages exposed to DE and treated with RvD1. We also evaluated lysate concentrations to determine intracellular localization of IL-22 during the immune response to organic dust extract.

Supernate concentrations after 1 hour demonstrated significant differences between cells exposed to DE and cells exposed to DE and treated with 10 nM RvD1 ($p=0.0399$) and 100 nM RvD1 ($p<0.0001$). After 2 hours, these changes were also observed at both 10 nM and 100 nM RvD1 concentrations with the same significance ($p=0.0002$). **Table 10** displays all statistically significant comparisons for supernate and lysate IL-22 concentrations.

Table 10. Statistical Comparisons of *In Vitro* IL-22 Production

Timepoint	Comparisons	P-value
1hr supernate	Medium vs. 100 nM RvD1	0.0399
	Medium vs. 100 nM RvD1 + DE	0.0034
	100 nM RvD1 vs. DE	0.0004
	DE vs. 10 nM RvD1 + DE	0.0006
	DE vs. 100 nM RvD1 + DE	<0.0001
1hr lysate	Medium vs. 100 nM RvD1	0.0188
	Medium vs. DE	0.0357
	Medium vs. 100 nM RvD1 + DE	0.0318
2hr supernate	Medium vs. 10 nM RvD1	0.0019
	Medium vs. 100 nM RvD1	0.0002
	Medium vs. 10 nM RvD1 + DE	<0.0001
	Medium vs. 100 nM RvD1 + DE	<0.0001
	10 nM RvD1 vs. DE	0.0302
	100 nM RvD1 vs. DE	0.0044
	DE vs. 10 nM RvD1 + DE	0.0002
	DE vs. 100 nM RvD1 + DE	0.0002
24hr lysate	Medium vs. 10 nM RvD1 + DE	0.0289
	Medium vs. 100 nM RvD1 + DE	0.0018

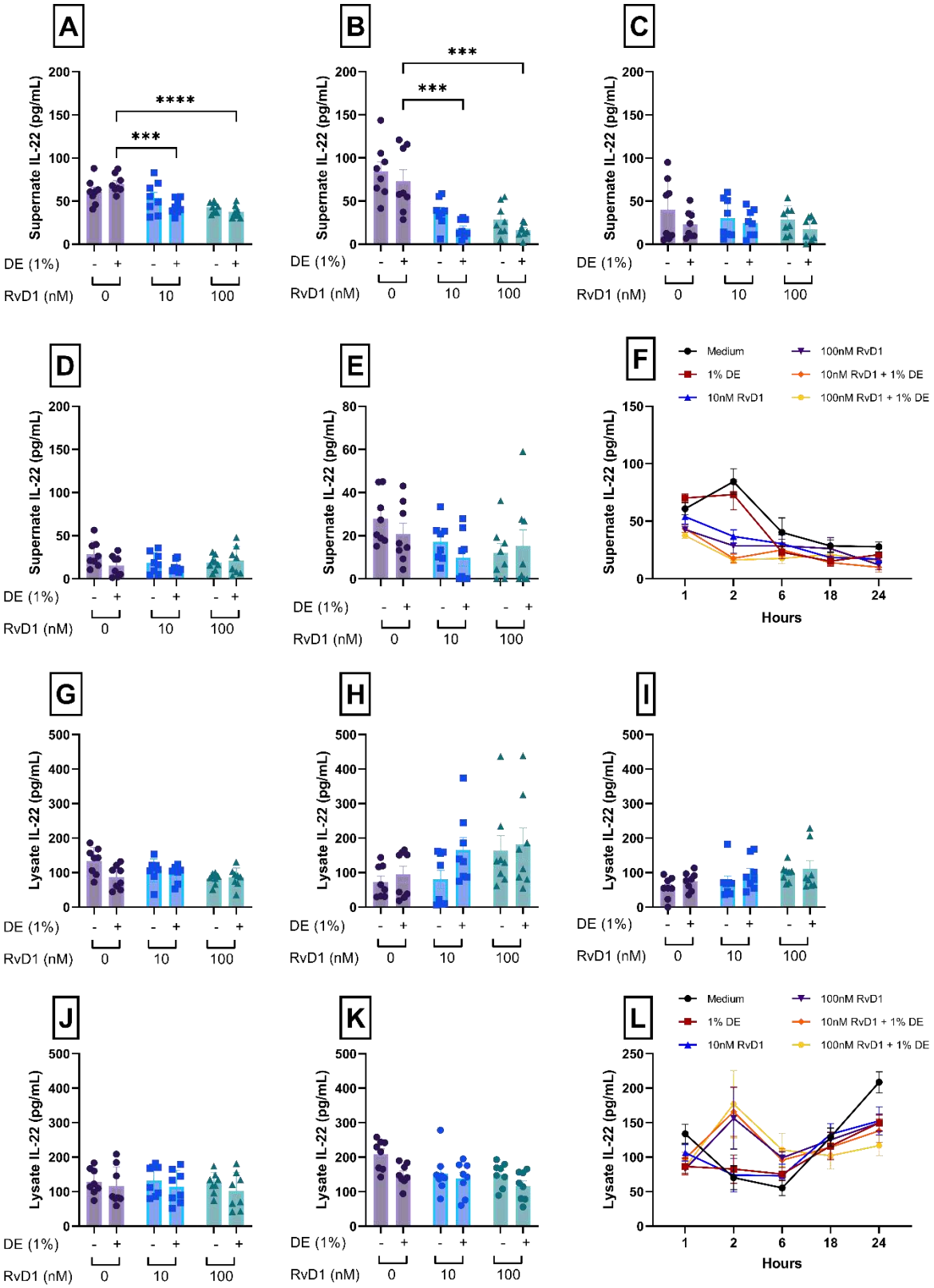
A
B
C
D
E
F
G
H
I
J
K
L
Supernate IL-22 (pg/mL)
Lysate IL-22 (pg/mL)
DE (1%)
RvD1 (nM)
0
10
100
***
****
Medium
1% DE
10nM RvD1
100nM RvD1
10nM RvD1 + 1% DE
100nM RvD1 + 1% DE
Hours
1
2
6
18
24

Figure 12. Effects of DE exposure and RvD1 treatment on IL-22 induction in alveolar macrophages over 24 hours
(A) Supernate Concentrations 1 hour post co-exposure; **(B)** supernate concentrations 2 hours post co-exposure; **(C)** supernate concentrations 6 hours post co-exposure; **(D)** supernate concentrations 18 hours post co-exposure; **(E)** supernate concentrations 24 hours post co-exposure; **(F)** supernate temporal relationships of treatment groups. **(G)** lysate concentrations 1 hour post co-exposure; **(H)** lysate concentrations 2 hours post co-exposure; **(I)** lysate concentrations 6 hours post co-exposure; **(J)** lysate concentrations 18 hours post co-exposure; **(K)** lysate concentrations 24 hours post co-exposure; **(L)** lysate temporal relationships of treatment groups over 24 hours.

3.1.5 Effects of DE Exposure and RvD1 Treatment on IL-6, TNF-α, IL-10, and IL-22 in Alveolar Macrophages

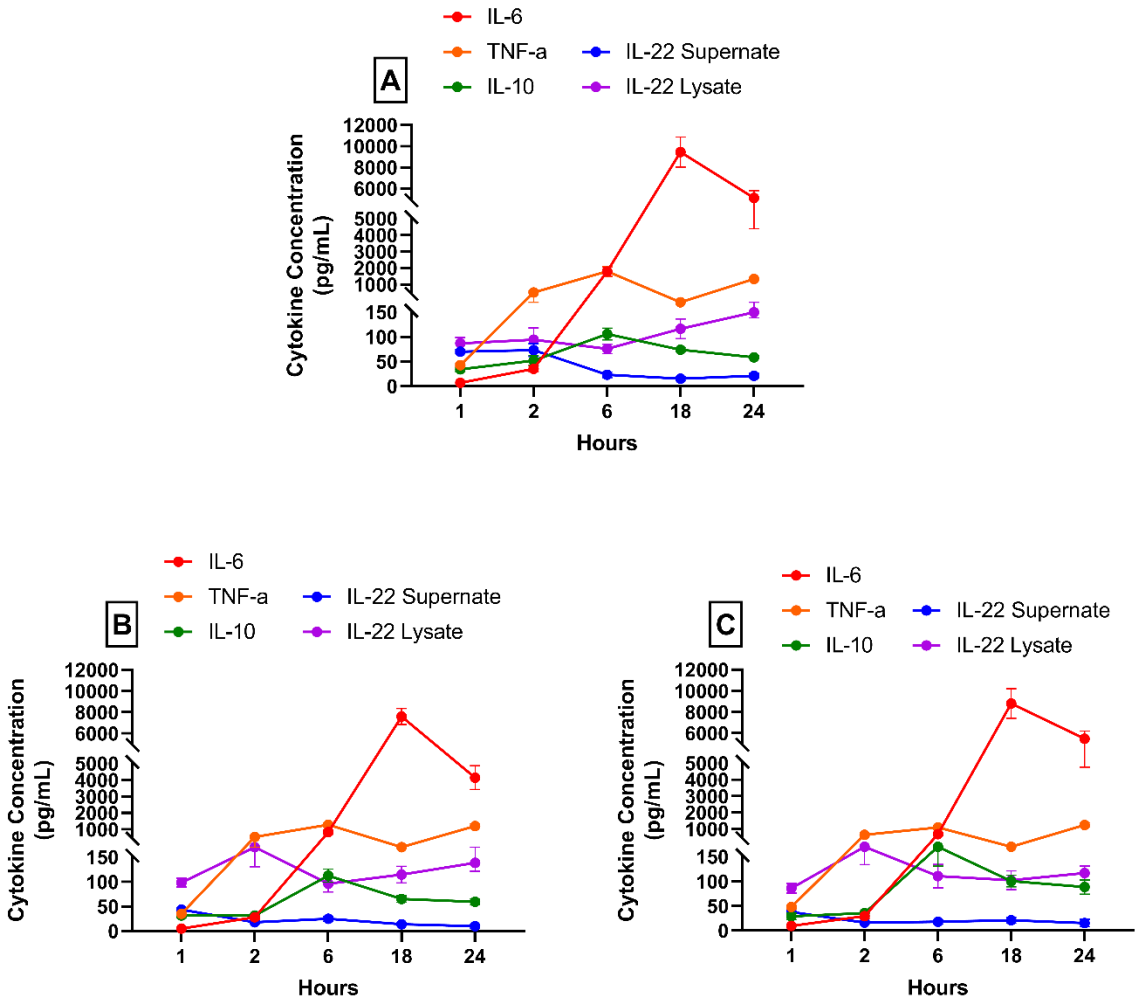

Figure 13. Effects of DE exposure and RvD1 treatment on IL-6, TNF-α, IL-10, and IL-22 in alveolar macrophages over 24 hours
(A) IL-6, TNF-α, IL-10, IL-22 supernate, and IL-22 lysate concentrations in cells exposed to 1% DE without RvD1 over 24 hours; **(B)** IL-6, TNF-α, IL-10, IL-22 supernate, and IL-22 lysate concentrations in cells co-exposed to 10 nM RvD1 with 1% DE over 24 hours; **(C)** IL-6, TNF-α, IL-10, IL-22 supernate, and IL-22 lysate concentrations in cells co-exposed to 100 nM RvD1 with 1% DE over 24 hours.

3.2 *In Vivo* Pilot Repetitive DE Exposure Model

3.2.1 Effects of DE Exposure and RvD1 Treatment on Bronchoalveolar Lavage Fluid Cell Infiltrates

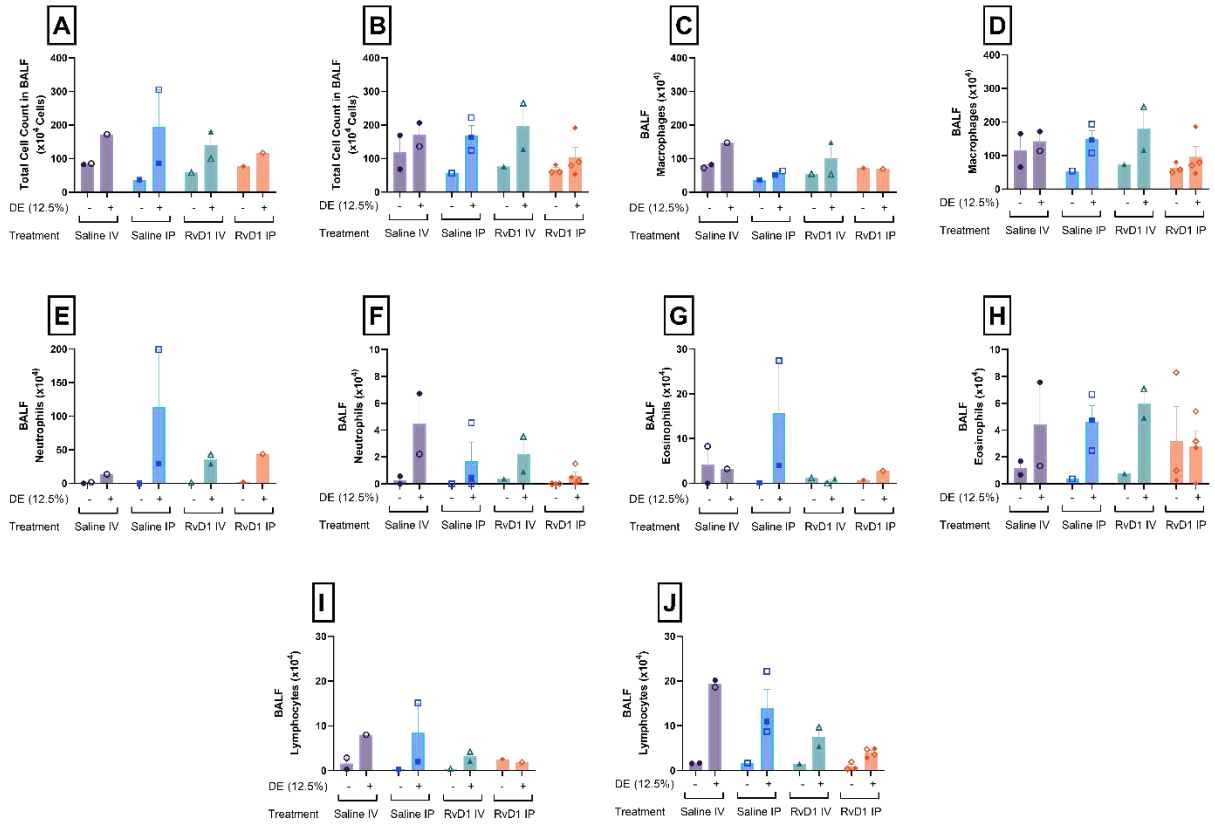

Figure 14. Effects of RvD1 on cell infiltrates in DE-exposed mice with recovery of 5 hours and 3 days
(A) Total cell counts in animals sacrificed with a recovery of 5 hours; **(B)** Total cell counts in animals sacrificed with a recovery of 3 days; **(C)** Macrophage counts in animals sacrificed with a recovery of 5 hours; **(D)** Macrophage counts in animals sacrificed with a recovery of 3 days; **(E)** Neutrophil counts in animals sacrificed with a recovery of 5 hours; **(F)** Neutrophil counts in animals sacrificed with a recovery of 3 days **(G)** Eosinophil counts in animals sacrificed with a recovery of 5 hours; **(H)** Eosinophil counts in animals sacrificed with a recovery of 3 days; **(I)** Lymphocyte in animals sacrificed with a recovery of 5 hours; **(J)** Lymphocyte in animals sacrificed with a recovery of 3 days.

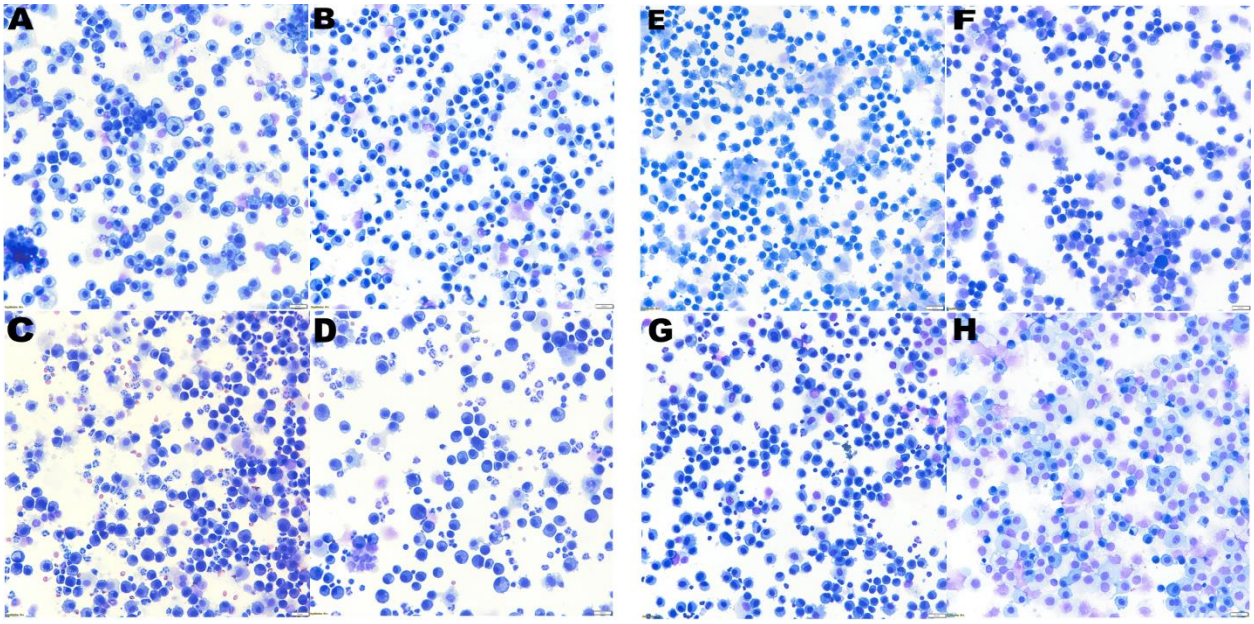

Figure 15. BALF cytospins of WT mice exposed to saline or DE and treated with saline or RvD1 with a recovery of 5 hours or 3 days
(A) saline/saline IV female 5 hours post last instillation; **(B)** saline/RvD1 IP male with a recovery of 5 hours; **(C)** DE/saline IP male with a recovery of 5 hours; **(D)** DE/RvD1 IV female with a recovery of 5 hours; **(E)** saline/saline IP male with a recovery of 3 days; **(F)** saline/RvD1 IP female with a recovery of 3 days; **(G)** DE/saline IV female with a recovery of 3 days; **(H)** DE/RvD1 IP male with a recovery of 3 days.

3.3 *In Vivo* Interleukin-22 Knock-Out Repetitive DE Exposure Model

3.3.1 Effects of DE Exposure and RvD1 Treatment on Bronchoalveolar Lavage Fluid Cytokines

BALF IL-6 concentrations demonstrated no statistical significance between animals exposed to DE and treated with saline and animals exposed to DE and treated with RvD1. Primary ANOVA results demonstrated significance for DE exposure at 5 hours ($p=0.0225$) and 3 days ($p=0.0238$) recovery. KO animals displayed significant effects between timepoint ($p=0.0253$) and exposure x timepoint interaction ($p=0.0337$).

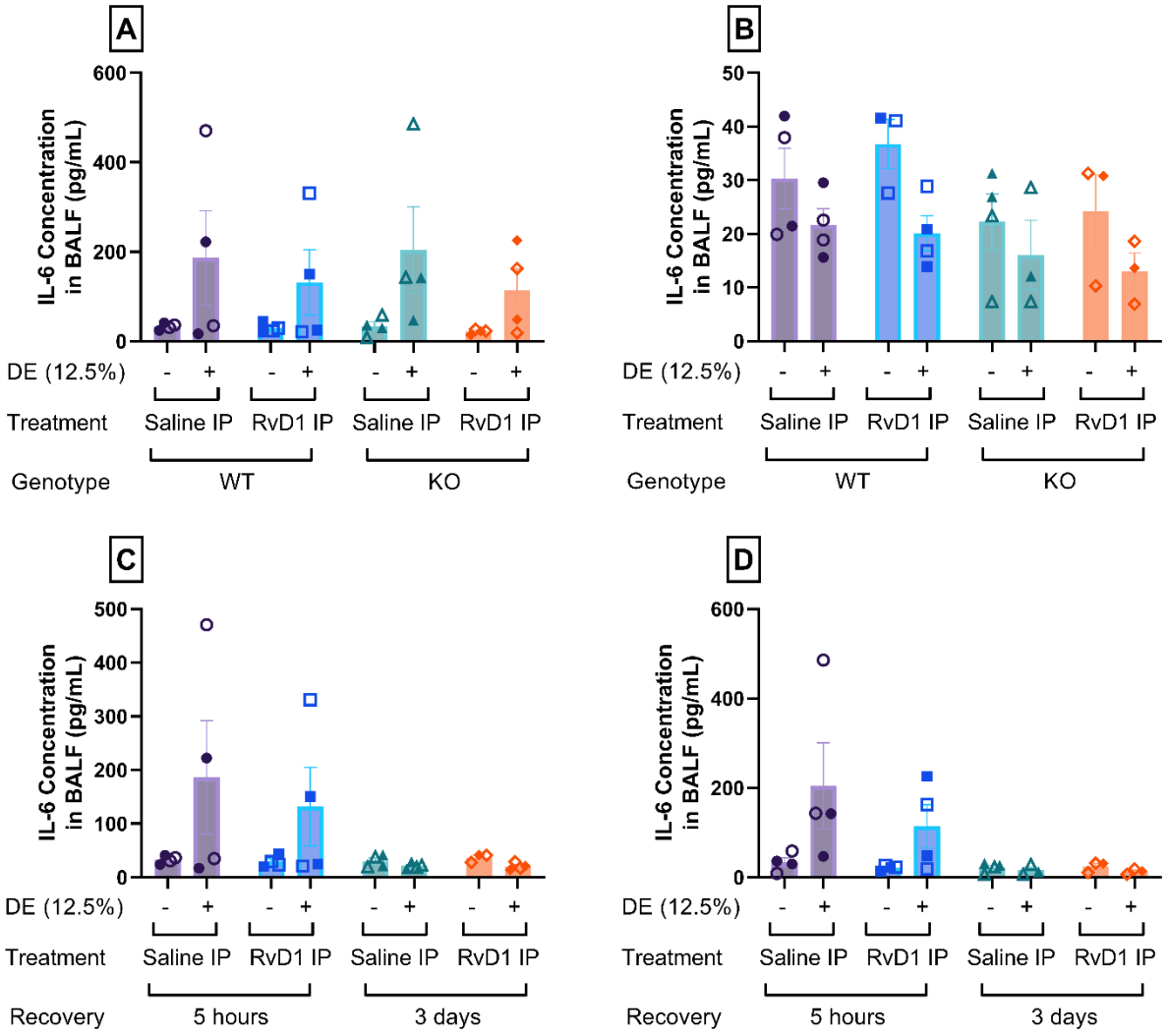

Figure 16. Effects of RvD1 on BALF IL-6 in DE-exposed WT and KO mice with recovery of 5 hours and 3 days

(A) IL-6 concentrations in BALF of WT and KO animals with a recovery of 5 hours; **(B)** IL-6 concentrations in BALF of WT and KO animals with a recovery of 3 days; **(C)** IL-6 concentrations in BALF of WT animals with a recovery of 5 hours or 3 days; **(D)** IL-6 concentrations in BALF of KO animals with a recovery of 5 hours or 3 days.

BALF TNF-α concentrations demonstrated no statistical significance between animals exposed to DE and treated with saline and animals exposed to DE and treated with RvD1. Primary ANOVA results of animals with a 3-day recovery demonstrated significance for DE exposure (p=0.0324). KO animals demonstrated significance for timepoint (p=0.0334) and exposure x timepoint interaction (p=0.0259).

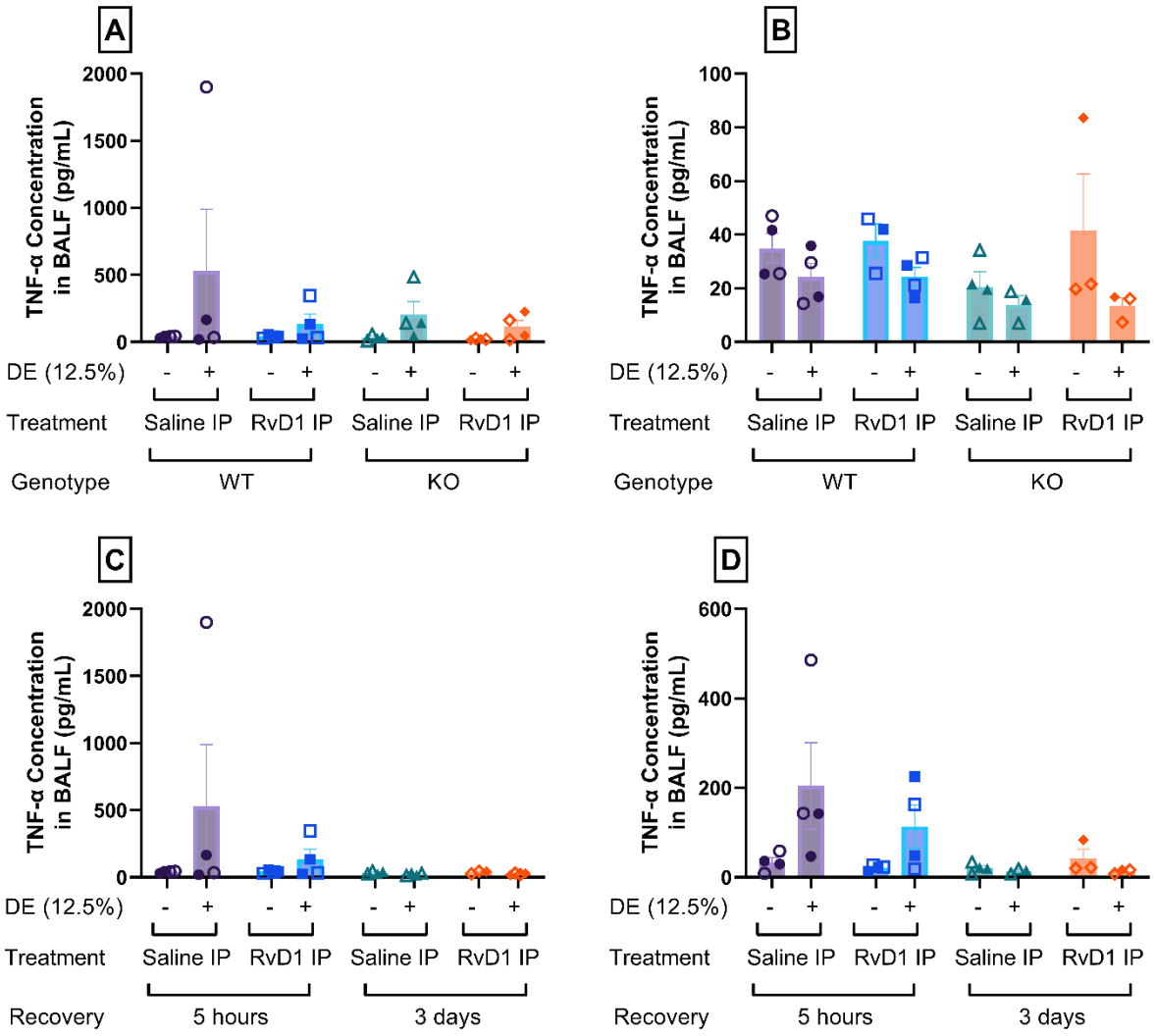

Figure 17. Effects of RvD1 on BALF TNF-α in DE exposed WT and KO mice with recovery of 5 hours and 3 days
(A) TNF-α concentrations in BALF of WT and KO animals with a recovery of 5 hours; **(B)** TNF-α concentrations in BALF of WT and KO animals with a recovery of 3 days; **(C)** TNF-α concentrations in BALF of WT animals with a recovery of 5 hours or 3 days; **(D)** TNF-α concentrations in BALF of KO animals with a recovery of 5 hours or 3 days.

BALF IL-10 concentrations demonstrated no statistical significance between animals exposed to DE and treated with saline and animals exposed to DE and treated with RvD1. ANOVA results revealed statistical significance for genotype ($p<0.0001$) and exposure x genotype interaction ($p= 0.0045$) at the 3-day recovery timepoint, and KO animals demonstrated significance for timepoint ($p=0.0002$). All statistically significant comparisons are displayed in **Table 11**.

Table 11. Statistical Comparisons of BALF IL-10 Concentrations

Comparison	**P-value**
Saline/saline 5hr vs. saline/RvD1 3d	0.0421
Saline/saline 5hr and DE/RvD1 3d	0.0190
Saline/RvD1 5hr and saline/RvD1 3d	0.0199
Saline/RvD1 5hr and DE/RvD1 3d	0.0089
Saline/saline 3d and DE/RvD1 3d	0.0411
Saline/RvD1 3d and DE/saline 3d	0.0244
DE/RvD1 5h and saline/RvD1 3d	0.0198
DE/saline 5hr and DE/RvD1 3d	0.0109
DE/RvD1 5hr and DE/RvD1 3d	0.0089

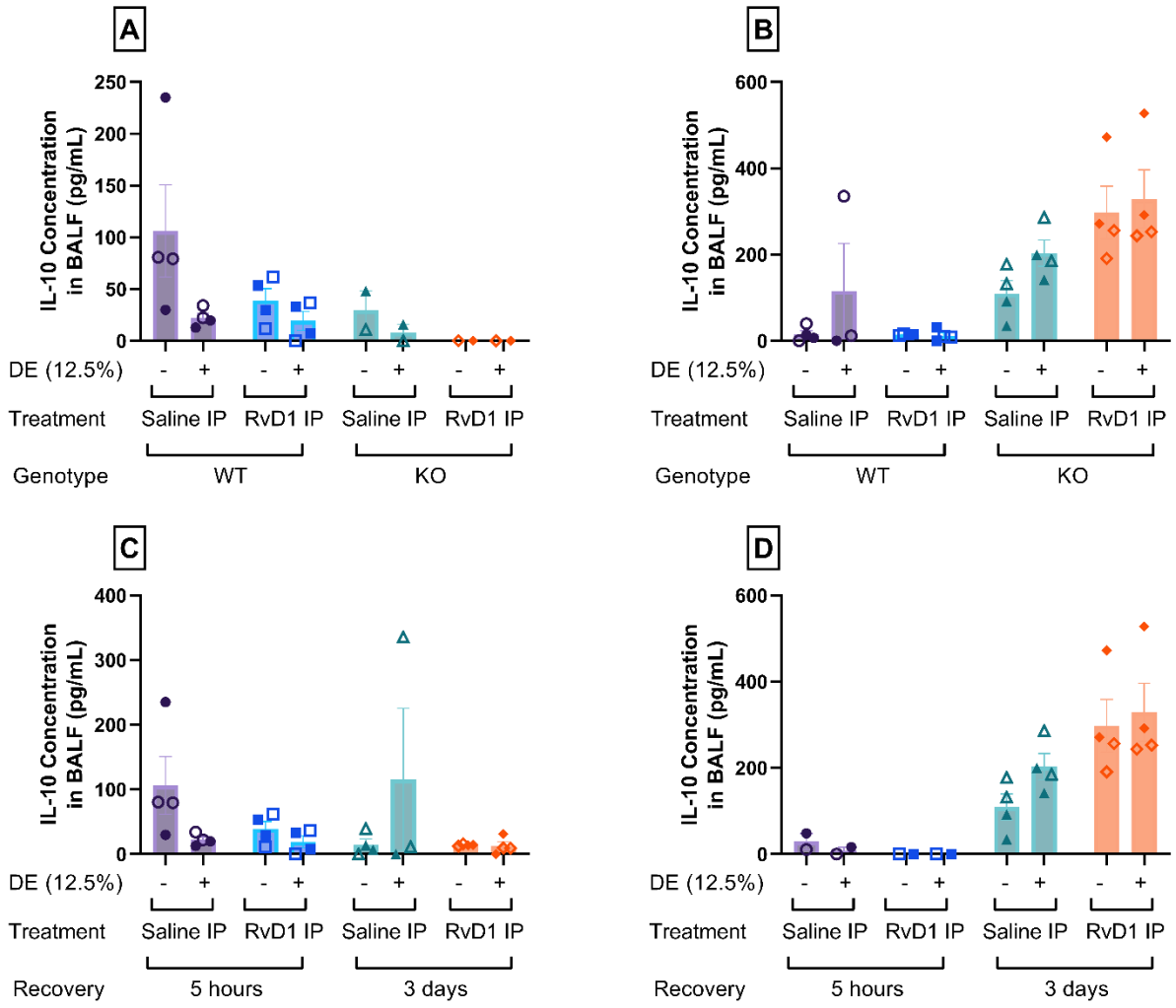

Figure 18. Effects of RvD1 on BALF IL-10 in DE-exposed WT and KO mice with recovery of 5 hours and 3 days

(A) IL-10 concentrations in BALF of WT and KO animals with a recovery of 5 hours; **(B)** IL-10 concentrations in BALF of WT and KO animals with a recovery of 3 days; **(C)** IL-10 concentrations in BALF of WT animals with a recovery of 5 hours or 3 days; **(D)** IL-10 concentrations in BALF of KO animals with a recovery of 5 hours or 3 days.

BALF IL-22 concentrations demonstrated no statistical significance between animals exposed to DE and treated with saline and animals exposed to DE and treated with RvD1. ANOVA results displayed significance for timepoint (p=0.0426) and treatment (p=0.0305).

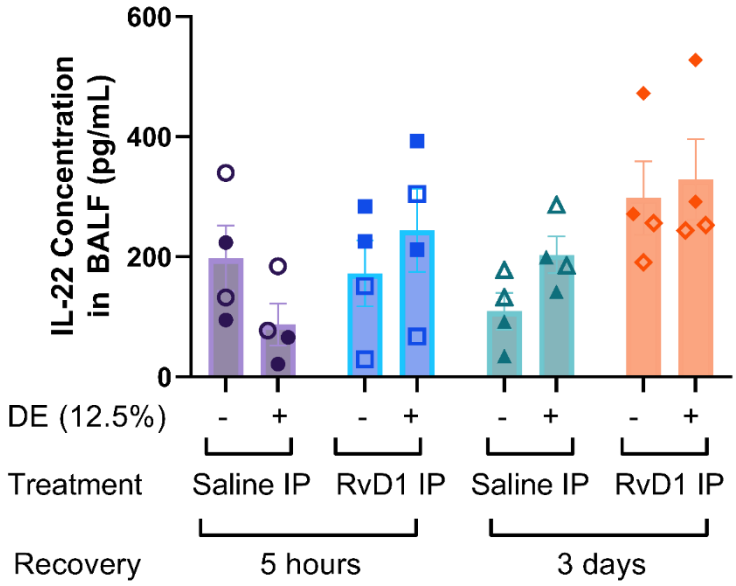

Figure 19. Effects of RvD1 on BALF IL-22 in DE-exposed WT mice with recovery of 5 hours and 3 days

3.3.2 Effects of DE Exposure and RvD1 Treatment on Bronchoalveolar Lavage Fluid Cellular Infiltrates

Total cell counts in BALF demonstrated no statistical significance between animals exposed to DE and treated with saline and animals exposed to DE and treated with RvD1. ANOVA results revealed a primary effect of DE exposure at 5 hours recovery ($p=0.0003$) and 3 days recovery ($p=0.0025$). Wild type animals demonstrated significant effects between exposure ($p<0.0001$) and exposure x timepoint interaction ($p=0.0047$). KO animals exhibited significance for exposure ($p=0.0051$). All statistically significant comparisons are displayed in **Table 12**.

Table 12. Statistical Comparisons of BALF Total Cell Counts

Comparison	**P-value**
WT saline/saline 5h vs. WT DE/saline 5h	0.0012
WT saline/saline 5hr vs. WT DE/RvD1 5hr	0.0309
WT saline/RvD1 5h vs. WT DE/saline 5h	0.0136
WT saline/RvD1 5hr vs. WT DE/RvD1 5hr	0.0381
WT DE/saline 5hr vs. WT saline/RvD1 3d	0.0099
WT DE/saline 5hr vs. WT saline/saline 3d	0.0122
KO DE/saline 3d vs. KO saline/RvD1 3d	0.0257
KO saline/RvD1 5hr and KO DE/EvD1 5hr	0.0443
KO DE/RvD1 5h and KO saline/saline 3d	0.0363
KO saline/RvD1 5hr vs. WT DE/saline 5hr	0.0200

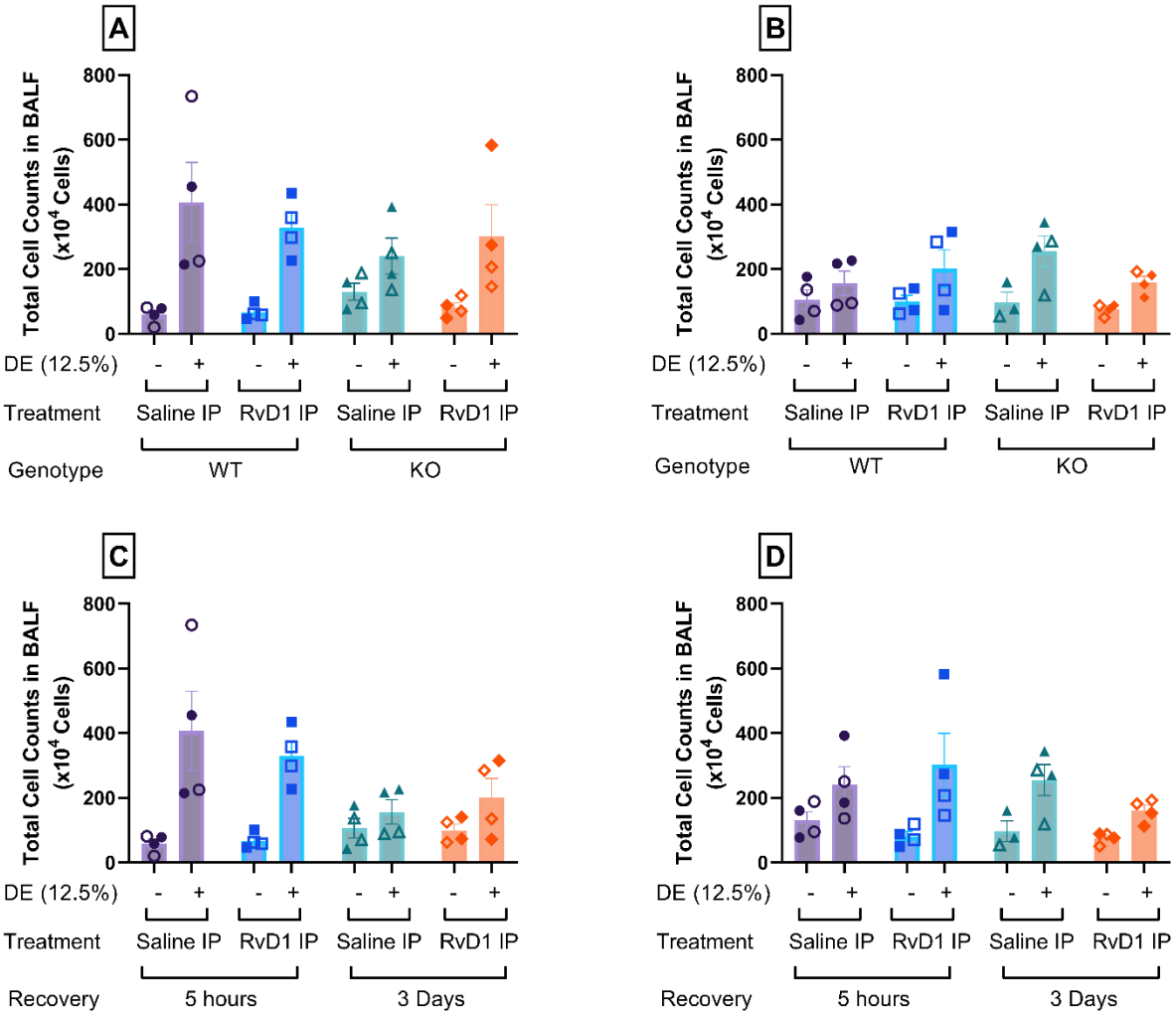

Figure 20. Effects of RvD1 on BALF total cell counts in DE-exposed WT and KO mice with recovery of 5 hours and 3 days

(A) Total cell counts in BALF of WT and KO animals with a recovery of 5 hours; **(B)** Total cell counts in BALF of WT and KO animals with a recovery of 3 days; **(C)** Total cell counts in BALF of WT animals with a recovery of 5 hours or 3 days; **(D)** Total cell counts in BALF of KO animals with a recovery of 5 hours or 3 days.

Macrophage counts in BALF demonstrated no statistical significance between animals exposed to DE and treated with saline and animals exposed to DE and treated with RvD1. ANOVA results demonstrated no significant effects.

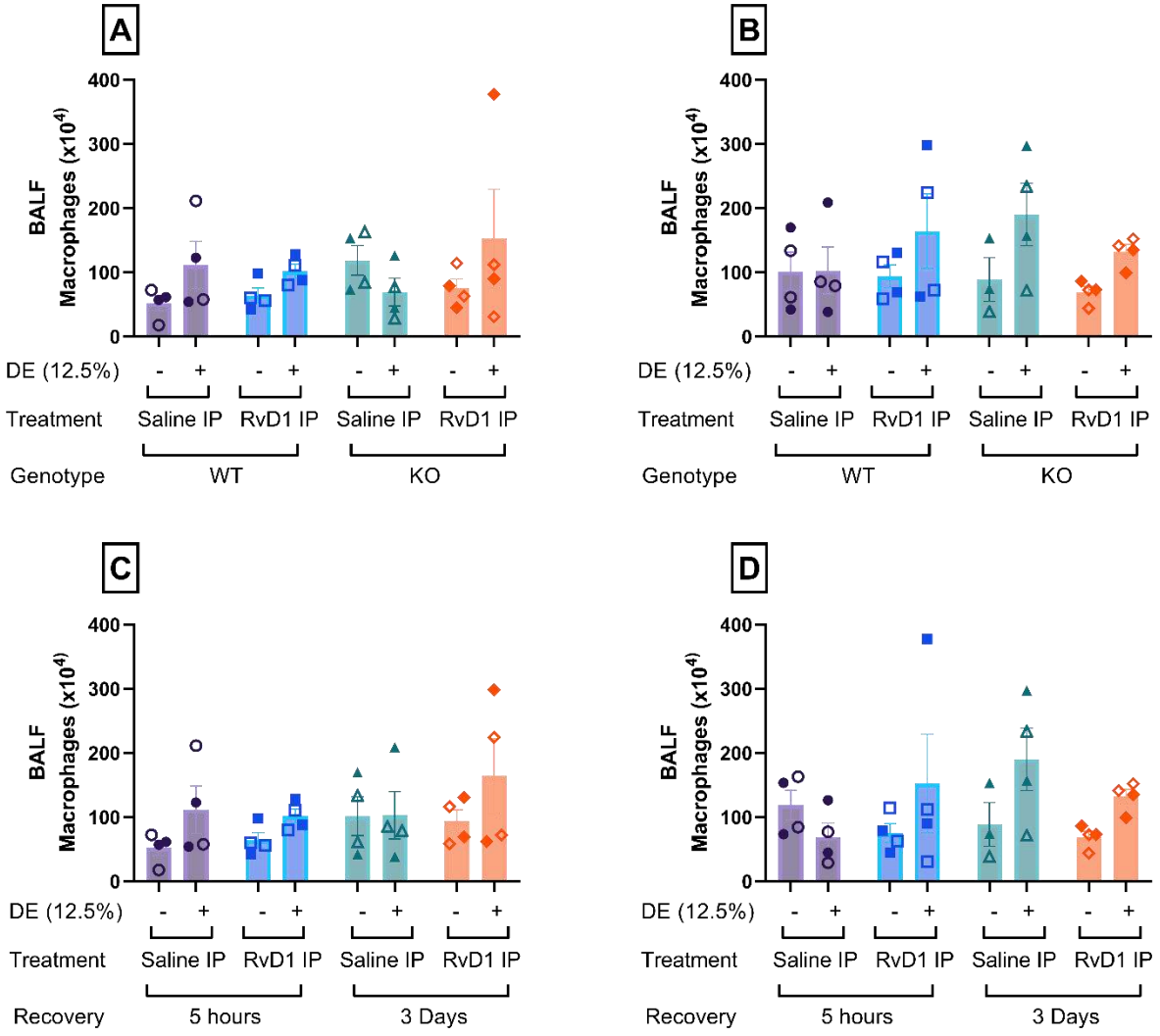

Figure 21. Effects of RvD1 on BALF macrophages in DE exposed WT and KO mice with recovery of 5 hours and 3 days
(A) Macrophage counts in BALF of WT and KO animals with a recovery of 5 hours; **(B)** Macrophage counts in BALF of WT and KO animals with a recovery of 3 days; **(C)** Macrophage counts in BALF of WT animals with a recovery of 5 hours or 3 days; **(D)** Macrophage counts in BALF of KO animals with a recovery of 5 hours or 3 days.

Neutrophil counts in BALF demonstrated no statistical significance between animals exposed to DE and treated with saline and animals exposed to DE and treated with RvD1. ANOVA results demonstrated significant effects of exposure ($p<0.0001$) and exposure x genotype interaction ($p=0.0144$) at 5 hours recovery. WT animals displayed significance for DE exposure ($p<0.0001$), timepoint ($p<0.0001$), and DE exposure x timepoint interaction ($p<0.0001$). KO animals displayed significance for DE exposure ($p=0.0292$) and timepoint ($p=0.0054$). All statistically significant comparisons are displayed in **Table 13**.

Table 13. Statistical Comparisons of BALF Neutrophil Counts

Comparison	**P-value**
WT saline/saline 5hr vs. WT DE/saline 5hr	0.0041
WT saline/saline 5hr vs. WT DE/RvD1 5hr	0.0372
WT saline/RvD1 5hr vs. WT DE/saline 5hr	0.0039
WT saline/RvD1 5hr vs. WT DE/RvD1 5hr	0.0353
WT DE/saline 5hr vs. WT saline/saline 3d	0.0002
WT DE/RvD1 5hr vs. WT saline/saline 3d	0.0044
WT DE/saline 5hr vs. WT saline/RvD1 3d	0.0002
WT DE/RvD1 5hr vs. WT saline/RvD1 3d	0.0043
WT DE/saline 5hr vs. WT DE/saline 3d	0.0003
WT DE/saline 5hr vs. WT DE/RvD1 3d	0.0003
WT DE/RvD1 5hr vs. WT DE/saline 3d	0.0057
WT DE/RvD1 5hr vs. WT DE/RvD1 3d	0.0052
KO saline/saline 5hr vs. WT DE/saline 5hr	0.0042
KO saline/saline 5hr vs. WT DE/RvD1 5hr	0.0379
KO saline/RvD1 5hr vs. WT DE/saline 5hr	0.0346
KO DE/RvD1 5hr vs. KO saline/RvD1 3d	0.0457
KO DE/RvD1 5hr vs. KO DE/RvD1 3d	0.0500

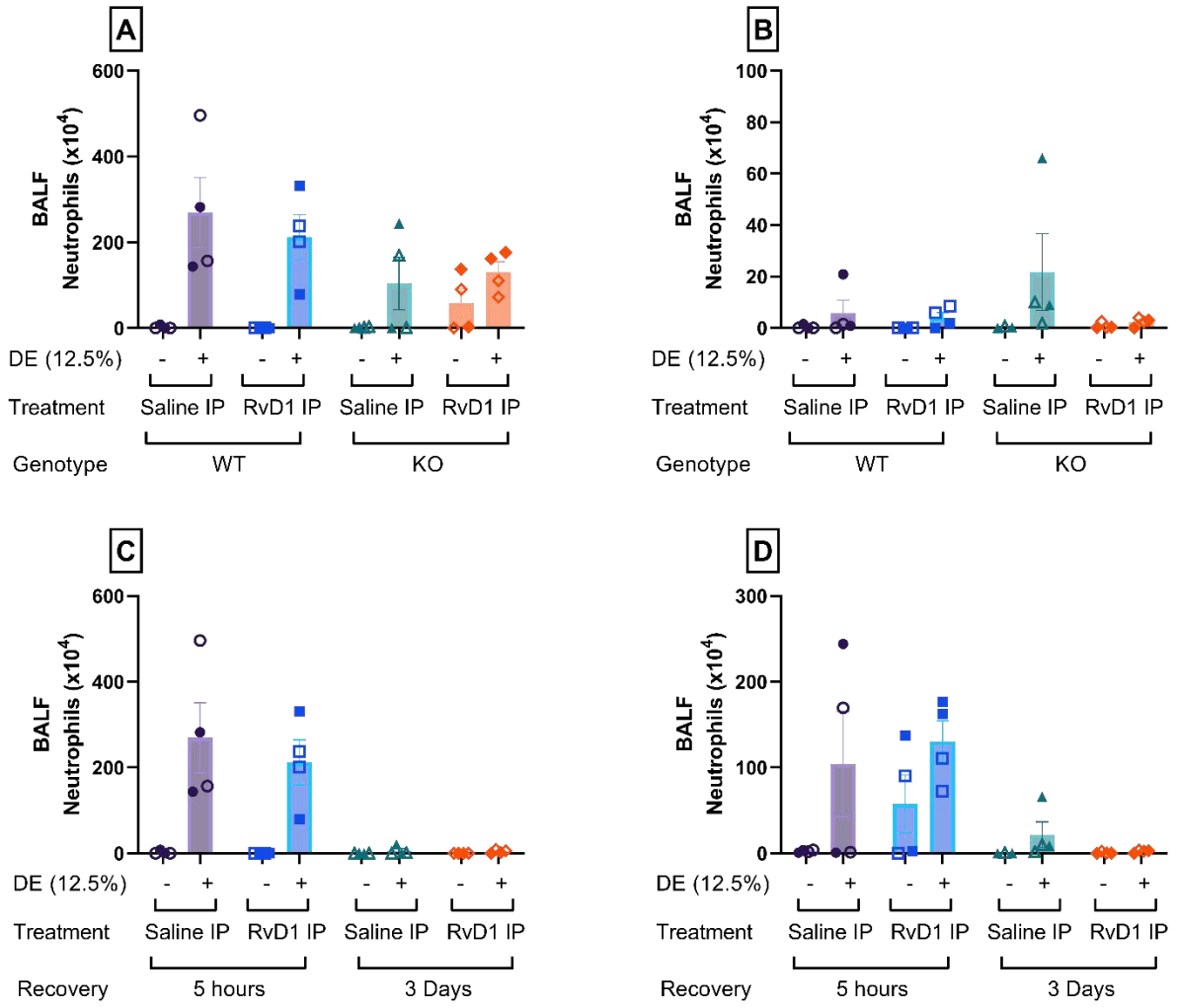

Figure 22. Effects of RvD1 on BALF neutrophils in DE-exposed WT and KO mice with recovery of 5 hours and 3 days

(A) Neutrophil counts in BALF of WT and KO animals with a recovery of 5 hours; **(B)** Neutrophil counts in BALF of WT and KO animals with a recovery of 3 days; **(C)** Neutrophil counts in BALF of WT animals with a recovery of 5 hours or 3 days; **(D)** Neutrophil counts in BALF of KO animals with a recovery of 5 hours or 3 days.

Eosinophil counts in BALF demonstrated no statistical significance between animals exposed to DE and treated with saline and animals exposed to DE and treated with RvD1. ANOVA results displayed significance for DE exposure (p=0.0336) at 5 hours recovery.

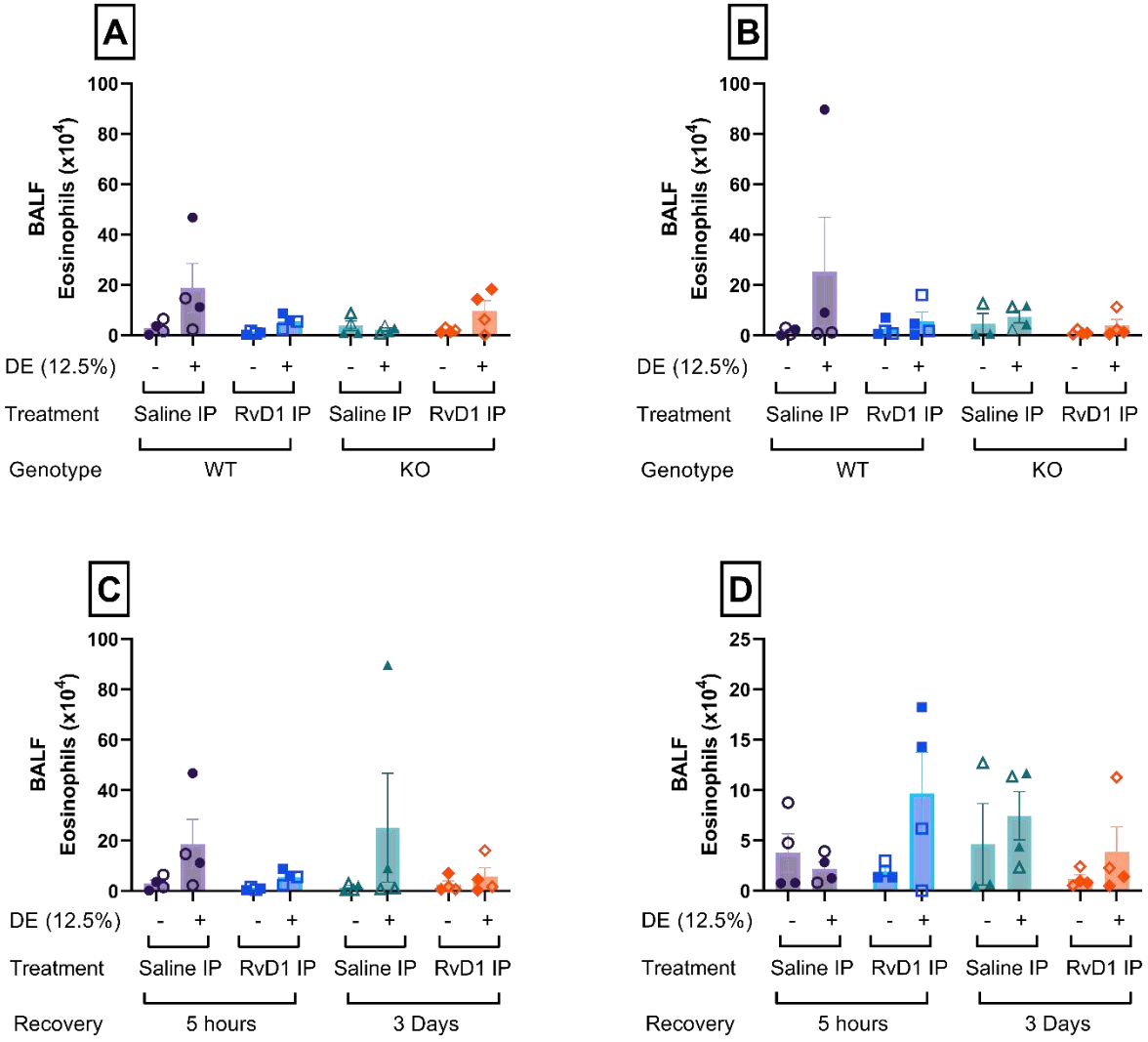

Figure 23. Effects of RvD1 on BALF eosinophils in DE-exposed WT and KO mice with recovery of 5 hours and 3 days
(A) Eosinophil counts in BALF of WT and KO animals with a recovery of 5 hours; **(B)** Eosinophil counts in BALF of WT and KO animals with a recovery of 3 days; **(C)** Eosinophil counts in BALF of WT animals with a recovery of 5 hours or 3 days; **(D)** Eosinophil counts in BALF of KO animals with a recovery of 5 hours or 3 days.

Lymphocyte counts in BALF demonstrated statistical significance between KO DE/saline mice with a recovery of 3 days and KO DE/RvD1 mice with a recovery of 3 days (p=0.0541). ANOVA results revealed significance for DE exposure at 5 hours recovery (p=0.0014) and 3 days recovery (p=0.0002). WT animals demonstrated significance for DE exposure (p=0.0009), timepoint (p=0.0147), and DE exposure x timepoint interaction (p=0.0379). KO animals also demonstrated significance for DE exposure (p<0.0001), timepoint (p=0.0002), and DE exposure x timepoint interaction (p=0.0002). All statistically significant comparisons are displayed in **Table 14**.

Table 14. Statistical Comparisons of BALF Lymphocyte Counts

Comparison	**P-value**
KO saline/saline 5hr vs. KO DE/saline 3d	<0.0001
KO saline/saline 5hr vs. KO DE/RvD1	0.0147
KO saline/RvD1 5hr vs. KO DE/saline 3d	<0.0001
KO saline/RvD1 5hr vs. KO DE/RvD1 3d	0.0047
KO saline/saline 3d vs. KO DE/saline 3d	<0.0001
KO saline/saline 3d vs. KO DE/RvD1 3d	0.0125
KO saline/RvD1 3d vs. KO DE/saline 3d	<0.0001
KO saline/RvD1 3d vs. KO DE/RvD1 3d	0.0105
KO DE/saline 5hr vs. KO DE/saline 3d	<0.0001
KO DE/RvD1 5hr vs. KO DE/saline 3d	<0.0001
KO DE/saline 3d vs. KO DE/RvD1 3d	0.0541

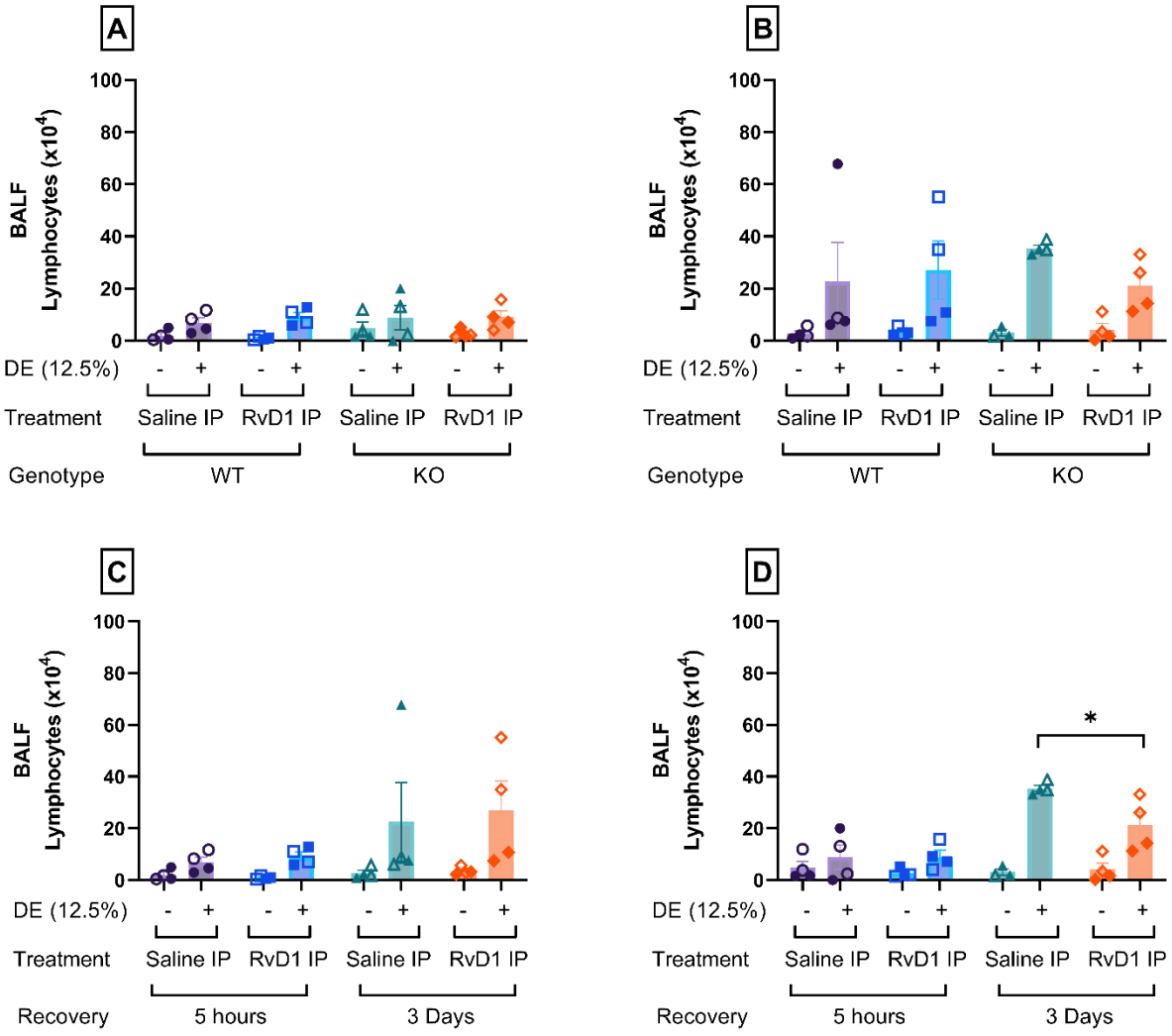

Figure 24. Effects of RvD1 on BALF lymphocytes in DE-exposed WT and KO mice with recovery of 5 hours and 3 days

(A) Lymphocyte counts in BALF of WT and KO animals with a recovery of 5 hours; **(B)** Lymphocyte counts in BALF of WT and KO animals with a recovery of 3 days; **(C)** Lymphocyte counts in BALF of WT animals with a recovery of 5 hours or 3 days; **(D)** Lymphocyte counts in BALF of KO animals with a recovery of 5 hours or 3 days.

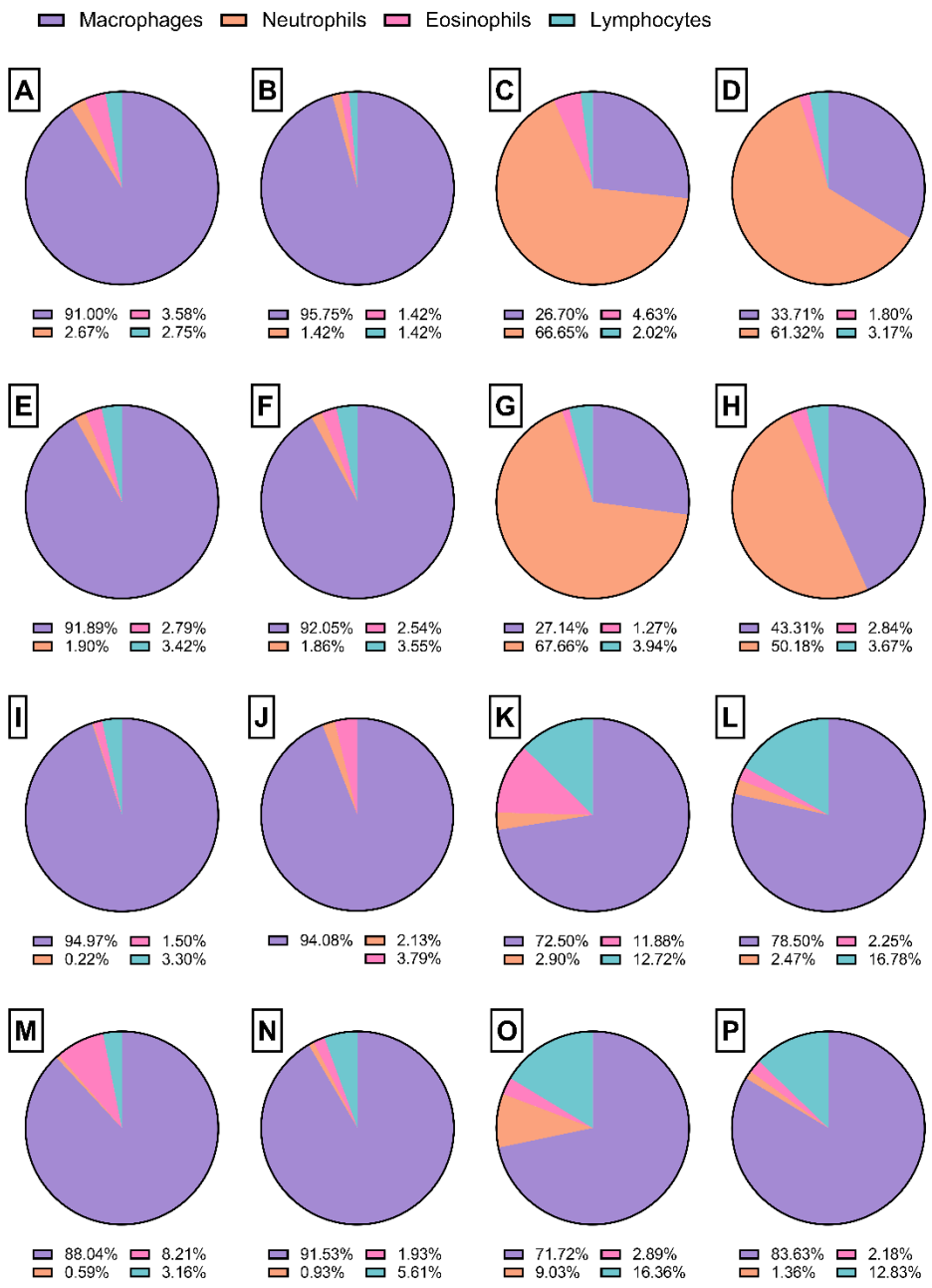

Figure 25. Effects of RvD1 on BALF cell infiltrate composition in DE-exposed WT and KO mice with recovery of 5 hours and 3 days

(A) WT saline/saline with a recovery of 5 hours; **(B)** WT saline/RvD1 with a recovery of 5 hours; **(C)** WT DE/saline with a recovery of 5 hours; **(D)** WT DE/RvD1 with a recovery of 5 hours; **(E)** KO saline/saline with a recovery of 5 hours; **(F)** KO saline/RvD1 with a recovery of 5 hours; **(G)** KO DE/saline with a recovery of 5 hours; **(H)** KO DE/RvD1 with a recovery of 5 hours; **(I)** WT saline/saline with a recovery of 3 days; **(J)** WT saline/RvD1 with a recovery of 3 days; **(K)** WT DE/saline with a recovery of 3 days; **(L)** WT DE/RvD1 with a recovery of 3 days; **(M)** KO saline/saline with a recovery of 3 days; **(N)** KO saline/RvD1 with a recovery of 3 days; **(O)** KO DE/saline with a recovery of 3 days; **(P)** KO DE/RvD1 with a recovery of 3 days.

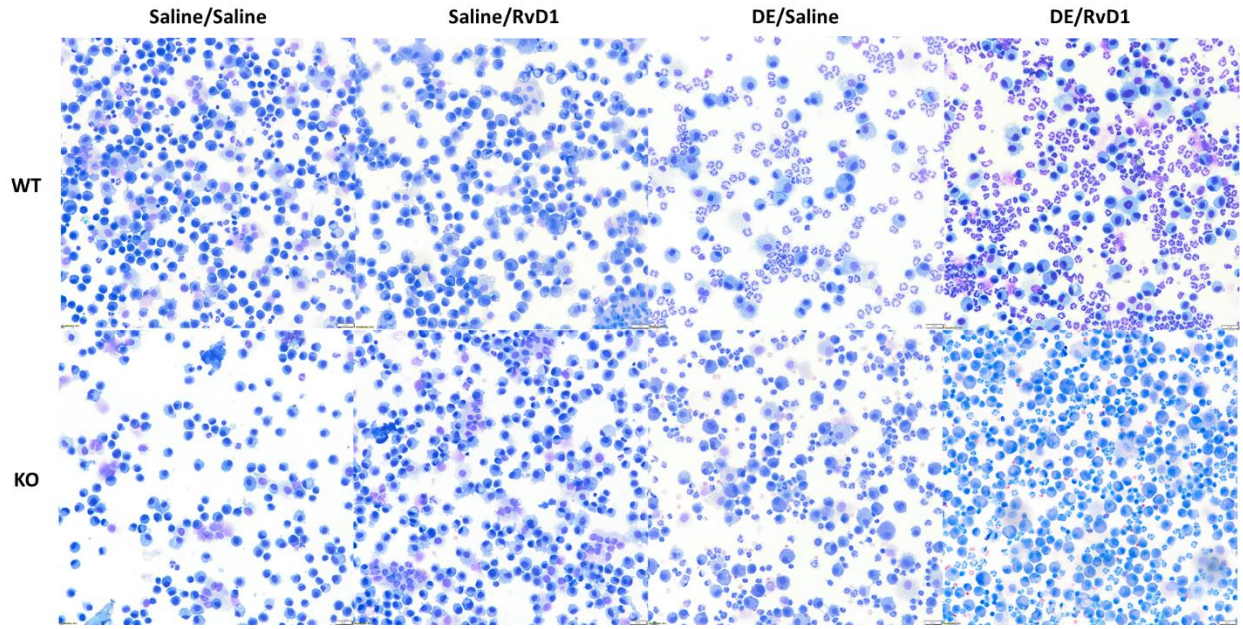

Figure 26. Effects of RvD1 on BALF cytospin cell composition in DE-exposed WT and KO mice with recovery of 5 hours

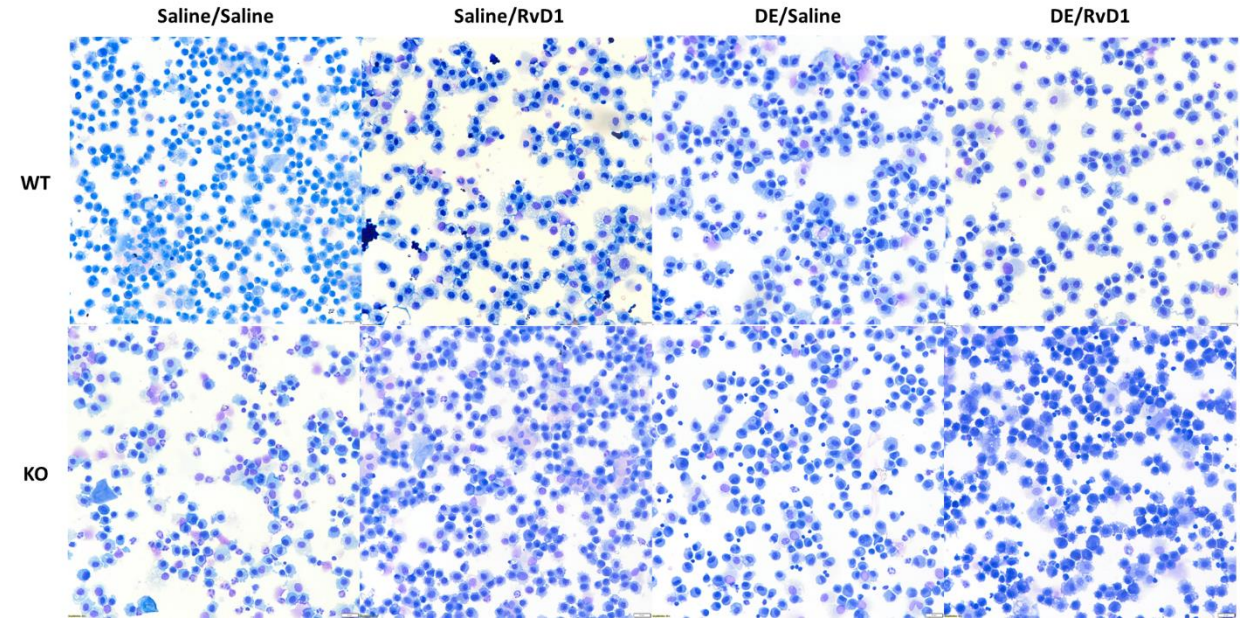

Figure 27. Effects of RvD1 on BALF cytospin cell composition in DE-exposed WT and KO mice with recovery of 3 days

3.3.3 Effects of DE Exposure and RvD1 Treatment on Lung Pathology

Histopathology of saline instillation mice revealed slight bronchiolar epithelial hyperplasia (★) with no observed alveolar inflammation or lymphoid aggregates and limited immune cells visible.

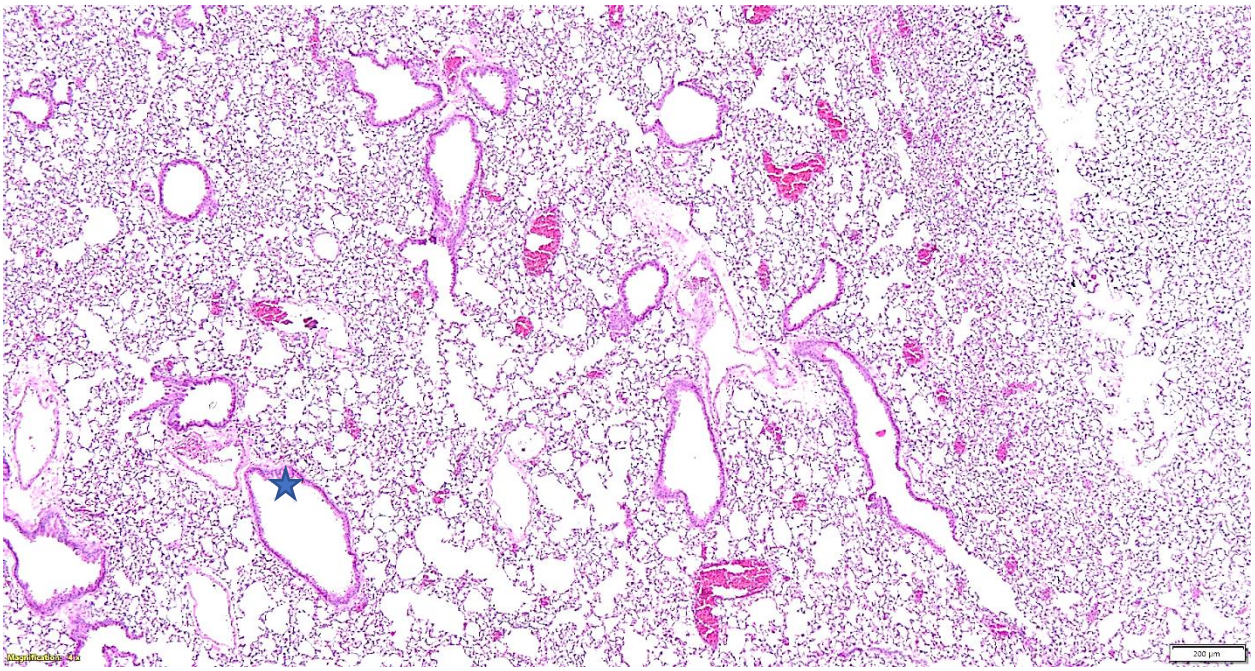

Figure 28. Effects of saline exposure and saline treatment in WT mice with a 5-hour recovery on lung histopathology

Animals exposed to DE without RvD1 treatment, displayed increased bronchiolar epithelial hyperplasia (★), and alveolar inflammation (●) compared to saline instilled animals. There are limited lymphoid aggregates (◆) observed, but those present are primarily perivascular.

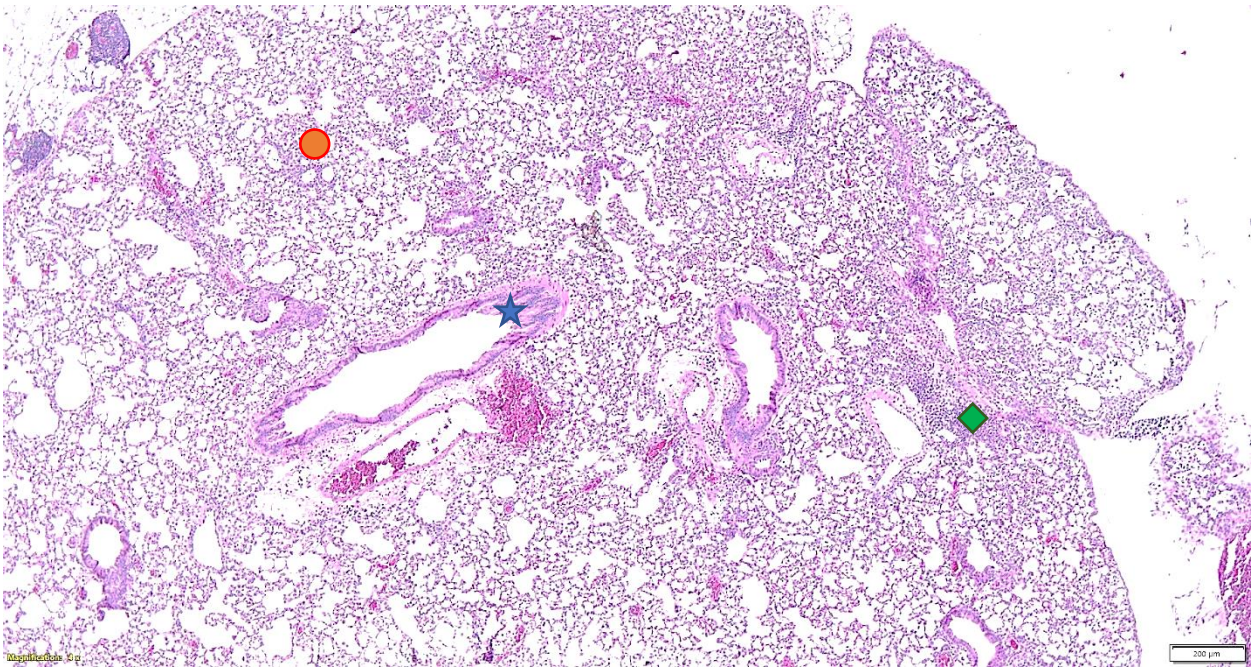

Figure 29. Effects of DE exposure and saline treatment in WT mice with a 5-hour recovery on lung histopathology

Animals exposed to DE and treated with RvD1 showed increased alveolar inflammation (●), bronchiolar epithelial hyperplasia, and peribronchiolar inflammation in the airway, as marked by (★) compared to DE animals treated with saline.

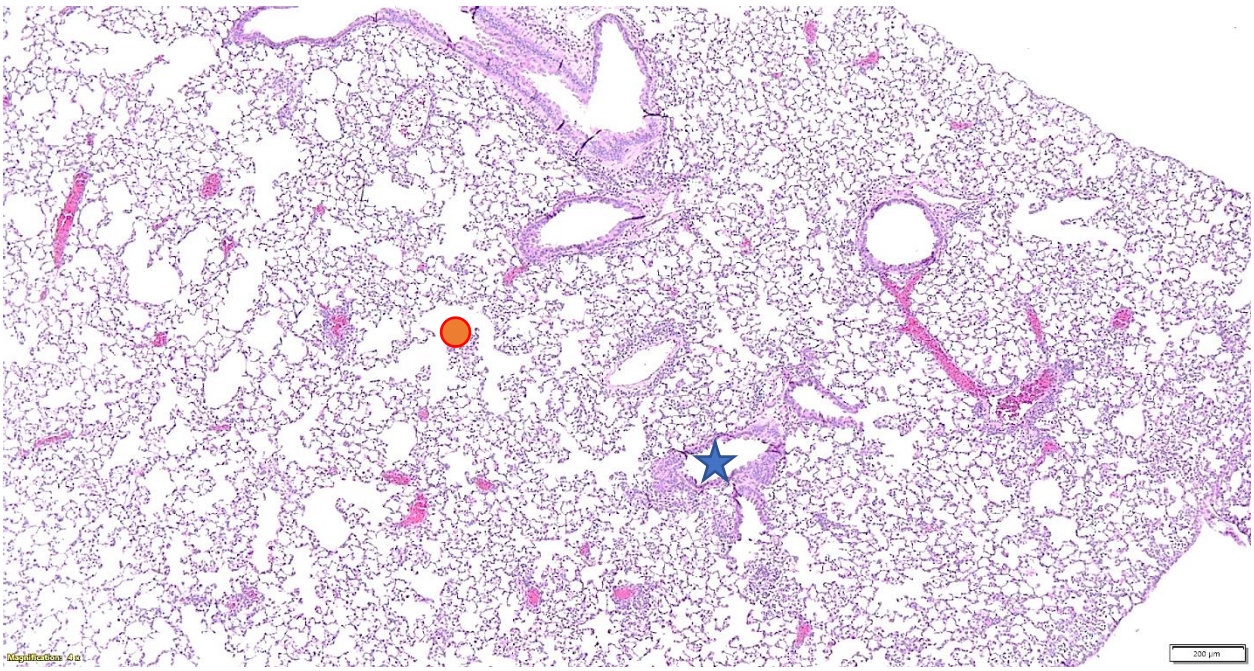

Figure 30. Effects of DE exposure and RvD1 treatment in WT mice with a 5-hour recovery on lung histopathology

IL22KO animals exposed to DE without RvD1 treatment exhibited increased bronchiolar epithelia hyperplasia (★), peribronchiolar/perivascular inflammation (▲), alveolar inflammation (●), and lymphoid aggregates (◆) compared to WT mice.

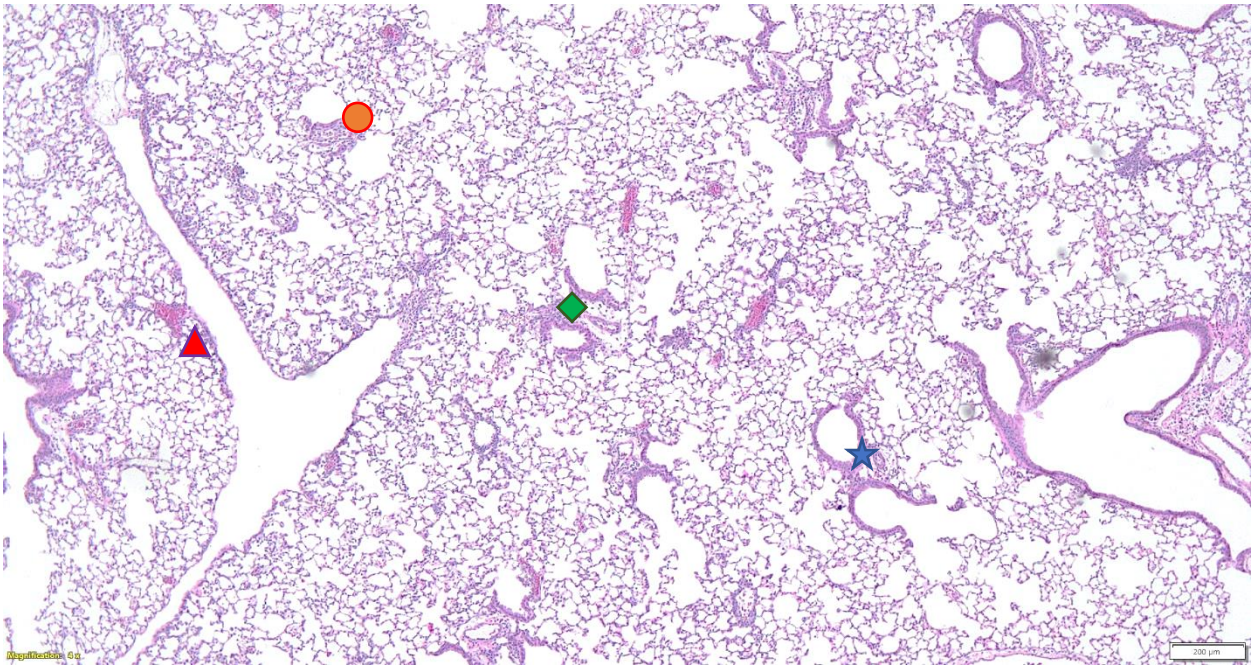

Figure 31. Effects of DE exposure and saline treatment in KO mice with a 5-hour recovery on lung histopathology

CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

4.1 Discussion

This study aimed to explore the pharmacological applications of RvD1 in the context of agricultural dust-induced chronic inflammation in both an *in vitro* alveolar macrophage model and an *in vivo* repetitive dust exposure murine model. It was predicted that treatment with RvD1 would reduce the inflammatory response by decreasing production of pro-inflammatory cytokines IL-6 and TNF-α and increasing production of anti-inflammatory, pro-resolution cytokines IL-10 and IL-22. It was also hypothesized that *in vivo* RvD1 treatment would reduce immune cell infiltration as well as IL-6 and TNF-α BALF concentrations in WT DE-exposed mice. We also predicted that knock-out of IL-22 *in vivo* would increase IL-6, TNF-α, and IL-10 cytokine production, as well as increase immune cell infiltration.

In investigating how RvD1 alters the inflammatory response to organic dust exposure in alveolar macrophages *in vitro*, the data demonstrated a modest effect of RvD1 in reducing IL-6 and TNF-α production and increasing IL-22 production. Alveolar macrophages exposed to DE with or without RvD1 had a robust inflammatory response by producing high levels of IL-6 and TNF-α. This is consistent with previous data that show organic dust initiates an inflammatory response in resident immune cell populations[48,119]. In cells exposed to DE without RvD1, secreted IL-10 and IL-22 concentrations were significantly higher than their medium-only controls. This was not hypothesized but would be consistent with alveolar macrophages being basally polarized to an anti-inflammatory state to assist in protecting the tissues from immune-mediated injury[39]. Cells exposed to DE and treated with RvD1 displayed decreased IL-6 and TNF-α production 6 hours post co-exposure compared to their DE-only control. IL-10

concentrations were significantly elevated in DE-exposed cells treated with 100 nM RvD1 compared to their DE only controls, indicating higher doses of RvD1 influence increased production of this anti-inflammatory/pro-resolution cytokine. IL-22 concentrations in cells exposed to DE and treated with RvD1 were significantly increased at 1 and 2 hours, with the peak occurring at 2 hours post co-exposure, compared to their DE-only controls. The temporal relationships between the peak of IL-22 production at 2 hours and the peak of IL-6 and TNF-α production at 6 hours may indicate IL-22 influences the inflammatory response to protect the tissues, and these parameters provide supporting evidence for this hypothesis. IL-22 secreted concentrations then stayed low in the subsequent timepoints investigated. Combined with previous data demonstrating IL-22 is Golgi-localized in macrophages following DE exposure, the data indicate it may participate in intracellular signaling or may be receptor bound by the macrophages for downstream signaling[148]. There appears to be variable outcomes between the 10 nM and 100 nM RvD1 doses, suggesting there may be an optimal dose between 10 nM and 100 nM to alter pro-inflammatory cytokine concentrations, improve clearance of organic dust, and enhance the repair process. *In vitro* evidence suggests RvD1 has a modest effect on the production of pro-inflammatory cytokines in response to organic dust. Contrary to accepted evidence, alveolar macrophages were determined as a source of IL-22 production[36].

A pilot *in vivo* murine study was performed to determine an appropriate RvD1 dosage and administration route. Animals were given 500 ng RvD1 IV through the tail vein and 250 ng IP. The IV dosage was chosen from previous studies utilizing a dosage of 500 ng RvD1 IV as a treatment in a murine chronic DE exposure model[34]. The IP dosage was chosen from a range of previous studies utilizing dosages ranging from 100 ng to 300 ng RvD1 IP as a treatment for acute and chronic lung injury murine models[38,58,79]. When evaluating immune cell infiltrations,

the dosage and route of administration did not have a significant impact on immune function, therefore IP administration route was chosen with a dose of 250 ng as it was a reasonable dosage between those previously used in the literature.

In evaluating the efficacy of RvD1 treatment in a murine model of repetitive dust exposure, RvD1 reduced BALF lymphocytes after 3 days in KO animals exposed to DE compared to animals treated with saline and exposed to DE. There was no statistical significance observed in BALF cytokine concentrations except for IL-10 production in IL22KO mice with a 3-day recovery. In this model, IL-10 was significantly increased in RvD1 treated animals compared to their saline controls and DE exposed animal treated with RvD1 had the highest production of IL-10. This observation may be due to compensation by IL-10 for the lack of IL-22, but further investigation is needed to evaluate this hypothesis. As seen in the *in vitro* model, IL-10 is upregulated in the response to agriculture dust. It is likely being produced early to assist in the transition to resolution and repair. RvD1 is known to increase immune activity and clearance by macrophages and neutrophils, accelerating the inflammatory response and allowing the transition to resolution sooner, which may account for the increased IL-10 concentrations as well[31].

Total immune cell infiltrate counts in BALF from animals exposed to DE were increased compared to their saline controls, but RvD1 treatment did not have a significant impact on immune cell infiltration. Previous data would suggest RvD1-treated animals should have decreased immune cell infiltration, which would contribute to the reduction of the total cell infiltrate counts[139]. Neutrophil counts in animals exposed to DE and treated with RvD1 were not significant compared to animals exposed to DE and treated with saline, contrary to previous evidence[31,34]. Lymphocyte counts in BALF demonstrated no statistical significance in WT

animals, but in KO animals, counts were significantly decreased in animals exposed to DE and treated with RvD1 compared to animals exposed to DE and treated with saline with a recovery of 3 days. This is consistent with lymphocytes primarily being recruited later in the inflammatory response and would be expected to be increased in later timepoints[97]. They are also known to secrete IL-22 and be polarized to a Th17 state in response to organic dust so understandably would be highly involved in the repair process[17,36]. Treatment with RvD1 is known to influence immune cell recruitment, and its role in lymphocyte recruitment would reduce the chronic inflammation typically seen in organic dust induced pathology[34,148]. Our lack of statistically significant findings in this study may be due to the low sample size, and more animals are needed to reach statistical significance.

Observed histopathology in this study is atypical of previous work using this model but displays some pathological changes between saline instillation and DE instillation mice as well as WT and IL22KO mice[34,148]. Lymphoid aggregates localized perivascularly is consistent with expected pathology, as lymphoid cells migrate from the vasculature into the interstitial tissue. There is a lack of peribronchial lymphoid aggregates in DE exposed animals compared to what is typically seen in this model. The increase in inflammation seen in RvD1-treated animals compared to their DE-only controls may indicate the dosage of RvD1 is not appropriate and is having undesirable effects on the immune response. IL22KO animals showed increased pathological changes, consistent with the hypothesis that knock-out of IL-22 impairs the resolution and repair processes following organic dust exposure. This is further evidence that IL-22 is critical to maintaining epithelial barrier integrity in organic dust exposure.

4.2 Limitations

In vitro data sets display high variability and error, which could be corrected by normalizing treatment groups as fold change compared to the media controls. Culture plates incubated for 18 and 24 hours after treatment may have experienced slight media evaporation, resulting in the possibility of increased cytokine concentration due to water loss. In addition, cytokine levels at the 24-hour timepoint may be elevated due to cell proliferation or cytokine accumulation in the medium. *In vitro* studies were performed as a co-exposure, which may alter the kinetics of the cytokines produced compared to pre- or post-treatment investigations.

During *in vivo* studies, some animals did not receive the full IV dosage of AT-RvD1 or EtOH/saline as some was accidentally administered in the tissues or leaked out when attempting to administer in the tail veins. The pilot study data also had a low sample size with high error, limiting the significance of the data. BALF cell counts and cytokines were highly variable, which would be expected to an extent in this model as some animals may have received a more complete instillation than others. Preliminary histology displays differences in previously seen pathological changes in this model, suggesting incomplete instillations or lack of potency with the hog dust extract. Performing bronchoalveolar lavages on the animals removes many of the immune cells in the airways, which would affect the number of immune cells visualized in the alveolar spaces and large airways. Lack of statistical significance is likely due to a low sample size. These studies will be used to perform a power analysis to determine an appropriate sample size to reach statistical significance.

4.3 Future Directions

Macrophages express the IL-22 receptor which may contribute to the increases in lysate concentrations of IL-22 *in vitro*. There is also previous evidence that IL-22 is localized to the Golgi apparatus[148]. To evaluate the localization of IL-22 in this model, immunocytochemistry staining could be performed at the various timepoints. The timepoints could also be expanded. Adding a 12 hour post exposure point to evaluate a broader range of the immune response, while expanding the incubation to 48 and 72 hours would assist in characterizing the repair process after dust exposure. Co-culture with mouse alveolar epithelial cells would be beneficial to determine how the production of cytokines influences epithelial barrier integrity. Supernate could also be evaluated for amphiregulin, an epithelial repair marker to determine how RvD1 and IL-22 influence epithelial repair after dust exposure[14,102]. These models could be adjusted to include a post-DE exposure RvD1 treatment to evaluate how RvD1 as a pharmaceutical alters the immune response. Droplet digital polymerase chain reaction (ddPCR) could also be performed on lysed cells to assess the cytokine transcript alterations compared to their protein concentrations.

In vivo studies could be improved by performing a power analysis to increase the sample size and increase statistical significance of the desired outcomes. A dose study should be conducted to determine if the current RvD1 dosage of 250 ng is appropriate for the desired outcomes. To expand on the model, RvD1 could be further evaluated in the repair process by implementing a 7-day recovery post-last-instillation followed by a microbial challenge. This would assist in determining the integrity of the mucosal barrier and the implications of dust-induced chronic pulmonary diseases on secondary pathologies in agriculture workers. ELISAs would be performed on the BALF of these animals to assess IL-6, TNF-α, IL-10, IL-22, and

amphiregulin production as another marker for epithelial repair. Real time polymerase chain reaction (RT-PCR) would be performed on all lung samples from previous and future studies to evaluate cytokine transcripts as well as amphiregulin and a custom wound healing array that encompasses many parameters to assess repair of the epithelial barrier.

4.4 Conclusions

The evidence obtained between both *in vitro* and *in vivo* studies suggest that RvD1 influences the production of IL-6, TNF-α, IL-10, and IL-22. It suggests that IL-22 may influence the immune response to organic dust exposure. The data support alveolar macrophages as a source of IL-22 in the pulmonary immune response to DE and that RvD1 influences its production during inflammation. Knock-out of IL-22 in a murine model influences the increased production of IL-10, which warrants further investigation.

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