TITLE PAGE

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concentrated animal feeding operations (CAFOs)
chronic obstructive pulmonary disease (COPD)
protein kinase C (PKC)
protein kinase A (PKA)
dust extract (DE)
tumor necrosis factor-alpha converting enzyme (TACE or ADAM 17)
amphiregulin (AREG)
epidermal growth factor receptors (EGFR)
bronchoalveolar fluid (BALF)
bronchus associated lymphoid tissue (BALT)
diacylglycerol (DAG)

ABSTRACT

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Farmers and workers in concentrated animal feeding operations (CAFOs) experience work-related respiratory disease, particularly chronic bronchitis and chronic obstructive pulmonary disease (COPD). Although multiple substances in CAFOs may contribute to disease, dust from these facilities is well recognized as an important respiratory health hazard. Our previous work has been focused on defining mechanisms by which CAFO dust results in lung inflammation. Importantly, we have identified three critical elements of this CAFO dust-induced lung inflammation mechanism that we propose make excellent therapeutic targets for treatment of this important occupational lung disorder: 1) cytokine release, focusing on the TNF-alpha–dependent airway epithelial cell release of IL-6 and IL-8 with sequential activation of the airway epithelial protein kinase C isoforms (PKC), alpha followed by epsilon; 2) the anti-inflammatory effects of the cyclic AMP dependent protein kinase (PKA); and 3) pro-inflammatory proteases as triggers present in CAFO dust.

This proposal outlines how we will use a pre-clinical animal model to decipher the relative value of targeting these three mechanistic elements that may dampen and/or reverse CAFO dust-induced lung disease. Toward this end, we have demonstrated that inhaled dust extract causes respiratory inflammation *in vivo* in a mouse model that has all of the prominent features of the pulmonary disorders seen in persons working in swine confinement facilities. In this renewal we propose a strategy to utilize this mouse model in preclinical studies aimed at determining which of the therapeutic targets outlined above are feasible and efficacious. We hypothesize that: **CAFO dust-induced lung inflammation is treatable by blocking PKC isoform-triggered airway cytokine release, activating PKA and inhibiting dust-derived proteases and their cellular targets.** We will test this hypothesis via three specific aims:

Aim 1: Establish how agents that specifically target TNF-alpha, IL-6, and IL-8 modulate dust extract-induced lung inflammation *in vivo*.

Aim 2: Determine how agents that augment PKA, especially therapeutic beta-adrenergic agonists, dampen dust extract-induced PKC isoform activation and attenuate lung inflammation *in vitro* and *in vivo*.

Aim 3: Determine the importance of proteases in dust extract-induced TNF-alpha/IL-6/IL-8 i*n vitro* and in tissue inflammation *in vivo* and identify potential targets for attenuating the dust extract protease-induced inflammatory changes.

Our proposal is designed to provide pre-clinical cell, lung slice, and animal data that will facilitate translational studies aimed at bringing potential interventions into the workplace.

SECTION ONE

Significant or Key Findings

The specific aims of the project have not changed from the original grant. Based on the known mechanisms of concentrated animal feeding operations (CAFO) dust-induced inflammation, we hypothesized that: **CAFO dust-induced lung inflammation is treatable by blocking protein kinase C (PKC) isoform-triggered airway cytokine release, activating protein kinase A (PKA) and inhibiting dust-derived proteases and their cellular targets.** We will test this hypothesis via three specific aims:

Aim 1: Establish how agents that specifically target TNF-α, IL-6, and IL-8 modulate dust extract-induced lung inflammation *in vivo*.

Aim 2: Determine how agents that augment PKA, especially therapeutic beta-adrenergic agonists, dampen dust extract-induced PKC isoform activation and attenuate lung inflammation *in vitro* and *in vivo*.

Aim 3: Determine the importance of proteases in dust extract-induced TNF- α /IL-6/IL-8 i*n vitro* and in tissue inflammation *in vivo* and identify potential targets for attenuating the dust extract protease-induced inflammatory changes.

Regarding **Aim 1**, a key observation of this year is that repeat experiments with IL-6 KO mice demonstrated that deficiency in IL-6 did not reduce the dust extract (DE)-induced lung inflammation. This work has been submitted for publication along with data from Dr. Poole's lab that showed that IL-6 KO mice were protected from DE-induced bone loss. Furthermore, our colleague, Dr. Poole, has previously made a similar observation with PKC-ε knock out mice despite that fact that PKC-ε is definitively upregulated by DE and initiates the cascade of TNF-alpha/IL-6/IL-8 (1). Collectively, these data suggested to us that DE not only influences the pulmonary inflammatory response but also the repair process after injury and that the absence of IL-6 did not allow repair to be initiated. Because of the central role of DE-induced tumor necrosis factor-alpha converting enzyme (TACE or ADAM 17) in regulating TNF-alpha/IL-6/IL-8, we initiated studies to see if DE-induced TACE also modulates repair molecules. In this context, we now have substantial preliminary data in primary human airway epithelial cells and from BALF from mice exposed to DE in vivo that DE upregulates amphiregulin (AREG) and that the time course of AREG production appears to correlate with repair processes. This work is critical preliminary data for our renewal application which addresses the hypothesis that AREG-mediated signaling following organic dust exposures promotes lung repair activities, and dysregulation of these normative lung repair processes leads to lung disease in agriculture workers. In addition, there is a manuscript under review that describes initial features of repair following DE.

Regarding **Aim 2**, we completed work on the role of beta 2-adrenergic agonists in attenuating DE-induced inflammation in the mouse model and this work has been published (2). Importantly, we have previously reported on the ability of PKA activing agents such as beta adrenergic agonists in attenuating DE-induced inflammatory cytokine release via reducing activity of TACE. Preliminary experiments show that PKA agents modulate DE-induced epithelial cell release of AREG. These experiments support the mechanistic studies regarding the regulation of AREG activities following DE exposure that we propose in our renewal application.

Regarding **Aim 3**, we finished work on the importance of proteases in DE as a component of dust that initiates inflammatory responses *in vitro* and *in vivo*. This work is now published (3). We also continued to expand the work regarding the possibility that the role of proteases in dust may involve DE-induced activation of epidermal growth factor receptors (EGFR) with experiments utilizing the EGFR. We presented an oral presentation at the American Thoracic Society demonstrating that erlotinib, an EGFR antagonist used clinically, attenuates both DE-induced inflammatory cytokines (IL-6/IL-6) as well as AREG which is also an endogenous EGFR agonist in airway epithelial cells and lung fibroblasts in culture as well as in BALF from mice exposed to a single dose of DE *in vivo*. These data contribute to our observations that both TACE and EGFR are central in mediating AREG and we will define mechanisms by which this occurs in our renewal application.

Translation of Findings

The work of this past year has set the stage that organic dust mediates not only lung inflammation but also the recovery from inflammatory injury. Our data continue to support the translational potential of the work in that our data contributes to a better understanding of how beta-adrenergic agonists that are clinically available, diets enriched in PUFA, and EGFR antagonists mediate dust-induced airway. The overall goal of the studies is to use this information to help guide eventual clinical evaluations aimed at helping prevent airway disease or to better treat disease in those workers who have progressed to significant disease. We also

propose that there is potential for monitoring protease content in dust environments of CAFOs to help reduce risk for airway disease.

Research Outcomes/Impact

The major outcomes of this year are reflected in the manuscripts which are found in the publications list below. In addition, because of our continued observations regarding the potent inflammatory effects of dusts from CAFOs, we partner with the NIOSH-funded Central States Center for Agriculture Safety and Health to keep respiratory protection as one of our ongoing outreach efforts. I serve as the PI for outreach for the CS-CASH.

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SECTION TWO (50-page limit)
Scientific Report

Background

(B.1) *Background*:

Respiratory Disease in Swine CAFO Workers

Farmers and workers in animal confinement facilities have increased risk of lung disease, including chronic bronchitis/chronic obstructive pulmonary disease (COPD).[1, 3, 4] Persons working in these facilities experience excessive loss of lung function, even in younger workers.[5, 6] Livestock farmers have been reported to be more likely to have COPD than crop farmers.[1] Increasingly, animals are raised in concentrated animal feeding operations (CAFOs) in the United States and around the world. In the US, over 40% of swine are raised in facilities with > 5,000 animals.[7]

Airway inflammation in persons working in swine facilities has been partially characterized. Normal volunteers without previous swine facility exposure demonstrate a remarkable increase in neutrophils in bronchoalveolar fluid (BALF) in response to acute exposure to a facility. This swine facility dust-triggered lung neutrophilia is associated with an increase in IL-8.[8, 9] In other studies, changes in IL-1α, IL-1β and IL-6 are observed following swine facility dust exposure.[10] In contrast to acute exposure, airway neutrophilia is more variable following chronic exposure in confinement facility workers, with some studies reporting minimal change in the BALF cellularity while other studies observe an increase in both neutrophils and lymphocytes.[11-13] Some studies also associate a systemic inflammatory response with inhalation of swine confinement dust.[10, 14] For example, Wang et al. described an increase in TNF-α and IL-6 in serum of naïve subjects exposed to hog barns for only 3 hours.[10] Our published mouse model of swine facility dust extract exposure has several similarities to inflammatory responses reported in people, including significant increases in neutrophils in BALF and increases in IL-8 homologues, IL-6 and TNF-α. Importantly, the mouse model also shows a very brisk cytokine response with just one exposure (similar to naïve persons exposed to swine facility for the first time) that is somewhat attenuated with repeat exposures (similar to some persons working in swine facilities), but with evidence of persistent inflammation.[2] We proposed to utilize this mouse model in each of our original aims to provide pre-clinical data for future studies aimed at modulating airway inflammation in persons working in CAFOs to attenuate respiratory illnesses and/or reduce risk of airways disease.

Animal Models for Respiratory Disease in CAFO Workers

Animal models provide a valuable tool to help identify important mechanisms underlying essential features of human diseases. We and others have worked to develop models to provide insights into respiratory disease experienced by persons working in CAFOs.[2, 15, 16] During our previous funding period, we developed and published our model that utilizes intranasal dust extract exposure.[2] Our key findings were:

1) A single exposure of dust extract caused marked increases of cytokines including TNF-α, IL-6, KC and MIP-2 (mouse homologues of IL-8) in BALF. These high cytokine levels diminished with repeated exposures after 2 weeks, but still remained significantly increased compared to intranasal saline controls.
2) Total cells in BALF were increased with a single intranasal exposure and continued to increase after 2 weeks of repeated exposure with BALF showing a predominance of neutrophils (PMNs).
3) Lung histology at two weeks clearly demonstrated bronchiolar and alveolar inflammation with peribronchial and perivascular mixed mononuclear cell aggregates (predominantly T cells and macrophages, but also including B cells).
4) We observed increased airway hyperresponsiveness to methacholine following just a single dust exposure that abated after 2 weeks of exposure, suggestive of adaptation.
5) Isolated tracheal epithelium from animals after a single dust extract exposure showed an increase in both PKC-α and PKC-ε activity. At 2 weeks, the PKC- ε isoform remained increased. This confirms our *in vitro* observations of PKC regulation of dust extract-stimulated cytokine release in the mouse model.
6) Alveolar macrophage function from animals exposed to dust extract for 2 weeks was abnormal with reduced phagocytic function and altered cytokine release.

Our model utilizing intranasal exposure of dust extract produced results that are very similar to models using nebulized dust extract[16] and a model where rodents are taken directly into confinement facilities.[15] Compared to other models where histology has been demonstrated, the mixed mononuclear cell aggregates in our model are a new finding related to swine facility dust exposure. However, rats exposed directly to swine

barns showed increased activation of bronchus associated lymphoid tissue (BALT). Our mixed mononuclear cell aggregates provide another histological feature for quantifying tissue inflammation that was useful as we compared effectiveness of specific interventions in this proposal.

CAFO Dust Alters Intracellular Signals: PKC and PKA

Our previous work demonstrated that swine CAFO dust potently stimulates airway epithelial cell IL-6 and IL-8 release that is mediated via activation of protein kinase C (PKC).[17] PKC is an important intracellular serine/threonine kinase in a variety of cell types, serving as an activator of diverse downstream signaling pathways and traditionally thought to be regulated by second messengers diacylglycerol (DAG), lipid, and calcium.[18] PKC consists of a family of isoenzymes that have been identified to co-exist in many cell types[19, 20, 21] and are categorized in three groups. The classical forms (cPKCs: α, β, and γ) are activated by calcium, phorbol ester, and phosphatidylserine. The calcium-insensitive but phorbol ester- and phosphatidylserine-activated isoforms make up the novel isoforms (nPKCs: δ, ε, θ, η, and μ). Additionally, there are phosphatidylserine-dependent but calcium- and phorbol ester-insensitive atypical forms (aPKCs: $\lambda\iota$ and ζ). PKCs are capable of influencing an array of intracellular activities by mechanisms that involve autophosphorylation and translocation, including phosphorylation of PKC isoenzymes by phosphoinositide-dependent protein kinases and tyrosine kinases. In addition, individual PKC isoforms can be targeted to different intracellular compartments by specific protein-protein interactions, and numerous studies implicate specific isoenzymes in mediating diverse responses to PKC activation.[22]

During the previous funding period, we reported that activation of PKC-ε activity of airway epithelial cells *in vitro* by another type of CAFO dust obtained from the air of large cattle feedlots is essential for dust extract-induced IL-6 and IL-8.[23] Furthermore, we have reported that swine CAFO dust extract sequentially augments PKC-α and PKC-ε activation of airway epithelial cells that is dependent on TNF-α release and results in IL-6 and IL-8 release[24]. Others have demonstrated that activation of PKC isoforms of airway epithelial cells are important with other agents that induce airway inflammation such as RSV infection.[25] Thus, strategies aimed at attenuating PKC isoform activation show promise in modulating airway inflammation and other disease processes. Therapeutic agents focused on specific PKC isoforms are under development[26-28] but are not readily available.

Interestingly, we and others have observed that agents that activate another important intracellular signal, cAMP-dependent protein kinase (PKA) often have the ability to attenuate cellular responses regulated by PKC in airway epithelium. For example, swine CAFO dust extract slows airway epithelial cell migration via a PKC-α mechanism[29] whereas agents that activate PKA stimulate epithelial cell migration.[30, 31] Similarly, PKA activation stimulates ciliary beat frequency (CBF) but swine CAFO dust attenuates the ability of PKA activating agents, like beta-adrenergic agonists, to upregulate CBF.[32] Burvall et al. have demonstrated a role for cAMP-mediated inhibition of CAFO dust-induced inflammation using an alveolar epithelial cell line.[33] In Aim 2, we evaluated if PKA activating agents such as beta-adrenergic agonists that are in widespread use for airway disease are able to substantially attenuate CAFO dust extract-induced PKC activation and associated inflammatory responses in both *in vitro* and *in vivo* models.

Components of Swine CAFO dust

CAFOs house several hundred to thousands of animals in close quarters. This is a dusty environment that has air-borne bacteria, molds, ammonia, hydrogen sulfite, and numerous other potential irritants. Endotoxin, a cell wall constituent of Gram-negative bacteria, has long been recognized as an important component in CAFOs and organic dust. Despite the importance of endotoxin in human disease in general, there is no consensus that endotoxin is the major causative agent of respiratory disease in CAFOs. Certainly endotoxin has been associated with respiratory symptoms in workers in some studies.[5, 34] Other studies have not shown a dose-response regarding endotoxin and respiratory disease in this environment.[35, 36] In terms of biologic agents, endotoxin is more easily measured in the environment than other bacteriologic components such as peptidoglycan (and its chemical marker, muramic acid). Our previous work has demonstrated that endotoxin is *not* responsible for CAFO dust extract-induced IL-6 and IL-8 in airway epithelial cells.[17, 23] While the role of endotoxin in inflammatory responses is important and is under investigation by others, we are focused on identifying other agents in the dust that have causative roles in airway inflammation in this environment. It is likely that CAFO dust is a significant respiratory irritant because it is a complex substance with multiple agents that are contributing to airway inflammation.

We have performed a number of analyses of our swine CAFO dusts. Semi-quantitative elemental analysis by inductively coupled plasma-mass spectrometry revealed trace metals (B, Mg, Ti, Mn, Fe, Co, Ni, Cu, Rb, Mo, Zn). However, dust that is heat-inactivated at 120°C (a process that inactivates the biologics and leaves the metals intact) did not result in inflammation or immune changes, indicating that metals and particulates are likely not playing a critical role. Bacterial counts of the dust revealed that approximately 98% of the colonies were Gram-positive bacteria (Staphylococcus, Bacillus, Streptomycetes, Enterococci), while the remaining 2% were Gram-negative bacteria. Mass spectrometry analysis demonstrated that CAFO dust had 10 times more muramic acid (reflecting peptidoglycan) than house dust, elevated levels of 3-hydroxy fatty acids indicating endotoxin (two fold) and no difference in ergosterol (reflecting fungi).[37] Others have also reported the importance of Gram-positive bacteria in swine CAFOs.[38, 39]

The importance of peptidoglycan in CAFO dust is likely related to our observation that dust extract from swine CAFOs augments expression of Toll-like receptor 2 (TLR2) on airway epithelial cells in culture and in lungs from mice with repeated exposures to dust extract.[40] TLR2 is one of a family of receptors that recognize pathogen-associated molecular patterns (PAMP). TLR2 recognizes peptidoglycan, lipoteichoic acid, and zymosan and is thought to be central in cellular responses to Gram-positive bacteria.[41] Interestingly, CAFO dust extract-induced TLR2 expression of airway epithelial cells was dependent on dust extract induced IL-6.[40] TLR2 antagonists are not yet available clinically, but therapeutic agents aimed at IL-6 are available and were utilized in our studies in Aim 1.

In addition to peptidoglycan, our dust characterization studies during this funding period have provided preliminary data that proteases are present in dust extract. Proteases are involved with airway inflammation caused by house dust mites.[42, 43] However, the role of proteases in occupational settings such as CAFOs is poorly defined. In Aim 3, we examined how proteases in CAFO dust mediate cellular and tissue inflammation. Protease inhibitors are a rapidly expanding class of therapeutic agents and could be important potential therapeutic targets in modulating CAFO dust extract-induced inflammation.[44] Furthermore, our preliminary data suggested that proteases in dust extract may be activating epidermal growth factor receptors (EGFRs). EGFRs are known to mediate airway inflammatory responses[45] and to be critical players in the pathogenesis of lung cancer.[46] Therapeutic agents targeted at EGFRs are in clinical use and also represent agents that have potential to be modified for treatment of airway disease induced by CAFO dust.[47]

(B.2) *Significance*: Our proposal was designed as a pre-clinical assessment of potential therapeutic targets aimed at controlling airway inflammation induced by dust extract of swine CAFO facilities. Based on our previous work identifying mechanisms of airway inflammation induced by dust extract, we have selected three specific targets: cytokines induced by CAFO dust extract (TNF-α, IL-6, IL-8), antagonism of cellular PKC with PKA activating agents (beta-adrenergic agonists), and proteases that are present in the dust. Therapeutic agents for these targets are largely already in clinical use. Determining that these targets and agents effectively modulate airway inflammation in our *in vivo* mouse model sets the stage for future translational studies with workers. Although attenuating airway inflammation after it has caused disease is one goal, we may find that a therapeutic agent such as beta-adrenergic agonists could have a role in preventing excessive inflammation before disease has developed. Additionally, determining the role for proteases in dust in this occupational setting may be important not only for identifying a potential therapeutic target, but also for identifying substances that could be monitored more closely in the environment to provide additional protection for workers.

Specific Aims

Farmers and workers in concentrated animal feeding operations (CAFOs) experience work-related respiratory disease, particularly chronic bronchitis and chronic obstructive pulmonary disease (COPD).[1] Although multiple substances found in CAFOs may contribute to disease, dust from these facilities is well recognized as an important respiratory health hazard.

During our first funding period, we focused our work on defining mechanisms by which CAFO dust results in lung inflammation. Importantly, we identified three critical points in the CAFO dust-induced inflammatory cascade to target for treatment of this important occupational lung disorder: 1) **Cytokine release**: IL-6, IL-8 and TNF-α play prominent roles in pulmonary inflammation induced by CAFO dust. Blocking these cytokines will likely result in diminished inflammation. 2) **PKC activation**: CAFO dust is a potent activator of PKC, which signals epithelial cells to release TNF-α, IL-6, and IL-8. Activation of the cyclic AMP-dependent kinase (PKA) can attenuate PKC activation and inflammatory responses caused by the activation of PKC. Therefore, activating PKA will likely dampen inflammation caused by CAFO dust. 3) **Protease activity**: Characterization of the CAFO dust led to the unexpected finding that proteases may act as a trigger of the pro-

inflammatory cascade. Diminishing protease activity may also lead to decreased pulmonary inflammation (Figure 1).

This report outlines work done from our most recent funding (2010-2016), which focuses on how we used a pre-clinical animal model to decipher the relative value of targeting these three points in the inflammatory cascade that may dampen and/or reverse CAFO dust-induced lung disease. Toward this end, we demonstrated that inhaled dust extract causes respiratory inflammation *in vivo* in a mouse model that has all of the prominent features of the pulmonary disorders seen in persons working in swine confinement facilities.[2] We utilized the mouse model in preclinical studies aimed at determining which of the therapeutic targets outlined above are feasible and efficacious. We specifically chose to examine these three elements in the above order because the outcome, inflammation, is the key feature of this disease and lends itself to several treatment opportunities.

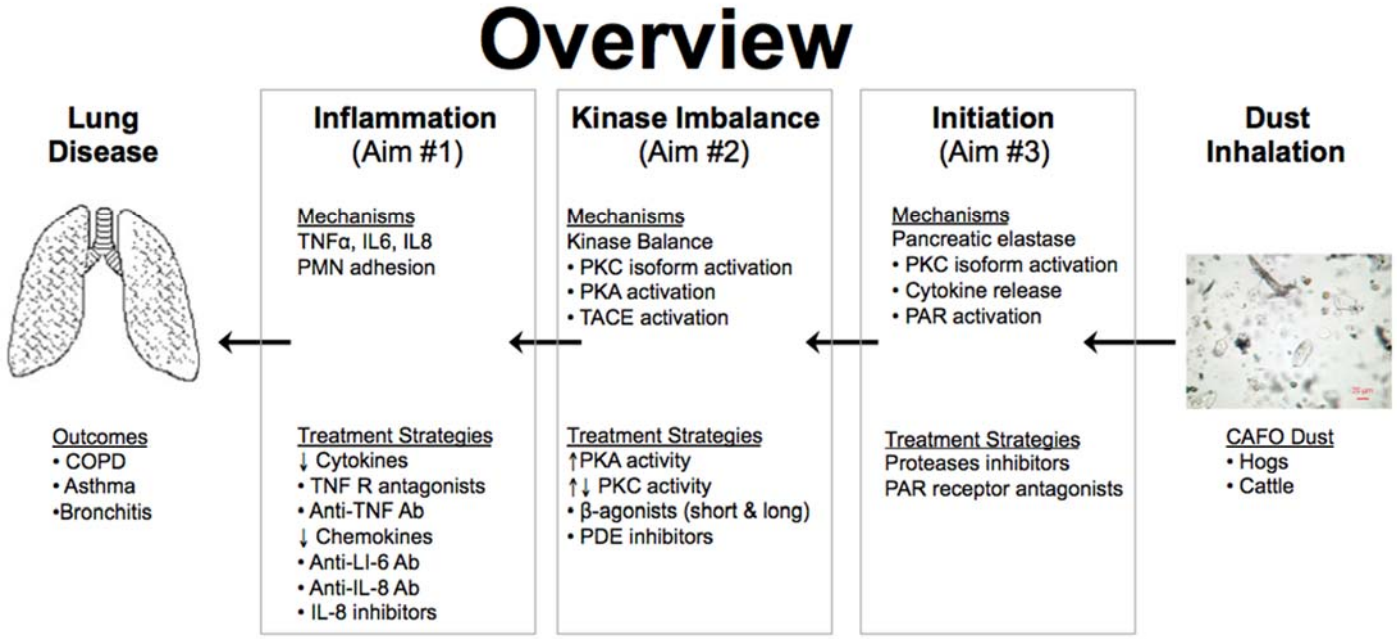
Based on the known mechanisms of CAFO dust inflammation, the hypothesis of this funding cycle was that: **CAFO dust-induced lung inflammation is treatable by blocking PKC isoform-triggered airway cytokine release, activating PKA and inhibiting dust-derived proteases and their cellular targets.** We tested this hypothesis via three specific aims:

Aim 1: Establish how agents that specifically target TNF-α, IL-6, and IL-8 modulate dust extract-induced lung inflammation *in vivo*.

Aim 2: Determine how agents that augment PKA, especially therapeutic beta-adrenergic agonists, dampen dust extract-induced PKC isoform activation and attenuate lung inflammation *in vitro* and *in vivo*.

Aim 3: Determine the importance of proteases in dust extract-induced TNF- α /IL-6/IL-8 i*n vitro* and in tissue inflammation *in vivo* and identify potential targets for attenuating the dust extract protease-induced inflammatory changes.

Our proposal was designed to provide pre-clinical animal and cell data that will facilitate translational studies aimed at bringing promising interventions into the workplace.



Methodology

Preparation of Dust Extract*:* Settled surface dust from hog confinement facilities is obtained for use in these studies as previously published.[2, 17] The dust of each facility is processed separately, and experiments repeated with different sources of dust. We have obtained dust from multiple facilities each having ~500-700 animals. For consistency, samples are collected in the winter. The extract is prepared in a fashion similar to that for grain dust extracts utilized in previously published work as well as by others studying DE.[71, 72] Dust extract is prepared by placing 1 g of dust in 10 ml of Hanks' balanced salt solution (Biofluids, Rockville, MD) without calcium. The mixture is vortexed and allowed to stand at room temperature for 1 h. The mixture is centrifuged for 10 min and the supernatant recovered and centrifuged again. The final supernatant is filter-sterilized with a pore size of 0.2 μM (Nalgene, Rochester, NY) and either used immediately or frozen in aliquots.

Murine Model: As per our standard protocol, mice receive intranasal instillation of 12.5% dust extract or saline control once (single exposure) or once daily for 2 weeks (repetitive exposure).[2] Animal food intake and body weight is monitored to ensure that the changes in lung pathology are not due to weight loss as opposed to actual treatment effects. We have not found changes in dust-exposed WT mice. Side-by-side experiments with WT, WT with relevant pharmacologic agents, and genetically deficient (for Aim 1) mice will be performed. The mice needed are commercially available and all of the genetically deficient mice to be examined are available on C57Bl/6 background that is the strain we have used in our previous work. We performed the experiments with mice 6-8 weeks old.

Bronchoalveolar Lavage (mice): Five hours after the final exposure, mice are sacrificed and BALF fluid is obtained. A tracheotomy is performed and a catheter inserted into the airway. BALF is performed three times (1 mL saline x 3) and recovered lavage fluid are cooled to 4°C, centrifuged, and cell-free first lavage fraction are divided into equal volumes. Aliquots are frozen at -80°C for later assay of cytokines/chemokines. Total cell number from first lavage and pooled lavage are determined by counting on a hemocytometer and differential cell counts done on Cytospin-prepared slides stained with DiffQuik. Counts of the cells determine the differential ratio of cell types in 200 cells per slide per mouse.

Cytokine/Chemokine Assays: Murine TNFα, IL-6, keratinocyte chemoattractant (KC; CXCL1), and macrophage inflammatory protein-2 (MIP-2; CXCL2) concentrations in BALF fluid supernatant are determined according to manufacturer's instructions using commercially available ELISA kits (R&D Systems, Minneapolis, MN). Human TNFα, IL-6, IL-8 from epithelial cell cultures are assayed by ELISA as previously published and routinely performed in our laboratory.[17, 24]

Mouse Lung Histology: To determine histology, the whole lung is excised and inflated to 10 cm H_20 pressure with 4% paraformaldehyde solution to preserve the pulmonary architecture. The inflated lungs are embedded in paraffin, stained with hematoxylin and eosin. The semi-quantitative histology and image analysis was performed by Dr. William West, Co-investigator, as previously published [2]. These parameters include: 1) alveolar compartment inflammation, 2) bronchiolar compartment inflammation, and 3) intrapulmonary mononuclear cellular aggregates. Each parameter is independently assigned a value from 0-3 (the greater score the greater the inflammatory changes in the lung). Slides were coded and Dr. West was blinded to the coding of all slides.

Immunohistochemistry of lung tissue: We have previously reported novel mononuclear cellular aggregates after 2 weeks of repetitive dust exposure. These aggregates are heterogeneous, but predominately characterized as T-lymphocytes and macrophages, and to a lesser degree B-lymphocytes.

Formalin-fixed, paraffin-embedded sections of 4-5 µm-thick tissues are deparaffinized through 2 exchanges of xylene, and rehydrated using a graded series of alcohol washes (100%, 95%, 80%, 50% ethanol) and rinsed twice in PBS. Antigen unmasking is performed using heat-induced epitope retrieval method.[73] Slides are immersed in pre-heated antigen retrieval solution (DIVA Decloaker solution; Biocare Medical, Concord, CA) and steamed for 30 minutes at 95°C in a vegetable steamer. After cooling, slides are rinsed with TBST washing buffer (Tris-based with sodium chloride and Tween-20). Endogenous peroxidase activity is quenched with 3% hydrogen peroxide for 15 minutes. After washing, slides are blocked for 30 minutes in a humidity chamber in 10% normal goat serum or 10% normal rabbit serum (Vector, Burlingame, CA) before application of primary antibodies. Slides are incubated with the following primary antibodies for one hour in humidity chamber: rabbit anti-CD3 (Pan-T-cell marker; dilution 1:300, Dako; Carpinteria, CA), rat anti-CD45R/B220 (Pan-B cell marker; clone RA3-6B2, dilution 1:200; BD Pharmigen; San Jose, CA), anti-Mac-3 (Mononuclear phagocytes; clone M3/84, dilution 1:50; BD Pharmigen; San Jose, CA).

After washing, slides are incubated with appropriate biotinylated goat-anti-rabbit IgG (dilution 1:500) or biotinylated anti-rat IgG mouse absorbed (dilution 1:500) secondary antibody in humidity chamber. Biotinylated antibodies were purchased from Vector. After one hour, slides are rinsed and primary antibody binding was detected using the avidin-biotin-immunoperoxidase method (Vectastain Elite ABC ready-to-use kit; Vector). Chromogen substrate (IMMPACT DAB; Vector) developer was used and slides were counterstained with 1% Meyers Hematoxylin. Slides were dehydrated through a series of ethanols, fixed with xylene,

Airway hyper-responsiveness measurements: Airway hyperresponsiveness (AHR) is measured by the linear first-order compartment model, an invasive model. This model provides dynamic resistance such that

increased resistance values signal constriction of the lungs. Mice are anesthetized with a ketamine (166 mg/kg) and xylazine (8 mg/kg) cocktail, tracheostomized with a steel 18-guage cannula, and mechanically ventilated at a rate of 160 breaths/min, and tidal volume of 0.15 ml, using a computerized small animal ventilator (Finepoint; Buxco Electronics, Wilmington, NC) as previously described [2 75]. Mice are allowed to stabilize on the ventilator for 5 minutes before measurements commenced.

We have found that three hours following intranasal instillation of dust extract is the optimal time point to measure changes in respiratory mechanics following exposure condition. In these studies, dose-responsiveness to aerosolized methacholine (1.5–48.0 mg/ml) will be obtained and reported as total lung resistance (R_L). These studies are conducted in conjunction with Dr. Todd Wyatt's laboratory.

Human Airway Epithelial Cell Cultures: We use primary human bronchial epithelial cells obtained from the core cell facility coordinated by Dr. Bailey. Lungs from human donors rejected for transplant are obtained through the International Institute for the Advancement of Medicine (IIAM). The IIAM is a non-profit organization to facilitate the recovery and placement of donated non-transplantable human organs and tissues with researchers worldwide. This research has approval from the IIAM ethics committee as well as the University of Nebraska Medical Center IRB.

Donor lungs are accepted from males and females under the age of 65, who are life-long non-smokers, are free from pulmonary disease, and have been on the ventilator <3 days. We do not accept specimens from individuals with known infection with human immunodeficiency virus, hepatitis B, hepatitis C, or tuberculosis.

The lungs are shipped on ice in UW (University of Wisconsin) or HTK (Histadine-Tryptophan-Ketoglutarate) solution, and processed within 24 hours of procurement. The first step in isolating primary airway epithelial cells is dissecting out the mainstem, lobar and segmental bronchi. Once the airway is dissected, it is slit longitudinally and cut into 1-2 cm segments. Mucous is removed from the airway by immersing the airways in Dithiothreitol (0.5μg/ml) and DNAse (10μg/ml) for 20 minutes. The airways are then washed 3 times and submerged in a digesting solution containing: DNase (10μg/ml), Protease (0.1%), Fungizone (2% v/v) and Pen/Strep (2% v/v). The airways are placed on a shaker at 4°C. After 48 hours, 10% fetal bovine serum is added to stop the protease/DNAse action. The airway epithelial cells are carefully scraped off the luminal side of the tissue. The resulting cells are centrifuged then washed in cold PBS and plated on collagen-coated plates in Bronchial Epithelial Growth Media (Lonza). Cells are grown to near confluence before being used in experiments.

Lung Slice Preparation: Mice are sacrificed by sodium pentobarbital injection. Tracheas are cannulated and the lungs are injected with approximately 1.3 mL low melting point agarose followed by 0.3 mL of air. The lungs are then chilled to 4°C. The lung lobes are then dissected and attached to the slicer stand of an EMS OTS-4500 oscillating tissue slicer (Electron Microscopy Sciences, Hatfield, PA). Lung slices (170-240 μm) are then added to DMEM with antibiotics and incubated at 37°C until the agarose has melted from the slice. Media is replaced and the lung slices are then incubated at 37°C until used in experiments. Slices are not used for cytokine experiments until maintained in culture for 48 hrs. to eliminate cytokine release related to the slicing.

Neutrophil preparation*:* Peripheral blood neutrophils are obtained from normal volunteers (as outlined in the human studies section) by a sedimentation method using Histopaque 1077 and Histopaque 1119 (Sigma-Aldrich). The resulting cells are consistently >95% viable as assessed by trypan blue exclusion and >95% pure. Neutrophils are labeled with BCECF-AM (Molecular Probes) for 15 minutes at 37°C. The cells are centrifuged and rinsed twice in RPMI medium.

Neutrophil adhesion assay*:* Human bronchial epithelial cells are rinsed three times with RPMI medium after exposure to various agents; fluorescently labeled neutrophils are added at a concentration of $1x10^5$ cells/well and cells allowed to adhere for 30 min at 37°C. Non-adherent inflammatory cells are removed by rinsing the wells one time with RPMI and two times with Dulbecco's PBS. Adherent cells are lysed with 1% Triton X-100 and BCECF fluorescence read on a fluorometer (Fluorolite 1000, Thermo LabSystems Inc.) with an λ_{ex}= 490 nm and λ_{em}= 530 nm. Fluorescence values are converted to number of adherent cells per well using a standard curve of fluorescently labeled neutrophil or lymphocyte cell numbers vs. fluorescence intensity. We have routinely performed the lymphocyte adhesion assay in our laboratory[48] and have modified it slightly for use with neutrophils.

Western Blotting: Lung homogenates will be used for analysis as needed, especially to examine TLR2 protein. The concentration of protein in each sample is determined using the Bradford method. Total protein lysate (50 μg) is resolved in a 10% polyacrylamide gel. It is then electroblotted onto nitrocellulose using semi-dry transfer at 10V for 45 minutes. The blot is blocked for at least 2 hours in 5% blotto w/v in PBS at room temperature. The blot is probed with rabbit anti-human primary antibody in 5% blotto overnight at 4°C. The blot is then washed 5 times in PBS with 0.5% Tween and incubated in a dilution of HRP-conjugated secondary antibody in 5% blotto for 2 hours at room temperature. The blot is then washed 5 times in PBS-Tween and developed using Pierce SuperSignal kit (ThermoScientific) The blots are then exposed to X-ray film and developed. Beta-actin is used as a loading control. Densitometry is performed using ImageJ software (Downloaded from http://rsbweb.nih.gov/ij/).

PKC Activity: PKCα and PKCε activity are determined in whole lung tissue including lung slices, isolated epithelial cells from murine trachea, and human airway epithelial cells in culture. Epithelial cells are isolated from trachea after whole lung lavage by exposing the lumen of the trachea and gently scraping off the epithelial cells by sterile cell lifter (Fisher Scientific) as previously described.[76] [77] The whole lung and the epithelial cell lysate are placed in 50 mM Tris-HCl (pH 7.4) lysis buffer with protease inhibitors as previously described[78] and then immediately flash-frozen in liquid nitrogen and stored at -80°C until assayed for PKC activity.

PKCα and PKCε activity is determined as previously described.[76] [77] Briefly, epithelial cell lysates and whole lungs are sonicated, centrifuged, the supernatant removed (cytosolic fraction), and the pellet resuspended in cell lysis buffer containing 0.01% Triton X-100 and sonicated again (particulate fraction). To measure PKC isoform activity specifically, 24 μg/mL PMA, 30 mM dithiotreitol, 150 μM ATP, 45 mM Mg-acetate, PKCα or PKCε isoform-specific substrate peptide (Calbiochem, San Diego, CA) and 10 μCi/mL [γ-^{32}P]-ATP are mixed together to form an activation. Chilled cell lysate (cytosolic or particulate) samples are added to the activation mix and incubated. This mixture is then spotted onto P-81 phosphocellulose papers (Whatman, Clinton, NJ) to halt incubation, and papers subsequently washed with phosphoric acid (75 mM) for 5 minutes, washed once in 100% ethanol, dried, and counted in nonaqueous scintillant (National Diagnostics, Atlanta GA). PKC activity is expressed in relation to the total amount of cellular protein assayed as picomoles of phosphate incorporated per minutes per milligram. PKC activity is reported as fold-increase from baseline.

Flow cytometry for ICAM-1: Epithelial cells are grown to 90% confluency on type I collagen-coated 6-well tissue culture plates. Epithelial cell monolayers are pretreated with various pharmacologic agents for 30 -60 min., rinsed once, and then challenged with 5% dust for 24 hours at 37ºC. Cell layers are then rinsed once before being removed from the plates by treatment with 0.025% trypsin/EDTA, followed by soybean trypsin inhibitor (*Invitrogen*, Grand Island, NY). Cells are washed twice more, counted, and cell pellets (1 x 10^6 cells) are resuspended in 1 mL PBS containing either 20mL of phycoerythrin conjugated (rabbit) anti-human CD 54 antibody or PE conjugated pre-immune rabbit IgG_2 (*Biolegend*, San Diego, CA), and incubated at 4ºC for one hour. After immunostaining, cells are washed twice, and fixed in 1% paraformaldehyde/PBS before being analyzed on a FACSCalibur flow cytometer (*Becton Dickinson*, San Jose, CA). Forward and side-scatter morphological gates are set to select the most homogeneous population of viable cells, and routinely omitted less than 10% of the total population. Ten thousand gated events are analyzed for each experimental condition, and the data were analyzed using Cellquest Pro software (*BD* SJ, CA).

Research Design

Aim 1: We used the same basic design to evaluate each sub-Aim determining how TNF-α, IL-6, and IL-8 impact inflammatory responses to dust extract. Experiments were done in cultured airway epithelial cells, mouse lung slices and in our mouse model of DE-induced inflammation. As per our standard protocol, mice received intranasal instillation of dust extract (12.5%) or saline control once (single exposure) or daily for 2-3 weeks (repetitive exposure). Animal food intake and body weight was monitored to ensure that the changes in lung pathology were not due to weight loss as opposed to actual treatment effects. Side-by-side experiments with WT mice, WT pretreated with pharmacologic inhibitors, and respective genetically deficient (KO) mice with and without dust extract exposure were conducted. In comparison to pharmacologic inhibitors, WT mice were exposed to the relevant vehicles for each inhibitor. The outcome measures assessed included bronchoalveolar (BALF) lavage for total cell count and differential and cytokine assay (TNF-α, IL-6, KC/CXCL1, MIP-2/CXCL2); lung tissue for histology and immunohistochemistry (including stains for cell markers in mononuclear cell aggregates; ICAM-1 expression); lung tissue for mRNA (TNF-α, IL-6, KC, MIP-2, TLR2, ICAM-1); and airway hyper-responsiveness measurements.

Aim 1a) Role of TNF-α

We used the pharmaceutical recombinant TNF-α receptor fusion protein (etanercept) and monoclonal anti-TNF-α antibody to attenuate dust extract-induced TNF-α, IL-6 and IL-8 of airway epithelial cells *in vitro.*[24] were examined for comparison. We then moved into experiments that utilized the pro-resolvin mediator Maresin-1 and omega-3 polyunsaturated fatty acid (DHA).

Aim 1b) Role of IL-6

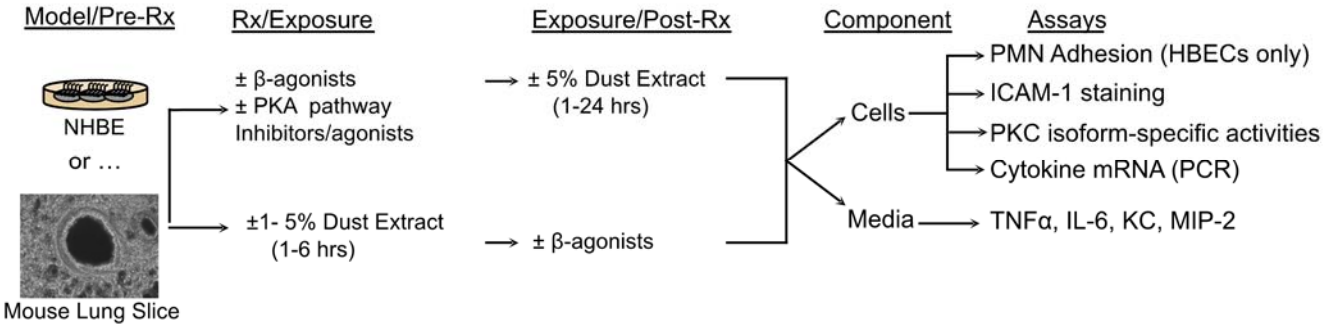
We know that dust extract augments IL-6 release *in vitro* and *in vivo*.[2, 17] In addition, dust extract induced TLR2 expression of epithelial cells is mediated, at least in part, by IL-6 *in vitro.*[40] In order to determine the degree to which various features of dust extract induced inflammation are mediated by IL-6 *in vivo*, we utilized IL-6 KO mice that were on the C57/BL6 mouse strain.

Aim 1c) Role of IL-8

To examine the role of IL-8 in the mouse model, we used antibodies to KC and MIP-2 in combination in mice with and without dust extract exposure. Initial concentrations of antibodies were based on examples from the literature [60]. Since KC and MIP-2 are thought to share a common receptor (a homologue of human CXC chemokine receptor 2), another approach utilized antileukinate, a hexapeptide inhibitor of human IL-8 activity, instead of KC and MIP-2 antibodies, to inhibit HDE-induced lung inflammation.[61] Animals treated with antileukinate alone were included for comparison.

An alternative approach for antagonizing IL-8 was to use a newly developed compound CXCL-8 (3-73)K11R/G31P (G31P) that blocks ELR-CXC chemokine binding to human neutrophils (gift of Dr. John Gordon)[62] with demonstrated effectiveness in a swine model of airway inflammation.[62] This agent targets neutrophils as opposed to targeting the chemokines.

Overview of Aim 2a



Aim 2: In this aim, we determined how beta-adrenergic agonists attenuate dust extract-induced PKC activity and subsequent inflammatory responses using cultured airway epithelial cells and precision lung slices *in vitro* and determine if beta-adrenergic agonists can attenuate dust-induced inflammatory changes *in vivo* in our mouse model.

Aim 2a) Determine how beta-adrenergic agonists attenuate dust-induced PKC activity and subsequent inflammatory responses using cultured airway epithelial cells and precision lung slices *in vitro.*

Aim 2ai) Do beta-adrenergic agonists inhibit PKC-mediated dust extract induced cytokine release, ICAM-1 expression, and neutrophil adhesion?

Cytokine release: We extended our earlier data that beta-adrenergic agonists attenuate airway epithelial cell PKC-mediated dust extract induced TNF-α, IL-8 and IL-6 release. We did this using both human bronchial epithelial cells in culture and precision mouse lung slices in culture. The lung slice model allows us to determine whether beta-adrenergic agonists remain effective in attenuating dust extract-induced TNF-α, IL-8 (KC and MIP-2 in mice), and IL-6 when epithelial cells are in relationship with other mesenchymal cells.

Human bronchial epithelial cells were cultured in growth medium (without epinephrine which can influence beta adrenergic receptors, ßAR) until nearly confluent. We used primary human bronchial epithelial cells obtained from rejected lung transplant donor lungs provided by Co-investigator, Dr. Bailey. Cells were exposed to beta-adrenergic agonists salbutamol, salmeterol, formeterol, and isoproterentol for 30-60 minutes. Cell layers were washed and then exposed to dust extract (5%) in medium that also included the beta agonists for several time points between 1-24 hours. Our previous work demonstrates that dust extract augments PKC alpha activity within 1 hour and returns to baseline by 2 hours whereas PKC epsilon activity is maximally augmented at 6 hours[24] (Figure 2). Cell culture supernatants were harvested for TNF-α, IL-8 and IL-6 measurements by ELISA. Cell layers were harvested to assay for ICAM-1 by flow cytometry or PKC isoform

activity, focusing on PKC-α and ε that we have previously demonstrated are altered by dust extract exposure. PKC isoform assays (beta, delta and zeta) were performed to assess the specificity of alpha and epsilon action and to ascertain whether beta-adrenergic agonists might have effects on other PKC isoforms present in epithelial cells. For comparison, cells exposed to control medium alone, 5% dust extract alone, and the beta-adrenergic agonists alone were included.

We also assessed whether beta-adrenergic agonists can attenuate dust-induced epithelial cell PKC activity and subsequent cytokine release when cells are exposed *first* to dust extract and then to beta adrenergic agonists by treating nearly confluent monolayer cultures with 1% and 5% dust extract for 1-6 hours, rinsing cell layers, and then subsequently exposing cell layers to various concentrations of beta adrenergic agonists for various time points. Cell layers were harvested and assayed for PKC isoform activity and cell supernatants harvested for cytokine measurements.

We also used the precision lung slice model to assess the modulation of dust-induced cytokine release. The lung slices were prepared from C57Bl/6 mice as these are the mice utilized in our initial dust-extract exposure model. Three lung slices were used per condition in each experiment (approximately 30 lung slices obtained per mouse). Lung slices were exposed to beta-adrenergic agonists for 30-60 minutes in media used to maintain lung slices in culture (DMEM with antibiotics). Media was changed to include 5% dust extract for 24-48 hours with beta-adrenergic agonists. The lung slice supernatants were collected and assayed for mouse TNF-α, IL-6, and KC and MIP-2 (CXCL-1 and CXCL-2, mouse homologues of human IL-8). The lung slices were harvested for measuring total protein in tissue and for extracting RNA to evaluate cytokine expression at mRNA level. Comparison conditions included lung slices exposed only to control medium, to beta-adrenergic agonists alone, and dust extract alone. We also examined lung slices that were exposed first to dust extract and then to beta-adrenergic agonists to determine if these agents can also attenuate cytokine release after dust extract exposure. We will first establish the concentrations of beta-adrenergic agonists and the time course for changes in cytokine expression in response to dust using the lung slices. We also determined whether or not repeated exposures to beta-adrenergic agonists have a similar effect on dust extract-induced cytokine expression since lung slices can be maintained in culture for up to 2 weeks with similar functional responses.

We also included lung slices in these experiments that were harvested to measure PKC activity including activity of PKC isoforms in the tissue. This allowed us to correlate changes in dust extract-induced tissue response with PKC isoform activity to determine whether this is similar in tissue involving several cell types as seen in the epithelial cell cultures.

ICAM-1 expression and neutrophil adhesion: In addition to assessing cytokine release and mRNA expression in human airway epithelial cells, we also performed experiments with similar exposure conditions to beta-adrenergic agonists and dust extract as described in the preceding cytokine section and harvest cell layers and evaluate for ICAM-1 (CD54) expression using flow cytometry as routinely performed in our laboratory[48]

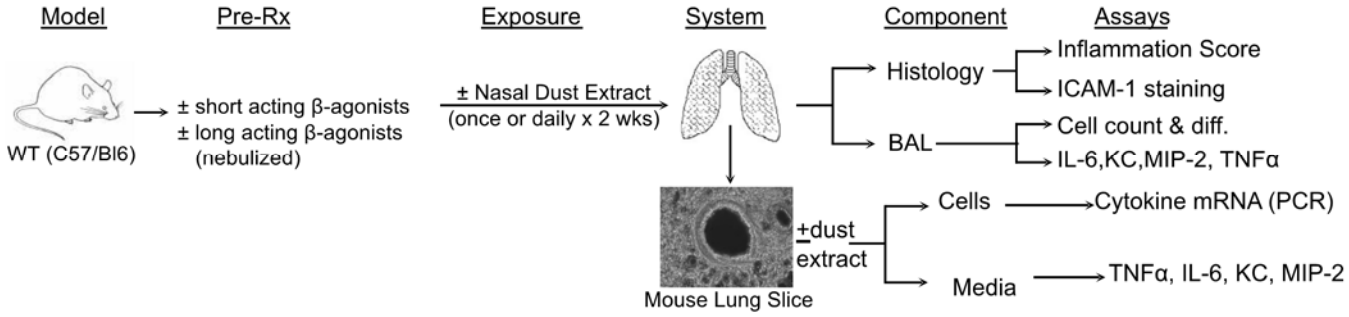
We also evaluated the effect of beta-adrenergic agonists on dust-induced neutrophil adhesion to epithelial cells. Using an adhesion assay demonstrated, airway epithelial cells were pretreated with beta adrenergic agonists for 30 minutes, washed, then exposed to dust extract with agonists for 24 hours. Epithelial cells were then washed, and co-cultured with neutrophils for 30 minutes. Neutrophils were obtained from normal volunteers and labeled with vital fluorescent chromogen calcein AM prior to co-culture. After co-culture, non-adherent neutrophils were washed from plates and adherent neutrophils with epithelial cells were removed and fluorescence measured via spectrophometer. We also evaluated the effect of exposing neutrophils to beta-adrenergic agonists (as opposed to epithelial cells) immediately prior to the adhesion assay to make sure that changes in neutrophil adhesion is not due to modulation of neutrophil function as opposed to epithelial cell function.

Aim 2aii) Confirm the role of the beta-adrenergic receptor (ßAR) and the other key downstream mediators of the PKA pathway (ßAR→G_s→adenylyl cyclase→cAMP→PKA) in beta-agonist attenuation of dust extract-induced cytokine release.

We confirmed that the effect of beta-adrenergic agonists in attenuating dust extract-induced cytokine release (TNF-α, IL-8, and IL-6) of airway epithelial cells and mouse lung slices is indeed acting through beta-adrenergic receptors and involves the downstream mediators that would be expected with ßAR stimulation. Although it is expected, it is important to ascertain that beta-adrenergic agonists are not mediating changes in dust extract-exposed cells and tissues through non-specific mechanisms.

We did these experiments utilizing the optimal experimental conditions for beta-adrenergic agonist attenuation of cytokines in the two systems in Aim 2ai and focused on the outcome measure of cytokine release and associated PKC isoform activity.

Overview of Aim 2b

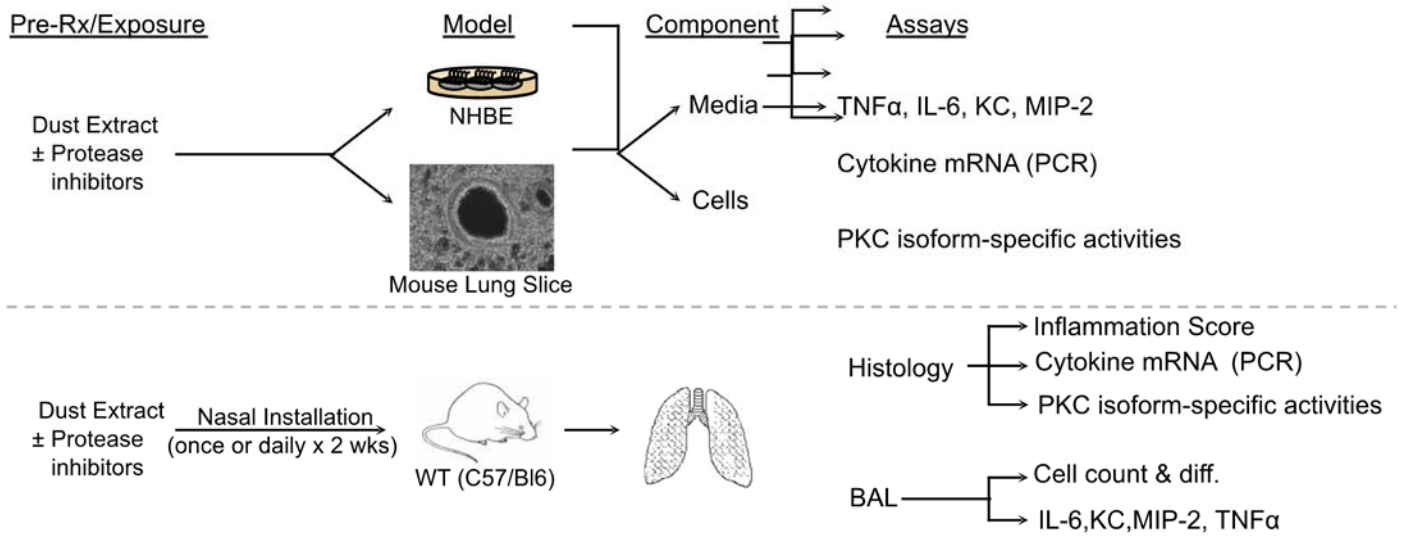


Aim 2b: Determine in vivo effectiveness of beta-adrenergic agonists in regulating dust extract-induced inflammatory responses in the mouse model.

Similar to experiments in Aim 1, we exposed C57Bl/6 mice with a single instillation and once daily for 2-3 weeks of 12.5% dust extract intranasally. Thirty minutes prior to each dust extract exposure, groups (n=7) of mice were exposed to beta-adrenergic agonists using nebulized agents. We will use one longer acting beta-adrenergic agonist (salmeterol) and one shorter acting agent (salbutamol/albuterol). Another sham group of mice were exposed to the inhalation chamber but without beta-adrenergic agonist exposure. We also included a group of mice exposed to PBS intranasally instead of dust-extract and exposed to beta-adrenergic agonists.

Similar to Aim 1, we euthanized animals after the dust extract exposure at 1 day and 2 or 3 weeks to obtain BALF fluid for cell count and cytokines and inflate and preserve lung tissue for histology assessments. We obtained tracheal epithelium to use to measure PKC isoform activity and lung tissue for mRNA. We harvested the lung of one mouse in each experimental condition to obtain lung slices to culture *ex vivo*. We were then able to compare cytokine release in lung slices of animals exposed to dust extract to lung slices only exposed to dust extract *ex vivo* as described Aim 2a. This allowed us to determine how well the lung slice model replicates *in vivo* effects of beta-adrenergic agonists on dust extract induced cytokine release.

Overview of Aim 3a



Aim 3 a: Determine the importance of proteases in dust extract inducing TNF- α/IL-6/IL-8 release *in vitro* and in the mouse model *in vivo.*

We inhibited proteases in dust extract using known inhibitors of serine proteases, phenylmethylsulfonyl fluoride (PMSF) and 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), that have been used to attenuate house dust mite protease stimulation of epithelial cell IL-8.[67] The porcine pancreatic elastase that is present in the dust extract is a serine protease and should be inhibited by these agents. Our strategy was to pretreat the dust extract with various concentrations of PMSF and AEBSF to destroy serine proteases and then use this "protease-inactivated" dust extract in human airway epithelial cell cultures and mouse lung slice cultures. We

also mixed the protease inhibitors with cell culture media without dust extract for comparison. Experimental conditions also included dust extract without protease inhibitors and control medium. The half-life of PMSF is about 110 minutes, so we allowed PMSF to interact with dust extract for at least 6-8 hours prior to placing dust extract+PMSF on cells or tissues so that PMSF is no longer active. AEBSF is similar to PMSF, but more water-soluble. We evaluated aliquots of dust extract treated with PMSF and AEBSF by zymography to confirm that protease activity has been depleted.

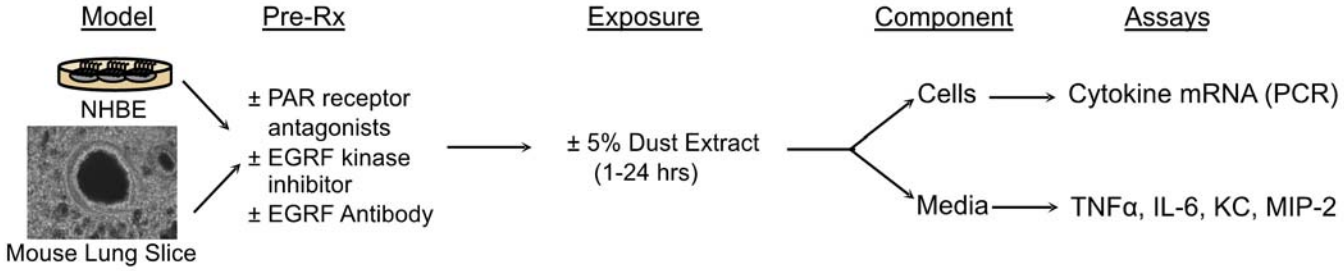
Human airway epithelial cells in culture and mouse lung slices in culture were exposed to dust extract pretreated with protease inhibitors for up to 24 hours. Culture supernatants were assayed for TNF- α, IL-6, and IL-8 (KC and MIP-2 from mouse lung slices). Cell layers were harvested for cytokine mRNA and for PKC isoform activity. For comparison, we evaluated the ability of commercially available elastase antibodies to inhibit the effects of "complete" dust extract exposure. We measured cytokine release and mRNA to determine whether blocking elastase has a similar effect as using the protease-inactivated dust extract.

To determine the importance of proteases in dust extract inducing lung inflammation *in vivo*, we used the same protease inactivation protocol with dust extract prior to exposing mice to dust extract intranasally. We determined the appropriate concentrations of protease inhibitors to use to neutralize dust extract proteases from the epithelial cell cultures and mouse lung slices experiments. Significant quantities of protease-inactivated dust extract were made at one time and aliquoted and frozen. The aliquots were thawed immediately prior to using for intranasal instillations. Mice were exposed to protease-inactivated dust extract for a single exposure or daily for 2-3 weeks as previously described in Aim 1 and 2. Similar outcome measures were assessed including BALF cell count and differential, BALF cytokines (TNF- α, IL-6, KC and MIP-2), lung histology, lung tissue for mRNA, and tracheal epithelial layers for PKC isoform activity. For comparison, groups of mice were exposed to usual dust extract, saline mixed with protease inhibitors, and saline alone.

Aim 3b: Determine potential cellular targets for attenuating dust extract protease-induced inflammatory changes.

We used our *in vitro* assay systems of human airway epithelial cell cultures and mouse lung slices to determine what receptors are involved with dust extract protease-mediated TNF-α /IL-6/IL-8 release as the first step in identifying relevant therapeutic targets. We determined that protease-activated receptors (PARs) are involved in mediating dust extract cytokine release. Alternatively, proteases in the dust are also involved with dust extract-induced activation of EGFRs that we have demonstrated in our preliminary data for the grant.

Overview of Aim 3b



Aim 3bi: Determine the role of protease-activated receptors (PARs) in mediating dust extract induced epithelial cell and mouse lung slice TNF-α /IL-6/IL-8.

PARs are G protein-coupled receptors known to be critically involved in mediating the activity of many proteases. Bronchial epithelial cells express PAR1, 2, 3, and 4[68] and PAR1 and PAR2 are central in mediating house dust mite protease induced IL-8 in airway epithelial cells.[67] We pre-treated primary human airway epithelial cells or mouse lung slices with commercially available antagonists to PAR1, PAR2, and PAR4 receptors for 30 minutes and then expose cells to 5% dust extract or control medium for up to 24 hours. Cell culture supernatants were assayed for TNF-α/IL-6/IL-8 by ELISA and cell layers harvested for evaluation of mRNA by quantitative RT-PCR. The PAR antagonists were used at concentrations reported to be effective in the literature. In addition to exposing cells to each antagonist separately, we evaluated combinations of the antagonists that show an effect on inhibiting dust-induced cytokine release or mRNA as others have shown that PAR 1,2 and 4 agonists additively augment IL-6 release and PAR 1 and 2 both augment IL-8 release.[68]

As an alternative approach, we utilized bronchial epithelial cells and transfect with siRNA to PARs that appear to be involved in dust extract induced cytokines. Our colleague Dr. Liu has experience with siRNA with airway epithelial cells.[69, 70] After differentially knocking down the relevant PARs, alone or in combination, we will

expose cells to 5% dust extract for up to 24 hours and then harvest cell culture supernatants for assay for TNF-α /IL-6/IL-8. Control siRNAs will be used for comparison.

Aim 3bii: Determine the role of EGFRs in mediating dust extract protease-induced TNF-α /IL-6/IL-8. Our preliminary data demonstrates that dust extract is capable of activating EGFRs and that inhibiting EGFRs attenuates IL-6/IL-8 release from cells. However, we have not definitively determined that proteases in dust extract are responsible for activation of EGFRs with associated changes in TNF- α/IL-6/IL-8. In these experiments, we used human airway epithelial cells in culture and mouse lung slices. We pretreated cells and slices with the EGFR kinase inhibitor AG1478 and with anti-EGFR antibody (commercially available) ± complete dust extract or protease-inhibited dust extract as prepared in Aim 3a. We harvested culture supernatants to assay for cytokines by ELISA. Control conditions included cells or tissues treated with anti-EGFR antibody without dust extract and control IgG.

Results and Discussion

Aim 1: We initially evaluated the role of TNF-alpha in more detail in our *in vitro* epithelial cell model to better understand how DE regulates TNF-alpha production in the airway. We chose this approach to determine if a more specific target than TNF-alpha itself could be an appropriate means of controlling TNF-alpha. We demonstrated that DE augments TNF-alpha converting enzyme (TACE) activity of airway epithelial cells that contributes to increased TNF-alpha as well as IL-6 and IL-8 release. Inhibition of TACE by pretreatment of cells with TACE inhibitor (TAPI) inhibits dust extract-induced release of TNF-alpha, IL-6 and IL-8 as well as subsequent epithelial cell ICAM-1 expression and functionally neutrophil adhesion to epithelial cells. Interestingly, in collaboration with Dr. Wyatt, we observed that pretreatment of airway epithelial cells with ethanol also reduces dust extract-induced TNF-alpha, IL-6 and IL-8 release along with ICAM-1 expression and neutrophil adhesion and pretreatment of cells with ethanol inhibits dust extract induced TACE activity. Thus, ethanol exposure can also attenuate airway epithelial dust extract-induced inflammatory responses via modulation of TACE activity. The results of these studies were presented at the annual meeting of the American Thoracic Society and subsequently, a manuscript based on this work was published.[79] To better assess the relevance of the alcohol regulation of DE-induced TNF-alpha, we utilized our mouse model. C57BL/6 mice were fed 20% alcohol or water ad libitum for 6 weeks and treated with 12.5% HDE by intranasal inhalation method daily during the final three weeks. DE-induced lung inflammatory aggregates were clearly present in the tissue from DE only exposed animals and were not visually detectable in the HDE/alcohol co-exposure group. Statistically significant weight reductions and 20% mortality were also observed in the mice co-exposed to HDE and alcohol. These data suggest that alcohol exposure depresses the ability of the lung to activate PKCε-dependent inflammatory pathways to environmental dust exposure. These data also define alcohol as an important co-exposure agent to consider in the study of inhalation injury responses. These results have been published.

We next expanded our understanding of dust extract regulation of transcriptional activity of human airway epithelial cells. We chose this approach to better define dust-induced airway inflammation targets that influence the production of cytokines TNF-alpha, IL-8 and IL-6. Our goal was to define the best ultimate targets for regulating these dust-induced cytokines so that we can regulate those targets with agents with potential to translate into clinical use. We first assessed binding at 45 transcription factor binding elements using a luciferase reporter system and demonstrated that the most significant dust-induced airway epithelial cell transcriptional changes involved NF-kB, AP-1, SP-1 and SRE (serum response element). These changes were confirmed using assays specific for each of these transcription factors and support that the potency of CAFO dust in inducing airway inflammation involves multiple pathways. Because of the growing understanding of the importance of poly-unsaturated fatty acids (PUFA), found in common foods and over-the-counter supplements that are broken down into various pro-resolving lipid meditators *in vivo*, we next evaluated the effect of the pro-resolving mediator Maresin-1 on dust-induced cytokine release and on dust-induced transcription factor activity. We demonstrated that Maresin-1 pre-treatment dose-dependently inhibits dust-induced airway epithelial cell IL-6 and IL-8 release, that the dust-induced transcriptional activity of SRE is specifically reduced by Maresin-1 pretreatment, and that total serum response factor protein (regulated by SRE) is also reduced by pretreatment with Maresin-1. Collectively, these data suggest that the pro-inflammatory response to DE can be abrogated by pre-treatment with Maresin-1 that may be functioning in part through modulating MAPK signaling (downstream of SRE). These data were presented in a poster session at the international American Thoracic

Society meeting. Dr. Tara Nordgren was added as key personnel as a postdoctoral fellow at this time and was centrally involved with this work and the subsequent manuscript.[80]

We next applied the observations regarding Maresin-1 to our mouse model of single and repetitive dust extract exposure. Mice were pretreated with MaR1 intraperitoneally 18 and 0.5 hours before a single intranasal instillation of 12.5% organic dust extract derived from hog confinement facilities. At 5 hours post-exposure, bronchial alveolar lavage fluid (BALF) was collected, and lungs harvested for immunohistochemistry. Low dose MaR1 (0.1 ng) significantly reduced DE-induced TNF-α and IL-6 levels in BALF ($p<0.01$). Higher dose MaR1 (1 ng) significantly lowered levels of KC (murine IL-8 homolog/neutrophil chemoattractant; $p<0.001$). By immunohistochemistry, DE-induced ICAM-1 expression in murine bronchial epithelial cells (BECs) was reduced with MaR1 pretreatment.[81] without altering repetitive DE-induced tissue inflammation or lymphoid aggregate formation typically seen in our animal model. However, lung tissue ICAM-1 expression was reduced in both single and repetitive exposure studies. Thus, MaR1 does modulate some key features, but not all, of dust-induced inflammation. This data has been published.

In order to explore an even more translational approach to modulating pro-resolving mediators than Maresin-1, we have performed experiments using the omega-3 fatty acid docosahexaenoic acid (DHA) which is a common nutritional supplement and which can contribute to production of MaR1 and other lipid mediators *in vivo*. In airway epithelial cells, DHA dose-responsively inhibited DE-induced IL-6 and IL-8 release, and blunted the dust-induced surface expression of ICAM-1. Likewise, DHA markedly reduced the DE-stimulated release of both IL-8 and TNF-alpha from THP-1 monocytes and TNF-alpha from mouse lung slice *ex vivo* cultures. To determine the in vivo significance of DHA, C57BL/6 mice were orally administered DHA for seven days prior to treatment with intranasal ODE or saline inhalations. Animals treated with 2 mg DHA demonstrated significant reductions in ODE-induced bronchial alveolar lavage neutrophil influx and pro-inflammatory cytokine/chemokine production compared to mice exposed to ODE alone. Collectively, these data demonstrate that DHA affects several lung cells to reduce the airway inflammatory response to organic dust exposures. Dietary supplementation with DHA may be an effective therapeutic strategy to reduce the airway inflammatory consequences in individuals exposed to agriculture dust environments. These results have been published.[82]

As a consequence of our finding that MaR1 attenuated DE-induced SRE noted above, we further explored the role of serum response factor (SRF) that is regulated by SRE. We demonstrated that SRF localizes to motile cilia of airway epithelial cells and alters its localization in response to DE. Additionally, inhibition of SRF signaling using the small molecule CCG-1423 reduces organic dust-induced IL-8 release from bronchial epithelial cells, and stimulates cilia beat frequency in ciliated mouse tracheal epithelial cells. Immunohistochemical data reveal SRF also localizes to the cilia of mouse brain ependymal and ovarian epithelial cells as well. These data reveal a novel mechanism by which a transcription factor localizes to motile cilia and modulates cell activities including cilia motility and an inflammatory response. This information challenges current concepts regarding motile cilia functioning, and may lead to significant contributions in understanding motile ciliary signaling dynamics. These observations have been published.[83]

The majority of the above data was done in pursuit of the role of influencing DE-induced regulation of TNF-alpha and replaced our initial plans to use etanercept or TNF-alpha antibodies *in vivo*. To directly address the role of IL-8 in DE-induced inflammatory response, we completed a series of *in vivo* studies. CXCL8 (3–74) K11R/G31P (G31P) is a highly effective ELR–CXC chemokine and neutrophil antagonist. We assessed whether G31P treatment blocks dust extract-induced lung inflammation. Mice were subcutaneously injected with G31P (100 μg/kg) daily for 6 weeks immediately prior to nasal instillation with saline or dust extract. Bronchoalveolar fluid cellular influx and cytokine levels, lung parenchymal histology, and PKC activity in tracheal and whole lung homogenates were assayed. Dust extract resulted in the formation of numerous large foci of peribronchiolar mononuclear cell lesions in the lungs as we have previously reported. Treatment of mice with G31P prevented the formation of these dust extract-induced lung mononuclear foci with only a small amount of peribronchiolar inflammation detected. G31P blocked neutrophil recruitment as detected in the lavage fluid from dust extract-treated mice. G31P treatment decreased dust extract-induced expression of CD54 in bronchial epithelium. Likewise, G31P treatment blocked dust extract-induced levels of keratinocyte cytokine (KC) and interleukin-6 (IL-6) in the lavage fluid. In both precision-cut whole lung slices and tracheal epithelium, G31P treatment of mice blocked dust extract-stimulated PKC alpha and PKC epsilon isoform activity required for chemokine release. These data demonstrate that dust-induced lung inflammation can be

effectively treated by ELR-CXC chemokine antagonists in an *in vivo* mouse model and provides early pre-clinical groundwork for exploring whether there could be a role for such agents in humans. These results were presented at the annual meeting of the American Thoracic Society and subsequently published after further replication of results.[84]

A key observation of this past year regarding Aim 1 is that repeat experiments with IL-6 KO mice demonstrated that deficiency in IL-6 did not reduce the DE-induced lung inflammation. This work has been submitted for publication along with data from Dr. Poole's lab that showed that IL-6 KO mice were protected from DE-induced bone loss. Furthermore, our colleague, Dr. Poole, has previously made a similar observation with PKC-ε knock out mice despite that fact that PKC-ε is definitively upregulated by DE and initiates the cascade of TNF-alpha/IL-6/IL-8.[85] Collectively, these data suggested to us that DE not only influences the pulmonary inflammatory response but also the repair process after injury and that the absence of IL-6 did not allow repair to be initiated. Because of the central role of DE-induced tumor necrosis factor-alpha converting enzyme (TACE or ADAM 17) in regulating TNF-alpha/IL-6/IL-8, we initiated studies to see if DE-induced TACE also modulates repair molecules. In this context, we now have substantial preliminary data in primary human airway epithelial cells and from BALF from mice exposed to DE *in vivo* that DE upregulates amphiregulin (AREG) and that the time course of AREG production appears to correlate with repair processes. This work is critical preliminary data for our renewal application which addresses the hypothesis that AREG-mediated signaling following organic dust exposures promotes lung repair activities, and dysregulation of these normative lung repair processes leads to lung disease in agriculture workers. In addition, there is a manuscript under review that describes initial features of repair following DE.[86]

Aim 2:
We have made progress in affirming our preliminary data regarding the ability of $beta_2$-adrenergic agonists that are used clinically (long acting salmeterol and short acting salbutamol) to attenuate dust extract-induced cytokine release (IL-8 and IL-6), ICAM-1 expression, and neutrophil adhesion to human bronchial epithelial cells in vitro. We have also compared the response of $beta_2$-adrenergic agonists to that of fluticasone (a steroid that is utilized clinically as an inhaled agent) as well as the combined effect of $beta_2$-adrenergic agonists and steroids. These data were presented as a poster presentation at the international meeting of the American Thoracic Society meeting.

We have also confirmed our hypothesis that augmenting protein kinase K (PKA) in dust extract-exposed airway epithelial cells does attenuate the dust extract-induced PKC epsilon activity, but not PKC-alpha activity. We have previously reported that dust extract induced PKC-epsilon is essential for regulating dust induced IL-8 production. Augmenting PKA activity does not attenuate exogenous TNF-alpha induced PKC-epsilon activity. Furthermore, PKA activation and not EPAC activation (exchange protein directly activated by cAMP and K+ channels that are additional downstream mediators of cAMP other than PKA) diminishes dust-induced IL-8 and IL-6, confirming the specificity of PKA on the process. These results have been published.[87]

We completed the *in vivo* work regarding the role of beta-adrenergic agonists in modulating lung inflammation. As expected, mice given DE intranasal in both the single and repeated exposure experiments exhibited a rapid and persistent inflammatory response characterized by a prominent cellular influx into the BALF, with marked accumulation of neutrophils. Mice pretreated with beta-agonist salbutamol prior to DE challenge displayed a significantly blunted inflammatory response in both total cells and neutrophilia (total cells decreased by 71%, neutrophils by 54%, single exposure; total cells down by 82%, PMN by 84%, repeated exposure). These effects were consistent with microscopic evaluation of parenchymal cellularity in representative lung sections after repeated exposures. BALF from mice pretreated with salbutamol before single or repeated DE exposure contained decreased levels of each of the four cytokines measured (IL-6, TNF-alpha, KC, and MIP-2) when compared to mice challenged with DE alone ($p<0.01$ for all comparisons). Immunochemical surface expression of the adhesion molecule ICAM-1 on the bronchial epithelium of lung tissue sections from mice given salbutamol before HDE exposure was reduced in comparison to mice exposed to DE alone.
In the model with repetitive DE exposure, we demonstrated that salbutamol pretreatment was able to significantly reduce bronchoalveolar lavage fluid (BALF) total cells and neutrophils, along with BALF TNF-alpha, IL-6, KC/CXCL1 and MIP-2/CXCL2 induced by DE. In addition, there was an attenuation of alveolar and

bronchiolar compartment inflammation in lung tissue as well as lymphoid aggregates. These results have been published.[88]

Preliminary experiments this past year show that PKA agents modulate DE-induced epithelial cell release of AREG. These experiments support the mechanistic studies regarding the regulation of AREG activities following DE exposure that we propose in our renewal application.

Aim 3: Analysis of dust extracts by zymography demonstrated that the extracts we utilize for all our experiments contains protease activity that is sensitive to protease inhibitors. Treatment of airway epithelial cells with various exogenous proteases or with dust extract induced release of IL-6/8 and TNF-α (5- to 18-fold increases vs. control). These effects were inhibited by pretreatment with PAR receptor antagonists (32 to 45% decreases vs. DE alone. Human bronchial epithelial cells and mouse lung slice cultures stimulated with 5% HDE released significantly more of each of the cytokines measured (IL-6, IL-8, TNF-α, keratinocyte-derived chemokine/CXC chemokine ligand 1, and macrophage inflammatory protein-2/CXC chemokine ligand 2) than controls, and these effects were markedly diminished by protease inhibition. Inhibition of PARs also blunted the HDE-induced cytokine release from BECs. In addition, protease depletion inhibited HDE-induced BEC intracellular PKCα and PKCε activation. C57BL/6J mice administered 12.5% HDE intranasally, either once or daily for 3 wk., exhibited increased total cellular and neutrophil influx, bronchial alveolar fluid inflammatory cytokines, lung histopathology, and inflammatory scores compared with mice receiving protease-depleted HDE. These data suggest that proteases in dust from CAFOs are important mediators of lung inflammation, and these proteases and their receptors may provide novel targets for therapeutic intervention in CAFO dust-induced airways disease. These results have been published.[89]

Work performed in support of Aim 3 contributed to our observation regarding the role of epidermal growth factor receptors (EGFR) in mediating epithelial cell responses to dust extract. Exposure of Beas-2B cells to dust extract caused EGFR phosphorylation and downstream ERK activation, and the EGFR-selective kinase inhibitor AG1478 blocked both responses. Both AG1478 and EGFR-neutralizing antibody reduced dust extract-stimulated IL-6 and IL-8 release by about half. Similar EGFR phosphorylation and requirement of EGFRs for maximal IL-6 and IL-8 release were found with primary isolates of human bronchial epithelial cells. Because dust extract-stimulated IL-6 and IL-8 release involve the Ca2+-dependent PKCα, we evaluated whether HDE would induce intracellular Ca2+ mobilization. HDE exposure induced intracellular Ca2+ mobilization in Beas-2B cells and in primary cell isolates, but this response was neither mimicked by EGF nor inhibited by AG1478. Thus, HDE activates EGFRs and their downstream signaling, and EGFR activation is required for some but not all airway epithelial cell responses to HDE. This work has been published.[90]

Recently, we also continued to expand the work regarding the possibility that the role of proteases in dust may involve DE-induced activation of epidermal growth factor receptors (EGFR) with experiments utilizing an EGFR antagonist. We presented an oral presentation at the 2016 American Thoracic Society demonstrating that erlotinib, an EGFR antagonist used clinically, attenuates both DE-induced inflammatory cytokines (IL-6/IL-6) as well as AREG which is also an endogenous EGFR agonist in airway epithelial cells and lung fibroblasts in culture as well as in BALF from mice exposed to a single dose of DE *in vivo*. These data contribute to our observations that both TACE and EGFR are central in mediating AREG and we will define mechanisms by which this occurs in our renewal application.

Collaborations: With co-investigator Dr. Jill Poole, we collaborated in evaluating the role of gram-positive bacterial products in mediating dust extract-induced lung inflammation. We have now published that Toll-like receptor 2 (TLR2) is involved in this process.[91] To summarize this work, we reported that lung macrophages isolated from TLR2 knock-out (KO) mice demonstrated reduced TNF-α, IL-6, MIP-2/CXCL2, but not KC/CXCL1 expression, following dust extract stimulation *ex vivo*. Using our mouse model of intranasal inhalation challenge, we evaluated bronchoalveolar lavage fluid and lung tissue in TLR2-deficient and wild type (WT) mice following single and repetitive dust extract exposure. Neutrophil influx and select cytokines/chemokines were significantly lower in TLR2-deficient mice at 5hr and 24hr after single dust extract challenge. After daily exposure to dust extract for 2 weeks, there were significant reductions in total cellularity, neutrophil influx, and TNF-α, IL-6, CXCL1, but not CXCL2 expression, in TLR2-deficient mice as compared to WT animals. Lung pathology revealed that bronchiolar inflammation, but not alveolar inflammation, was reduced in TLR2-deficient mice following repetitive exposure. In addition, airway inflammatory responses in WT mice following challenge

with a TLR2-agonist, peptidoglycan, were similar to dust extract-induced responses. Taken together, these results demonstrate the importance of gram-positive bacterial components and TLR 2 in lung inflammation from dusts of confinement facilities.

The importance of gram-positive bacterial organisms in swine confinement facilities was also seen in work done as a collaboration with Dr. Tricia LeVan. We performed shotgun pyrosequencing metagenomics analyses of DNA from dusts from swine confinement facilities or grain elevators, with comparisons to dusts from pet-free households. DNA sequence alignment showed that 19% or 62% of shotgun pyrosequencing metagenomics DNA sequence reads from swine facility or household dusts, respectively, were of swine or human origin, respectively. In contrast only 2% of such reads from grain elevator dust were of mammalian origin. These metagenomics shotgun reads of mammalian origin were excluded from our analyses of agricultural dust microbiota. The ten most prevalent bacterial taxa identified in swine facility compared to grain elevator or household dust were comprised of 75%, 16%, and 42% gram-positive organisms, respectively. Four of the top five swine facility dust genera were assignable (Clostridium, Lactobacillus, Ruminococcus, and Eubacterium, ranging from 4% to 19% relative abundance). The relative abundances of these four genera were lower in dust from grain elevators or pet-free households. These analyses also highlighted the predominance in swine facility dust of Firmicutes (70%) at the phylum level, Clostridia (44%) at the Class level, and Clostridiales at the Order level (41%). In summary, shotgun pyrosequencing metagenomics analyses of agricultural dusts show that they differ qualitatively and quantitatively at the level of microbial taxa present, and that the bioinformatics analyses used for such studies must be carefully designed to avoid the potential contribution of non-microbial DNA, e.g. from resident mammals.[92]

Additionally, we contributed to the work of co-investigator Dr. Jill Poole who has lead work on examining the effect of dust-induced inflammation in PKC-epsilon knockout mice as a strategy to better understand the essential features of dust-induced increases in PKC-epsilon that we have observed in our in vitro model system previously. In summary, these studies demonstrated that PKCε-deficient mice were hypersensitive to dust exposure, and suggest that PKCε is important in the normative lung inflammatory response to dust extract. Furthermore, we observed that dust extract failed to upregulate nitric oxide (NO) from *ex vivo* stimulated PKCε KO lung macrophages suggesting that dampening of dust extract-induced NO may contribute to these enhanced inflammatory findings in the PKC-epsilon knockout mice.[85]

Conclusions

In summary, we have published key findings from each of the three aims. An important observation from Aim 1 is that DE enhances TNF- α converting enzyme (TACE) activity resulting in TNF-α, IL-6, and IL-8 epithelial cell release and results in lung tissue inflammation *in vivo*. Furthermore,[93] studies aimed at understanding relevant DE-induced changes in epithelial cell transcriptional activity demonstrated the importance of serum response element (SRE) and downstream signaling through MAPK. As we sought to discover agents that could attenuate these DE-induced transcriptional alternations, we determined that certain pro-resolvin mediators such as Maresin-1 that are derived from PUFA in the diet had a substantial influence of TNF-α/IL-6/IL-8 from epithelial cells and lung tissue inflammation *in vivo*.[80,81,82] In our renewal proposal, we will more definitively address the role of PUFA in regulating repair following lung inflammation as we seek to determine if diet can be a strategy to reduce pulmonary complications of dust exposure in workers.

An important unanticipated finding from Aim 1 was that IL-6 KO mice had greater inflammatory response to DE and not attenuated as we expected.[94] This started our interest in how DE modulates repair after injury. In this context, we have preliminary data about DE regulation of AREG and its potential key role in the repair process and this is the central focus of the renewal proposal.

In Aim 2, we determined that agents that augment PKA especially beta-adrenergic agonists used clinically, attenuate PKC isoform activation by attenuating TACE and this results in downregulation of DE-induced inflammatory responses both *in vitro* and *in vivo*. Additionally,[87,88] an output of Aim 3 was the observation that DE results in EGFR activation and contributes to DE-induced epithelial cell inflammatory cytokine release as well as to *in vivo* inflammation. The work defining the important role of TACE and EGFR in DE-induced responses contributes to the background of the mechanistic studies proposed in our renewal application.[90] Our preclinical observations regarding the ability of beta-adrenergic agonists to downregulate inflammatory

processes in the mouse model suggest that clinical studies aimed at determining how to best utilize that category of therapeutic agent in the prevention and control of respiratory symptoms of CAFO workers could be considered.

In Aim 3, we demonstrated that proteases in DE contribute to both epithelial cell responses as well as *in vivo* inflammation and that both[89] PAR-1 and PAR-2 receptors are involved. We continue to better define which proteases are central to this response and how monitoring of protease activity may be useful in assessing dusty CAFO environments.

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Additional material

1) Cumulative inclusion enrollment table
    a. The human subjects component was obtaining blood from normal volunteers to use in neutrophil adhesion *in vitro* assays.

Cumulative Inclusion Enrollment Report

This report format should NOT be used for collecting data from study participants.

Study Title: Targeting airway inflammation from concentrated animal feeding operations dust

Comments:

Racial Categories	Ethnic Categories									Total
	Not Hispanic or Latino			Hispanic or Latino			Unknown/Not Reported Ethnicity			
	Female	Male	Unknown/ Not Reported	Female	Male	Unknown/ Not Reported	Female	Male	Unknown/ Not Reported	
American Indian/ Alaska Native										0
Asian										0
Native Hawaiian or Other Pacific Islander										0
Black or African American										0
White	3	1								4
More Than One Race										0
Unknown or Not Reported										0
Total	3	1	0	0	0	0	0	0	0	4