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The Impact of LCN2 in the Lung Inflammatory Response to Agricultural Dust Exposure

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ABSTRACT OF THE DISSERTATION

The Impact of LCN-2 in the Lung Inflammatory Response
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by

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Agricultural workers have increased risks of developing debilitating respiratory conditions such as chronic obstructive pulmonary disease (COPD) due to their repetitive exposure to organic dust particles. Lipocalin-2 (LCN-2) is an acute phase protein that plays crucial roles in mediating immune responses to inflammatory stimuli in multiple tissues. Its role in the lung immune response to inflammatory stimuli other than bacteria, however, has not been well studied. This research examines the paradoxical roles of LCN2 in the immune response to extracts of dusts collected from swine confinement facilities (DE), in an effort to identify protective vs. harmful roles of LCN2 in the context of lung inflammation. Additionally, the cross-talk between LCN-2 and the anti-inflammatory cytokine IL-10 in mediating the tissue repair process was also examined. Major research findings indicate an important function of LCN-2 in regulating tissue homeostasis, tissue repair, and anti-inflammatory cytokine interleukin-10 (IL-10) production in the lungs. Additionally, compartmentalized effects on T cell and macrophage levels were observed in BALF vs. lung tissue in

DE-exposed LCN-2 KO mice versus wildtype mice. The role of the LCN-2/IL-10 axis in lung tissue repair was further explored by administering recombinant IL-10 to LCN-2 KO mice, resulting in reduced pathology in the lungs. Ablation of LCN-2 resulted in deficits in recovery of mice exposed to DE, which was reversed with administration of IL-10. Lastly, at the population level, we show that higher bronchitis diagnosis rates are associated with natural dust exposure, and that intake of Omega-3 fatty acids is associated with decreased reports of bronchitis. Taken together, these investigations highlight a novel role for LCN-2 in promoting lung repair following organic dust-induced inflammation, mediated in part by IL-10. These findings may substantially impact the breadth of knowledge available for researchers in this field, and consequently help discriminate between the protective and detrimental roles exerted by LCN2 in response to inflammation in the lungs. This can further lead to the potential use of LCN-2 as a diagnostic marker or its therapeutic use in the treatment of debilitating lung inflammatory conditions.

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Chapter 4

Chapter 1: INTRODUCTION

The Agricultural Occupational Industry has been identified by the CDC as one of the most harmful occupations, in part due to its association with debilitating respiratory inflammatory conditions. Aerosolized particulate matter exposures elicit potent lung inflammatory responses, and individuals that are chronically exposed to aerosolized dusts have increased risk for numerous lung diseases, including asthma and COPD. Thus, the burden faced by agricultural industry workers is high [1]. Inflammatory mediators and proteins are critical players in the response to inflammation and the etiology of inflammatory diseases, thus emphasizing the need to research the effects of these key players in mediating the response to agricultural dusts. Here, we investigate the role of an immune mediator, LCN2, in the lung's immune response to agricultural dust exposures. Moreover, we investigate the cross-talk between LCN-2 and the anti-inflammatory cytokine, IL-10.

Lipocalin-2 (LCN2) is an acute-phase protein (APP) that is well-recognized in promoting innate immunity through its role as an iron chelator that limits bacterial growth via iron sequestration. In addition to its role as an iron chelator, emerging evidence suggests that LCN2 plays additional roles in immune response, including paradoxical roles in both promoting and limiting immune responses [2]. LCN2 is highly expressed in the lung even under homeostatic conditions, but there is a gap of understanding regarding LCN2's role in the lung during both homeostatic and inflammatory states. LCN2 has been proposed as a biomarker for numerous non-infectious lung diseases including chronic

obstructive pulmonary disease (COPD) [3] and blast lung injury [4] due to its significant elevation in these disease states. Although, the protective versus deleterious actions of LCN2 in these disease settings is unclear.

Using a non-infectious murine model of organic dust exposure, we have found that repetitive exposure to extracts of agricultural organic dusts (DE) leads to neutrophil influx and dramatic lung pathology including peribronchiolar and vascular inflammation and the development of striking lymphoid aggregates throughout the lung [5-9]. Lung pathology in WT mice exposed to DE decreases when the mice are allowed to recover for three days. However, deficits in recovery are observed in LCN-2 KO mice. Administration of the anti-inflammatory cytokine IL-10, to LCN-2 KO mice during the recovery period, resulted in a reduced inflammatory response and pathology.

The role of LCN2 in mediating the immune response to dust in the lungs remains elusive as limited research in this area has been done. This study may substantially impact the breadth of knowledge available for researchers in this field, and consequently help discriminate between the protective and detrimental roles exerted by LCN2 in response to inflammation in the lungs. This can further lead to the potential use of LCN-2 as a diagnostic marker or its therapeutic use in the treatment of debilitating lung inflammatory conditions.

In this dissertation, a review of LCN-2 will be discussed first and followed by studies investigating the role of LCN-2 in the inflammatory response against repetitive hog dust exposure at the in vitro and in vivo level. In vitro data will

show preliminary findings of the SPM Maresin-1, which is derived from Omega-3 fatty acids, and its relationship to mediating the resolution of inflammation. Lastly, the NHANES study will be used to investigate the effects of natural dust exposure on disease outcome and of the use of Omega-3 fatty acids as potential modifiers of disease outcome at the population level.

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I. Chapter 2: Comprehensive Review of Lipocalin 2-mediated Effects Lung Inflammation

ABSTRACT

Lipocalin-2 (LCN2) is an inflammatory mediator best known for its role as an innate acute-phase protein. LCN2 mediates the innate immune response to pathogens by sequestering iron, thereby inhibiting pathogen growth. Though LCN2 and its bacteriostatic properties are well studied, other LCN2 functions in the immune response to inflammatory stimuli are less well understood, such as its role as a chemoattractant and involvement in the regulation of cell migration and apoptosis. In the lungs, most studies thus far investigating the role of LCN2 in the immune response have looked at pathogenic inflammatory stimuli. Here, we compile data that explore the role of LCN2 in the immune response to various inflammatory stimuli in an effort to differentiate between protective versus detrimental roles of LCN2.

INTRODUCTION

LCN2, also known as neutrophil gelatinase-associated lipocalin (NGAL), is an acute-phase protein (APP) that is secreted by immune and epithelial cells in various mucosal tissues. LCN2 was first discovered in 1989 in granules of neutrophils as a 25 kDa protein covalently linked to matrix metalloproteinase 9 (MMP9), but is also expressed in macrophages, eosinophils, basophils, and epithelial cells. LCN2 is associated with acute inflammation and is involved in the

regulation of host responses to inflammation (1). Thus, it likely plays an inflammatory role at mucosal surfaces and is associated with a wide range of pathologies. Common pathologies associated with LCN2 inflammatory roles include heart failure, kidney disease, and gut inflammation, and LCN2 has been proposed as a biomarker for the identification of each of these diseases (2-6). LCN2 is also found in certain tissues such as the lungs under normal physiological conditions and has been shown to play a critical role in controlling the pathogenesis of bacterial infections (7-9). Current literature suggests that LCN2 plays paradoxical roles in lung inflammation by recruiting neutrophils and inducing pro-inflammatory cytokine signaling, while also promoting anti-inflammatory, alternatively activated or “M2-like” macrophage polarization (10). Given these seemingly contradictory roles that LCN2 plays in the lung immune response (Figure 1), the role of LCN2 in lung inflammation remains to be better characterized.

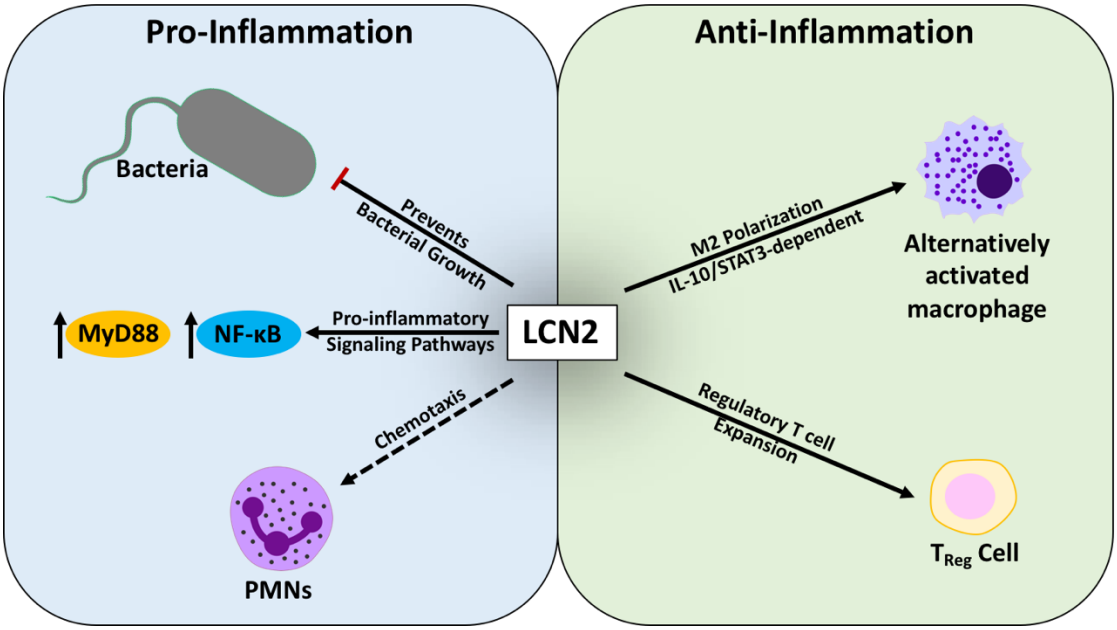

Figure 1. LCN2 Roles in Mucosal Surfaces. In response to inflammation induced by various stimuli, LCN2 levels increase and have been shown to mediate both pro- and anti-inflammatory responses. (A) Pro-inflammatory responses: 1) LCN2 is most commonly known for its innate response to bacteria and iron scavenging abilities, thereby preventing bacterial growth. 2) Additionally, LCN2 increases pro-inflammatory signaling pathways. 3) LCN2 has also been shown to recruit PMNs to the site of inflammation. (B) Anti-inflammatory responses: 1) LCN2 skews toward alternatively activated, "M2-like" macrophage polarization through an IL-10/STAT3 dependent manner. 2) LCN2 also plays a role in the expansion of regulatory T cells.

In this review, we compile information on LCN2 homeostasis and LCN2-mediated effects during lung inflammatory states. The structure and function of LCN2, as well as its association with lung inflammation and iron homeostasis in the lungs are discussed. Additionally, the use of LCN2 as a diagnostic biomarker of various pathologies as well as its potential as a biomarker in lung inflammatory conditions is explored. Together, this review aims to highlight our current knowledge regarding the protective versus harmful roles LCN2 may play in lung inflammation and immunity, as well as identify gaps in knowledge with an aim of spurring future research in this area.

LIPOCALIN 2 STRUCTURE AND FUNCTION

Lipocalin 2 Structure

LCN2 is also known as NGAL, 24p3, siderocalin, and uterocalin (11). LCN2 is a member of the lipocalin family, which consists of proteins that generally bind small hydrophobic ligands (12). The lower region of LCN2's cuplike structure (calyx) contains hydrophobic amino acid residues that act as binding sites for small lipophilic molecules. Additionally, unlike other lipocalins, the calyx of LCN-2 consists of a larger and shallower mouth lined with positively charged and polar residues that allow the binding of macromolecules and hydrophilic molecules (12, 13). In the lungs, the secretion of LCN2 by immune cells, such as neutrophils and macrophages, and airway epithelial cells is induced during an inflammatory response, while LCN2 secretion by various types of cells also occurs in response to oxidative stress (10, 14, 15). LCN2 can be secreted in three different forms: as a monomer, homodimer, or as a heterodimer with MMP9; the secreted conformation is typically dependent on the cell type secreting it (13). Due to the relatively small and stable nature of LCN2, it has been proposed as a candidate diagnostic marker for inflammatory lung conditions such as lung cancer (13, 14).

Lipocalin 2 receptors

There are five known LCN2 receptors: megalin (16), 24p3R (*Slc22a17*), melanocortin 4 receptor (17), melanocortin 1 receptor (18), and melanocortin 3 receptor (18) (17). Megalin is an endocytic receptor expressed primarily on the

apical membrane of epithelial cells in several tissues including the lungs, brain, kidney, placenta, endocrine glands, and the genital system (19, 20). Megalin is also expressed in non-epithelial cells such as oligodendrocytes in the spinal cord and neurons in the cerebellum of mice (21). The expression of megalin can be regulated by the nuclear receptor hepatic nuclear factor 4α (HNF4α) (22) as well as peroxisomal proliferator-activated receptors (16). The expression of megalin is critical in brain development and in various tissues of adults such as the blood-brain barrier, nervous system and gallbladder. PPARs serve as protective agents in all of the different physiological systems, highlighting the importance of understanding the mechanisms controlling the expression of megalin (16).

The receptor 24p3R belongs to the organic cation transporter family, is ubiquitously expressed, and plays a crucial role in regulating the expression of iron-responsive genes via its binding to LCN2 and subsequent endo- and exocytosis of LCN2. The endocytosis of both iron-bound and free LCN2 can be promoted by 24p3R (13). 24p3R has been implicated in LCN2-mediated apoptosis of human epithelial HeLa cells (2). In this study, HeLa cells were transfected with recombinant iron-loaded or iron-lacking 24p3R. The latter resulted in induced expression of the proapoptotic protein Bim and consequently, apoptosis. Furthermore, addition of intracellular iron was able to block the Bim induction and suppress apoptosis. Expression of 243pR receptor can be regulated by the Wnt signaling pathway at the transcriptional level, leading to alternative spliced isoforms of the receptor. Thus, the biological role of 24p3R in

cells with an active Wnt pathway may differ from its role in cells without an active Wnt pathway (23).

The melanocortin receptors are seven-transmembrane G protein-coupled receptors (18). These receptors are well known for their roles in steroidogenesis and skin pigmentation, but have also been shown to be potential modifiers of inflammatory diseases (18). The receptors are expressed throughout the body and exhibit a myriad of downstream effects, but all are known to activate MAPK and signal through the JAK-STAT pathways (18). Recently, it was discovered that bone-derived LCN2 can regulate food intake by binding to the melanocortin 4 receptor (17). LCN2 crosses the blood-brain barrier and binds to MC4R in the paraventricular ventromedial neurons in the hypothalamus (17), which results in suppression of appetite. It is important to note that MC1R and MC3R are also receptors that LCN2 can bind, however findings from this study showed that MC1R and MC3R do not regulate appetite, unlike the metabolic regulatory effects seen with MC4R. Several studies have shown that the expression of LCN2 receptors increases during inflammation, such as Shao et al, 2016 who showed increased levels of 24p3R expression in neutrophils of patients with psoriasis vs normal neutrophils (24). Most of the findings on changes of LCN2 receptor expression during inflammation have been shown at other mucosal sites, and remain to be investigated in the lungs.

Lipocalin 2 Function

LCN2 is involved with inflammatory response in multiple diseases and during infections; however, whether this modulation is beneficial or detrimental is context-dependent. For example, LCN2 can regulate chemokine levels during lung inflammation and the recruitment of polymorphonuclear leukocytes (PMNs) (11). Additionally, LCN2 can limit inflammatory responses, as seen in several studies including a systemic LPS challenge where $LCN2^{-/-}$ mice exhibited higher TNFα and IL-6 levels relative to WT controls (25). LCN2 is also involved in apoptosis modulation (12, 13, 26) and has been shown to play a role in cancer progression and chemoresistance depending on the type of tumor (14). Physiologically, monomeric LCN2 is cleared from circulation more rapidly than the dimeric form (27). Epithelial cells can secrete only the monomeric form, and immune cells predominantly secrete dimeric LCN2. In one study aimed at assessing the contribution of immune vs non-immune cells on systemic LCNs, LCN2 bone marrow chimeras were used to measure LCN2 expression, and they showed that non-immune cells were the major sources of systemic LCN2 during basal and inflammatory states (28). The two different forms of LCN2 have been used diagnostically to differentiate between acute kidney injury, chronic kidney injury, and urinary tract infection. Studies have shown that monomeric and dimeric LCN2 as well as their two sources can have different functions. For example, LCN2 is known to facilitate mucosal regeneration by promoting cell migration and forming a heterodimer with MMP-9, and one study showed that

immune cell-derived LCN2 molecules are the main contributors to mucosal protection again chronic colitis (28). The roles of immune- vs non-immune cell-derived and monomeric vs dimeric forms of LCN2 remain to be better characterized, however. LCN2 levels have been shown to be upregulated in both direct and indirect lung injuries, but, its exact role in the different lung injury types remains elusive. The levels of LCN2 in direct versus indirect lung injury have been investigated and shown to be increased in direct lung injury; Kangelaris et al, compared LCN2 gene expression levels in patients who had sepsis with and without acute respiratory distress syndrome (29), and found that the former had higher LCN2 gene expression levels (29). One of the roles that LCN2 is thought to have on direct lung injuries is the deactivation of macrophages (10). LCN2 was shown to skew toward M2-macrophage polarization, thus impacting host defense against bacterial pathogens. Mesenchymal stem cells (30) have been shown to enhance bacterial clearance in pneumonia, and Gupta et al, showed that bacterial clearance was mediated by LCN2, which is upregulated in MSCs in response to inflammatory stimuli (30). Several studies have shown that in sepsis-induced lung injury, increased LCN2 expression induced by epithelial cells as well as plasma NGAL levels can be predictive of multiple organ injuries induced by sepsis (31). Although LCN2 has been shown to increase in indirect injuries, is capable of binding to inflammatory mediators such as LPS, and binds MMP-9, its direct roles remain elusive. In the next sections, we highlight several of the key

functions of LCN2 in the context of immune response, iron homeostasis, and microbial pathogenesis.

LIPOCALIN 2 AND INFLAMMATION

LCN2 and its Role in Inflammation and Immune Response

LCN2 is an important inflammatory mediator in both acute and chronic inflammation. In the lung, LCN2 can be rapidly induced and secreted into the alveolar spaces by airway epithelial cells, alveolar macrophages, and neutrophils in response to inflammation (9). LCN2 enhances and sustains the inflammatory response by serving as a chemoattracting factor for the recruitment of neutrophils (32). LCN2 recruitment of neutrophils has been shown in chronic inflammatory pain, alcoholic liver disease, and psoriasis studies (4, 6, 33). Upon recruitment, the PMNs then stimulate the production of pro-inflammatory cytokines and chemokines, including LCN2, creating a feedback loop that drives the innate immune response. Additionally, during inflammation, the increased levels of LCN2 expression lead to increased neutrophil infiltration and activation, which then further promote epithelial cell responses including the activation of MyD88 and NF-κB (33). LCN2 reacts with its receptors on various cell types in the lung to exert its biological effects including apoptosis, cell migration, morphological changes, and the amplification of inflammatory responses (33). LCN2 regulates iron metabolism and the Bim (2) pathway, allowing it to also regulate cell death and cell survival (34). The clearance of apoptotic immune cells such as neutrophils in response to inflammation is necessary for immune resolution, thus

highlighting the importance of the functional role of LCN2 on apoptosis and the prevention of a prolonged inflammatory response.

LCN2 also plays a role in the expansion of regulatory T cells (35). The aim of this study was to investigate how LCN2 modulates T-cell response, specifically, via its regulation of the human leukocyte antigen G complex (35). Peripheral blood mononuclear cells from healthy donors were treated with different concentrations of NGAL in iron-loaded and/or iron-free forms and the regulatory T-cell population was identified as $CD4^+CD25^+FoxP3^+$ cells using flow cytometry. The findings showed that LCN2 increases HLA-G expression during acute and chronic inflammatory responses and expansion of regulatory T cells, thus indicating a potential protective role of LCN2 as an immune activator.

Several studies have shown that the induction of LCN2 expression in response to inflammation is mediated by the MyD88 signaling pathway (11, 28, 33). One form of neutrophil activation is via the formation of neutrophil extracellular traps (33), a process of cell death. This process of cell death is known as ETosis and was first observed in neutrophils, however, other immune cells such as macrophages, eosinophils, and basophils have all been reported to undergo ETosis. ETosis consists of two major events: chromatin unfolding and the production of reactive oxygen species (ROS), which lead to the release of the decondensed chromatin along with the granular contents into the extracellular space (36). In keratinocytes of psoriasis patients, NETs stimulate epithelial cells to produce high levels of inflammatory mediators such as LCN2 and IL-36γ. IL-

36γ then induces TLR4 expression, and both synergize and signal through MyD88 and NF-κB to induce further production of LCN2 and IL-36γ. Consequently, the upregulated LCN2 levels modulate neutrophil migration and NET formation, thus contributing to the sustainment or enhancement of the inflammatory response, which is referred to as amplification loops in psoriasis (33).

Interestingly, MyD88 KO mice were found to be more susceptible to dextran sulfate sodium (37)-induced colitis than WT mice and to have an impaired LCN2 response, suggesting that MyD88-mediated LCN2 induction may actually help protect against inflammation in the gut (11). Microbiota present in a given tissue, such as the gut microbiota, can also regulate LCN2 expression in a MyD88-dependent manner. For example, a recent investigation measured LCN2 levels from germ-free and conventional WT mice before and after the introduction of fecal contents of conventional mice to germ-free mice. The investigators identified significantly increased LCN2 levels in the conventional WT and conventionalized germ-free mice compared to the germ-free mice, highlighting a role for the gut microbiota in maintaining normal levels of LCN2 . Singh et al showed that in MyD88 KO mice lacking signaling through all TLRs except TLR3, DSS-induced colitis resulted in lower levels of colonic and systemic LCN2. In comparison, they showed that when using adaptive immune response-deficient *Rag1* KO mice, colonic and systemic LCN2 levels were unaltered (28). These results highlight the potential protective role of LCN2 against gut inflammation, as

shown by the increased susceptibility to DSS-induced colitis and impaired LCN2 response in the MyD88 KO mice. These data are seemingly contrary to other data indicating that LCN2 plays a detrimental or pathological role in response to inflammation, e.g. as seen by the amplification loop in psoriasis mentioned previously (33). A pathological role of LCN2 has also been observed in the brain, where LCN2 contributed to neuronal damage in the brain caused by the HIV viral glycoprotein, gp120. Here, the researchers showed that the ablation of LCN2 leads to the amelioration of neuronal damage, prevention of behavioral deficits, and an increase in the expression of a neuroprotective CCR5 ligand (38). Taken together, there is evidence to suggest that LCN2 could be playing either a protective or detrimental role in the immune response to inflammation. The role of LCN2 in the lungs has been studied primarily using pathogenic and carcinogenic models, as outlined in the microbial pathogenesis section below. However, other known insults of lung inflammation include allergens and particulate matter exposures. Future studies looking specifically at the role of LCN2 in both the innate and adaptive arms of the immune system across different types of inflammation in the lungs are needed. In addition, it is important to consider that LCN2 could potentially be involved in the resolution of the immune response to inflammation, thus emphasizing the need for studies looking into its impact on resolution markers as well as pathological changes at different time points.

LCN2 and its Role in Microbial Pathogenesis

One of the best-known roles of LCN2 is its impact on microbial pathogenesis. LCN2 inhibits bacterial growth by chelating bacterial siderophores and sequestering iron. Some of the most well-studied bacteria in LCN2 investigations are *E. coli* and *M. tuberculosis*, which cause various conditions including UTIs and respiratory illnesses (22, 39, 40). While studies of bacterial infection in the lung have provided very useful information that help elucidate the role of LCN2, bacterial studies looking at other organs can also provide insight into the role of LCN2 in the lung. For example, Moschen et al, 2016 showed that an LCN2 deficiency in IL10 -/- mice, leads to the compensatory expression of antimicrobial peptides (41). They also showed that altered microbial signatures led to increased growth of pathogenic bacteria and to the progression from colitis to colorectal cancer.

Siderophores are iron chelators essential for the interaction of iron with LCN2. Siderophores are classified into three groups according to their functional chemical group: catecholates, carboxylates, and hydroxamates (13). Endogenous siderophores such as lactoferrin and transferrin help transport iron throughout the body. Bacteria have evolved siderophores that have a higher affinity for iron than host endogenous molecules, such as various *Enterobacter* species (13). As a result, the host has evolved to produce proteins such as LCN2, which bind to bacterial siderophores heavily loaded with iron and limit the iron availability needed for bacterial growth. During certain bacterial infections in

the lungs, host cells can sequester iron and help prevent bacterial growth, which is often described as nutritional immunity and refers to the ability of a host to sequester trace minerals to prevent pathogenicity (20). When the iron supply is scarce, bacteria increase siderophore production to meet their iron requirements and outcompete host siderophores. In response to this, host cells such as airway epithelial cells and neutrophils secrete additional LCN2 to sequester and neutralize the bacteria (13). Interestingly, several reports show that bacteria can evolve to express modified siderophores that can evade recognition by LCN2 (13).

Furthermore, the effects of LCN2 in the pathogenesis of bacterial infections have been shown to be particularly important in the lungs and specifically alveolar epithelial cell responses. Saiga et al showed that LCN2-deficient mice infected with *Mycobacterium tuberculosis* have higher levels of bacteria in alveolar epithelial cells than WT mice, suggesting LCN2 limits the growth of *M. tuberculosis* (7, 9). In contrast, Guglani et al showed that LCN2 KO mice did not exhibit a higher bacterial burden than WT mice when infected with *M. tuberculosis*. However, LCN2 KO mice had reduced PMN levels and increased granuloma sizes, thus LCN2 played a protective role by driving PMN infiltration, as well as reducing the size of granulomas. As a result, animals expressing LCN2 had a lower mortality rate than LCN2-deficient mice (8). Additionally, this group also showed that LCN2 can regulate the infiltration of lymphocytes by inhibiting inflammatory chemokines, and that LCN2 promoted the

accumulation of neutrophils during mycobacterial infection (8). Another *M. tuberculosis* chronic infection model provides additional support for the innate role of LCN2, where the investigators found an increased inflammatory response in LCN2 KO mice compared to WT mice as shown with increased number and size of granulomatous lesions in the LCN2 KO mice, possibly due to the progression of *M. tuberculosis* (9). Furthermore, in the highly susceptible LCN2 KO mice, the impaired mycobacterial clearance was from alveolar epithelial cells, not alveolar macrophages, as assessed by measuring mRNA levels of lung fibroblasts, alveolar macrophages and tracheal epithelial cells (7, 8).

Similar effects of LCN2 on lung inflammation and bacterial growth were observed in studies using *E. coli* and *Klebsiella pneumonia* infection models (39, 40). Chan et al showed that in response to *K. pneumonia*, LCN2 KO mice had impaired lung clearance, but that reconstitution with LCN2 rescued the phenotype. Additionally, LCN2 also contributes to skewing towards M2-like polarization and worsens pneumococcal pneumonia outcomes, as shown with elevated levels of LCN2 and IL-10, which were associated with detrimental outcomes (10). In one study looking at the effects of a specialized pro-resolving lipid mediator (SPM), aspirin-triggered resolvin D1 (42), in the resolution of *E.coli*-induced pneumonia (42), LCN2 bronchoalveolar lavage (BAL) levels were elevated in mice infected with *E. coli*. Moreover, AT-RvD1 further increased LCN2 levels in BAL. In addition to these observations, reduced pro-inflammatory cytokine levels and improved bacterial clearance were observed, thus

highlighting potential protective roles in mucosal host defense of LCN2 and SPMs. The roles of LCN2 in lung infections outside of *M. tuberculosis*, *Klebsiella pneumonia*, and *E. coli* remain largely unexplored, and even less is known regarding its role in non-infectious, inflammatory lung conditions, such as asthma, chronic obstructive pulmonary disease, and cancer. For example, LCN2 has been shown to promote lung metastasis of murine breast cancer cells as well as to induce cancer cell apoptosis (2). Lastly, LCN2 levels are also elevated in response to dust-induced lung inflammation, with one example being elevated serum LCN2 levels in patients exposed to house dust mite extracts (43). After undergoing sublingual allergen immunotherapy, LCN2 serum levels were restored to normal levels, and the authors concluded that clinical reactivity in allergic patients may be assessed by serum LCN2 (43). Investigations into the role of LCN2 in these other settings could yield important biological insights considering the roles LCN2 plays in inflammation and the immune response in other inflammatory settings.

LIPOCALIN 2 AND IRON HOMEOSTASIS

Nearly all aerobic animals require iron for cell growth and proliferation, and the required iron is derived from dietary sources and recycled daily to maintain iron homeostasis. Enterocytes in the duodenum of the small intestine absorb nutritional iron. Ferric reductases in the lumen of the intestine reduce the ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}), which is then transported across the apical

membrane of the duodenal enterocytes (44). Ferrous iron can be oxidized by ferric protein within the enterocytes, which leads to storage of iron. Iron can also be exported across the basolateral membrane of enterocytes to the bloodstream depending on the body's requirements of iron (5, 44). Iron plays a crucial role in providing oxygen to all cells in the body, which is needed for oxidative metabolism and is essential for the proper functioning of many iron-containing proteins. LPS-induced hypoferremia has been shown to be facilitated by LCN2, resulting in the ability of a host to regulate its iron levels based on inflammatory conditions (45). Srinivasin et al, 2012 showed that LCN2 acts as an antioxidant by regulating iron homeostasis, which was evident by the protection of an iron chelator known as Desferroxamine from LPS-induced toxicity in LCN2 KO mice (45). Another example showing the regulation of LCN2 on iron homeostasis was shown by Lim et al, who showed that in the brains of patients with dementia, LCN2 regulates iron homeostasis. Authors speculate that as a consequence of LCN2 modulation on iron transport an cell death signaling, LCN2 likely regulates cell differentiation (46). Because iron is required throughout the body, transport of iron to various tissues can be mediated by endogenous siderophores. Lung cells are able to acquire iron from the circulation and exogenously (44). The ability of LCN2 to serve as a scavenger for iron via its binding to siderophores allows for its accumulation of iron, impacting iron equilibrium during both homeostatic and inflammatory/infectious settings. The rapid increase of LCN2 seen in various

tissues in response to acute inflammation modulates iron homeostasis, consequently affecting the function of neutrophils (47).

In addition to regulating tissue-specific iron availability, LCN2 also plays a role in preventing deleterious impacts of having high free iron levels. Specifically, iron has the ability to shift between oxidation states and is therefore known as a transition metal. However, this also results in the potential high reactivity and toxicity of free iron. The production of ROS is catalyzed by iron and these free radicals can damage tissue. The binding of iron to proteins can counteract iron's chemical reactivity. Iron levels are carefully balanced and necessary to ensure the systemic and cellular metabolic needs as well as in the prevention of harmful iron excess (44).

The role of LCN2 in iron homeostasis may be particularly relevant in the respiratory and cardiovascular systems because gas exchange between the atmosphere and the bloodstream occurs through a collaborative effort between numerous cell types in the lungs and vasculature (44). Here, the lungs play a crucial role in the transport of oxygen to all other tissues in the body via the diffusion of oxygen that is transported by red blood cells (RBCs) containing heme groups that bind oxygen through a central iron atom. Iron is needed for not only RBC production and oxygen transport, but for energy production in the mitochondria, DNA synthesis and repair, and transcription (44). Lung cells, like many others, require iron to maintain their metabolic demands and ensure their survival and function. Imbalances of iron levels are known to lead to respiratory

problems such as chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), and acute respiratory distress syndrome (29) (44). Abnormal iron metabolism and catalytically active metals can cause oxidative stress and lead to cell and tissue damage. Common symptoms of ARDS include severe shortness of breath and rapid breathing, and it is important to note that patients with ARDS have higher levels of iron and iron-related proteins in their lower respiratory tracts (48). One hallmark characteristic in patients with CF is the colonization at an early age by *Pseudomonas aeruginosa* in the lungs. Airways secretions/sputum of CF patients as described in (49) consist of high levels of iron, thought to be of importance in the bacterial load. Interestingly, high iron levels are also observed in CF patients without *P. aeruginosa* infection, suggesting that the CF lungs may potentially be primed for the bacterial infection (49). Additionally, iron accumulation in the lungs of COPD patients have also been observed which is postulated to contribute to the generation of ROS and to bacterial pathogenesis and COPD exacerbation (50, 51).

LCN2 USE AS A BIOMARKER

LCN2 is rapidly induced in response to stress and inflammation, while also being constitutively produced by certain cell types in different tissues. In the heart and kidneys, LCN2 is constitutively produced by pre-adipocytes and mature adipocytes, thus LCN2 has been proposed to be a reliable biomarker in the diagnosis of obesity-related metabolic complications, kidney disease, and heart

failure (5). LCN2 has been shown to play a protective role during kidney inflammation through its activation of autophagy, which Qui et al showed is mediated by HIF-1alpha. This group also showed that LCN2 reduced the NF-κb-p65 signaling pathways, suggesting a potential crosstalk between HIF-1alpha and NF-κb (52). Mishra et al showed that in acute kidney injury, LCN2 can tilt the fate of tubule cells toward survival, thus making it a desirable candidate for therapeutic interventions (53).

Immune cell infiltrates are widely used to diagnose inflammatory conditions; in “low grade inflammation”, modest elevations in proinflammatory genes are observed. LCN2 is stable, and fecal LCN2 is an effective and non-invasive biomarker in diagnosing various “low grade” inflammatory conditions such as intestinal inflammation (6) as well as Crohn’s inflammatory bowel disease (54). Additionally, urinary LCN2 is used as a biomarker to diagnose Lupus nephritis (4). Plasma levels of LCN2 have been postulated as a biomarker to detect mild cognitive impairments in Alzheimer’s disease (55), and to predict clinical outcomes in ischemic stroke patients (56). Additionally, LCN2 levels in the cerebrospinal fluid have been shown to be a sensitive biomarker for vascular dementia (57) and neuropsychiatric lupus (58). Lastly, LCN2 levels are useful in detecting multiple types of cancers such as colorectal cancer, breast cancer, ovarian cancer, and pancreatic cancer (2, 4). In terms of lung inflammation, LCN2 has been proposed as a potential biomarker candidate to diagnose different types of lung cancers (11, 14, 59). The role of LCN2 in lung

inflammation and as a potential marker of inflammatory lung diseases is a less well-defined research area that warrants further evaluation.

In cancer, the function of LCN2 appears to be context dependent. In breast cancer, LCN2 contributes to tumor growth, and higher levels of metalloproteinase-9 (MMP9) (60). Interestingly, LCN2 promotes lung metastasis of breast cancer possibly by inhibiting the PI3K/Akt pathway (61). In the context of lung cancer, Steiling et al, the identification of COPD-related processes that potentially impact lung carcinogenesis was done by investigating the effects of COPD on lung cancer-specific gene expression (62). From this study, LCN2 was one of the genes associated by COPD in smokers. By using mice with the Gprc5a (G protein-coupled receptor family C type 5A) gene knockout and exposing them to tobacco carcinogen, Treekitkarnmongko et al showed that the Gprc5a knockout mice develop lung tumors with somatic driver mutations in *Kras*, unlike WT mice. Furthermore, they showed that early in lung adenocarcinoma (LUAD) development, LCN2 was increased (63). Additionally, Treekitkarnmongko et al found that LCN2 was elevated in human LUAD, however, not in lung squamous cell carcinoma (LUSCs) compared with normal lung tissues (63). This study also found a strong association between LCN2 and COPD among patients with LUAD and lung cancer-free smokers (63). Overall, the study showed that LUAD development was increased in Gprc5a knockout mice and that mice exhibited elevated protumor inflammatory signaling and reduced antitumor immunity, suggesting that LCN2 antagonizes LUAD

development and can be identified as a molecular feature of COPD (63). Consistent with an antitumor effect of LCN2, a proteomic analysis analyzing the bronchoalveolar lavage (BAL) of patients with COPD, COPD and lung cancer, lung cancer without COPD, and healthy controls found that LCN2 was significantly downregulated in patients with lung cancer. Although contrary to Treekitkarnmongko et al, this publication found LCN2 was also significantly decreased in COPD patients (64). However, the mechanism driving LCN2's antitumor effect in lung cancer, and whether the LCN2-PI3K/Atk pathway is also involved in this disease reminds to be elucidated.

CONCLUSIONS

LCN2 is an important inflammatory mediator at mucosal surfaces throughout the body. It is well known for its iron-chelating properties that prevent bacterial growth, while also serving as an important regulator of iron transport, tissue-specific iron availability, and ROS production. LCN2 has also been shown to mediate inflammation and the immune response in different ways. Increased levels of LCN2 that result from inflammatory conditions leads to the recruitment and thus sustained activities of neutrophils, while emerging evidence suggests additional roles for LCN2 in regulating immune response, including the regulation of lymphocyte activation and functioning. Clinically, LCN2 has proven to be a promising biomarker candidate for various inflammatory conditions and cancers, warranting continued studies to better understand LCN2's roles in each of these

pathologies. Since the lung inflammatory role of LCN2 in response to non-bacterial insults remains understudied, research investigating the role of LCN2 in lung inflammation using different insults is needed. These types of studies, such as ones done by our lab using organic agricultural dust extracts (65), will help us gain a better understanding of the underlying role of LCN2 in the lung immune response to different type of exposures and help identify any differences (if any) in LCN2 roles observed using different types of exposures. Overall, the upregulated levels of LCN2 in direct and indirect lung injuries play a protective role against bacterial pathogenesis, although macrophage deactivation worsens infections in the lungs and other organs. Moreover, the ability of LCN2 in binding to other microbes or microbial components, such as LPS, could also determine injury outcomes. In any case, additional research in this area is needed. Taken together, it will be critical to identify both the protective and detrimental roles of LCN2 in the lung comparable to what has been found elsewhere in the body. These investigations will support a deeper understanding of LCN2 functions, including potential translational opportunities that could improve the treatment and/or diagnosis of inflammatory lung pathologies.

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II. Chapter 3: Lipocalin-2 Regulates IL-10 in a Murine Model of Repetitive Organic Dust Exposure

ABSTRACT

Agriculture industry workers are frequently exposed to harmful dust particles and consequently have a higher risk of developing chronic lung inflammatory conditions such as chronic obstructive pulmonary disease (COPD). Lipocalin-2 (LCN-2) is an innate immune protein with paradoxical roles in both pro- and anti-inflammatory responses at mucosal sites; however, it's role in lung inflammation induced by dust remains unknown. Here, we investigated the protective role of LCN-2 in a murine model of organic dust exposure-induced lung inflammation using wildtype C57Bl6 and LCN-2 knockout (KO) mice exposed repetitively to extracts of dusts (DE) collected from swine confinement facilities. Repetitive DE exposure consisted of fifteen days of intranasal instillations with and without a three-day recovery period. Results indicated an important function of LCN-2 in regulating tissue homeostasis, tissue repair, and anti-inflammatory cytokine interleukin-10 (IL-10) production in the lungs. Additionally, compartmentalized effects on T cell and macrophage levels were observed in BALF vs. lung tissue in DE-exposed LCN-2 KO mice versus wild-type mice. Ablation of LCN-2 resulted in deficits in recovery of mice exposed to DE, which was reversed with administration of IL-10. The role of the LCN-2/IL-10 axis in lung tissue repair was further explored by administering recombinant IL-10 to LCN-2 KO mice, which resulted in reduced pathology in the lungs, including

decreased lymphoid aggregate formation and extracellular matrix deposition compared to mice not given IL-10 administration. Taken together, these investigations highlight a novel role for LCN-2 in promoting lung repair following organic dust-induced inflammation, mediated, at least in part, by IL-10.

INTRODUCTION

Agricultural industry workers have been recognized by the Centers for Disease Control and Prevention (CDC) as individuals who are at a higher risk of developing inflammatory lung diseases such as asthma and chronic obstructive pulmonary disease (COPD) [1]; [2]. Workers in these industries are in frequent exposure to harmful particulate matter (dusts) and vapors [1]; [2] . Prolonged exposure to these respirable dusts, and particularly dusts generated in concentrated animal feeding operations (CAFOs) including those housing swine, can cause severe inflammation leading to debilitating chronic lung diseases[1], [2] . Since COPD is a chronic condition that causes limited airflow in the lungs, other complications such as cardiovascular diseases can arise and are common causes of death resulting from COPD[1] [2] . Currently, COPD is the third leading cause of death worldwide, and although preventable, there is no known cure. Current therapeutic options treat disease symptoms symptoms but not the cause of underlying disease and are not curative, thus providing no aid in reversing the decline of lung function associated with COPD[3]. As a result, it is crucial to find a treatment approach that will promote and utilize processes involved with lung tissue repair.

LCN-2 is an innate protein with paradoxical roles in both pro-inflammatory and anti-inflammatory processes that is significantly elevated at mucosal sites under inflammatory conditions[4, 5] **(Figure 1)**. One well-documented role of LCN-2 in innate immunity is the prevention of bacterial growth via its ability to bind to

iron and consequently limit iron availability necessary for bacterial growth[6]. Additional roles of LCN-2 in promoting inflammation include its promotion of MyD88 and NF-kB activities and its ability to act as a chemokine to recruit neutrophils to sites of inflammation[7]. The anti-inflammatory roles of LCN-2 include its ability to skew towards an M2-like (alternatively activated) macrophage polarization[5] and the enhancement of regulatory T cells[4]. Although these roles have been shown at mucosal sites of various tissues, it remains unclear if LCN-2 is protective or detrimental in the context of lung inflammation induced by organic dusts (such as those found in agricultural environments), as most studies looking at lung inflammation were performed using models of bacterial infection. LCN-2 has been shown to be elevated in the lungs in response to inflammatory insults, including during exposure to dust extracts (DE) [8-11].

To further explore the role of LCN-2 in modulating lung tissue responses to DE, we performed repetitive dust exposures in WT and LCN-2 KO mice **(Figure 2)**. To evaluate the role of LCN-2 in lung tissue repair, these studies included collections at time points five hours post last instillation as well as during the recovery period at 3 days following the last instillation. Additionally, to investigate the crosstalk between LCN-2 and IL-10 in tissue repair, we performed repetitive exposure to DE in LCN-2 KO mice followed by IL-10 administration during the recovery period. Our results identified that LCN-2 KO mice had deficits in recovery and that IL-10 administration reduced tissue damage induced by DE.

These findings show a protective role of LCN-2 in the lung immune response to DE and add to the breadth of knowledge of LCN-2 as an immune regulator in lung inflammation.

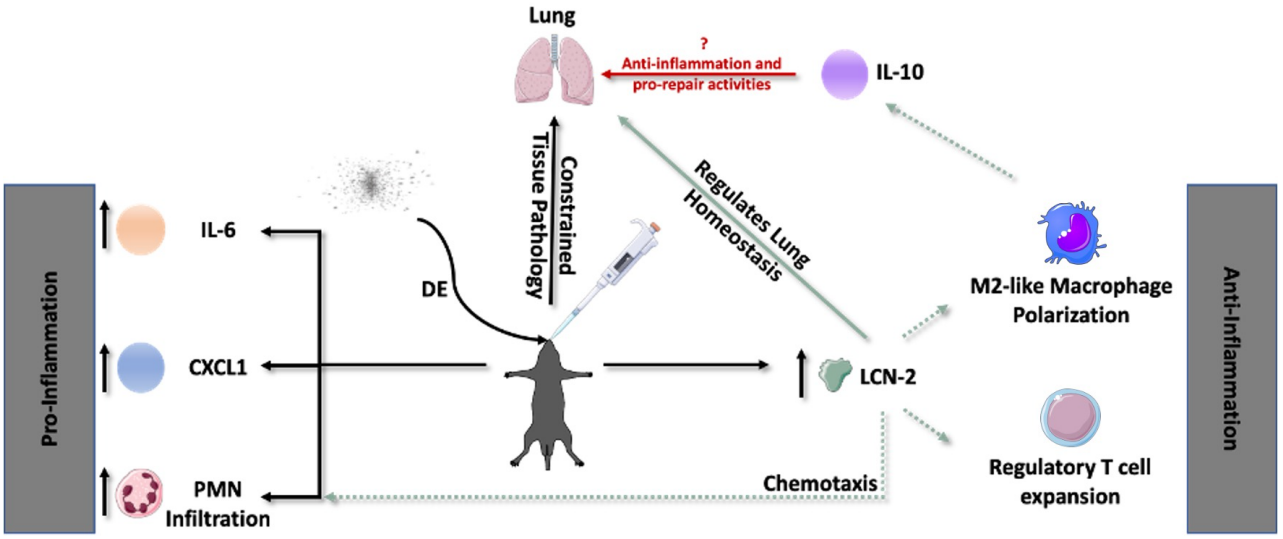


Figure 1. LCN-2 and IL-10-Mediated Effects in Lung Inflammation Induced by DE: Pro-inflammatory effects of DE exposure and anti-inflammatory mechanisms promoted by DE. DE leads to increased LCN-2, pro-inflammatory cytokine IL-6, pro-inflammatory chemokine CXCL1, neutrophil (PMN) infiltration, and lung tissue pathology (denoted with black solid arrows). Effects of LCN-2 in regulation of inflammation and IL-10 are denoted by green dashed arrows, and their interplay with the tissue pathology are shown with the solid green arrow. Portions of this figure were prepared using images from Servier Medical Art. Servier Medical Art by Servier is licenses under a Creative Commons Attribution 3.0 Unported License.

MATERIALS AND METHODS

Preparation of DE and IL-10

The DE used for these experiments was derived from settled surface dusts collected from swine concentrated animal feeding operations (CAFOs) housing ~500-700 animals, as previously described [12-14]. The extracts were prepared by dissolving 1 g of dust in 10 ml of Hanks' balanced salt solution (Biofluids, Rockville, MD, USA) without calcium. After vortexing, the mixture was

incubated at room temperature for 1 hour. The mixture was then centrifuged for 10 minutes, and the supernatant fraction was recovered. The mixture was then centrifuged once more, and the final supernatant fraction was filter-sterilized and aliquoted and stored at -80^{o}C until used. Recombinant IL-10 (Biolegend, San Diego, CA USA; Catalog #575804) was diluted to a concentration of 0.02 μg/μl with 1X PBS.

Animal Care and Housing

Male 6- to 8-week-old C57Bl/6 mice were obtained from Jackson Laboratories (Bar Harbor, Maine) and placed in group-housing cages under pathogen-free conditions. Male LCN-2 knockout (KO) mice were obtained from breeding male and female LCN-2 homozygous KO mice (B6.129P2-*Lcn2tm1Aade*/AkiJ). Mice were fed *ad libitum* standard mouse chow diet and water. All experimental procedures and protocols involving animals were in accordance with NIH guidelines and approved by the University of California, Riverside, Institutional Animal Care and Use committee and mouse health and diet were supervised by the University of California, Riverside campus veterinarian.

In vivo DE Exposure and Sample Collection

WT and LCN-2 KO mice were chronically exposed to DE prepared from dusts collected from swine confinement facilities or their saline vehicle control. Dust extract solution was prepared as described previously [15-17] and given as a 50 μl intranasal instillation (IN) of 12.5% DE or saline under light isoflurane anesthesia [16]. Male mice of 8-13 weeks old were given IN instillations in the mornings for 15 weekdays. For this work, we evaluated two time points after chronic DE exposure: 1) five hours after last IN (no recovery period), and 2) 72 hours after last IN (recovery period). For studies that included IL-10 administration, 1 μg of recombinant mouse IL-10 per 25g mouse body weight was administered on the day of the final DE IN and at 48 hr post final DE IN. Animals were euthanized with isoflurane (five hours post final DE exposure in no-recover groups, or at 3 days following the final DE instillation in the recovery groups). BALF and lung tissue collections were performed as described below. For BALF collection, a cannula was first inserted into the trachea and secured by tying a knot with a suture around the trachea. A syringe was used to rinse/wash the whole lung with 1 ml of sterile 1X PBS, which was collected and used for enzyme-linked immunoassays (ELISAs). The lungs were washed two additional times, with each wash consisting of 1 ml PBS. After BALF collection, the left lung lobe was tied off, excised and frozen in RNA later to be used for qPCR and Nanostring analysis of various mRNA transcripts. The right lung lobes were inflated with 0.5 ml of 10% formalin and hung with a constant pressure of 20 cm

H_2O for 24 hours while submerged in formalin for optimal lung morphology preservation.

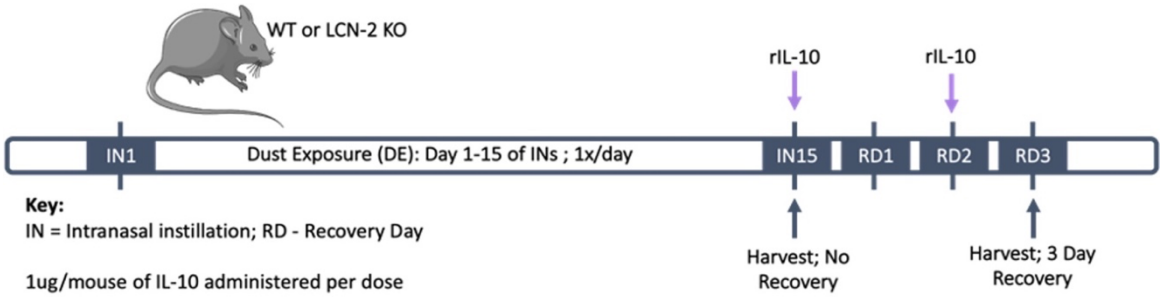


Figure 2. Murine Model Timeline of DE Exposures, Harvests, and/or IL-10 Administration: Mice received intranasal instillations (IN) for 15 consecutive weekdays. For experiments with IL-10 administration, 1 μg/25 g dose was given to mice on the last IN and on the 2nd recovery day. Harvests were performed 5 hours or 3 days post last IN. Portions of this figure were prepared using images from Servier Medical Art. Servier Medical Art by Servier is licenses under a Creative Commons Attribution 3.0 Unported License.

Staining for Differential Cell Counts Using BALF and Lung Tissue

Cell pellets from all three washes were used to process BALF for cytospins and flow cytometry. BALF was centrifuged for 5 minutes at 1500 rpm to pellet cells. The three fractions of 1 mL BALF were centrifuged for 5 minutes at 1500 rpm and the first collected fraction was used for cytokine/mediator analysis. Supernatant fractions were removed from the second and third BALF fractions, and cells from all three fractions were pooled together. Red blood cells were lysed in ACK lysing buffer (Thermo Fisher Scientific, Waltham, MA, USA; Catalog #8485741) for 5 minutes at room temperature and cells were resuspended in 1X PBS. A hemacytometer was used to enumerate cells in BALF samples and cytospins were made using an Epredia Cytospin Centrifuge (VWR, Radnor, PA, USA; Catalog# 89370-864). Percent macrophage and neutrophil populations were enumerated per 300 cells per mouse. Cytospins were stained with a Diff-

Quik protocol (Siemens Diff-Quik Stain Set; Newark, Delaware) for cell differential analyses. Additionally, processed BALF and lung homogenates were stained using a panel of antibodies for flow cytometry analyses of various cell populations, as described below.

LCN-2 and Cytokine Measurements in BALF

Collected BALF samples were centrifuged and the supernatant fractions were used for detecting mouse LCN-2 (Catalog #DY1857), IL-6 (Catalog #DY406), IL-10 (Catalog #DY417), and CXCL1 (Catalog #DY453) levels using R&D Systems (Minneapolis, MN, USA) Duoset ELISA kits following instructions of manufacturer. A VarioSkan microplate reader (Thermo Scientific, VL0000D0) was used to read ELISA plates at the wavelengths recommended by the manufacturer (R&D Systems). The flow cytometry panel consisted of the following antibodies purchased from Biolegend (San Diego, CA, USA), Thermo Fisher Scientific (Waltham, MA, USA), BD Biosciences (San Jose, CA, USA), and Bio-Rad (Hercules, CA, USA): MERTK-FITC (Catalog #151503), CDllb-PE (Catalog #12-0112-82), SiglecF-PE-Texas Red (Catalog #562757), CD3-PerCP-Cy5.5 (Catalog #45-0031-82), Ly6C-PE-Cy7 (Catalog #25-5932-82), CD206-AF647 (Catalog #MCA2235A647), MHC II-AF700 (Catalog #107621), B220-A780 (Catalog #47-0452-82), Ly6G-BV510 (Catalog #127633), Cd11c-QDOT605 (Catalog #117334), and CD86-QDOT655 (Catalog #50402970). Briefly, cells were stained with Zombie Violet (Biolegend, San Diego, CA, USA; Catalog

#423114) LIVE/DEAD stain at conditions recommended by manufacturer, blocked with 1:50 Fc blocking buffer (BD Biosciences, San Jose, CA, USA; Catalog #553142), and stained with a master mix containing all antibodies at a final concentration of 1:100. Compensation was performed using the Thermo Fisher Scientific ABC Total Antibody Compensation Bead Kit (Catalog #A10497). Samples were read in a BD Biosciences LSR Flow Cytometer and data was analyzed using the FlowJo software (Ashland, OR, USA).

Scoring of Lung Pathology

All formalin-fixed lungs used for histological assessment were the same lungs used for BALF collections. After fixation (described above), lungs were placed in 70% ethanol and shipped to the University of California Irvine Pathology Research Services Core for paraffin embedding and sectioning. Lungs tissue sections were subsequently stained with hematoxylin and eosin or with the Richard Allan Scientific Masson's Trichrome Kit (Thermo Fisher Scientific, Waltham, MA, USA; Catalog #22-110-648) for extracellular matrix (ECM) deposition scoring. Observers were blinded to exposure and treatment conditions for each slide. Evaluation of lung inflammation was done by scoring for alveolar inflammation, bronchiolar inflammation, epithelial hyperplasia, and lymphoid aggregates in H&E-stained slides whereas Masson's Trichrome-stained slides were used to quantify pro-fibrotic changes. Scoring for lymphoid aggregates, alveolar and bronchiolar inflammation was done by scanning each slide at 20x

and 40x followed by enumerating cells at 40X for 10 images per mouse total. Each slide was scored for the degree of inflammation using scores (0-3) with higher scores representing the most severe inflammation. For epithelial hyperplasia and lung pro-fibrotic changes, each slide was scored for the degree of inflammation using scores (0-4) with higher scores representing the most severe inflammation [12, 13].

NanoString Gene Expression

A mouse NanoString Fibrosis V2 panel (Catalog #115000388; NanoString Technologies, Seattle, WA, USA) was used for quantification of 778 RNA transcripts using an nCounter Sprint Profiler. Three male mouse lungs were used per condition from independent experiments. Upon harvest and proper storage of mouse lungs described previously, mouse lungs were thawed for RNA extraction. Lungs were rinsed with PBS followed by homogenization in 1 ml Trizol by using a 7 cm polypropylene pellet pestle in a microcentrifuge tube. A Purelink RNA Mini kit (Catalog #12183025; Invitrogen, Carlsbad, CA, USA) was used for the RNA extraction, which was performed per the manufacturer's instructions. RNA integrity number (RIN) was acquired from the UC Riverside Institute for Integrative Genome Biology Core Facility by using an Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). All samples used had a RIN of 7-9. A codeset probe provided by the kit was hybridized for 18 hours with 50 ng RNA. Samples were brought up to volume with nuclease-free water and 32 μl were loaded onto

an nCounter Sprint Cartridge (Catalog #100078; NanoString Technologies, Seattle, WA, USA). A total of 12 samples fit in one cartridge, thus a total of 12 samples were used for chronic studies with no recovery or IL-10 administration, 12 samples for chronic studies with recovery but no IL-10 administration, and 12 samples for chronic studies with recovery and IL-10 administration. All samples passed the NanoString system quality control (QC) test. Data were analyzed using nSolver 4.0 and by performing a NanoString Advanced analysis. The following housekeeping genes showing the strongest correlation with each other were used for normalization of gene expression: *Acad9, Cnot10, Mtmr14, Nol7, Nubp1, Pgk1, Ppia, and Rplp0*. The agglomerative analysis on nSolver was used to generate heatmaps. Per NanoStrings' recommendation, 50 transcript counts were taken as "count threshold". The 20 most upregulated genes among all treatment were identified by the differential expression analysis, while the gene set analysis and pathway scores identified which pathways the most upregulated genes are related to. The STRING database (https://string-db.org) was used to explore protein-protein interactions among differentially regulated genes from NanoString that were statistically significant based on the unadjusted p-values [18].

RNAscope

RNAscope® experiments were performed using the formalin-fixed paraffin-embedded (FFPE) tissue protocol for the RNAscope® Multiplex Fluorescent Reagent Kit v2 Assay (Document Number: 323100-USM). FFPE were prepared as previously described and sectioned at 5 μm. Three mouse lungs were used per condition from independent experiments. RNAscope® 3-plex Negative Control (Catalog #320871), RNAscope® 3-plex Positive Control (Catalog #320881), RNAscope® LCN-2 (Catalog #313971), and RNAscope® IL-10 (Catalog #317261-C2) probes were purchased from Advanced Cell Diagnostics, Inc. (Newark, CA, USA). Opal 570 (Catalog #FP14880001KT; 1:750) and Opal 690 (Catalog # FP14970001KT; 1:750) dyes from Akoya Biosciences (Marlborough, MA, USA) were used to visualize hybridized probes. Upon completion of the RNAscope® staining protocol, tissues were blocked with 10% goat serum for 90 minutes and incubated with 1:150 diluted Ym-1 (Catalog #60130; Stem Cell Technologies, Cambridge, MA, USA) in 5% goat serum at 4^{o}C overnight. The following day, tissues were incubated with 1:500 of goat anti-rabbit-488 antibody for 1 hour at RT, then stained with Hoechst 33342 dye (Catalog # B2261; Sigma) for 5 minutes at RT. Slides were mounted with Vectashield (Catalog #H1000; Vector Laboratories, Burlingame, CA 94010, USA), cured overnight, and stored at 4^{o}C in the dark until the following day. An Axiovert 200M fluorescence microscope (Zeiss) with filters for DAPI, FITC, Cy3 and Cy5 was used to acquire Z-stacks of images. The Slidebook software

(Version 6, Intelligent Imaging Innovations, Denver, CO) was used to analyze the images and quantification of mRNA was performed by enumerating the sum of intensity per dots.

Statistical Analysis

GraphPad Prism software 7 (San Diego, CA, USA) was used to perform two-way ANOVA or one-way ANOVA tests for statistical analysis followed by Tukey's post-hoc analysis for multiple comparisons. Prism was also used to generate all graphs, with a cut-off for all statistical analyses set at a *P* value of 0.05. *P* values equal to or below 0.05 were considered statistically significant.

RESULTS

Repetitive DE Exposure Activates Airway Inflammatory and Reparative Responses and Increases Lipocalin-2 Levels in Lung BALF

Previous findings from our lab using DE for a 24-week chronic study identified LCN-2 as one of the most differentially upregulated genes using a NanoString Cancer panel [8]. LCN-2 protein levels in BALF of WT significantly increased in response to DE with and without a recovery period after the exposures to DE **(Figure 3A, Top).** The basal levels of LCN-2 protein were surprisingly high (~140,000 pg/ml), which increased to (~660, 000 pg/ml) in response to DE. This is an approximated three-fold increase in comparison to basal levels of LCN-2 in serum (~50,000 pg/ml), which increase to ~77,000 pg/ml

in response to DE **(Figure 3A, Bottom)**. In the mice that were allowed to recover for three days post-exposures, LCN-2 levels for both saline- and DE-exposed mice were lower than the mice that did not have a recovery period (**Figure 3A**). To assess the lung immune response to DE exposure and determine the contribution of LCN2 to the immune response, WT and LCN-2 KO mice we exposed to DE repetitively for three weeks. We evaluated immune response to DE by measuring neutrophil recruitment, pro-inflammatory cytokine IL-6, and chemokine CXCL1. Elevated levels of neutrophils in the WT and LCN-2 KO mice exposed to DE in comparison to their respective saline controls were observed (**Figure 3C**). A three-day recovery period reduced neutrophil infiltration levels in mice, though no differences in neutrophil infiltration were observed between WT and LCN-2 KO mice (**Figure 3C**). WT and LCN-2 KO mice exposed to DE with no recovery period showed significantly higher levels of IL-6 in the BALF compared to their respective saline controls, and CXCL1 was trending higher in WT animals and significantly increased in LCN-2 KO mice (**Figure 3F-G**). Interestingly, BALF of saline- and DE-exposed mice had elevated levels of IL-6 protein during the recovery period while CXCL1 protein levels were restored to basal levels for both saline- and DE-exposed mice (**Figure 3F-G**). These findings show striking increases in LCN-2 in response to DE, though no effect was observed of LCN-2 on pro-inflammatory cytokine IL-6 or chemokine CXCL1. This led us to investigate the role of LCN-2 in promoting anti-inflammatory or pro-resolution programs in response to DE.

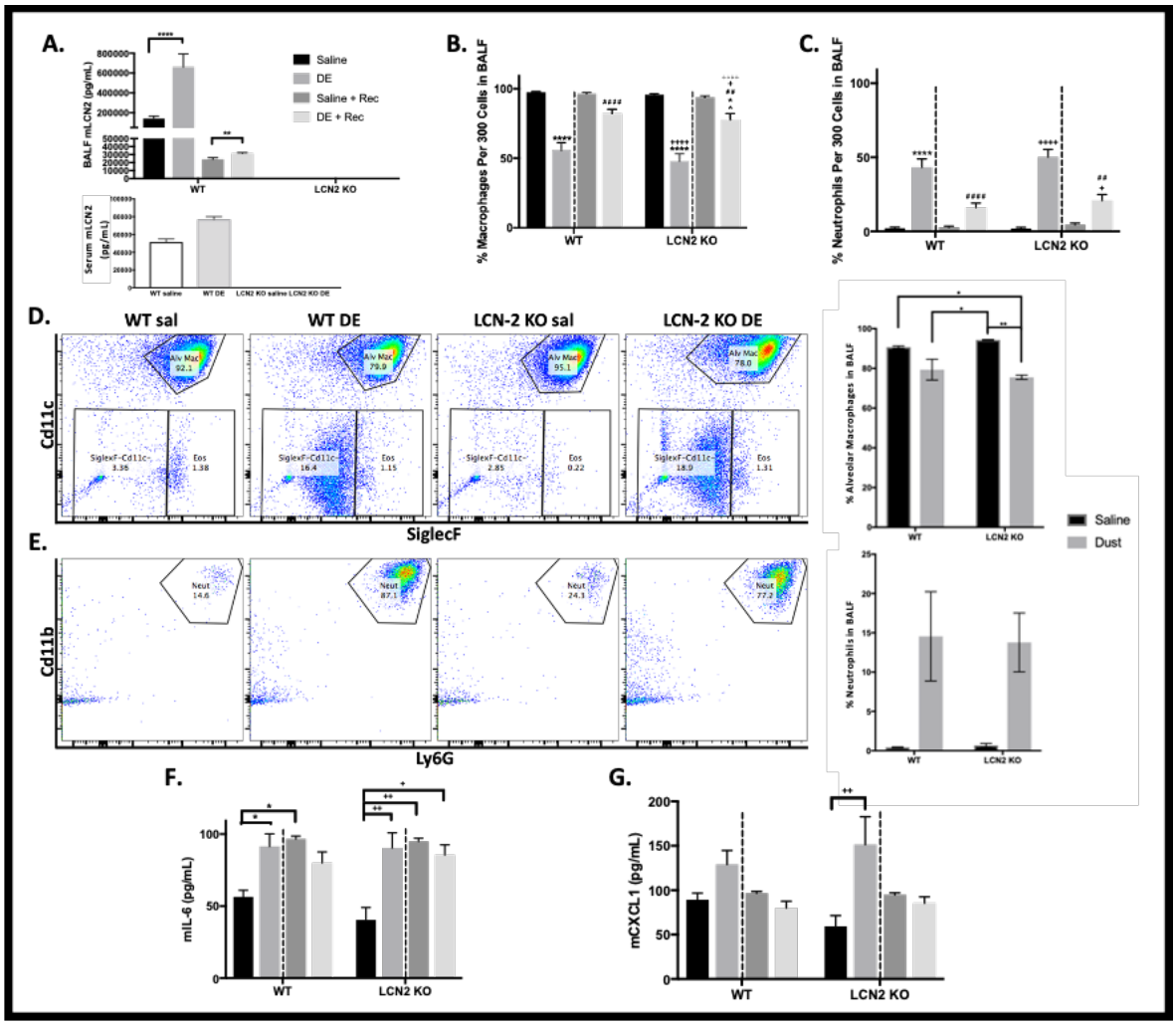


Figure 3. Effects of repetitive DE exposure on pro-inflammatory indices in BALF in wild type and LCN-2 KO mice. (A) LCN-2 protein quantification was performed using an LCN-2 Duoset kit. Percent macrophage per 300 cells (B) and percent neutrophils per 300 cells (C) were quantified using cytospins and flow cytometry was utilized to complement these findings (D, E). Pro-inflammatory cytokine IL-6 (F) and chemokine CXCL1 (G) protein levels were also measured via Duoset ELISA kits. A-C, F-G: N=6-8; D-E: N=3. Significant *P* values were $\leq$ 0.05 as noted by symbols and determined based on 2-way ANOVA.

Compartmentalized Effects of Lipocalin-2 on Alternatively Activated Macrophage Polarization and T Cell Recruitment in BALF vs Lung Tissue

DE-induced inflammation has been shown to alter macrophage polarization and function [14, 16, 19-21]. To examine if LCN-2 influences macrophage

function and/or polarization, flow cytometry was performed to explore the activation status of alveolar vs interstitial and classically vs alternatively activated macrophages. After gating out for alveolar and interstitial macrophages, M2-like macrophages were identified as $CD86^{-}CD206^{+}$ cells. There were no differences identified in BALF populations of $CD86^{-}CD206^{+}$ alveolar macrophages **(Figure 4A; p = 0.1535)**, but we did observe elevated T cell levels in in LCN-2 KO mice in comparison to their saline controls and to WT mice **(Figure 4B)**. However, in the lung tissue, we did see significantly elevated levels of alveolar and interstitial macrophages in the LCN-2 KO mice in comparison to WT mice **(Figure 4C, D)**. In the lung tissue, we also saw differences in T cell levels between WT and LCN-2 KO mice **(Figure 4E).** Since LCN-2 is also known to promote anti-inflammatory programs, and macrophages and T cells are both sources of IL-10, we next examined the effects of LCN-2 on IL-10 protein levels via ELISA **(Figure 4F)**. The IL-10 decreases in WT mice exposed to DE in comparison to their saline controls was consistent with other findings using our same murine DE exposure model [22]. The LCN-2 KO mice exposed to DE did not exhibit a significant decrease in IL-10 protein levels in comparison to their saline controls, suggesting a potential disruption in IL-10 regulation that may be mediated by LCN-2. Although significant differences in IL-10 protein were not observed between WT and LCN-2 KO mice, the increases in CD86-CD206+ alveolar and Interstitial macrophages suggested that the LCN-2 KO mice could be recruiting a larger

number of M2-like macrophages to the site of inflammation in comparison to the WT mice.

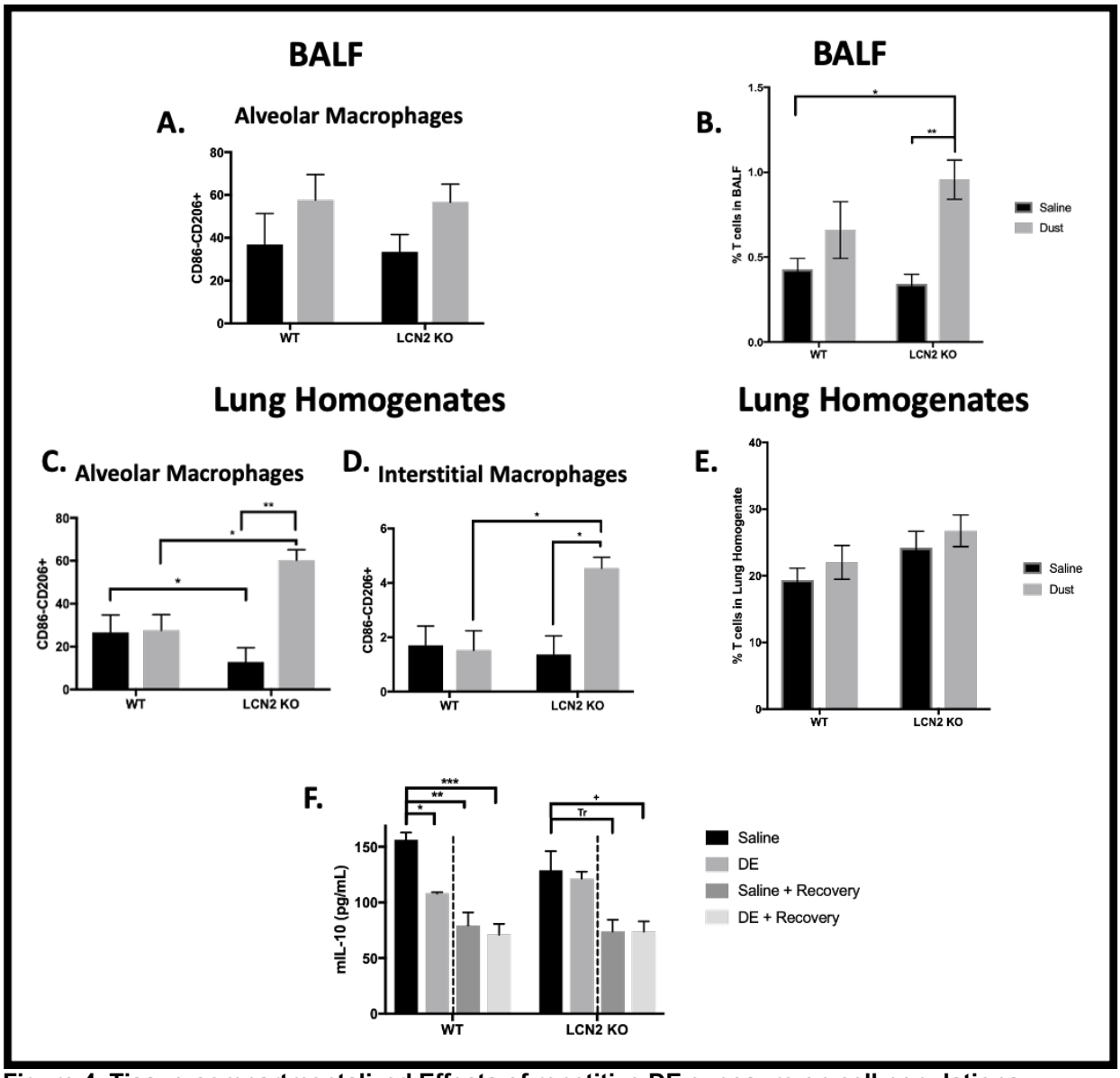
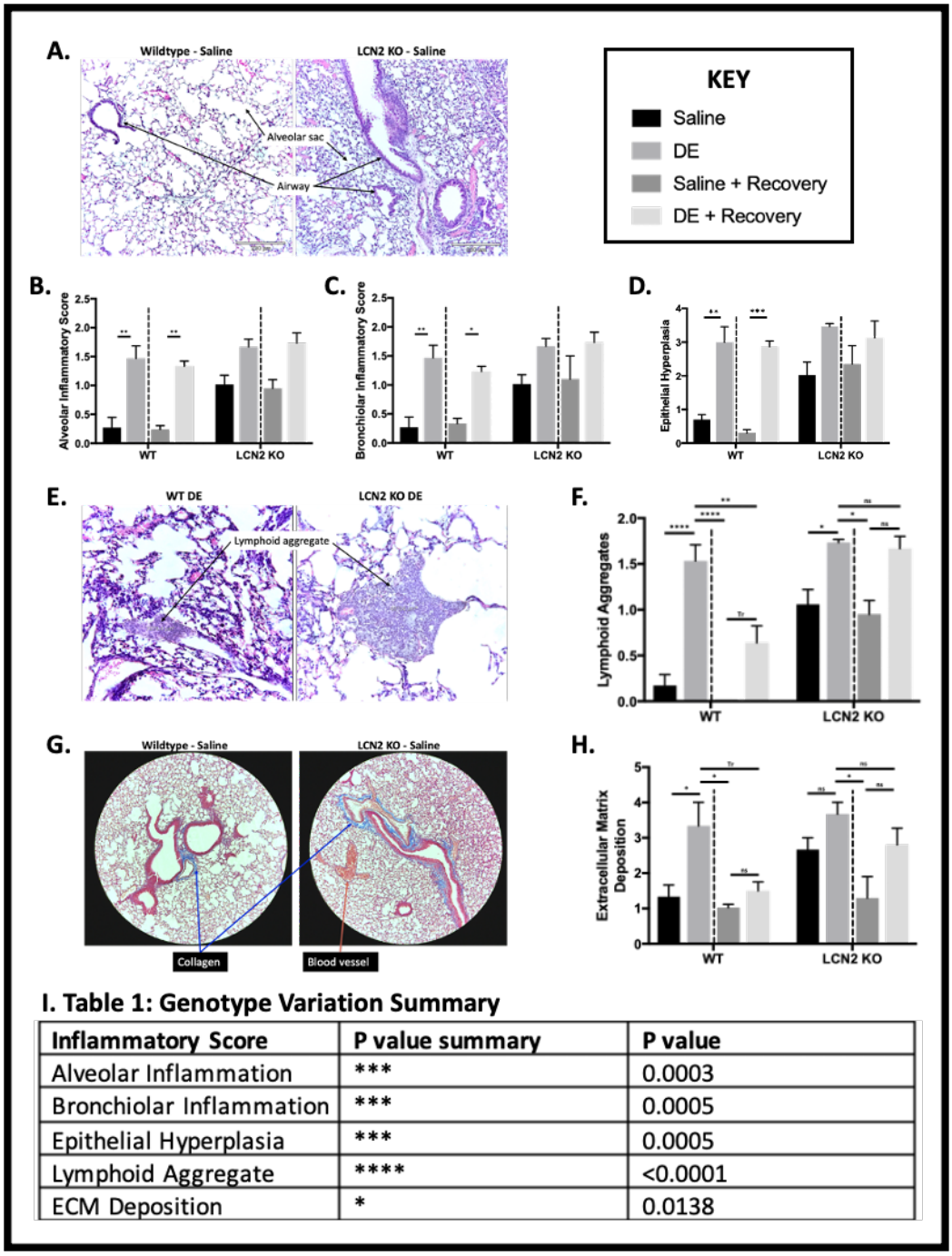


Figure 4. Tissue-compartmentalized Effects of repetitive DE exposure on cell populations and IL-10 levels in wild type and LCN-2 KO mice. Flow cytometry was used to quantify $CD86^{-}CD206^{+}$ alveolar and interstitial macrophages in BALF and lung tissue homogenates **(Figure A, C, D)**. After gating for alveolar and interstitial macrophages, CD86 was used to identify M1-like (Classically activated) and CD206 for M2-like (alternatively activated) macrophages. M2-like macrophages were identified as $CD86^{-}CD206^{+}$ cells. T cell levels were also assessed via flow cytometry **(Figure 4B, E)**. (F) IL-10 protein levels were quantified by ELISA. A-E: n=3; F: n=6-8. Significant *P* values were ≤ 0.05 as noted by symbols and determined based on 2-way ANOVA. Nonsignificant trends (p = 0.05-.1) are indicated as Tr.

Lipocalin-2 Ablation Disrupts Lung Tissue Homeostasis and Prolongs Lymphoid Aggregate Formation and Extracellular Deposition

To determine if LCN2 plays a role in tissue repair after DE exposure, we analyzed the following inflammatory parameters: alveolar inflammation, bronchiolar inflammation, epithelial hyperplasia, lymphoid aggregates, and extracellular matrix deposition. Strikingly, LCN-2 KO mice exposed to saline showed significant differences in cellular infiltration to the lung tissue compared to WT mice exposed to saline, suggesting an important function of LCN-2 in regulating homeostasis in the lungs during basal conditions **(Figure 5A)**. For all inflammatory scores assessed, the WT and LCN-2 KO mice exposed to DE had increased levels of inflammation in comparison to their saline controls (Figure 5A-H). Alveolar inflammation, bronchiolar inflammation and epithelial hyperplasia scores for the WT and LCN-2 KO mice allowed to recover post DE-instillations were similar to mice without a recovery period for both and no differences were observed between WT and LCN-2 KO mice **(Figure 5A-5D)**. However, a significant main effect of genotype was observed with $P \leq 0.0005$ (**Figure 5, Table 1)** when performing a multiple comparisons test with the two-way ANOVA, indicating that differences in means observed between the groups are due to the genotype/ LCN-2 ablation. Here, WT mice exposed to DE showed significant decreases in lymphoid aggregate formation and extracellular matrix (ECM) deposition in the recovery period, whereas LCN-2 KO mice exhibited no resolution of these inflammatory pathologies during the recovery period **(Figure**

5E-5H). It is important to note that for all inflammatory scores, there was a main effect of genotype, $p \leq 0.0138$ **(Figure 5, Table 1)**, indicating that the ablation of LCN-2 itself is responsible for differences being observed.



I. Table 1: Genotype Variation Summary

Inflammatory Score	P value summary	P value
Alveolar Inflammation	***	0.0003
Bronchiolar Inflammation	***	0.0005
Epithelial Hyperplasia	***	0.0005
Lymphoid Aggregate	****	<0.0001
ECM Deposition	*	0.0138

Figure 5. Inflammatory Scores of Mouse Lung Tissues with and without repetitive DE exposure +/- recovery: A) Lung tissue sections of unchallenged Wildtype vs LCN-2 KO mice. B-D) Inflammatory scores for alveolar inflammation, bronchiolar inflammation and epithelial hyperplasia. E-F) Lymphoid aggregate formation in mouse lungs; images of lungs on left panel, quantification of aggregate formation scores on right panel. G-H) Collagen deposition; Masons trichrome stain for collagen on left panel; quantification of collagen deposition on right panel. A-H: N=6-8. Significant *P* values were ≤ 0.05 as noted by symbols and determined based on 2-way ANOVA. Nonsignificant trends (p = 0.05-.1) are indicated as Tr.

RNA Pathway Analyses Identify Alterations in ECM Remodeling with Lipocalin-2 Ablation in DE-exposed Mice

To further investigate the signaling pathways and specific genes altered by LCN-2 ablation, we performed NanoString analysis; differential expression observed between groups is summarized in **Figure 6**. In summary, genes involved with ECM Degradation and ECM Synthesis were differentially expressed between WT and LCN-2 KO mice exposed to DE **(Figure 6B-G)**, which supports the differences in ECM deposition observed in mice based on histopathological analysis **(Figure 5)**. It is important to note that because the DE induces a potent inflammatory response, it was important to include mice with and without a recovery in order to detect changes in WT vs LCN-2 KO mice exposed to DE. Thus, **Figure 6D and 6F** represent Log2 fold changes of differentially expressed genes involved with ECM degradation and ECM synthesis across all groups, whereas **Figure 6E and 6G** compares only WT and LCN-2 KO DE-exposed mice with a recovery period. Results indicated an increase in LCN-2 KO mice exposed to DE in genes that encode for collagen subunits and collagen-binding proteins, Col1a2 and Ctsl, in comparison to WT mice exposed to DE. Increases in genes that encode for a matrix metalloproteinase (Mmp) inhibitor, Timp2 as well as Mmp-11 were also observed in LCN-2 KO mice (**Figure 6D-E**). Lastly, genes involved in ECM synthesis, such as ones that encode for alpha and beta-chains of integrins and for elastic fibers were also increased in LCN-2 KO mice exposed to DE in comparison to WT mice exposed to DE **(Figure 6F-G)**. Interestingly,

although LCN-2 did not alter neutrophil infiltration, the highest number of differentially expressed genes identified were involved in the ‘Neutrophil Degranulation’ pathway, supporting the potential role of LCN-2 in regulating the tissue pathology observed in the model **(Figure 6H-I)**. Moreover, the increases observed in ECM synthesis and degradation between the WT and LCN-2 mice highlight a role of LCN-2 in ECM remodeling and tissue repair.

A. Table 2: Differentially Regulated Genes

Experimental Group		Number of Samples per group	Differentially Regulated Genes		Total Number of Differentially Regulated Genes
			Up	*Down*	
3 Day Recovery vs No Recovery		12	19	30	49
DE vs Saline		12	34	16	50
3 Day Recovery vs No Recovery Exposed to DE		12	113	107	120
3 Day Recovery KO vs No Recovery Exposed to DE	KO	6	33	36	69

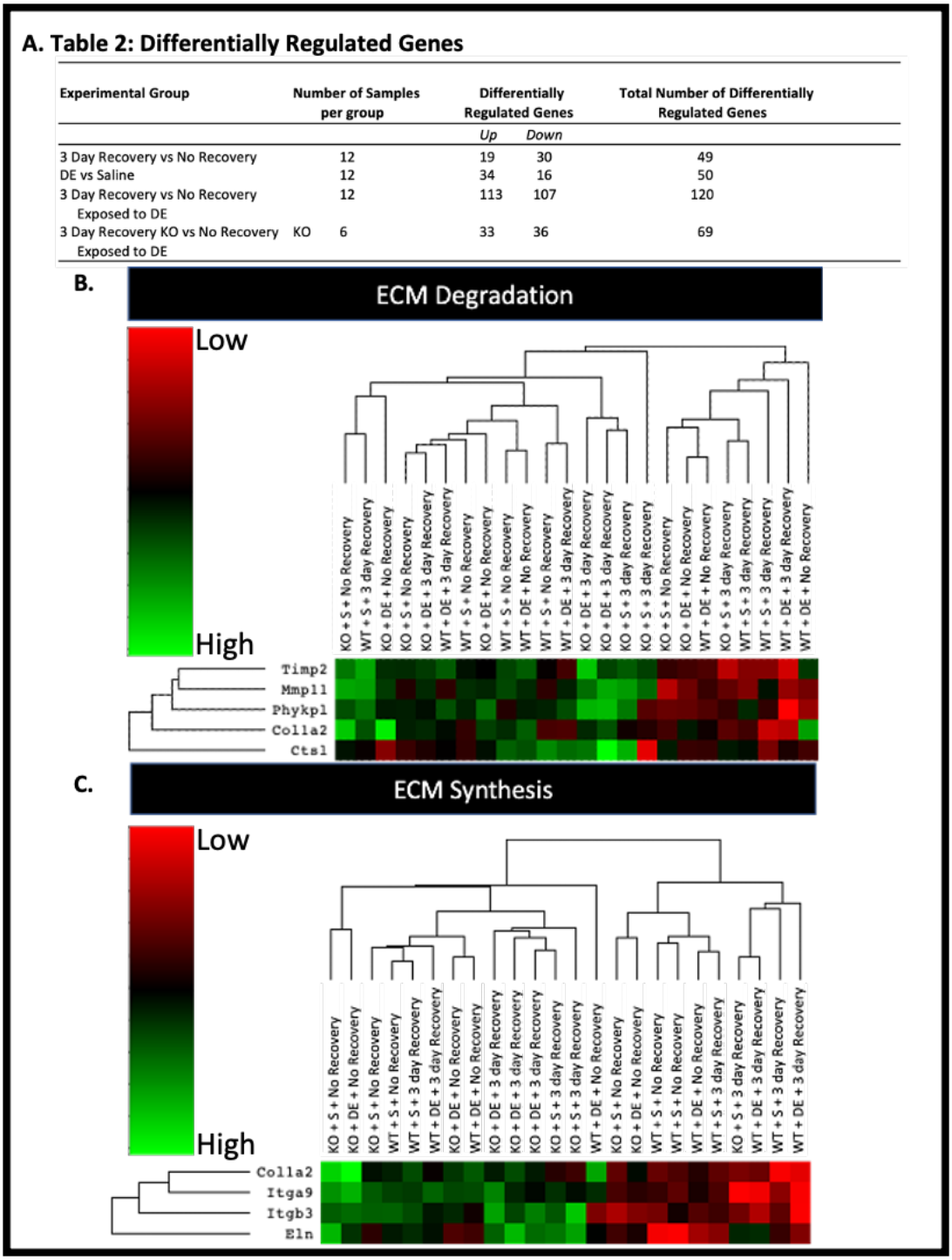


Figure 6A-C. NanoString Analysis of Differentially Regulated Genes in Mouse Lungs of DE WT and LCN-2 KO mice with and without a recovery period. A) Summary of differentially regulated genes. B) Heatmaps of genes associated with ECM Degradation. C) Heatmaps of genes associated with ECM Synthesis.

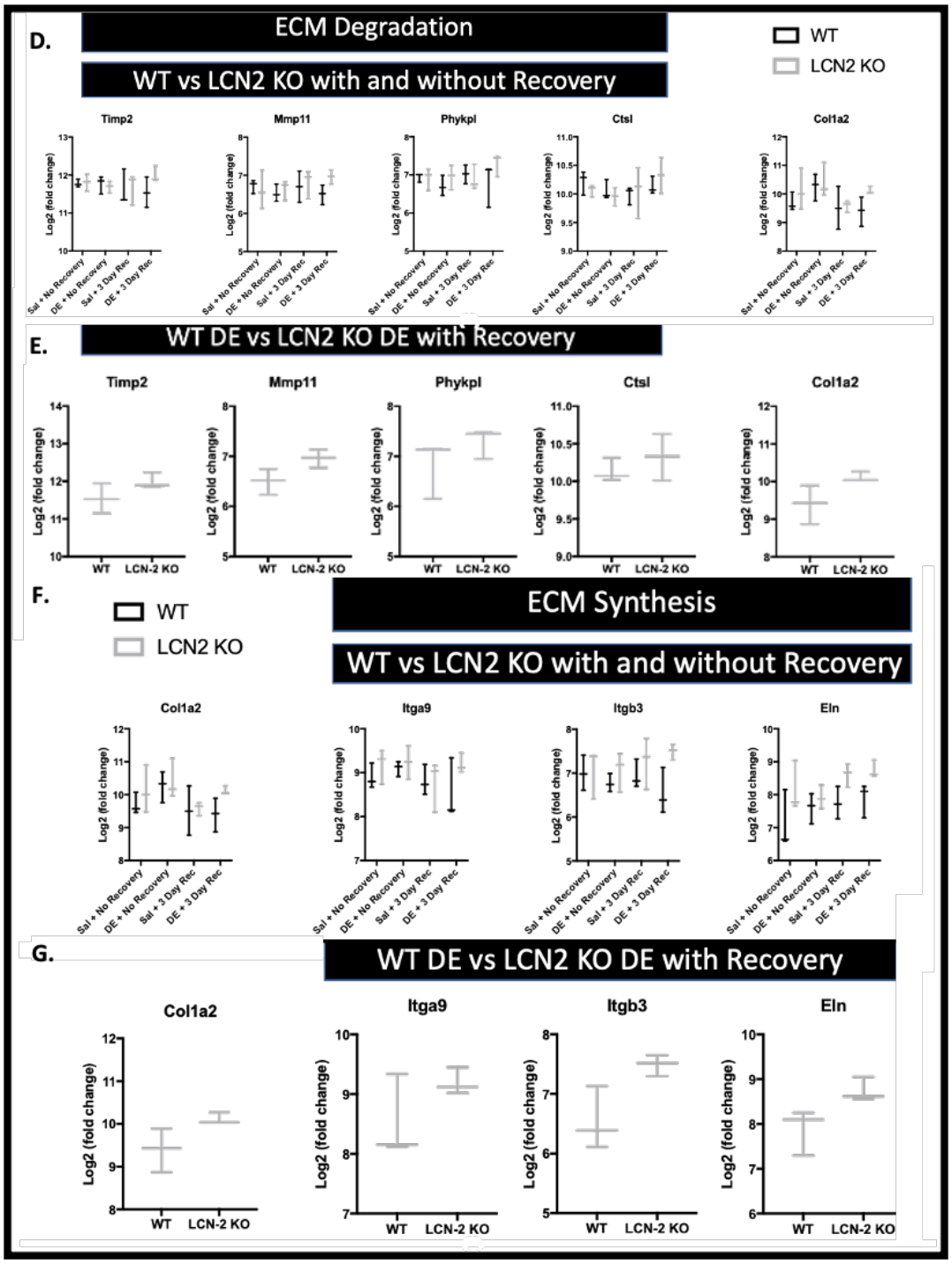


Figure 6D-G. NanoString Analysis of Differentially Regulated Genes in Mouse Lungs of DE WT and LCN-2 KO mice with and without a recovery period. D-E) Log 2 values of genes in (B) for all groups, F-G) Log 2 values of genes in (C) for all groups.

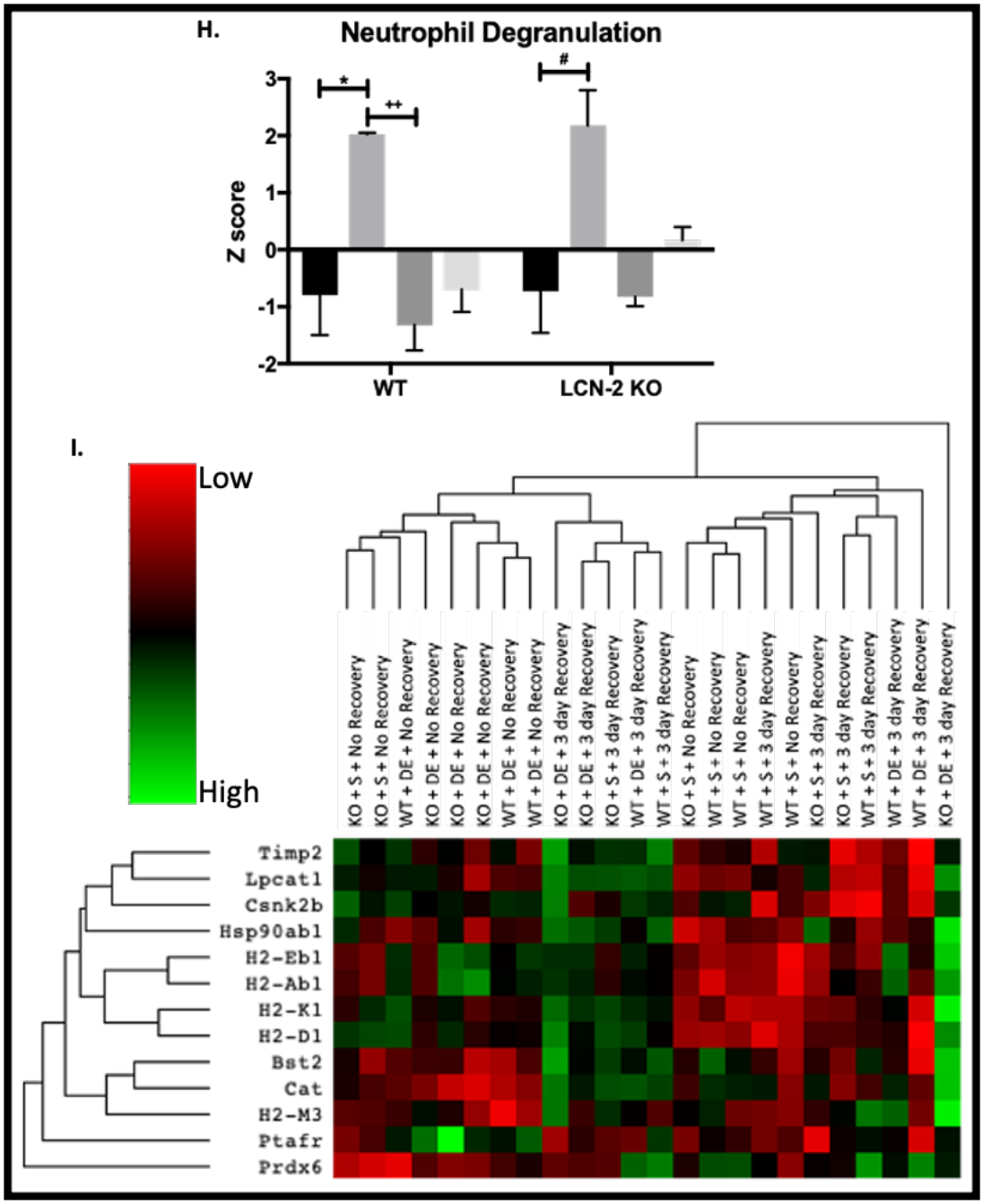


Figure 6H-I. NanoString Analysis of Differentially Regulated Genes in Mouse Lungs of DE WT and LCN-2 KO mice with and without a recovery period. H) Pathway score for Neutrophil Degranulation, I) Heatmap of genes associated with Neutrophil Degranulation. A P-value of $\leq$0.05 based on 2-way ANOVA was considered significant. N=3 for all groups in NanoString analyses.

Enhanced LCN-2 Levels Induced by DE Drive Production of IL-10

To further explore the potential role of LCN-2 in anti-inflammatory IL-10 regulation, we used an RNAscope-IHC stain for LCN-2 mRNA, IL-10 mRNA, and a marker for M2-like macrophages, YM-1 **(Figure 7-8)**. Striking increases of LCN-2 mRNA were observed in cells of the airway epithelium and parenchyma in response to DE **(Figures 7-8)**, corroborating our BALF protein findings. Thus, to illustrate the pattern of puncta and range selected for LCN-2 mRNA quantification, **Figure 7A & 8** depict low, medium and high ranges of LCN-2 mRNA. Representative images used for LCN-2 mRNA and IL-10 mRNA quantification are shown in **Figure 7B**. Quantification of LCN-2 and IL-10 in lung parenchyma and airways are shown in **Figure 7C**. Images at a lower magnification were used to quantify YM-1 protein levels **(Figure 7C)** and for better representation of a larger field of view **(Figure 8)**. Increases in IL-10 mRNA production of cells were observed in the airway epithelium and parenchyma (**Figures 7-8**) in response to DE with and without a recovery period. Additionally, IL-10 mRNA for DE-instilled mice during recovery was statistically higher than mice without recovery in comparison to the saline controls. Increased levels of cells staining positive for Ym-1 were also found in the airway epithelium and parenchyma (**Figures 7-8**) of DE-instilled mice, and interestingly, in the LCN-2 KO mice. Mice exposed to DE showed increased formation of lymphoid aggregates **(Figure 7-8)** as previously demonstrated by histopathology. The decreased levels of IL-10 in LCN-2 KO mice and increases in WT mice in

response to DE during recovery suggests a potential role of IL-10 in tissue repair that is regulated at least in part by LCN-2. Thus, to further evaluate the role of IL-10 in tissue repair, we administered recombinant mouse IL-10 to LCN-2 KO DE-instilled mice during the recovery period.

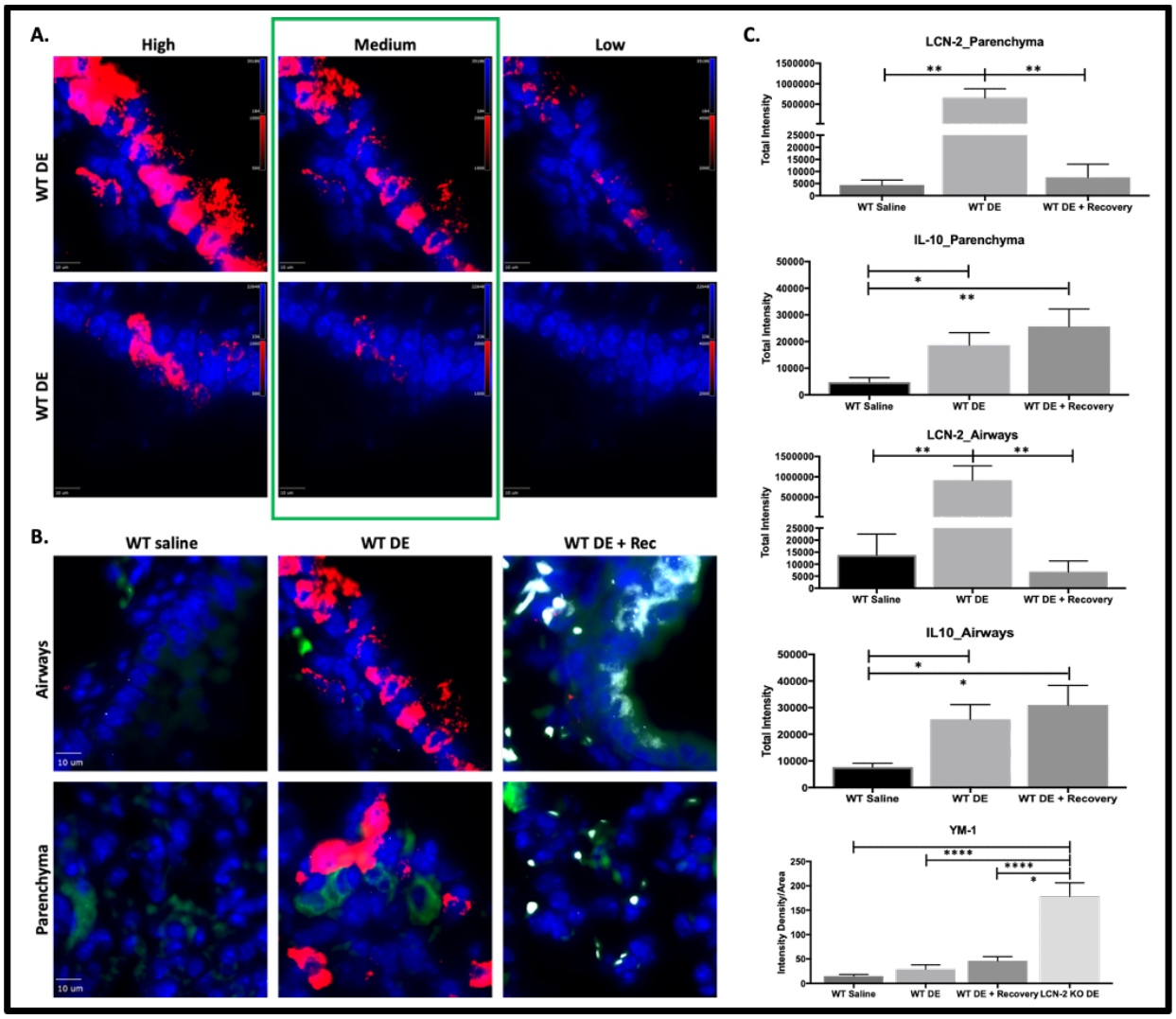


Figure 7. RNAscope LCN-2 and IL-10 mRNA analysis and YM-1 protein quantification. A) Three ranges of LCN-2 mRNA fluorescence signal are shown for two WT DE mouse lungs. Note the wide range of fluorescence intensities for the LCN-2 mRNA signal. The green box indicates the fluorescence intensity range chosen for quantification of LCN-2 mRNA. Mouse WT DE samples chosen to select range was based off of samples showing LCN-2 mRNA signal on the lower and higher cutoffs. B) Representative images staining for LCN-2 mRNA (red), IL-10 mRNA (white) and YM-1 protein (green) per condition shown for mouse lung parenchyma and airways. C) Quantification of LCN-2 and IL-10 mRNA in parenchyma and airways and quantification of YM-1 protein. A-C: N=3. Significant *P* values were $\leq$ 0.05 as noted by symbols and determined based on 1-way ANOVA.

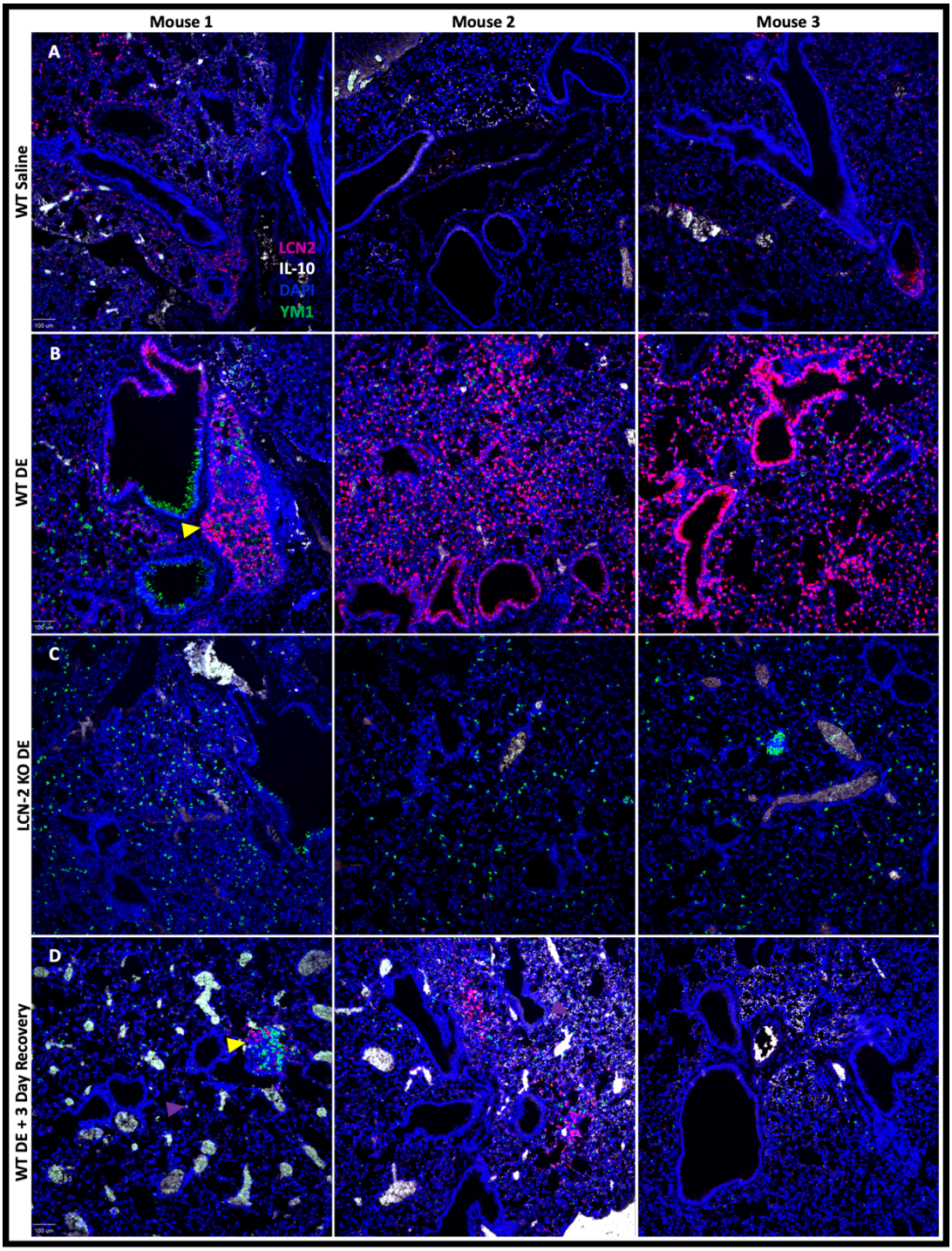


Figure 8. RNAscope representative images of mouse lungs stained for LCN-2 mRNA (red), IL-10 mRNA (white) and YM-1 protein (green) at a lower magnification (10X). A) One representative image per mouse (n=3) of WT saline condition, B) One representative image per mouse (n=3) of WT DE, C) One representative image per mouse (n=3) of LCN-2 KO DE, D) One representative image per mouse (n=3) of WT DE + 3 day recovery.

Lipocalin-2 Ablation Prevents Repair of Lung Tissue Damage Induced by DE

To further investigate how LCN-2 may be involved with the lung pathology observed in mice exposed to DE, we were interested in the potential crosstalk of LCN-2 and IL-10, as IL-10 is known to be involved with tissue repair and anti-inflammatory processes. Thus, we administered recombinant mouse IL-10 during the recovery period of a chronic + 3-day recovery study in LCN2 KO mice (**Figure 2**). To confirm successful administration of IL-10 in mice, IL-10 protein levels in BALF were measured by ELISA. As expected, higher levels of IL-10 were observed in animals with recombinant IL-10 compared to vehicle control **(Figure 9A)**. Significant decreases in IL-10 were observed in DE exposed mice with and without IL-10 administration in comparison to the saline-treated mice that received the IL-10 administration **(Figure 9A)**. To assess the effects of IL-10 administration on IL-6 and CXCL1, we measured protein levels via ELISA. Similar to what was observed with the chronic DE studies during the recovery period with no administration of IL-10 **(Figure 3F)**, the DE exposed mice in this study that did not receive the IL-10 administration had significantly decreased IL-6 protein in comparison to their saline controls **(Figure 9B)**. Administration of IL-10 did not result in any significant changes in IL-6 protein levels **(Figure 9B)**. There were no significant changes in CXCL1 protein levels observed in saline or DE-exposed mice during the recovery period as a result of IL-10 administration

(Figure 9C). Overall, these results suggest that IL-10 does not affect the BALF pro-inflammatory response to DE in our exposure model.

To help further characterize the immune response to DE, macrophage, neutrophil, and lymphocyte levels in the BALF of saline and DE-exposed mice with and without IL-10 administration were also quantified. There were no significant changes in macrophage levels during the recovery period between mice that received IL-10 and ones that did not **(Figure 9D)**. Mice that received IL-10, however, did exhibit significantly reduced neutrophil levels during the recovery period in comparison to mice that were not given IL-10, suggesting an anti-inflammatory, protective role of IL-10 **(Figure 9E)**. Lymphocyte levels in mice were significantly increased in response to DE with and without IL-10 administration **(Figure 9F)**. However, there were no significant differences observed in lymphocyte levels between DE-instilled mice with and without IL-10 administration **(Figure 9F)**. To identify changes in tissue repair that may result from IL-10 administration, we scored ECM deposition and lymphoid aggregates (LA) for all mouse lung tissues since those inflammatory scores showed the most robust changes in LCN-2 KO mice during the recovery period without IL-10 administration in the previous studies. There were significant differences in ECM between saline and DE-exposed mice that did not receive IL-10 during the recovery period **(Figure 9G)**. Administration of IL-10 resulted in significant decreases in ECM deposition for DE-treated mice in comparison to DE with no IL-10 administration, although saline mice with IL-10 in comparison to saline with

no IL-10 showed no statistical difference **(Figure 9G)**. Lastly, significant increases in LA formation in response to DE were seen during the recovery period of mice that did not receive IL-10, but the formation of LA was significantly reduced in response to IL-10 administration after DE exposure **(Figure 9H)**. Overall, these findings show that IL-10 may potentially be an important regulator of the tissue repair process during the recovery period of our murine DE exposure model.

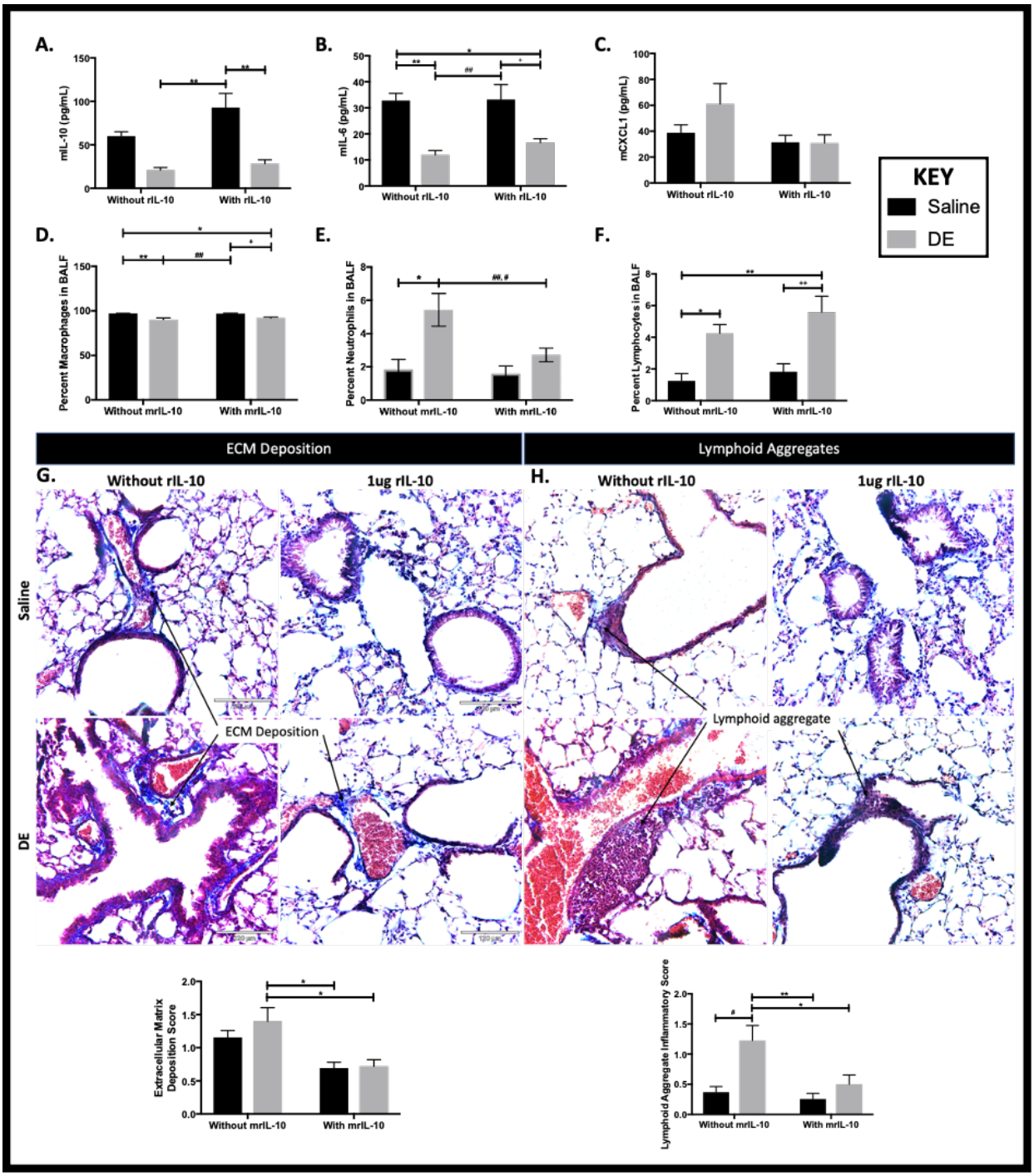


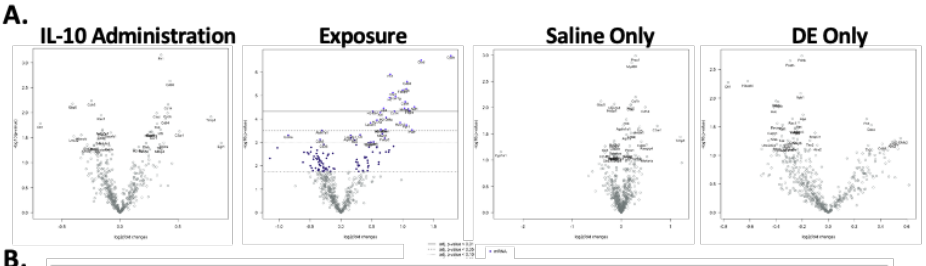
Figure 9. Lung immune response to DE in LCN-2 KO mice with and without IL-10 administration. A) Assessment of anti-inflammatory cytokine IL-10 protein levels, B) pro-inflammatory cytokine IL-6, C) pro-inflammatory chemokine CXCL1. Cytospin differential analysis of macrophage (D), neutrophils (E) and lymphocyte levels (F). G) Histopathology in mouse lungs and quantification showing ECM deposition (Left) and lymphoid aggregate formation (right). A-H: N=3. Significant *P* values were ≤ 0.05 as noted by symbols and determined based on 2-way ANOVA.

Differential Expression of Fibrosis-related Genes is Downregulated by IL-10

An nCounter Fibrosis V2 NanoString panel was used to help identify pathways affected by IL-10 during the recovery period of lung inflammation induced by DE. Volcano plots of the overall gene expression changes in groups comparing rIL-10 treatment with and without DE exposure, saline only and DE only samples are shown in **Figure 10A**. The differentially regulated genes for all groups are summarized in **Figure 10B**. The most significantly up-and down-regulated genes across each group comparison were uploaded to the STRING Database to allow for identification of most relevant pathways **(Figure 10C)**. In the group comparison exposures to saline and DE, the most relevant pathway for genes that were upregulated was the cellular response to triacyl and diacyl bacterial lipopeptide, similar to the group comparing rIL-10 treatment. Up-regulated genes in saline only mice comparing rIL-10 treatment to no rIL-10 treatment were most-closely related to neural crest cell migration involved in autonomic nervous system development. Down-regulated genes for the DE only group comparing administration of rIL-10 vs no IL-10 were most-closely related to positive regulation of ovarian follicle development. **I**n the DE only group, it was observed that IL-10 administration resulted in more downregulated vs upregulated differentially expressed genes. Additionally, genes that were downregulated were also closely associated with the development of glomerular endothelium. Lastly, DE-exposed mice with and without rIL-10 had higher levels, in comparison to saline controls, of differentially regulated genes involved in the ECM synthesis,

ECM degradation and neutrophil degranulation pathways **(Figure 10D)**. Moreover, elevated pathway scores were observed in DE-instilled mice with administration of rIL-10 vs no IL-10.

A.



B.

Experimental Group	Number of Samples per group	Differentially Regulated Genes		Total Number of Differentially Regulated Genes
		Up	*Down*	
1ug IL-10 vs No rIL-10	6	21	19	40
DE vs sal	6	30	2	32
1ug IL-10 vs No rIL-10 sal Only	3	18	6	24
1ug IL-10 vs No rIL-10 DE Only	3	3	23	26

C.

Table 4: STRING Database Analysis of Differentially Expressed Genes and their associated Biological Pathways

	Up-regulated genes	STRING database biological process	P	Down-regulated genes	STRING database biological process	P
1ug IL-10 vs no IL-10	21	Cellular response to triacyl bacterial lipopeptide	< 1.0e-16	19	Pos regulation of ovarian follicle development; regulation of transforming growth factor beta3 production	n.s. = 0.068
DE vs Saline	30	Cellular response to triacyl bacterial lipopeptide; Cellular response to diacyl bacterial lipopeptide	< 1.0e-16	2	n.a.	n.s. = 1
Saline, 1ug IL-10 vs no IL-10	18	Neural crest cell migration involved in autonomic nervous system development	2.22e-16	6	**Non-alcoholic fatty-liver disease**	n.s. = 0.396
DE, 1ug IL- 10 vs no IL-10	3	n.a.	n.s. = 1	23	Pos regulation of ovarian follicle development; glomerular endothelium development	5.36e-11

D.

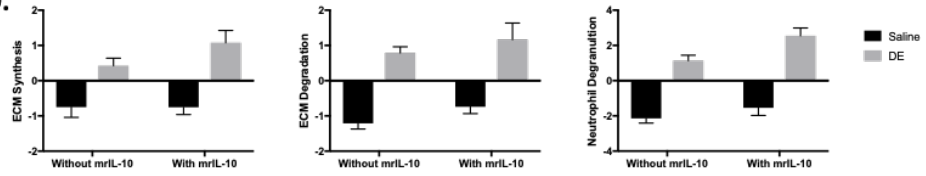


Figure 10. Changes in gene expression and related pathways altered after administration of recombinant mIL-10 to LCN-2 KO mice exposed repetitively to DE. Differentially expressed genes across four different parameters, with exposure to DE being the cause of most differential expression indicated by higher adjusted p values: 1) IL-10 administration vs no IL-10 administration for saline and DE Mice, 2) Exposure to DE vs exposure to saline for mice without and with IL-10 administration, 3) Administration of IL-10 to saline exposed mice vs no administration of IL-10 to saline-exposed, and 4) Administration of IL-10 to DE-exposed mice vs no administration of IL-10 to saline-exposed (Figure 10A). A summary of differentially regulated genes for all groups is provided in Figure 10B. The STRING Database was used to identify the most significant pathways implicated for each of the four parameters, which are summarized in Figure 10C. Pathway scores for ECM Synthesis, ECM Degradation and Neutrophil Degranulation are shown in Figure 10D. C: N=3. Significant *P* values were $\leq$ 0.05 as noted by symbols and determined based on 2-way ANOVA.

DISCUSSION

Byproducts of the agricultural industry such as harmful dusts, vapors and fumes can cause serious respiratory diseases including asthma, interstitial lung disease, COPD and potentially lung cancer[2]. Some of these harmful substances include but are not limited to dust, pesticides, fertilizers, fumes, and animal wastes. COPD is a leading cause of death worldwide and is most commonly caused by smoking. However, there is growing concern for individuals in the agricultural industry as they are more susceptible to acquiring COPD. This susceptibility to respiratory conditions results in part from the frequent exposure to harmful particulate matter. It is estimated that 8-32% or agricultural industry workers develop COPD in comparison to 4% of non-farming workers[1]. Thus, it is crucial to gain a better understanding of the molecular mechanisms involved in the progression and resolution of chronic lung inflammation caused by organic dusts. In the investigations described herein, we aimed to determine the role of LCN-2 in the context of lung injury driven by chronic organic dust inhalation, using a murine model of repetitive DE exposure.

Through these investigations, we identified a striking increase in lung LCN-2 levels in response to DE. Furthermore, we found that LCN-2 was necessary for lung recovery following DE exposure, evidenced by LCN-2 KO mice having enhanced histopathological injury, and an increase in ECM synthesis and degradation pathways activated after repetitive exposure to DE. The LCN2-associated alterations in ECM pathways was of particular interest; the

lung ECM is composed of proteins that are essential for normal lung development and organ health, providing structural integrity, shaping cell behavior, and acting as a reservoir for cytokines and growth factors[23]. In addition to serving as a physical scaffold for cellular constituents, it initiates critical cues that are required for tissue homeostasis, morphogenesis, and differentiation[24]. ECM synthesis is a coordinated process that is critical in balancing the unique ECM environment via the production of new ECM molecules by surrounding cells. ECM turnover via ECM degradation also plays a crucial role in regulating the ECM environment. In our investigations, we identified that LCN-2 KO mice had increased levels of genes involved with both ECM synthesis and degradation in comparison to WT mice. Genes associated with ECM degradation included Timp2, Mmp11, Phykpl, Ctsl, Col1a2. Metalloproteinases (MMPs) such as Mmp11 are matrix degrading enzymes involved in the remodeling and breakdown of ECM and basement membrane components[25-29]. Inhibitors of MMPs, such as Timp2, prevent the breakdown of the ECM and have been shown to diminish cell proliferation mediated by growth factors and to diminish neo-angiogenesis[30]. In addition to regulating the inhibition of MMPs, Timp-2 has also been identified as being important for the activation of MMPs[26-29] and helps promote ECM deposition[31]. It is notable that dysregulation of ECM remodeling pathways can have pathological consequences. In example, increases in MMPs that can be activated intracellularly, such as Mmp-2, have been associated with fibrosis[31] while Timp-

2 has been shown to regulate the activity of Mmp-2[32, 33]. It is unknown if the increases in Timp-2 and Mmp-11 observed in our studies are directly related or independent of each other; however, like Mmp-2, Mmp-11 is also activated intracellularly and thus capable of interacting in the same manner with Timp-2 as Mmp-2. Ctsl is another proteolytic protein that can degrade the ECM[34], and interestingly has also been shown to regulate the levels of ECM components in lymphoid organs, consequently having a broader effect on the immune system[35]. Col1a2 encodes one of the subunits of Type I collagen, a prominent ECM component[36] that is degraded by Mmps. Transcripts of a number of integrin proteins, involved in regulating cell-cell and cell-ECM interactions, were also found to be dysregulated. Overall, our NanoString findings imply that LCN-2 plays a critical role in regulating the repair process in the lung after exposure to DE via modulating ECM synthesis, deposition, and degradation processes that are important for injury response and repair processes.

The NanoString findings of altered ECM-related pathways are supported by the prolonged tissue damage observed in the LCN-2 KO mice, which showed deficits in recovery with enhanced histopathology **(Figure 5)**. The high LCN-2 levels in the lungs even at basal levels as compared to serum implies an important role of LCN-2 in regulating lung homeostasis even in the absence of an insult. In support of this homeostatic role, recent findings of LCN-2 highlight its pleiotropic actions as not only being pro-inflammatory, but in regulating repair and anti-inflammatory processes as well. In example, LCN-2 has been shown to

play an important role in dampening levels of pro-inflammatory cytokines and chemokines in the brain in response to inflammation [37, 38], thus it is possible that in other tissues such as the lung, LCN-2 is regulating inflammation physiology and/or recruitment of immune cells as a protective mechanism. It has been shown by others that LCN-2 is an important regulator of iron metabolism and iron homeostasis[39] and that iron can regulate both innate and adaptive immunity[40]. It is also possible that this increased cellular infiltration observed in the LCN-2 KO mice is due to a disturbance in iron regulation, and this is consequently leading to the recruitment of immune cells to help produce proteins that help regulate iron levels.

Furthermore, the enhanced histopathology observed with lymphoid aggregate formation and ECM deposition in LCN-2 KO mice in our model lend support to the hypothesis that LCN-2 is involved in the repair process in the lungs. Lymphoid aggregates, also known as secondary lymphoid tissues and Bronchus-Associated Lymphoid Tissue (BALT) induced in response to DE have previously been shown by others to increase with inflammation [41]. In WT mice, these aggregates start to disappear during the recovery period. Other studies using the same *in vivo* DE-exposure model show that the aggregates primarily consist of T cells and B cells, but also contain other immune cells[16]. However, future characterization of these aggregates via immunolabeling or flow cytometry may help us understand how LCN-2 is interacting with these different immune cells in the lymphoid aggregates. Taken together, these results in combination

with our NanoString findings show a role of LCN-2 in regulating lung tissue repair of damaged tissue induced by DE.

An unexpected finding in our investigations was the observed compartmentalized effects of LCN-2 in BALF versus lung tissue **(Figure 4)**. In lung tissue, LCN-2 KO mice instilled with DE had enhanced levels of alveolar and interstitial macrophages with positive staining for M2-like macrophage markers (CD86 = M1-like; CD206 = M2-like; $CD86^{-}CD206^{+}$). In contrast to macrophage findings, we found T cells elevated in BALF but not in the lung homogenates of LCN-2 KO mice. Based on these alterations in cell populations that regulate anti-inflammatory pathways and altered tissue pathology in DE-exposed LCN-2 KO mice, we chose to further investigate anti-inflammatory signaling mechanisms including potential crosstalk between LCN-2 and anti-inflammatory IL-10 signaling pathways. Indeed, our data show evidence that the crosstalk of LCN-2 and IL-10 is necessary for tissue repair, as we found a reduction of tissue damage seen in recovery when LCN-2 KO mice were administered recombinant IL-10 **(Figure 9)**. These findings help support the hypothesis that IL-10 is mediating tissue repair in this model, and that LCN-2 may be necessary to trigger the sufficient induction and regulation of IL-10. In response to repetitive exposure to DE, IL-10 levels in the lungs of mice increased, however, more robust increases of IL-10 levels are observed after DE during the recovery period. This increase during the recovery period is not surprising, considering the well-known role of IL-10 in anti-inflammation and tissue repair [43, 44]. It is also important to

note that this increase of IL-10 mRNA coupled to decreased LCN-2 mRNA transcript levels during the recovery period occurs after the initial significant increases of LCN-2 of mice repetitively exposed to DE. This suggests that the significant increases of LCN-2 in response to DE are needed for anti-inflammation and pro-repair activities to occur. Using the NanoString Fibrosis V2 panel, we were also able to determine which pathways were most affected by IL-10 administration in mice during the recovery period. While overarching differentially expressed pathways observed were related to responses to bacterial components (likely due to the diverse microbial components found in DE), the NanoString results indicated that IL-10 administration altered transcript levels in pathways related to developmental processes. Overall, these findings support our hypothesis of LCN-2 playing a protective role against lung injury induced by DE, in part by regulating IL-10-mediated anti-inflammatory and pro-repair programs to promote recovery after repetitive DE exposure.

Furthermore, using RNAscope we identified that cells in the airway epithelium and parenchyma can be sources of LCN-2 and IL-10. Here, we observed LCN-2 being produced by cells with and without positive staining for Ym-1 basal levels. It is interesting to note that in comparison to WT mice exposed to DE, the LCN-2 KO mice exposed to DE had higher levels of YM-1, which is a known marker for M2-like macrophages. This increase in YM-1 as a result of LCN-2 ablation may indicate a compensatory response. We hypothesize that if LCN-2 is playing a protective role in the lung immune response to DE via

IL-10 crosstalk and promoting anti-inflammatory pathways, its ablation could cause the polarization of more M2-like macrophages in an effort to help promote anti-inflammation and/or tissue repair, although this hypothesis requires further investigation. It is also important to note that other sources of LCN-2 such as neutrophils or dendritic cells were not stained for and remain to be considered in future studies. Similarly, alternative sources of IL-10 such as T cells were not included in our stains and need to be considered as important sources of IL-10 production and potential mediators of tissue repair in future studies. Additionally, given LCN-2's known role in iron homeostasis acting as an iron scavenging protein [42], it is also possible that in the absence of LCN-2, there is polarization of more M2-like macrophages to produce other proteins that help regulate iron homeostasis in the lungs.

With regards to the macrophage populations observed in our model, It is worth noting that other investigations using the same murine repetitive exposure model have found that DE can increase the number of macrophages staining positive for M1-like markers [14, 16, 19-21]. It is becoming increasingly recognized that the M1/M2 polarization paradigm is ill-fitted to accurately reflect the dynamic activation of macrophages, and together with previous study findings, our currently results warrant more in-depth explorations of macrophage activation during environmental dust exposure.

Overall, our findings highlight a protective role of LCN-2 in lung inflammation associated with DE exposure, which we have identified is in part

due to its regulation of the anti-inflammatory cytokine, IL-10. Further studies to identify additional potential sources of LCN-2 and IL-10 and the interactions between these two mediators with other cells will help elucidate specific mechanisms involved with the repair process to environmental dust exposures. These investigations would support feasibility studies aimed as assessing the therapeutic utility of LCN-2 in the future in patients with chronic lung conditions.

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Conflicts of interest

There are no conflicts of interest to report.

Author contributions

Conception and Experimental Design: TMN, MK, EG; Sample collection, Data Acquisition and Data Analysis: SG, DOJ, AU, SS, SP, ED, TMN, MK; Manuscript Drafting: SG, TMN, MK; Manuscript Editing: SG, DOJ, AU, TMN, MK.

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Data Availability Statement

Results for NanoString experiments can be found in the NCBI Gene Expression Omnibus (GEO) repository (will be uploaded upon acceptance).

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III. Chapter 4: Occupational Dust Exposure and Fatty Acid Intake in Patients with Asthma, Bronchitis, Emphysema and Chronic Obstructive Lung Disease (COPD): NHANES Analysis 2007-2012

ABSTRACT

Agriculture industry workers are exposed to harmful toxins, dusts and vapors on a daily basis. This frequent exposure to harmful particulate matter and vapors has been known in to increase the susceptibility to developing debilitating inflammatory lung conditions. Organic dust has been linked to upper respiratory lung conditions such as asthma, chronic bronchitis, emphysema and chronic obstructive pulmonary disease (COPD). Due to the increased risk of occupational workers in developing inflammatory lung conditions, it is of importance to identify any associations between occupational dust exposure and chronic lung diseases. Additionally, Omega-3 fatty acids (FAs) can be protective against diseases driven by inflammation. Thus, this study was conducted to identify protective benefits exerted by Omega-3 FAs EPA and DHA intake against chronic lung disease outcomes in individuals with organic dust exposure. NHANES data identified. The total number of eligible participants included in the analysis was 9651. Univariate analysis showed that exposure to natural dust was associated to significant increased reports of bronchitis ($p = 0.0350$) and emphysema ($p=0.0009$) and COPD ($p=0.0029$). Additionally, EPA+DHA intake significantly decreased incidents of self-reported asthma ($p=0.0079$) and bronchitis ($p=0.0392$). After adjusting for the confounding variables age, gender, race, BMI weight, BMI height, smoking status, poverty class, and education,

multivariate logistic regression models showed trends in natural dust exposure increasing self-reported bronchitis (p=0.0657; OR: 1.288; 95%CI: 0.983, 1.686) and emphysema (p=0.0677; OR: 1.503; 95%CI: 0.970, 2.331). Additionally, combined effects of natural dust exposure and EPA+DHA intake significantly showed decreased incidents of self-reported bronchitis in individuals with higher EPA+DHA intake (p=0.0136).

INTRODUCTION

Obstructive lung diseases are a major public health problem and carry significant social and economic burdens. Workers in the agricultural industry are more likely than the general population to develop obstructive lung diseases due to higher exposure to harmful particulate matter such as dust particles [1]. COPD and asthma are the two major diagnoses associated with obstructive lung disease worldwide [2]. Of these, COPD is the third leading cause of death worldwide and approximately 15% of COPD cases are attributed to occupational history [3]. Asthma has also been linked to occupational exposure and is a growing concern as it has been on the rise and currently considered the most prevalent chronic respiratory disease, affecting 262 million people worldwide [4].

While COPD and Asthma are both diseases of inflammation, their pathophysiology varies. In asthma, airway smooth muscle cell constriction, airway hyper-reactivity to allergens, and increased eosinophils and activation of T-cells lead to airway obstruction. Airway obstruction is usually reversible with treatment or spontaneously. Use of inhaled corticosteroids helps to stop lung

function deterioration. Patients can present with symptoms even when they have near normal lung function. COPD, however, is not typically associated with constriction of airway smooth muscle cells. Mucus hypersecretion and mucosal infiltration of inflammatory cells leads to cellular damage and the loss of the alveolar structure. The destruction of cells and structural changes can then alter the pulmonary circulation and lead to problems with oxygenation. In COPD, symptoms often start to appear with decline of FEV1 and lung function deterioration continues despite treatment. Bronchitis and emphysema both fall under the umbrella term of COPD and are characterized based on inflammation affecting the lining of the airways versus the air sacs in the lungs, respectively. Numerous studies have shown that the use of nutrients such as Omega-3 polyunsaturated fatty acids (n-3 PUFA) improves COPD and Asthma outcomes [1, 5-7].

The human body is unable to synthesize essential fats n-3 PUFAs, thus highlighting the importance of nutritional intake. Sources of n-3 PUFAs include but are not limited to: fish, flaxseeds, nuts, and vegetable oils. The three main n-3 PUFAs include Eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and alpha-linolenic acid (ALA). Although n-3 PUFAs have been shown to directly improve COPD and Asthma outcomes, their use as a mediator between occupational dust exposure and obstructive lung diseases has never been investigated. Data available from the National Health and Nutrition Examination Survey (NHANES) was utilized to help fill this gap in knowledge [8].

NHANES is a unique program of studies that includes physical examinations and interviews of the participants [8]. This allows for the careful examination of the health AND the nutritional status of participants. NHANES has been a useful tool in helping to identify associations between risk factors and obstructive lung diseases [9]. In this study, we use the NHANES cross-sectional studies from waves 2007-2012 to identify associations between natural dust exposure and disease prevalence of asthma, bronchitis, emphysema, and COPD. Additionally, we investigate the use of EPA+DHA intake as a potential modifier of obstructive lung disease outcome.

MATERIALS AND METHODS

Study Design

This study was a cross-section analysis using NHANES survey years 2007-2021. The methods and selection of participants for the NHANES cross-sectional studies are publicly available through the CDC.gov website [8]. Included in these studies are the NHANES survey years 2007-2012 with lung function measurements as well as asthma, bronchitis and emphysema questionnaires. The primary outcomes of this study were the self-reported asthma, bronchitis, or emphysema and COPD, as defined by lung function measurements. The following NHANES questionnaire variables were used to identify self-reported asthma, bronchitis and emphysema: MCQ010, MCQ160k, MCQ160g. forced expiratory volume in one second (FEV1) and FEV1 and forced vital capacity (FVC) lung outcome functions were obtained using SPXNFEV1 and

SPXNFVC codes. Distribution of current COPD was defined as “Yes” if the FEV1/FVC ration was less than 0.7. All data used was acquired by the National Center for Health Statistics (NCHS) during home health interviews and mobile examination centers (MEC). Pre-bronchodilator spirometry measurements were collected in the NHANES waves 2007-2012, thus these same waves were used for data analysis. Lung function measurements included: FEV1, FVC, FEV1/FVC ratio, FEV1 percent predicted, and FVC percent predicted. An FEV1/FVC ratio of less than 0.7 was used to diagnose COPD.

Participants

Male and non-pregnant female adults 20+ y, who had occupational dust history, dietary intake available and spirometry measurements will be included. Individuals with energy intake greater than or less than reasonable intake were excluded (<600 or >6,000 kcal/day for women; <800 or >8,000 kcal/day for men). Pregnant female adults were also excluded. NHANEs cycles from 2007-2012 will be included that have information on natural dust exposure, lung function and lung disease diagnosis (asthma, bronchitis, emphysema). Criteria, criteria codes, and descriptions for data sets used are outlined in **Table 1**.

Table 1: List of National Health and Nutrition Examination Survey codes for exposures, outcomes and covariates.

Criteria	Criteria code	Description
Occupational	OCQ530	Ever had work exposure to organic dusts?
EPA & DHA Fatty Acid intake	DR1TP205	PFA 20:5 (Eicosapentaenoic) (gm)
	DR1TP226	PFA 22:6 (Docosahexaenoic) (gm)
Spirometry lung function	SPXNFEV1	Baseline 1st Test Spirometry, Forced Expiratory Volume in the first 1.0 second, in mL
	SPXNFVC	Baseline 1st Test Spirometry, Forced Vital Capacity, in mL
Asthma diagnosis	MCQ010	Ever been told you have asthma
	MCQ025	Age when told you had asthma
	MCQ035	Still have asthma
Chronic bronchitis diagnosis	MCQ160k	Ever been told you have chronic bronchitis
	MCQ170K	Age when told you had chronic bronchitis
	MCQ180k	Still have chronic bronchitis
Emphysema diagnosis	MCQ160g	Ever been told you have emphysema
	MCQ180g	Age when told you had emphysema

Criteria codes used for information on natural dust exposure, EPA+DHA FA intake, lung function measurements and self-reported obstructive lung disease diagnosis are listed in Table 1. Descriptions for all codes are also provided in Table 1.

Exposure Assessment

Exposure to organic dusts was determined by using criteria code (OCQ530), which provides data from surveys of all participants asked if they "Ever had work exposure to organic dusts?". Participants were then categorized into "no" and "yes" groups based on their self-reported exposure to organic dust. Two interviewer-administered 24-hr recalls that were developed and validated by the U.S. Department of Agriculture were used to determine dietary intake. Our primary intake of interest were n=3 PUFAs EPA (criteria code: DR1TP205) and DHA (criteria code: DR1TP226), which are reported as grams/day. Mean intake of EPA + DHA was used in the final analysis and was divided into the following tertiles: 0-20mg, 20-70mg, and 70+mg.

Outcome Assessment

The primary outcome for this study was self-reported asthma, self-reported bronchitis, self-reported emphysema, and an FEV1/FVC ratio of less than 0.7 for current COPD. Assessment of lung disease outcome was done by using self-reported diagnosis of asthma, bronchitis, and emphysema. Surveys consisted of questions asking: 1. Ever been told you have disease? 2)Age when told you have disease? and/or 3) Do you still have disease? Based on answers provided, participants were categorized into “No history of disease” or “Yes, have a history or still have history of disease”. Lung function measurements for all participants were obtained from the physical examination portion. Pulmonary function tests serve as a diagnostic tool for lung conditions including asthma, bronchitis, emphysema and COPD, with its FEV1/FVC ratio being useful in diagnosing obstructive lung disease [10]. Additionally, FEV1% and FVC% can provide clinical information about factors known to impact lung function including but not limited to gender, age and race.

Other covariates

Potential confounding variables include age, race, gender, BMI height, BMI weight, smoking, poverty index and education, which were selected based on previous associations found in the literature [11-14]. Male and non-pregnant females over 20 years of age with natural dust history, EPA+DHA intake, and spirometry measurements available were included. Individuals with energy intake greater than or less than the plausible intake (<600 or >6,000 kcal/day for women

and <800 or >8,000 kcal/day for me) were excluded from this study. The average of two 24-hr recalls were calculated for both EPA and DHA and the average EPA and DHA concentration (provided by NHANES in grams) for each participant was used to calculate the total sum of EPA+DHA. The EPA+DHA was used in the final analysis and divided and participant EPA+DHA levels were divided into three quartiles: 0-0.02g, 0.02-0.07g, 0.07g and above. Participants were divided into four different race categories: hispanic, non-hispanic white, non-hispanic black and other race. BMI groups included BMI Weight and BMI Height. For BMI Weight, participants were categorized into the three following groups: Underweight/Normal: <25.0kg, Overweight: 25.0-29.9kg and Obesity: >30kg. BMI Height consisted of tertile groups: 135-160cm, 160-174cm and 174cm and above. Other confounding variables include smoking, which consisted of a non-smoker, former-smoker and smoker groups. Additionally, people were categorized by a poverty-income ratio (PIR) of poor (0-1.35), nearly poor (1.36-1.85) and poor (1.86+). Lastly, education levels were taken into account with less than 12th grade, high school/GED, some college and college or above groups. A summary of NHANES participant characteristics can be found in **Table 2**.

Statistical Analysis

To describe the study population from the NHANES cohort for the years 2007-2012, a descriptive statistics analysis was done to obtain counts and percentages for categorical data and means for continuous data (Table 2). The

results of the descriptive analysis for the categorical variable are represented by counts, weighted percentages and 95% confidence intervals (CI). For continuous variables, means, standard errors and 95% CI were used. To characterize the participant population broken down by past exposure to occupational natural dust, a Rao-Scott chi-square test was performed. Univariate regression analysis allowed for direct comparisons between one given factor and self-reported asthma, bronchitis, emphysema or COPD. After adjusting for confounding variables, multivariate regression analysis was used for direct comparisons between Dust or EPA+DHA and lung disease outcome. A mediation analysis was also performed using a multivariate regression analysis to determine the direct effects of EPA+DHA in mediating the effects of dust on lung disease outcome. SAS version 9.4 was used for all analyses and a p value of <0.05 was considered statistically significant.

RESULTS

Descriptive Analysis of Eligible Participants among the NHANES Cohort

Overall, the population included a total of 9651 eligible participants with an average age of 45 years. Approximately 50.6% were females, 71.7% were non-hispanic whites, 35.2% were obese, 54.6% were non-smokers, 70.8% had a high PIR, 20.4% had a college education, 49.22% were 160-174 cm in height, 22% had previous exposure to organic dust, and 37.17% had an intake of 20-70mg of EPA+DHA **(Table 2)**.

Table 2: Characteristics of NHANES participants stratified by self-reported asthma, chronic bronchitis, emphysema and COPD.

Disease		Asthma	Chronic Bronchitis	Emphysema	COPD
Characteristic	ALL (n = 9614, 9630, 9646, 9651) Mean (SE) or %	No History (n = 8279), Yes Have a History or still have Disease (n = 1335); *p* value, Mean (SE) or %	No History (n = 9149), Yes Have a History or still have Disease (n = 481); *p* value, Mean (SE) or %	No History (n = 9537), Yes Have a History or still have Disease (n = 109); *p* value, Mean (SE) or %	No History (n = 8366), Yes Have a History or still have Disease (n = 1285); *p* value, Mean (SE) or %
Average Age (year)		**0.0002**	**<0.0001**	**<0.0001**	**<0.0001**
	45 (0.40)	45 (0.40), 43 (0.60)	45 (0.41), 51 (0.93)	45 (0.40), 60 (1.05)	43 (0.37), 56 (0.57)
Age (year)		**0.0005**	**<0.0001**	**<0.0001**	**<0.0001**
20-29	20.1	19.2, 25.3	20.6, 12.3	20.4, 1.2	22.5, 5.3
30-39	19.4	19.3, 20.0	19.9, 11.5	19.6, 4.7	21.3, 7.8
40-49	21.1	21.4, 19.5	21.0, 21.5	21.1, 19.2	21.8, 16.3
50-59	20.4	20.9, 17.7	20.2, 24.6	20.5, 18.6	19.3, 27.6
60-69	12.8	19.2, 17.6	18.4, 30.0	18.5, 56.4	15.1, 43.0
Gender		**<0.0001**	**<0.0001**	0.0701	**<0.0001**
Female	50.6	49.0, 60.1	49.6, 68.8	50.7, 40.6	52.1, 41.4
Male	49.4	51.0, 40.0	50.4, 31.2	49.3, 59.4	47.9, 58.6
Race		**<0.0001**	**<0.0001**	**0.0007**	**<0.0001**
Hispanic	12.5	13.0, 9.3	12.8, 6.9	12.6, 1.2	13.8, 4.4
Non-Hispanic White	71.7	71.4, 73.6	71.2, 81.5	71.5, 89.1	69.7, 84.5
Non-Hispanic Black	9.9	9.6, 12.0	10.0, 8.3	10.0, 4.6	10.4, 7.2
Other Race	5.9	6.0, 5.1	6.0, 3.3	5.9, 5.1	6.2, 3.9
BMI Average (kg/m^2)		**0.0002**	**<0.0001**	**0.0493**	**<0.0001**
	29	*27.5 (7.8), 28.4 (9.2)*	*27.5 (7.8), 29.5 (10.1)*	*27.6 (7.9), 29.2 (9.7)*	*27.8 (8.1), 26.5 (6.7)*
BMI Groups		**0.0002**	**0.0003**	0.0806	**<0.0001**
Underweight/Normal: <25.0	31.1	31.6, 28.1	31.4, 23.8	31.1, 21.4	30.2, 36.8
Overweight: 25.0-29.9	33.8	34.4, 29.7	34.0, 28.3	33.8, 30.9	33.3, 36.7
Obesity: > 30	35.2	34.1, 42.2	34.5, 47.9	35.1, 47.7	36.5, 26.5
Smoking		0.5161	**<0.0001**	**<0.0001**	**<0.0001**
Non-Smoker	54.6	54.8, 53.1	55.4, 39.2	55.1, 7.7	58.5, 29.4
Former Smoker	24.1	24.2, 24.1	24.0, 27.9	24.0, 37.9	22.3, 35.8
Smoker	21.3	21.0, 22.8	20.6, 33.0	21.0, 54.5	19.2, 34.8
Poverty Income Ratio		**0.0002**	**<0.0001**	**0.0007**	**0.0312**
Poor: 0-1.35	20.4	19.7, 24.7	19.8, 30.1	20.2, 31.6	20.9, 17.2
Nearly poor: 1.36-1.85	8.8	8.5, 10.2	8.6, 13.0	8.7, 16.9	8.7, 9.1
Not poor: 1.86$^+$	70.8	71.8, 65.2	71.7, 56.9	71.0, 51.5	70.4, 73.6
Education		0.1532	**<0.0001**	**<0.0001**	**0.0023**
Less than 12th grade	9.7	14.9, 13.5	14.4, 20.0	14.5, 34.2	14.1, 18.4
High school/GED	13.8	21.7, 21.2	21.6, 24.1	21.5, 34.0	21.2, 24.8
Some college AA	19.6	31.3, 34.8	31.6, 36.7	31.9, 23.1	32.3, 29.0
College or above	20.4	32.1, 30.5	32.5, 19.2	32.0, 8.6	32.4, 27.8
Height (cm)		0.1439	0.1014	0.1056	**0.0173**
135, 160	17.4	17.9, 16.1	17.4, 21.9	17.5, 26.9	18.1, 14.3
160, 174	49.2	39.3, 37.8	39.2, 37.9	39.1, 42.8	38.6, 42.7
174+	33.4	42.8, 46.1	43.5, 40.1	43.4, 30.2	43.3, 43.0
Natural dust		0.8355	**0.0350**	**0.0009**	**0.0029**
No	77.0	77.0, 77.4	77.2, 72.9	77.2, 59.8	77.6, 73.1
Yes	22.0	23.0, 22.6	22.8, 27.1	22.8, 40.2	22.4, 27.0
EPA+DHA (gm)		**0.0079**	**0.0392**	0.8416	0.7374
[0, 0.02)	32.5	31.7, 37.3	32.1, 39.9	32.5, 34.1	32.6, 31.5
[0.02, 0.07)	37.2	37.3, 36.0	37.5, 31.2	37.1, 38.8	37.0, 38.5
0.07+	30.4	31.0, 26.7	30.2, 28.8	30.4, 27.1	30.4, 30.0

p values represent the overall p values associated with the history (yes have a history or still have a history) against no history of asthma, bronchitis, emphysema and COPD. P values are provided to the right side of each group and bolded for those reaching statistical significance ($p<0.05$). Characteristics are listed in first column. Column headings for other columns define samples analyzed and statistics represented. Italicized values are showing median and (inter-quartile ranges). Definitions: Body Mass Index (BMI), Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA).

Univariate and Multivariate Regression Analysis: Associations between exposure to natural dust and/or EPA+DHA consumption tendencies to prevalence of self-reported asthma.

Univariate regression analysis (Table 2) showed that increases in EPA+DHA ($p=0.0079$) were inversely correlated with higher reports of asthma. Increases in age ($p<0.0001$) and weight ($p=0.0002$) and decreases in PIR ($p=0.0002$) are associated with increased reports of asthma. Gender ($p<0.0001$) and race ($p<0.0001$) were also shown to impact outcome with increased reports amongst females and non-hispanic whites and non-hispanic blacks. Multivariable regression analysis of associations between organic dust exposure or EPA+DHA intake with asthma outcome was performed to adjust for confounding variables (Table 3, Independent analysis; Figure 1). Results showed that after adjusting for confounding variables, neither natural dust nor EPA+DHA intake significantly altered asthma outcome.

Multivariate regression analysis was also performed to assess the combined effects of natural dust and EPA+DHA on asthma outcome (Table 3:– Mediation Analysis). Results showed that the combined effects of natural dust exposure and EPA+DHA intake on asthma outcomes were not significant ($p=0.4743$).

Table 3: Multivariate Logistic Regression Analysis: Independent and Dependent effects Natural Dust Exposure on self-reported Asthma, Bronchitis, Emphysema, and COPD.

	Multivariable Regression Analysis; *p* value[1] OR[2] [95% CI][3]			
Independent	**Asthma**	**Chronic Bronchitis**	**Emphysema**	**COPD**
Natural Dust	0.6645	0.0657	0.0677	0.3384
No	1	1	1	1
Yes	1.050 [0.839, 1.313]	1.288 [0.983, 1.686]	1.503 [0.970, 2.331]	1.113 [0.891, 1.392]
EPA+DHA	0.1331	0.1792	0.8604	0.4509
0-20mg	1	1	1	1
20-70mg	0.858 [0.700, 1.036]	0.743 [0.533, 1.034]	1.130 [0.620, 2.061]	1.135 [0.910, 1.417]
70+mg	0.802 [0.641, 1.004]	0.954 [0.662, 1.375]	0.966 [0.452, 2.065]	0.991 [0.803, 1.221]
Dependent – Mediation Analysis	**Asthma**	**Chronic Bronchitis**	**Emphysema**	**COPD**
Natural dust_EPADHA	0.4743	**0.0136**	0.3781	0.3511
No: 0-20mg	1	1	1	1
No: 20-70mg	0.858 [0.691, 1.065]	0.637 [0.439, 0.924]	1.295 [0.706, 2.374]	1.192 [0.954, 1.490]
No: 70+mg	0.812 [0.632, 1.044]	0.979 [0.621, 1.543]	1.051 [0.519, 2.126]	1.003 [0.806, 1.248]
Yes: 0-20mg	1.077 [0.789, 1.471]	1.113 [0.748, 1.657]	1.801 [0.853, 3.802]	1.202 [0.887, 1.629]
Yes: 20-70mg	0.898 [0.653, 1.235]	1.187 [0.835, 1.688]	1.636 [0.679, 3.944]	1.198 [0.871, 1.648]
Yes: 70+mg	0.825 [0.565, 1.204]	0.952 [0.537, 1.688]	1.557 [0.654, 3.710]	1.174 [0.832, 1.656]

Models were adjusted for age, gender, race, BMI weight, smoking, PIR, education, and height. p values represent the overall p values associated with each characteristic and self-reported disease. P values reaching statistical significance ($p<0.05$) are bolded, odd ratio point estimate for each given group is listed next to its 95% CIs in brackets. Definitions: Confidence Interval (CI), Body Mass Index (BMI), Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA).

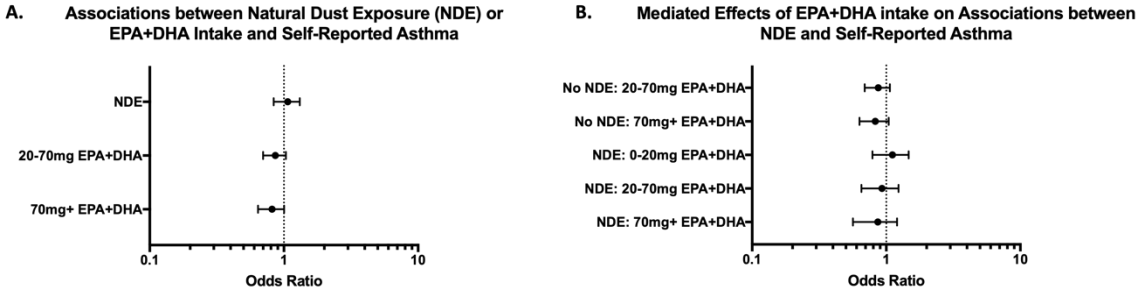

Figure 1. Association between natural dust exposure and/or EPA and DHA consumption tendencies with self-reported asthma (A – Independent Analysis; B – Mediation Analysis) in the NHANES population. Odds ratios for reporting current asthma compared to the total NHANES population based on identifying an individual with: A) Top: Exposure vs no expo-sure to natural dust, Middle: 20-70mg vs 0-20mg intake of EPA+DHA, and Bottom: 70mg+ vs 0-20mg intake of EPA+DHA. B) from top to bottom and all compared to "No exposure to natural dust + 0-20mg intake of EPA+DHA": No exposure to natural dust + 20-70mg intake of EPA+DHA, No exposure to natural dust + 20-70mg intake of EPA+DHA, Exposure to natural dust + 0-20mg intake of EPA+DHA, Exposure to natural dust + 20-70mg intake of EPA+DHA, and Exposure to natural dust + 70mg+ intake of EPA+DHA.

Univariate and Multivariate Regression Analysis: Associations between exposure to natural dust and/or EPA+DHA consumption tendencies to prevalence of self-reported bronchitis.

Univariate regression analysis **(Table 2)** showed that increases in exposure to organic dust ($p=0.0350$) and decreases in EPA+DHA ($p=0.0392$) were correlated with increased reports of bronchitis. Increases in age ($p<0.0001$), weight ($p=0.0003$), smoke use ($p<0.0001$), and decreases in PIR ($p<0.0001$), education ($p<0.0001$), and height ($p<0.0001$) were also associated with increased reports of bronchitis. Gender ($p<0.0001$) and race ($p<0.0001$) were also shown to impact outcome with increased reports amongst females and non-hispanic whites.

After adjusting for confounding variables, multivariable regression analysis **(Table 3; Independent; Figure 2A)** of associations between organic dust exposure or EPA+DHA intake with bronchitis outcome was performed. Results showed that after adjusting for confounding variables, natural dust was trending ($p=0.0657$) towards significantly altering bronchitis outcome with increased reports of bronchitis in individuals with exposure to organic dust in comparison to no exposure, but no significant impact of EPA+DHA intake ($p=0.1792$) was observed on bronchitis outcome. However, multivariate regression analysis was also performed to assess the combined effects of natural dust and EPA+DHA on bronchitis **(Table 3: Dependent; Figure 2B)**. Results showed that the combined effects of natural dust exposure

and EPA+DHA intake on bronchitis outcome were significant (p=0.0136). Moreover individuals with no dust exposure and 20-70mg EPA+DHA intake showed the most robust decreases in self-reported bronchitis in comparison to individuals with no dust exposure and 0-20mg EPA+DHA intake.

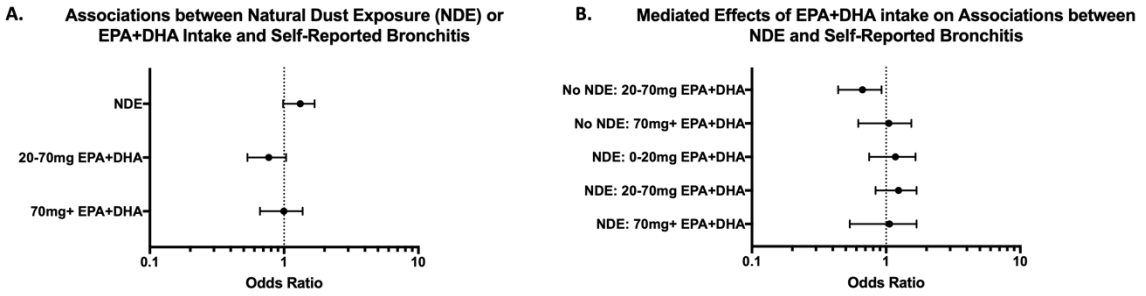

Figure 2. Association between natural dust exposure and/or EPA and DHA consumption tendencies with self-reported bronchitis (A – Independent Analysis; B – Mediation Analysis) in the NHANES population. Odds ratios for reporting current bronchitis compared to the total NHANES population based on identifying an individual with: A) Top: Exposure vs no exposure to natural dust, Middle: 20-70mg vs 0-20mg intake of EPA+DHA, and Bottom: 70mg+ vs 0-20mg intake of EPA+DHA. B) from top to bottom and all compared to "No exposure to natural dust + 0-20mg intake of EPA+DHA": No exposure to natural dust + 20-70mg intake of EPA+DHA, No exposure to natural dust + 20-70mg intake of EPA+DHA, Exposure to natural dust + 0-20mg intake of EPA+DHA, Exposure to natural dust + 20-70mg intake of EPA+DHA, and Exposure to natural dust + 70mg+ intake of EPA+DHA.

Univariate and Multivariate Regression Analysis: Associations between exposure to natural dust and/or EPA+DHA consumption tendencies to prevalence of self-reported emphysema.

Univariate regression analysis **(Table 2)** showed that increases in exposure to organic dust (p=0.0009) were correlated with increased reports of emphysema. Additionally, increases in age (p<0.0001) and smoke use (p<0.0001), and decreases in PIR (p=0.0007) and education (p<0.0001) were associated with increased reports of bronchitis. Race (p<0.0001) was also shown to impact outcome with increased reports amongst non-hispanic whites.

After adjusting for confounding variables, multivariable regression analysis **(Table 3: Independent; Figure 3A)** of associations between organic dust exposure or EPA+DHA intake with emphysema outcome was performed. Results showed that after adjusting for confounding variables, natural dust was trending (p=0.0677) towards significantly increasing reports of emphysema in individuals with exposure to organic dust in comparison to no exposure, but no impact of EPA+DHA intake (p=0.8416) was observed on emphysema outcome. Multivariate regression analysis was also performed to assess the combined effects of natural dust and EPA+DHA on emphysema **outcome (Table 3: Dependent; Figure 3B)**. Results showed that the combined effects of natural dust exposure and EPA+DHA intake on emphysema were not significant (p=0.3781).

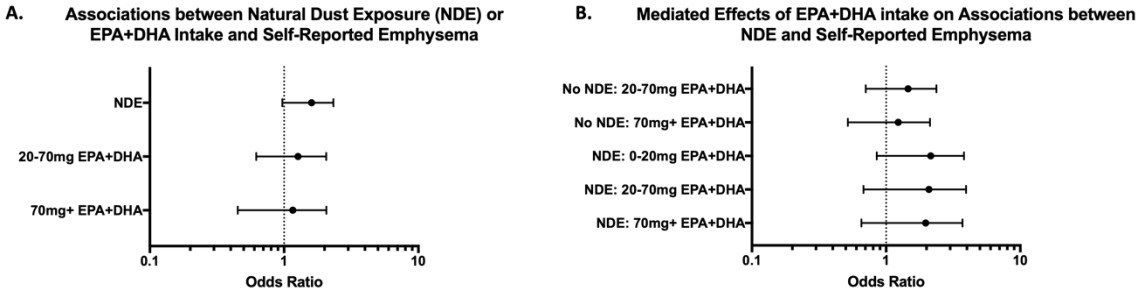

Figure 3. Association between natural dust exposure and/or EPA and DHA consumption tendencies with self-reported emphysema (A – Independent Analysis; B – Mediation Analysis) in the NHANES population. Odds ratios for reporting current emphysema compared to the total NHANES population based on identifying an individual with: A) Top: Exposure vs no exposure to natural dust, Middle: 20-70mg vs 0-20mg intake of EPA+DHA, and Bottom: 70mg+ vs 0-20mg intake of EPA+DHA. B) from top to bottom and all compared to "No exposure to natural dust + 0-20mg intake of EPA+DHA": No exposure to natural dust + 20-70mg intake of EPA+DHA, No exposure to natural dust + 20-70mg intake of EPA+DHA, Exposure to natural dust + 0-20mg intake of EPA+DHA, Exposure to natural dust + 20-70mg intake of EPA+DHA, and Expo-sure to natural dust + 70mg+ intake of EPA+DHA.

Univariate and Multivariate Regression Analysis: Associations between exposure to natural dust and/or EPA+DHA consumption tendencies to prevalence of COPD.

Increases in natural dust exposure were associated with significantly ($p=0.0029$) increased incidents of COPD **(Table 2)**. Increases in age ($p<0.0001$), smoking ($p<0.0001$), PIR ($p=0.0312$) and height ($p=0.0173$) and decreases in weight ($p<0.0001$) and education ($p=0.0023$) were significantly associated with increases in COPD outcome. Additionally, gender ($p<0.0001$) and race ($p<0.0001$) were also shown to significantly alter COPD outcome, with highest incidents observed in males and non-hispanic whites.

After adjusting for confounding variables, multivariable regression analysis **(Table 3: Independent; Figure 4A**) of associations between organic dust exposure or EPA+DHA intake with COPD outcome was performed. Results showed that after adjusting for confounding variables, neither natural dust ($p=0.3381$) nor EPA+DHA ($p=0.4509$) intake significantly impacted reports of COPD. Furthermore, multivariate regression analysis was also performed to assess the combined effects of natural dust and EPA+DHA intake on COPD outcome **(Table 3: Dependent; Figure 4B)**. Results showed that the combined effects on COPD outcome were not significant ($p=0.3511$).

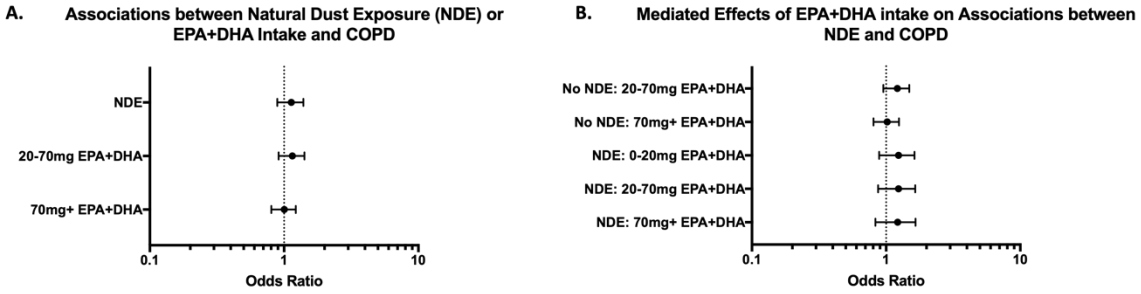

Figure 4. Association between natural dust exposure and/or EPA and DHA consumption tendencies with COPD (A – Independent Analysis; B – Mediation Analysis) in the NHANES population. Odds ratios for reporting current COPD compared to the total NHANES population based on identifying an individual with: A) Top: Exposure vs no exposure to natural dust, Middle: 20-70mg vs 0-20mg intake of EPA+DHA, and Bottom: 70mg+ vs 0-20mg intake of EPA+DHA. B) from top to bottom and all compared to "No exposure to natural dust + 0-20mg intake of EPA+DHA": No exposure to natural dust + 20-70mg intake of EPA+DHA, No exposure to natural dust + 20-70mg intake of EPA+DHA, Exposure to natural dust + 0-20mg intake of EPA+DHA, Exposure to natural dust + 20-70mg intake of EPA+DHA, and Exposure to natural dust + 70mg+ intake of EPA+DHA.

DISCUSSION

Respiratory diseases that are associated with the agricultural industry were one of the first-recognized occupational hazards [15]. Obstructive lung diseases are characterized by chronic inflammation and can lead to debilitating effects in individuals. Asthma is the most prevalent chronic respiratory disease worldwide, has been on the rise worldwide [4] and has been linked to occupational exposure. COPD is the third leading cause of death worldwide and approximately 15% of COPD cases are attributed to occupational exposure [16]. Health disparities in patients with obstructive lung diseases include but are not limited to race, socioeconomic status, poverty-income ratio, education, and diet, and can significantly impact the disease outcome. Diet is an important and necessary source for many nutrients that the human body requires to survive,

and nutrients such as n-3 PUFAs can be protective against diseases driven by inflammation [17, 18]. This protection by n-3 PUFAs against airway inflammation has been shown to be mediated by special pro-resolving lipid mediators (SPMs), which are known to be involved in the resolution of inflammation [19, 20]. Although n-3 PUFAs have been shown to have protective effects against obstructive lung diseases, their potential use as mediators in driving inflammation induced by natural dust exposure has never been studied. Here, we investigate the effects of natural dust exposure on asthma, bronchitis, emphysema and COPD disease outcome. Moreover, we investigate the effects of n-3 PUFAs EPA+DHA in mediating the diseases outcomes. Our findings show significant associations between natural dust exposure and increased reports of bronchitis and emphysema, significant associations between EPA+DHA intake and decreased reports of asthma and bronchitis, and significant decreases in bronchitis outcome mediated by EPA+DHA intake. These findings add to breadth of knowledge in our field and help aid in the therapeutic discovery of potential drugs to use against obstructive lung diseases.

Organic settled dusts collected (DE) from Swine confinement facilities has been shown to induce a potent immune response in the lungs of mice and early pathological signs similar to chronic lung diseases [21-23]. When given intranasally and repetitively, the use of the dust is a well-established model for the study of chronic lung diseases [24-29]. SPMs play important roles in the resolution of the inflammatory response to the dust. SPMs such as Maresins and

Resolvins are derived from omega-3 fatty acids and have been shown to play various roles in inflammation including but not limited to: maintenance of lung epithelial permeability [30], prevention of neutrophil survival [31], suppression of oxidative stress [32], modulating T cell responses [33, 34], modulating macrophage responses [35, 36]. SPMs have been shown to be protective against inflammatory responses induced by DE both in vitro and in vivo [24, 37, 38]. Given the link between organic dust with chronic lung disease and the protective effects observed from SPMs against inflammation induced by the organic dust, we utilized the NHANES survey to assess this further at the population level.

Our results show that EPA+DHA intake is associated with increased self-reported bronchitis in individuals with no exposure to natural dust. However, in individuals with previous exposure to natural dust, EPA+DHA intake is not associated with self-reported bronchitis. We hypothesize that EPA+DHA intake in individuals who are chronically exposed to natural dust have a robust inflammatory response to the dust that cannot be mediated by EPA+DHA. Although not significant, it is important to note that in individuals with exposure to natural dust, higher EPA+DHA intake leads to decreases in self-reported bronchitis. Higher intake of n-3 PUFAs has been linked to decreased morbidity of several inflammatory diseases, however, their effects on respiratory diseases such as COPD remain understudied [5]. n-3 PUFAs can be beneficial when used therapeutically, as they have been previously shown to help with weight gain [39]

and improving physical endurance [40] in patients with COPD [41], though contradicting studies demonstrating no benefit have been reported as well [42, 43]. Additionally, in COPD patients, n-3 PUFAs and their metabolites have also been shown to increase in response to high levels of n-6, which is hypothesized to be a compensatory mechanism to resist pro-inflammation [44]. n-3 PUFAs and their metabolites have also been shown to regulate airway inflammation in asthma by counter-regulating airways eosinophilic inflammation and promoting the resolution [45, 46]. Oral administration of n-3 PUFAs in patients with asthma have shown improved symptoms, improved spirometry indices, and decreased pro-inflammatory cytokines IL-17A and TNF-α [47]. Clinical and human studies investigating the protective effects of n-3 PUFAs on obstructive lung diseases are limited by multiple factors such as poorly or heterogeneously defined populations and small sample sizes. Thus, highlighting the need to use tools such NHANES to study the mediated effects of n-3 PUFAs on disease outcome associated with natural dust exposure.

Strengths of this study include the adjustment of the confounding factors: age, gender, race/ethnicity, smoking status, poverty level, BMI weight and BMI height, as these factors themselves are associated with chronic lung diseases. Our analysis allowed us to look at the effects of natural dust and EPA+DHA intake as a potential modifier for multiple types of chronic lung conditions that are characteristic in this population. Some of our limitations include lack of access to participants symptoms, limiting a thorough comparison of the effects of natural

dust on disease diagnosis as it inhibits us from identifying overlaps of symptoms for different disease types as well as direct associations between natural dust or EPA+DHA on self-reported disease. Another limitation includes that most diagnoses reported in NHANES are “self-reported” and not diagnosed by a physician at the time of the physical examination and survey. An exception of this is our criteria for COPD, which was based on spirometry data. Lastly, another limitation is the variability amongst the individuals that answered “yes” to exposure to natural dust, as exposure can range from i.e. minimal exposure to exposure on a daily basis for 30+ years. Analysis of natural dust effects and the use EPA+DHA as a modifier of overall lung function may also be a valuable consideration, as this can provide insightful information as well. Lastly, while our investigation focused only on occupational exposure to natural dusts, other dust exposures (e.g. mineral dust exposure) would also be valuable.

In conclusion, we show here that self-reported occupational natural dust exposure is trending towards a significant association with increased reports of bronchitis and emphysema, but not asthma or COPD. Our findings show that Omega-3 FA intake is associated with decreased bronchitis reports, but only in individuals with no previous natural dust exposure, suggesting an overactive inflammatory response in individuals with repetitive natural dust exposure that cannot be resolved with Omega-3 FA intake. Overall, our findings provide important information on the detrimental effects of repetitive natural dust exposure on the health of the lungs, specifically on disease prevalence.

Moreover, we show here that EPA+DHA Omega-3 FA acids can potentially be protective against lung diseases, however, protection is dependent on overall previous exposure to natural dust. These findings contribute to the overall understanding of the consequences of dust exposure in lung health, and aid in the development or preventative or therapeutic strategies against chronic lung diseases.

Author Contributions: Conceptualization and methodological strategizing was done by T.M.N. and C.H. Data curation was done by J.M. Formal analysis was done by T.M.N., C.H., J.M., and S.G. Original draft preparation was done by S.G. All authors have read and agreed to the pub-lished work.

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Institutional Review Board Statement: The National Health and Nutritional Examination was approved by the National Center for Health Statistics Institutional Review (Protocols #2005-06 and #2011-17 covering the periods of 2007-2012 from which our studies were derived). IRB ap-proval and conduct details can be found online at cdc.gov/nchs/nhanes/irba98.htm.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Study details are available online at cdc.gov/nchs/nhanes/irba98.htm.

Data Availability Statement: The NHANES dataset is publicly available online, accessible at cdc.gov/nchs/nhanes/index.htm.

Conflicts of Interest: The authors declare no conflicts of interest.

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IV. CONCLUSIONS

In conclusion, workers in the agricultural industry are more susceptible to developing chronic lung conditions, such as COPD due to their exposure to harmful particulate matter. Thus, studies investigating the inflammatory response to these dust particles are crucial to our overall understanding of the underlying mechanisms responsible as well as in the aid of the development of therapeutic drugs for patients with obstructive lung diseases. Exposure to DE elicits a potent inflammatory response *in vitro* and *in vivo*, and results in a striking increase of LCN-2. LCN-2 is an innate protein most known for its role in preventing bacterial growth by serving as an iron scavenging protein.

Limitations in this field include limited studies looking at the role of LCN-2 in inflammation induced by pathogens other than bacteria. Additionally, since LCN-2 plays paradoxical roles in inflammation, more studies looking at the role of LCN-2 in both pro- and anti-inflammation are necessary. LCN-2, as seen under many different inflammatory settings, increases significantly in the lungs in response to DE. The levels of LCN-2 at basal and under inflammatory conditions are striking and suggest an important role of LCN-2 in regulating lung homeostasis as well as the inflammatory response to DE. The findings show that ablation of LCN-2 leads to a disruption of homeostasis in the lungs and in deficits in recovery from inflammatory pathology due to exposure to DE.

Given that LCN-2KO mice had deficits in recovery and more severe lung pathology, our studies also included experiments where we administered IL-10

during the recovery period, a cytokine most known for its role in anti-inflammation. Administration of IL-10 resulted in a reduction of the inflammatory response and pathology in LCN-2 KO mice.

Lastly, nutrients such as Omega-3 fatty acids have been shown to improve outcomes in people with COPD. Using the NHANES 2007-2012 cohorts, we showed at the population level an association between natural dust exposure to increases in bronchitis diagnosis. Moreover, we showed that EPA+DHA intake can modify the outcome of bronchitis.

Overall, these findings suggest an important and protective role of LCN-2 in the lung immune response to hog dust extracts. This protection is in part due the role that LCN-2 seems to play on IL-10 levels and emphasizes the importance of additional studies investigating the cross-talk of LCN-2 with IL-10. The findings also highlight the importance of other factors that can mediate the inflammatory response to DE, such as SPMs derived from Omega-3 fatty acids. The findings help support the hypothesis that LCN-2 plays a protective role in the inflammatory response to dust, but also emphasizes the importance that nutrients play in mediating protection or recovery from inflammation.