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14. ABSTRACT

Since 2001, more than 2.8 million military personnel, DoD contractors, and US government and NGO employees supporting the war effort have been deployed to Southwest Asia. They have been exposed to a variety of hazardous conditions during deployment including direct physical lung injury from explosions as well as chronic exposures from inhalation of airborne PM and other harmful chemicals, including smoke from burn pits, sand, and geogenic dust containing potentially toxic metals such as titanium, cadmium, aluminum, and lead. Exposure to cigarette smoke is an additional risk factor for respiratory disease in these individuals. Consequently, mounting evidence demonstrates that military personnel returning from Southwest Asia have increased rates of respiratory symptoms compared to non-deployed military personnel.

15. SUBJECT TERMS
None listed.

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1. INTRODUCTION:

The overarching goal of this grant is to discover how inhalation of airborne particulate matter (PM: 'desert dust') and other noxious substances by military personnel deployed to Southwest Asia causes lung damage and to develop strategies to accurately diagnose and repair the injured lung. Since 2001, more than 2.8 million military personnel, contractors, and US government and NGO employees supporting the war effort have been deployed to Southwest Asia. They have been exposed to a variety of hazardous conditions during deployment including direct physical lung injury from explosions as well as chronic exposures from inhalation of airborne PM and other harmful chemicals, including smoke from burn pits, sand, and geogenic dust containing potentially toxic metals such as titanium, cadmium, aluminum, and lead. Mounting evidence demonstrates that military personnel returning from Southwest Asia have increased rates of respiratory symptoms compared to non-deployed military personnel. Our **hypothesis** is that chronic exposure of deployed military personnel to airborne PM from Southwest Asia predisposes ('primes') the respiratory epithelium for enhanced injury to a subsequent harmful stimulus ('two-hit hypothesis') such as mechanical stress, exposure to toxic chemicals, cigarette smoke, allergens, or viral infection. **Project 1** will characterize the spectrum of deployment-related respiratory diseases and describe clinical findings (including chest imaging and lung function abnormalities) in our cohort of over 200 previously deployed military personnel followed in the Center for Deployment Lung Disease at National Jewish Health. The project also explores noninvasive tools for diagnosis of small and large airways diseases that occur in military deployers. Clinical specimens (nasal and airway brushings, lung biopsies), pulmonary function testing, chest imaging and questionnaires from these personnel will be acquired, characterized, stored, and distributed to Projects 2, 3, and 4. **Project 2** will investigate the effects of PM on alveolar epithelial cells and the additive effects of a second stimulus (physical, chemical, viral) imposed on the primed epithelium leading to dysfunction of the epithelial cells in cultured cells and in a mouse model. Advanced mass spectrometry and electron microscopy technologies will be used to identify the metal content present in the lung. Project 2 will also test the therapeutic effectiveness of two small molecules that target the WNT/β-catenin pathway in healing the injured lung. **Project 3** will focus on the effects of PM on the bronchial epithelial cells lining the more proximal airways, the role of oxidative stress, the effects of cigarette smoke resulting in damage to the cells lining the airways, and how these stimuli interact, leading to asthma and bronchiolitis. **Project 4** will study the effects of PM on gene expression profiles of cells lining the nose and airways and the superimposed effects of cigarette smoke and allergens. This project will also determine if nasal epithelial gene expression profiles can be used to monitor effects of airborne PM exposure on military personnel.

2. KEYWORDS:

Airborne particulate matter; deployment lung disease; acute lung injury; asthma, bronchiolitis, pulmonary fibrosis; gene expression profile; expression quantitative trait loci; oxidant; electron microscopy; high-resolution computed tomography; inductively coupled plasma mass spectrometry; airway epithelial cells; alveolar epithelial cells

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Major Task 1: Establish clinical infrastructure and processes for recruitment of study subjects (deployers and controls) from NJH Deployment Lung Clinic.

Major Task 2: Establish *in vitro* and animal models of exposure of alveolar epithelial cells to airborne PM and combined effects of physical, chemical, and infectious stimuli.

Major Task 3: Establish *in vitro* and animal model of exposure of bronchial epithelial cells to airborne PM.

Major Task 4: Establish 'omics' approaches to analyze the transcriptome and genetics of *in vivo* and *in vitro* airway epithelium from subjects with deployment-related lung diseases to determine mechanisms of these diseases, the molecular effects of PM exposures, and to identify minimally invasive biomarkers for these diseases and exposures.

What was accomplished under these goals?

PROJECT 1. Exposure Characterization and Identification of Noninvasive Methods for Diagnosis of Deployment-Related Lung Disease

Major Task 1: Establish clinical infrastructure and processes for recruitment of study subjects (deployers and controls) from NJH Deployment Lung Clinic.

Accomplishments: The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project identify these dates and show actual completion dates or the percentage of completion.

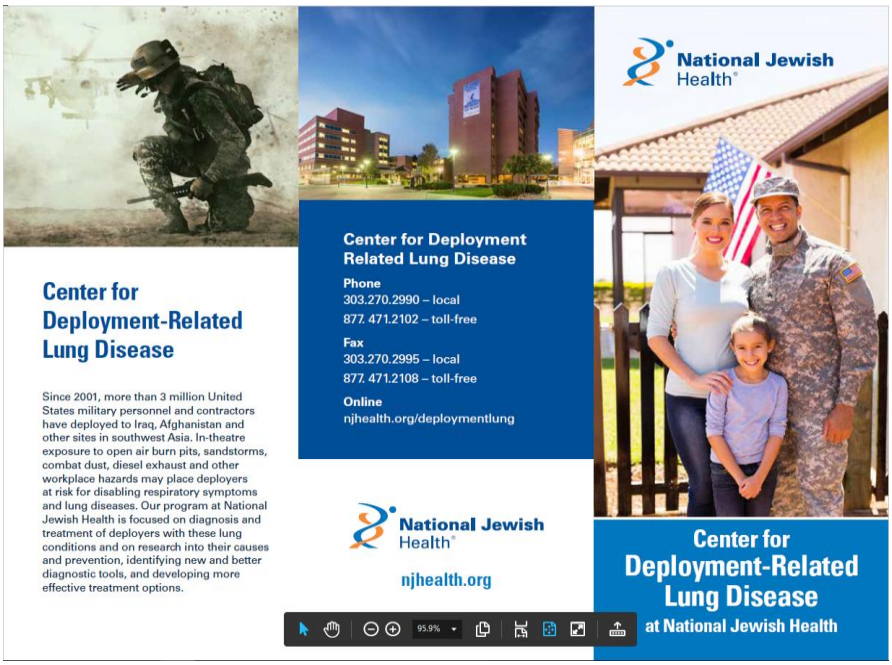
Major Task 1: Establish clinical infrastructure and processes for recruitment of study subjects (deployers and controls) from NJH Deployment Lung Clinic.

What was accomplished under these goals?
For this quarterly reporting period only describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided.

Subtask 1. Hire coordinators, obtain IRB/HRPO approval, develop project database, begin recruitment and consent patients and controls from our NJH Deployment Clinic.

Progress

1. NJH IRB approved the GLIDE Study in December 2016. GLIDE Study documents were submitted to HRPO in March 2017 and approved in August 2017. National Jewish Health switched to Biomedical Research Alliance of New York (BRANY) Institutional Review Board in June 2020. We recently received Continuing Review approval for the GLIDE Study from BRANY through 9/27/2021.
2. Our primary study coordinator, Jenna Wolff, left NJH in June 2021, and has been replaced by Kathy Pang, MPH. The replacement coordinator has been trained by other experienced Project 1 coordinators to recruit study subjects, consent, test and record data.
3. We continue to enter data from our Deployment Lung Disease Registry and Biorepository into a REDCap database.
4. We are using a printed Deployment-Related Lung Disease brochure for GLIDE Study recruitment (see below).



5. We have updated our website to highlight our Center for Deployment-related Lung Disease and to support deployer study subject recruitment (https://www.nationaljewish.org/doctors-departments/depts/medicine/environmental-occupational/deployment-related-lung-disease-center).
6. We created a recruitment tool on the NJH website to ensure that we are not missing deployers who are being seen in other clinics at our institution. (https://www.nationaljewish.org/clinical-trials/lung-injury-from-military-deployment) and who may be interested in study participation.
7. The GLIDE Study Outreach Committee continues to meet regularly to develop and implement plans for study subject recruitment.
8. GLIDE Study physicians and coordinators review and contact subjects from our existing Deployment Lung Disease Registry and Biorepository who meet GLIDE Study inclusion criteria to assess their willingness to participate.
9. The GLIDE Study coordinator facilitates scheduling deployer research subjects for GLIDE study participation, including for lung clearance index testing (LCI), chest CT image acquisition, research bronchoscopies and nasal epithelial cell brushings.
10. We continue to successfully consent and enroll deployers for LCI. We have met our target for LCI controls and are no longer actively recruiting control subjects.
11. Data for deployer study subject recruitment is indicated below. We defined **"screened"** as the total number of deployers who have signed consent and completed the deployment research questionnaire. We defined **"enrolled"** as deployers who signed informed consent and agreed to complete the research questionnaire and other Project 1 specific aims – that is, LCI, quantitative chest CT imaging, nasal epithelial brushing and/or fiberoptic bronchoscopy. We defined **"completed"** as those who completed testing after having been enrolled. There are a number of study subjects who have consented to GLIDE Study bronchoscopies and/or nasal brushings who have not completed these procedures because of the COVID-19 pandemic.

- Number of subjects **screened**/target: **235/250**
- Number of patients **enrolled**/target: **115/250**
- Number of patients **completed**/target: **103/250**

Difficulties encountered

Due to the COVID-19 pandemic, National Jewish Health implemented an institutional pause in recruitment of research study subjects in early March to minimize risk of viral transmission for subjects and study personnel. Recruitment resumed for some but not all components of the GLIDE Study, with new pandemic-specific protocols in place to protect study subjects and personnel, for example, during aerosol generating procedures such as LCI and pulmonary function testing.

Subtask 2. Establish protocols for bronchoscopies and BAL and collection of nasal and bronchial brushing. Develop live cell core protocols and procedures.

Progress

1. GLIDE Study staff and investigators have developed these protocols and procedures.
2. We work with the Minimally Invasive Diagnostic Center (MIDC) staff where these procedures are performed. GLIDE Study bronchoscopists use the compatible electronic medical record documentation process (using a study-specific NJH Provation program) to record research bronchoscopies performed in the MIDC, ensuring accurate documentation.
3. We continue to meet as needed with MIDC personnel who are involved in GLIDE Study bronchoscopies to familiarize them with research protocols and study staff.
4. We worked closely with MIDC personnel to address technical issues and refine our protocols during the first round of research bronchoscopies.
5. We communicate regularly with laboratory technicians who process study samples to ensure that sample collection yield is maximized.
6. Study bronchoscopists adjusted brushing techniques to enhance bronchial epithelial cell collection from deployer subjects based on feedback from laboratory-based co-investigators. However, these techniques have been on hold pending implementation of institutional pandemic-related procedures for analyzing bronchial brush cell samples.
7. We have worked with Drs. Downey and Chu (Project 2 and 3) and Dr. William Janssen to collaborate on obtaining bronchoalveolar lavage specimens from normal human volunteers (collected under a separate grant and IRB protocol). The samples are de-identified and stored in our institutional biobank under our Honest Broker protocol. These samples can be used as controls to compare with deployer bronchoalveolar lavage specimens.
8. We are currently finalizing changes in protocols for collecting and analyzing nasal and bronchial brushing samples during COVID-19 to assure that study subjects and staff are protected from viral exposure risk.
9. We have sent pilot deployer and control BAL samples to the University of Colorado Anschutz Medical Campus for preliminary flow cytometry (CyTOF) analysis to characterize dominant cell profiles.

Difficulties encountered

Due to the COVID-19 pandemic, National Jewish Health implemented an institutional pause in recruitment of research study subjects in early March 2020 to minimize risk of viral transmission for study subjects and personnel. Recruitment slowly resumed in July, with new and emerging protocols in place to protect subjects and study personnel. Policies and protections for aerosol-generating procedures such as LCI and nasal brushing are in place. Research bronchoscopy procedures now require bronchoscopists who have been specially trained in the use of controlled air-purifying respirators (CAPRs) and other COVID-19 prevention techniques and who work closely with anesthesiologists to assure optimal study subject airway and aerosol controls.

Subtask 3. Collect, analyze, and archive bronchial (B) and nasal (N) brushings through live cell core.

Progress

1. GLIDE Study staff established protocols for the collection and distribution of samples to the appropriate labs involved in GLIDE and with the USGS.
2. Samples have been delivered to the USGS for analysis and to assure adequate methods and quality based on current protocols.
3. Prior to the COVID-19 pandemic, we performed 38 research bronchoscopies on deployer study subjects and implemented processes for collecting bronchoalveolar lavage (BAL) fluid, bronchial epithelial cell (BEC) brushings, and nasal epithelial cell (NEC) brushings.
4. BAL, BEC brushings, and NEC brushings were delivered to the appropriate labs for storage and analysis of samples.

Difficulties encountered

Collection of deployer BAL, BEC and NEC samples was temporarily halted due to the COVID-19 pandemic while additional institutional safety policies were implemented. We are currently awaiting the completion of a fully equipped BSL2+ laboratory to assure that all study subject samples can be processed and analyzed safely by GLIDE investigators.

Subtask 4: Establish protocols for collection of samples including lung blocks and cells from Video-Assisted Thoracic Surgery (VATS) biopsies done for clinical diagnosis.

Progress

1. Protocols have been established and remain in place, including an IRB-approved consent form for collection of lung tissue blocks for GLIDE Study purposes from VATS biopsies performed for clinical diagnosis.

Difficulties encountered

None

Subtask 5: Collect, analyze and archive VATS biopsies from deployers and controls.

Progress

1. Using our Deployment Lung Disease Registry and Biorepository, we continue to track which subjects have had a VATS biopsy and meet other requirements for participation in the GLIDE study.
2. When possible, we obtain informed consent for participation in the GLIDE Study from Registry participants who have had a VATS biopsy. During this reporting period, we continued to collect and analyze clinical VATS lung biopsies on symptomatic deployers seen in our clinical center of excellence.
3. Using a standardized REDCap-based scoring form, the study pathologist has completed scoring on 61 GLIDE Study lung tissue samples.
4. We have obtained and the study pathologist has scored non-deployer VATS lung tissue samples, including 31 positive and 11 negative controls, for comparison with deployer samples.
5. Once USGS co-investigators have finalized analytical methods for BAL and brushings, we will send stored, non-deployer VATS lung biopsy tissue with which to pilot a revised lung tissue digestion process before beginning analysis of GLIDE Study lung tissue samples.

Difficulties encountered

There were two prolonged time periods (including during the COVID-19 pandemic) during the past grant year in which access to the USGS lab was limited due to federal restrictions. Work at the USGS now has resumed.

Subtask 6: Establish LCI using Multiple Breath Washout technique.

Progress

1. We continue to use the Procedures Manual for LCI measurements developed for this study.
2. We continue to use a dedicated LCI testing space in the Clinical Translational Research Center at NJH where we will also perform GLIDE study subject consenting.
3. Our study staff continues to perform LCI testing on deployers, allowing each staff member to gain additional proficiency in LCI performance and quality.
4. We confer regularly with LCI experts in Toronto to assure that testing is meeting the highest quality standards.
5. We developed a revised safety protocol for LCI testing (an aerosol-generating procedure) targeted to the COVID-19 pandemic to protect study subjects and staff. This included ensuring adequate room filtration/negative pressure ventilation, disinfection, training, and personal protective equipment as well as screening of study participants.

Difficulties encountered

None

Subtask 7: Complete LCI measurement from deployers and controls.

Progress

1. We continue to recruit GLIDE Study deployers for LCI testing.
2. We have completed recruitment of LCI control subjects.
3. We created and used a SAS program that allows us to easily match deployer subjects to controls and that aids in targeted study subject recruitment.
4. We recently submitted a manuscript for review and publication describing the utility of LCI as a noninvasive tool for diagnosis of airways disease in symptomatic military personnel following deployment to austere environments.

Difficulties encountered

None

Subtask 8: Establish protocols for chest CT, quantitative pulmonary analysis and textural analysis and analyze CT scans.

Progress

1. Working with the NJH Imaging Department staff, we developed and implemented protocols for scheduling and consenting GLIDE Study participants for study chest CT imaging.
2. We established a protocol for transmitting study imaging acquisition data with de-identified study subject numbers and linkage to the GLIDE Study database for future analysis.
3. We designed, pilot-tested, revised and implemented a scoring system for use in REDCap by the study radiologists for qualitative chest CT image interpretation.
4. All three GLIDE Study radiologists have scored 98 chest CT images (blinded to deployer vs control status). Based on preliminary review of findings, we are adding 35 additional chest CT images (all obtained using the GLIDE Study protocol and all with informed consent) to the total number for which independent scores will be obtained by the three study radiologists. This will enhance statistical power for data analysis.

Difficulties encountered

None

Subtask 9: Complete CT scans and quantitative analysis from deployers and controls.

Progress

1. We have queried the control CT scan database to establish demographic linkages (including age, smoking status and gender) to match with deployer study subject images.
2. We have acquired 48 (out of the targeted total of 50) deployer study subject chest CT images for use in quantitative analysis based on the GLIDE protocol.
3. Study investigators and research staff have created and implemented a process for matching deployers and controls and have devised methods to blind radiologists during batched scoring of chest CT scans.
4. Study radiologists scored 48 deployer scans and 48 controls matched for age, sex, and smoking status, and data have been recorded in our study database.

5. We will send 35 additional deployer study subject CT scans (obtained for clinical purposes and for which informed consent for study inclusion has been obtained) to load into the imaging system and prepare for scoring by study radiologists.
6. We have sent a total of 48 deployer CT images and 48 matched control CT images to Thirona for analysis of Pi10. We plan to send 35 additional images during the next quarter to enhance statistical power before proceeding with data analysis.

Difficulties encountered

There are no longer well-matched control CT images available through the COPDGene study. We have conferred with our study biostatistician who recommends increasing the number of deployer subject CT images used in these analyses (without increasing the number of control images) to enhance our sample size and improve statistical power.

Subtask 10: Establish methods for analyzing and characterizing elemental and particulate matter (PM) profiles in lung tissue and BAL.

Progress

1. We have established methods for PM profile analysis in lung tissue.
2. Pilot samples of BAL supernatants and cells have been analyzed by USGS to assure optimum methods are in place before completing analysis of deployer samples.
3. Methods to assure that BAL samples have been irradiated and present no infection risk to laboratory staff have been implemented.
4. The USGS analytical chemist continues to analyze BAL samples for the study and will identify any issues or problems with sample preparation, communicating with our study investigators about media and storage processes that may need to be adjusted to optimize analytical methods.

Difficulties encountered

There were two prolonged time periods (including during the COVID-19 pandemic) during the past grant year in which access to the USGS laboratory was limited due to federal restrictions. Work at the USGS now has resumed.

Subtask 11: Complete analysis of VATS lung biopsies using LA-ICP-MS and FE-SEM.

Progress

We plan to send GLIDE lung tissue samples to USGS for analysis after processing of study BAL and brush samples is completed and after test tissue samples from non-deployed individuals have been analyzed; see subtask 5 for details.

Difficulties encountered

See subtask 5.

Subtask 12: Prepare and submit manuscripts for publication.

Progress

1. A large case series manuscript on clinical findings in symptomatic deployers titled "Respiratory Diseases in Post-9/11 Military Personnel Following Southwest Asia Deployment" was published in the May 2020 issue of Journal of Environmental and Occupational Medicine (JOEM) (attached to this report).
2. A manuscript on the utility of a respiratory hazard exposure matrix and Military Occupational Specialty (MOS) codes to identify risk factors for respiratory symptoms and diseases in military deployers was published in the December 2019 issue of JOEM (attached to this report).
3. We submitted two abstracts to both the 2020 American Thoracic Society (ATS) International Conference and the 2020 Military Health System Research Symposium (MHSRS). The first described preliminary findings from quantitative analysis of airway wall thickness on chest CT imaging from deployers compared to controls; the second described severity, morphology and risk factors for expiratory central airways collapse (ECAC) in symptomatic deployers. Both ATS abstracts were published in the American Journal of Respiratory and Critical Care Medicine 2020 edition and the MHSRS website for conference attendees.
4. In October 2020, we submitted a manuscript for review and publication with findings from the lung clearance index testing to Respiratory Medicine, demonstrating the utility of LCI as a noninvasive tool to detect both small and large airways disease in deployers.
5. In October 2020, we submitted a manuscript for review and publication on findings of expiratory central airways collapse in symptomatic deployers with persistent exertional dyspnea.

Difficulties encountered

None

PROJECT 2. Acute Lung Injury in Deployed Military Personnel: Basic Mechanisms and Novel Therapeutic Approaches.

Major Task 2: Establish *in vitro* and animal models of exposure of alveolar epithelial cells to airborne PM and combined effects of physical, chemical, and infectious stimuli.

Subtask 1. Develop and refine *in vitro* cell culture models using cell lines to study combined effects of PM and physical, chemical, and infectious stimuli on alveolar epithelial cells.

Progress

In the previous year (Year 3), we extensively tested whether exposure to particulate matter (PM) induced cytotoxicity in the murine alveolar epithelial type II cell line, MLE-12, and whether there was enhanced cytotoxicity when combined with a second stimulus. We observed that at concentrations up to 25 µg/cm², PM from Iraq and Afghanistan induced only minor cytotoxicity and that there was evidence of mild enhanced cytotoxicity when PM exposure was combined with bleomycin. During Year 4, we focused our efforts on studying responses of primary human airway epithelial cells because we feel that these primary human cells are most relevant to human disease (see **Subtask 2** below). As will be discussed below, studies with primary human small and large airway epithelial cells, conducted in collaboration with Drs. Chu and Day (see below), suggested that higher concentrations of PM induced inflammatory cytokine production.

Difficulties encountered

None

Subtask 2. Develop and refine *in vitro* cell culture models using primary human alveolar epithelial cells to study combined effects of PM and physical, chemical, and infectious stimuli on alveolar epithelial cells.

Progress

Primary Lung Epithelial Cells. In collaboration with Drs. Chu, Day, Seibold, Janssen, and Fingerlin, we characterized the changes in global mRNA expression (transcriptional profile) of primary human airway and alveolar epithelial cells exposed to PM from Afghanistan, Iraq, and California using RNA-Seq. We have focused on the 4 hr and 24 hr time points after exposure to capture both early and more delayed alterations in gene expression based on our initial experiments. As outlined in the Year 3 progress report, we completed exposure studies on primary cells from five different non-smoking donors and analyzed the transcriptional responses using NextGen RNA-sequencing. We have conducted extensive bioinformatic analysis of these data sets using several tools including DAVID, Hallmark, KEGG, and Ingenuity Pathway Analysis (QIAGEN) with the goal to characterize the cellular signaling pathways that control the cellular responses to particulate matter exposure.

We identified several novel and physiologically important pathways that are activated in response to exposure to PM from Southwest Asia. Some of these pathways have been mentioned in previous reports and additional analysis has confirmed their importance, and that they are strongly altered by exposure to PM. As part of this analysis we compared transcriptional responses to PM exposure between primary large airway, small airway, and alveolar epithelial

cells. As illustrated in **Figure 1**, while there are common pathways activated in each cell type (e.g. IL-17, IL-10, COPD), there are also distinct pathways specific to each cell type.

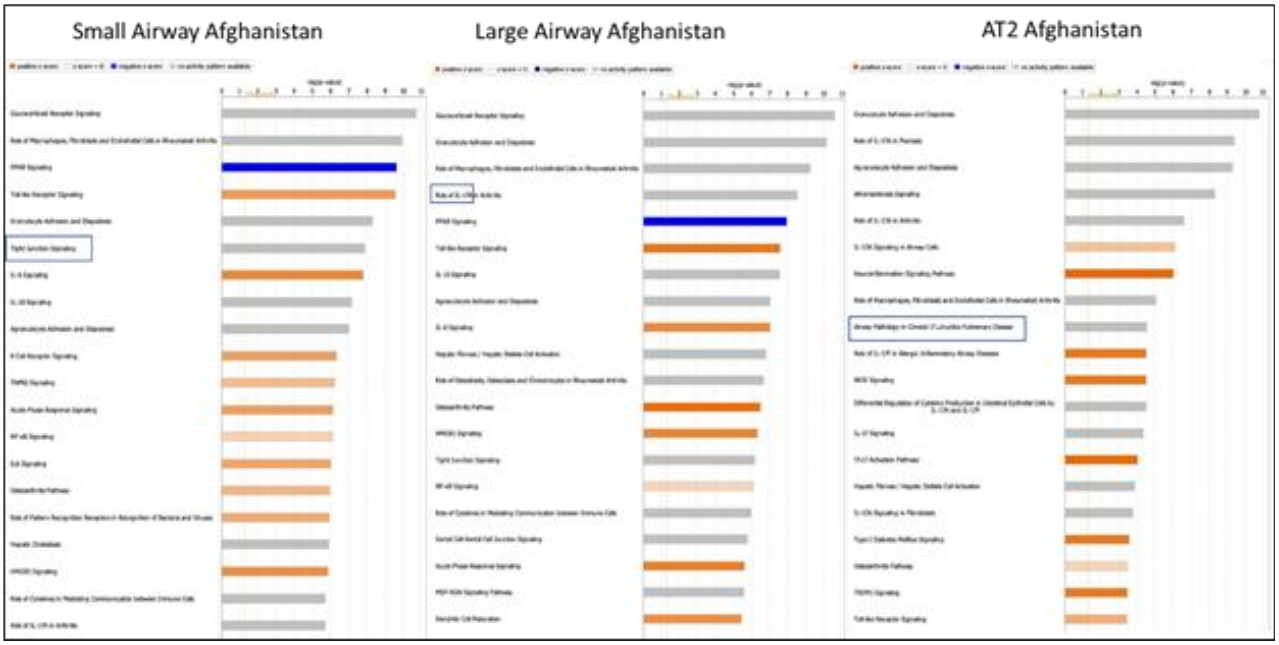


Figure 1. Ingenuity pathway analysis of changes in mRNA expression (RNA-seq) in human primary large airway epithelial cells, small airway epithelial cells, and alveolar type II epithelial cells exposed for 24 hr to PM (10 μg/cm^2) from Afghanistan *in vitro*. Note both common and distinct responses between the different cell types.

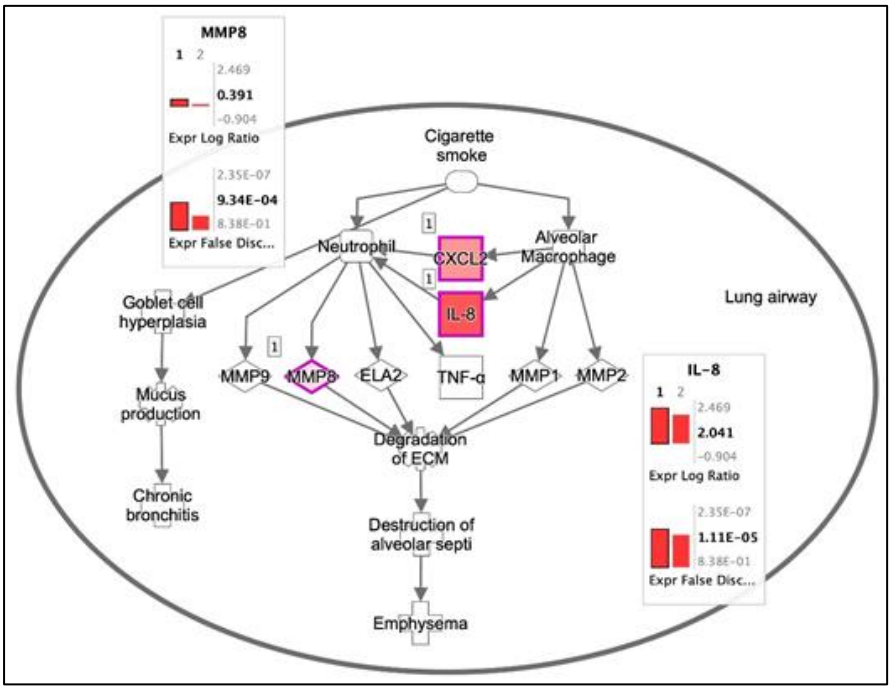


Figure 2. Ingenuity pathway analysis for human respiratory epithelial cells showing changes in the chronic obstructive pulmonary disease pathway induced by Afghan PM compared with control (China Lake) PM. There is less expression of MMP8 and IL-8 induced by PM from China Lake compared to PM from Afghanistan.

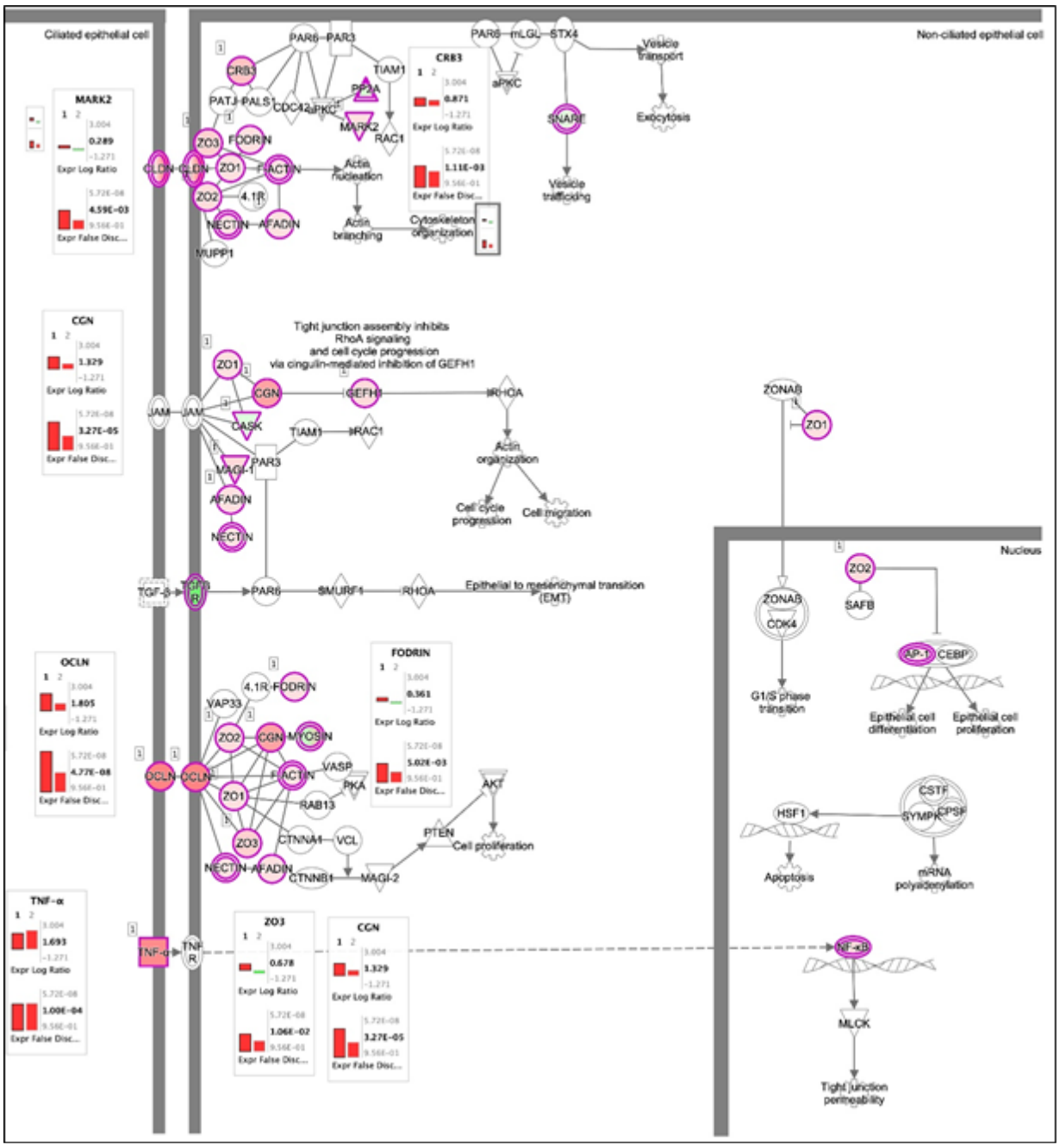


Figure 3. Exposure of human lung epithelial cells to PM from Afghanistan induces differential expression of key molecules in the tight junction signaling pathway, including CRB3, CGN, OCLN, TNF, ZO3, CGN, FODRIN, and more.

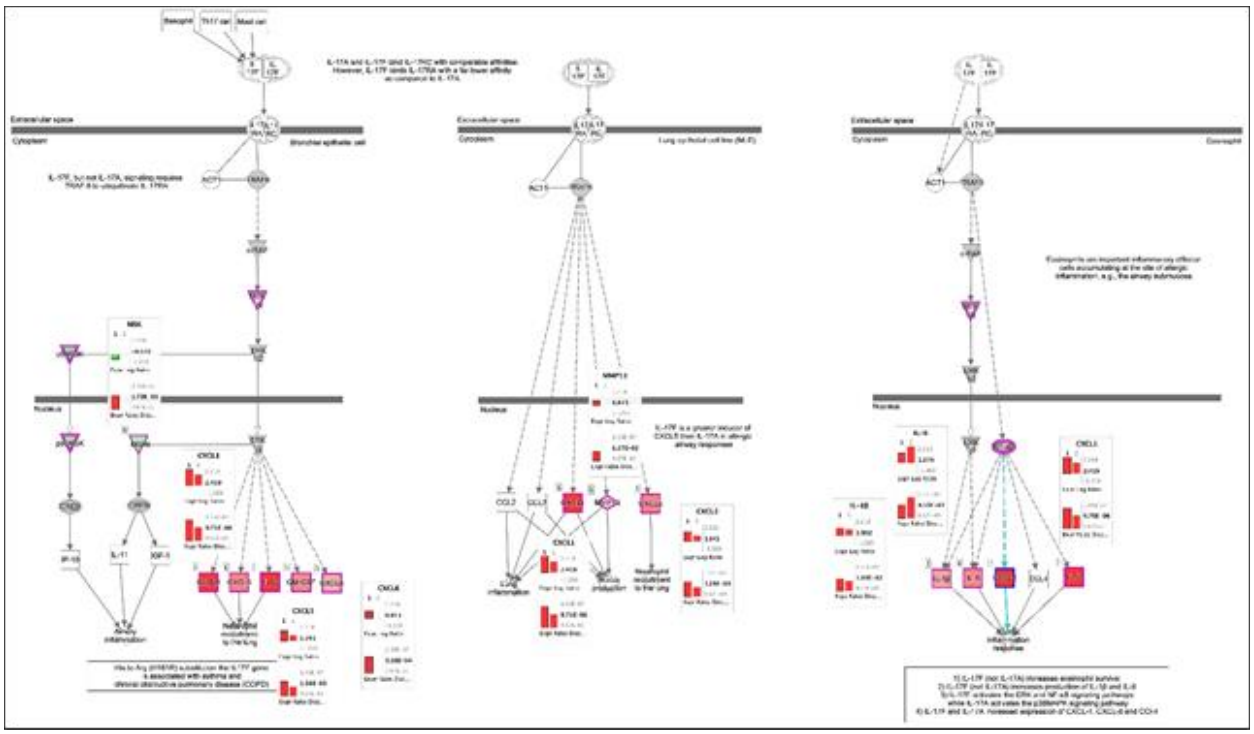


Figure 4. Exposure of human lung epithelial cells to Afghan PM induces alterations in the IL-17 pathway.

Figures 2 and 3 illustrate that exposure of human lung epithelial cells to Afghan PM induce complex changes in gene expression profiles that has overlap with gene expression profiles originally described in other diseases. **Figure 2** illustrates that a pathway that has been characterized in chronic obstructive pulmonary disease (COPD) induced by exposure to cigarette smoke is also activated in lung epithelial cells by exposure to particulate matter. **Figure 3** illustrates that exposure of human lung epithelial cells to Afghan PM alters the expression of molecules known to be important in regulation of epithelial intracellular junctions including occlusion, and zonular occlusions proteins. **Figure 4** illustrates induction of the IL-17 pathway by PM exposure. Collectively this pathway analysis helps us understand the basis for the pathological changes in lung epithelial cells induced by exposure to PM from Southwest Asia and also indicate similarities in alterations in transcriptional profiles induced by cigarette smoke exposure and those induced by PM exposure.

Human alveolar macrophages. We have completed our analysis of the effects of PM exposure on human alveolar macrophages. As summarized in the Year 3 progress report, the top pathways activated by exposure to PM include the TREM1, Stat3, and HMGB1 pathways. During this year, we have validated these observations using quantitative polymerase chain reaction (qPCR) analysis. We developed and validated TacMan probes from 8 genes in each pathway (e.g. IL-1, TNF, IL-6, IL-8, CXCL2, MMP-1, MMP-8, MMP-9). This analysis demonstrated that expression of these genes are indeed altered by exposure to PM and the magnitude of these alterations was similar when measured by RNA-seq or by qPCR. This was important to ensure that the alterations observed in the RNA-seq analysis were not artifactual.

Difficulties encountered
None

Subtask 3: Determine combined effects of PM and physical, chemical, and infectious stimuli on alveolar epithelial cells *in vitro*. Test effects of small molecule modulators of the WNT pathway on epithelial injury. Test effects of small molecules on epithelial injury.

Progress

In Year 4 of this project, we initiated studies to assess the effects of ICG-001, a small molecule modulator of the WNT/beta-catenin pathway, on wound repair in human airway epithelial cells. In our initial studies we used the electric cell-substrate impedance sensing (ECIS) system to measure electrical impedance of the epithelial monolayer as an indicator of the integrity of the interepithelial junctions (tight junctions and adherence junctions). The ECIS system provides a special cell culture inserts with an electrode that runs the full diameter of the culture insert. This electrode can be heated by an electrical current passing through it, thus killing the cells adjacent to the electrode and creating a linear wound in the bottom of the plate. Repair of this wound can be followed in real-time by measuring the electrical resistance of the monolayer using the ECIS instrument. We used this approach to study the effects of ICG-001 on repair of the epithelial wound. As illustrated in **Figures 5** (rat lung epithelial cells) and **6A** (murine lung epithelial cells)**,** ICG-001 slows healing of the epithelial wounds in a dose-dependent manner. **Figure 6B** illustrates that ICG-001 slows healing of the epithelial scratch wounds with human BEAS2B epithelial cells. We believe that this effect is predicted based on the known effects of ICG-001 to attenuate epithelial mesenchymal transposition (EMT) and thus decreased epithelial motility, a response that is required for healing epithelial wounds. This is only one of a complex series of responses involved in wound healing.

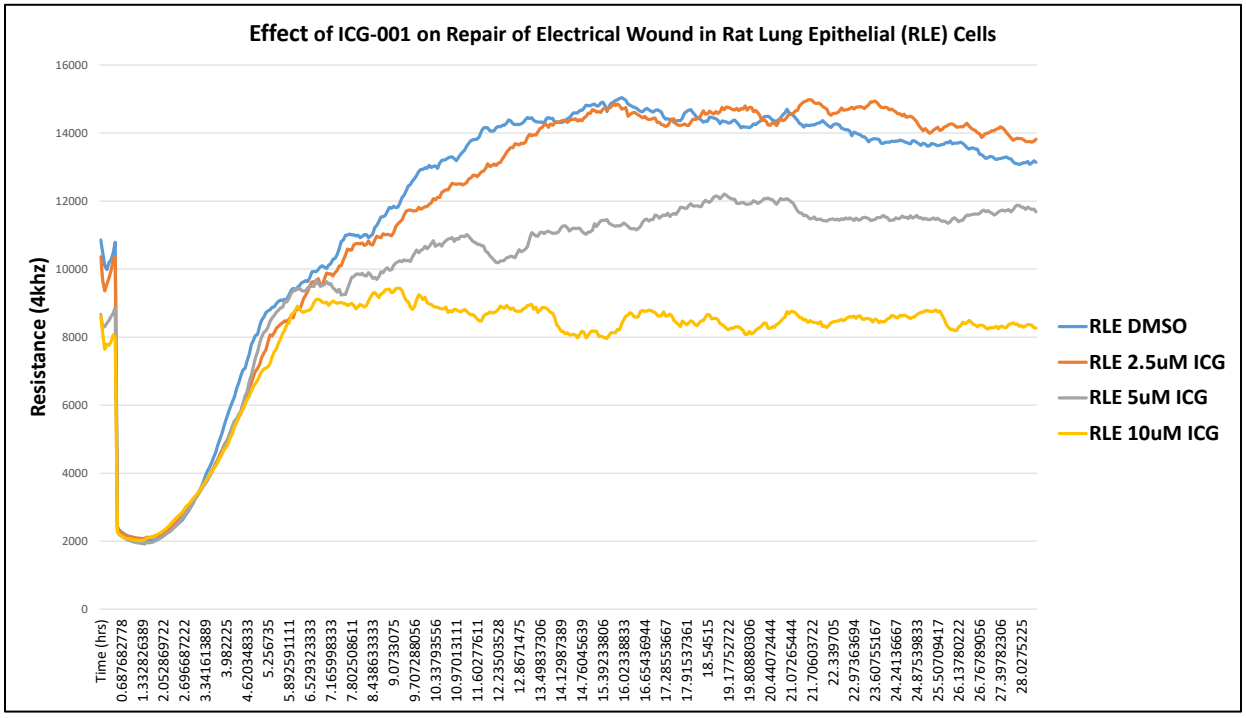


Figure 5. ICG-001 slows repair of an electrical wound in rat lung epithelial (RLE) cells.

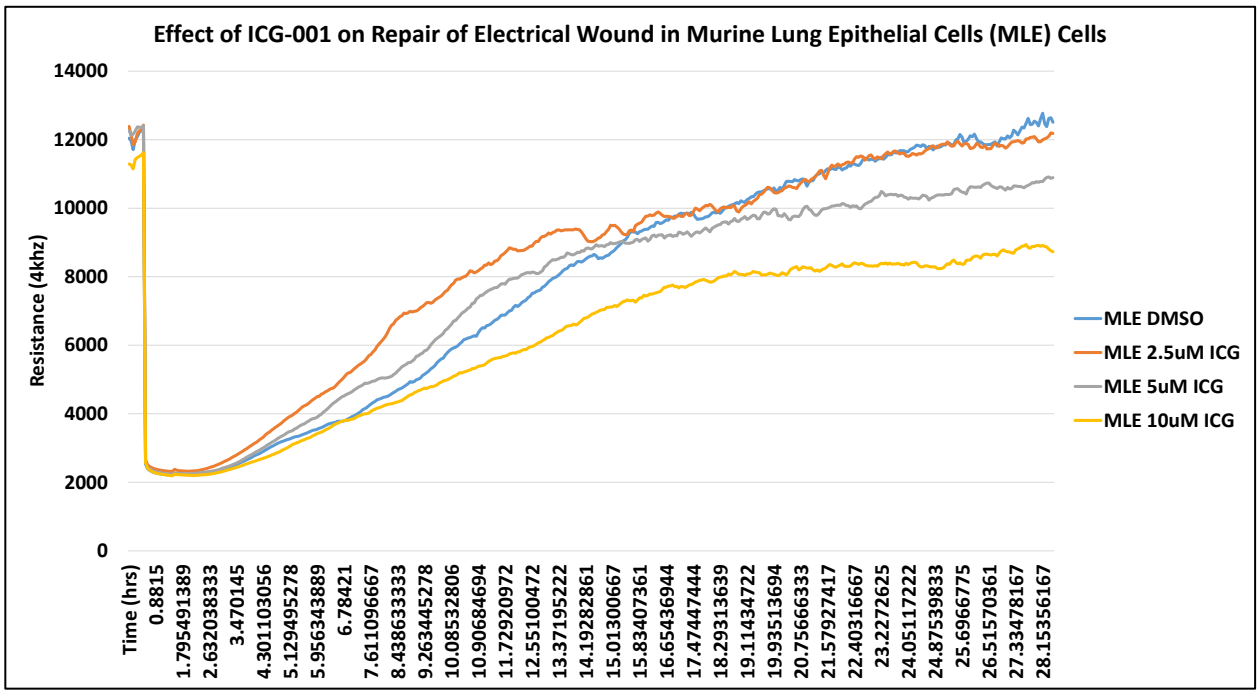


Figure 6A. ICG-001 slows repair of an electrical wound in cultured murine lung epithelial (MLE) cells.

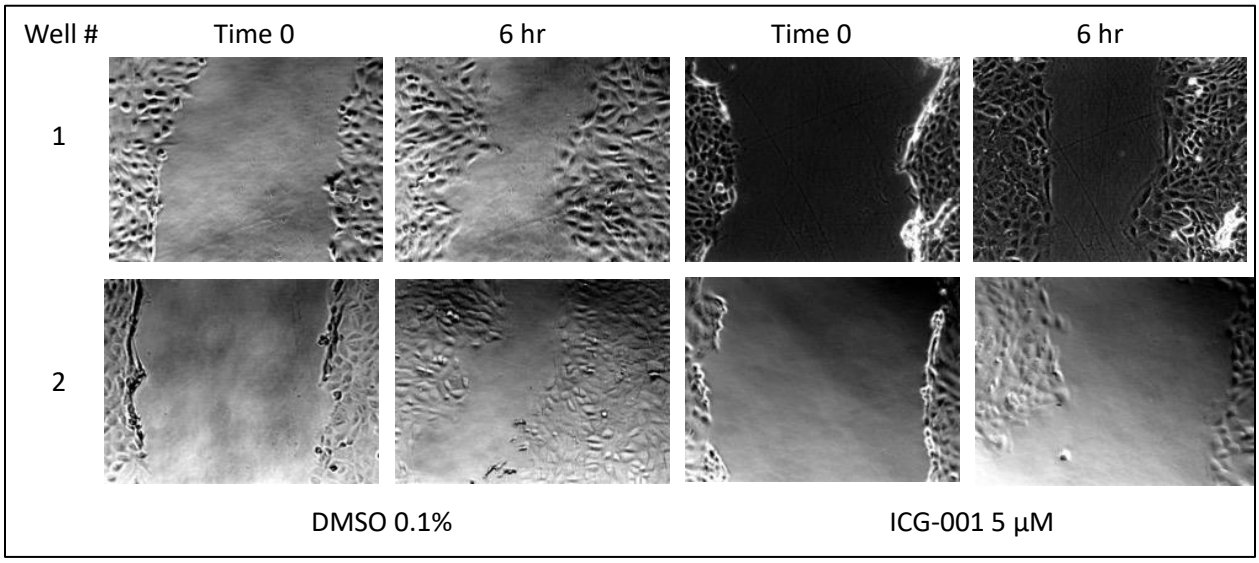


Figure 6B. ICG-001 slows repair of a scratch wound in cultured human BEAS2B lung epithelial cells.

Difficulties encountered

None

Subtask 4: Develop and refine animal (mouse) models of PM exposure and the effects of physical, chemical, and infectious stimuli on acute lung injury.

Progress

As described in the Year 3 progress report, we completed two large inhalational exposure experiments in which mice were exposed to aerosolized PM from Afghanistan and from California using whole body exposure chambers in the NAMRU-Dayton facility. Despite increasing the concentration of aerosolized PM to 75 mg/m³ given over 12 days, the mice developed only minor degrees of pulmonary fibrosis. This is in marked contrast to the exposures conducted using direct oropharyngeal aspiration that bypasses the nasopharynx. The oropharyngeal aspiration route of exposure resulted in significant increases in the lung collagen content with increased deposition of collagen around the small airways and in the lung parenchyma 35 days after the last dose of PM. We believe that the difference between oropharyngeal aspiration and aerosolization is due to the fact that the nasopharynx of the mice filters out most of the aerosolized PM during the whole body exposures so that the PM does not reach the lungs. By contrast, oropharyngeal aspiration bypasses the nasopharynx and delivers materials directly into the lung.

During Year 4, we completed the third set of experiments with aerosol exposure to purified crystalline silica (Minusil) using whole body exposure chambers at NAMRU-Dayton. We used crystalline silica (Minusil: 75 mg/m^3) because it is the most potent fibrogenic particulate matter that we have studied to date. We extended the observation period to 60 days after the completion of the aerosol exposures because of reports in the literature where the fibrotic response to inhaled silica was delayed up to 90 days. In our studies, we observed evidence of continuing pulmonary inflammation at the 60-day time point as indicated by an increase in total number of macrophages recovered by BAL (**Figure 7**). Importantly, at the 60-day time point there was an increase in BAL neutrophils indicating that the neutrophilic inflammation persisted out to 60 days after silica exposure. Thus there was evidence of both neutrophilic and myelomonocytic inflammation 60 days after the last exposure to silica. Biochemical analysis of lung collagen content using hydroxyproline revealed an increase in lung collagen content and a decrease in lung compliance in the mice exposed to the purified crystalline silica (**Figure 8**). These are both consistent with a fibrotic response in the lung. However, the histological analysis of these lungs using hematoxylin and eosin, trichrome, and picrosirius red staining did not show any evidence of pulmonary fibrosis. These histological sections of the lungs were independently examined by our lung pathologist, Dr. Steve Groshong, who also felt that there was minimal histological evidence of pulmonary fibrosis. We remain uncertain as to the reason for the discrepancy between the biochemical analysis of lung collagen content and the histological analysis pulmonary fibrosis. We believe that an important factor is that direct intrapulmonary administration of particulate matter results in inhomogeneous deposition of particulate matter in the lung and thus the parts of the lungs that we sampled for biochemical analysis may have been more heavily involved in fibrosis whereas the parts of the lung that were taken for histological analysis were less involved in the fibrotic process because of the uneven distribution of the PM.

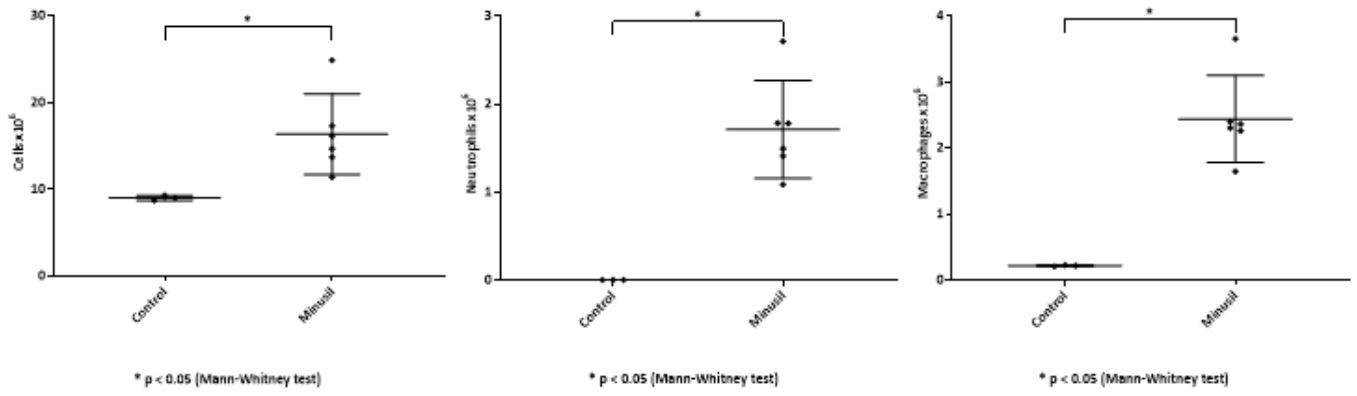


Figure 7. Exposure to aerosolized crystalline silica (Minusil) in whole body aerosolization chambers induces lung inflammation as judged by an increase in total cells, neutrophils, and macrophages in BAL Fluid. Data are from the first time point – 14 days after the last exposure.

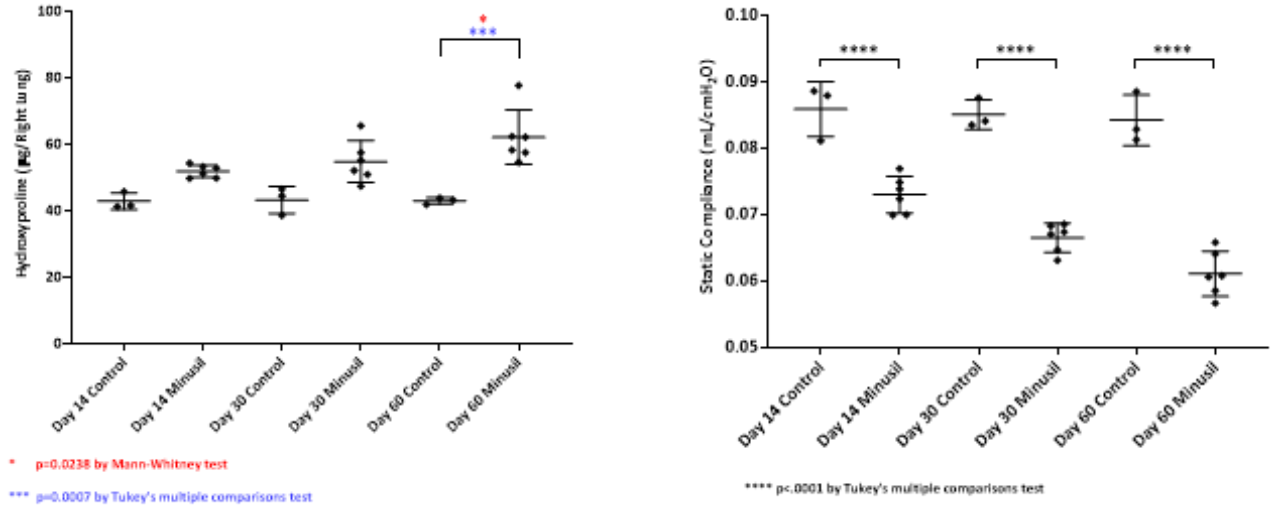


Figure 8. Effects of exposure to purified crystalline silica (Minusil) given by aerosolization using whole body aerosol chambers on lung collagen content measured using hydroxyproline (left panel) and lung compliance measured using the SciReq FlexiVent (right panel).

During Year 4, we completed another (independent) set of experiments characterizing the effects of particulate matter from Iraq, Afghanistan, and California on pulmonary fibrosis. We used a slightly higher concentration of particulate matter (25 mg/kg) with a total of 5 doses over a 10-day period. We chose to repeat this experiment because in the initial experiments in which we used lower concentrations of particulate matter (5 mg/kg and 10 mg/kg), while we observed an increase in lung collagen content, there was considerable variability between animals. This meant that only the highest dose of particulate matter resulted in a statistically significant increase in lung fibrosis.

The results of the repeat set of experiments are summarized in Figures 9 -12. Oropharyngeal aspiration of particulate matter from Afghanistan, Iraq, and China Lake, as well as crystalline silica (Minusil), resulted in a significant decrease in lung compliance (i.e. the lungs became stiffer) (**Figure 9**). Crystalline silica (Minusil) caused the largest decrease in compliance followed by particulate matter from Iraq and Afghanistan. As illustrated in **Figure 10**, administration of crystalline silica (Minusil) and particulate matter from Iraq, Afghanistan, and China Lake all caused a significant increase in lung collagen content as measured by hydroxyproline. Similar to compliance, crystalline silica and particulate matter from Iraq and Afghanistan cause a greater increase in lung collagen than particulate matter from China Lake. Collectively, these data indicate that multiple doses of inhaled particulate matter from a variety of sources including California and Iraq, and Afghanistan induced significant pulmonary fibrosis in mice.

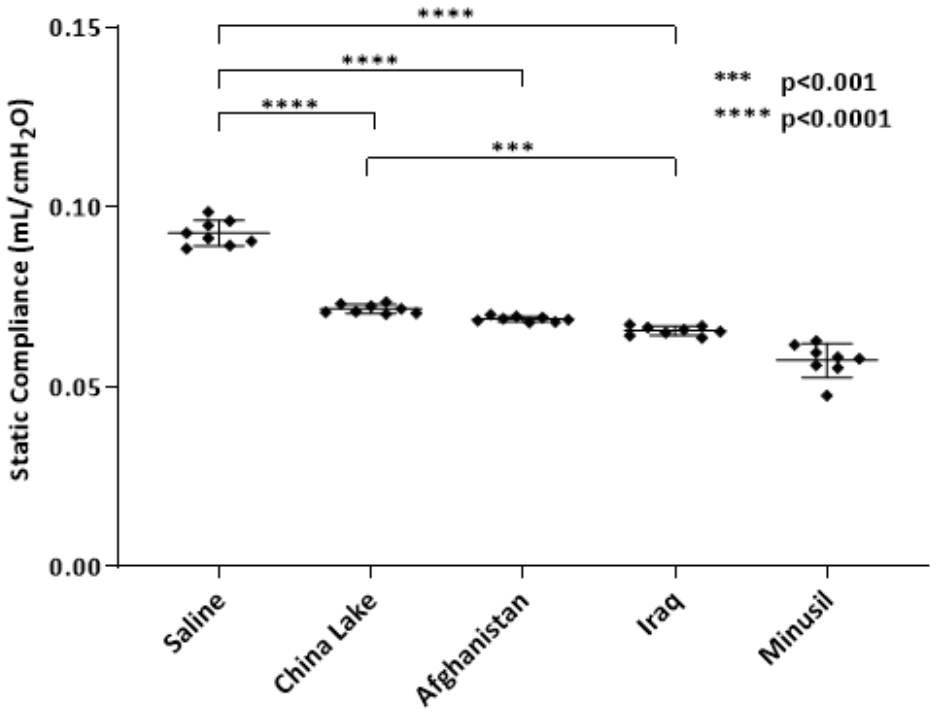


Figure 9. Effects of exposure to PM from Afghanistan, Iraq, or China Lake, or to purified silica (Minusil) on lung compliance measured using the SciReq FlexiVent.

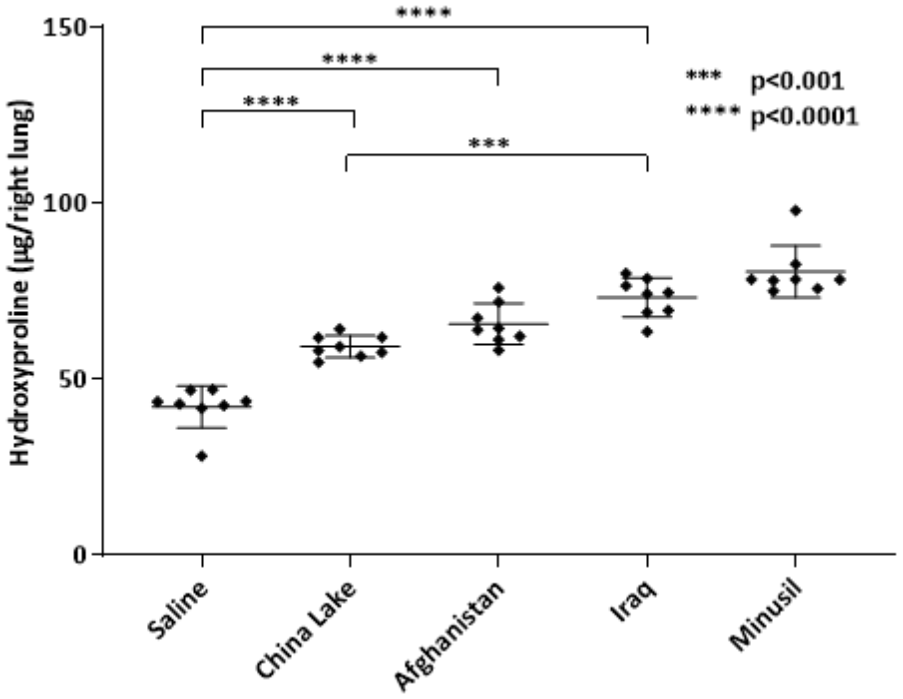


Figure 10. Effects of exposure to PM from Afghanistan, Iraq, or China Lake, or to purified silica (Minusil) on lung collagen content measured using the hydroxyproline assay.

For this repeat set of experiments, our pathologist Dr. Steve Groshong conducted a blinded morphometric assessment of lung pathology using the Ashcroft scoring system. This series of experiments yielded much more reproducible results and demonstrated that respiratory exposure to particulate matter from Iraq, Afghanistan, and California resulted in an increase in lung fibrosis as determined by histological assessment (**Figures 11 and 12**). The particulate matter from Afghanistan was the most potent in inducing lung fibrosis with particulate matter from Iraq and California inducing less but still statistically significant pulmonary fibrosis.

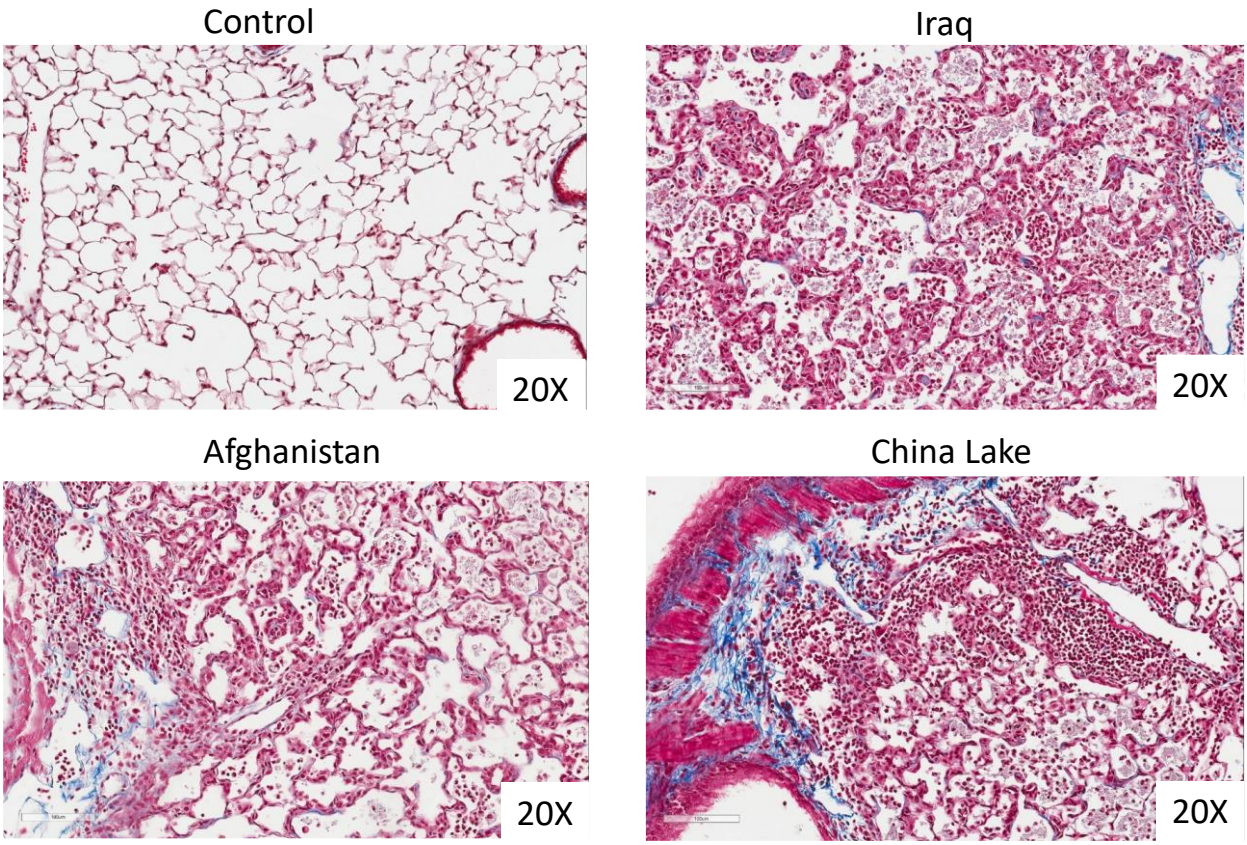


Figure 11. Respiratory exposure to PM from Afghanistan, Iraq, or China Lake resulted in pulmonary fibrosis as assessed by Trichrome staining (collagen stains blue).

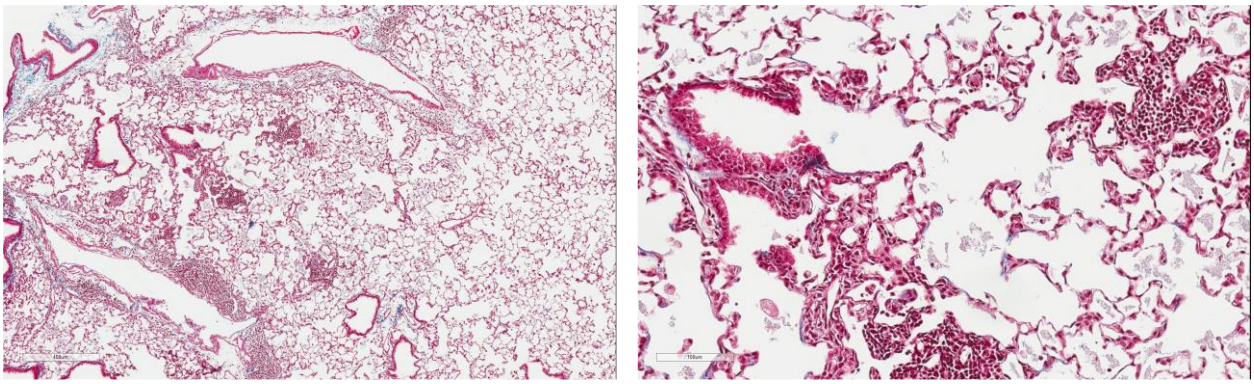


Figure 12. Respiratory exposure to purified crystalline silica resulted in pulmonary fibrosis as assessed by Trichrome staining (collagen stains blue).

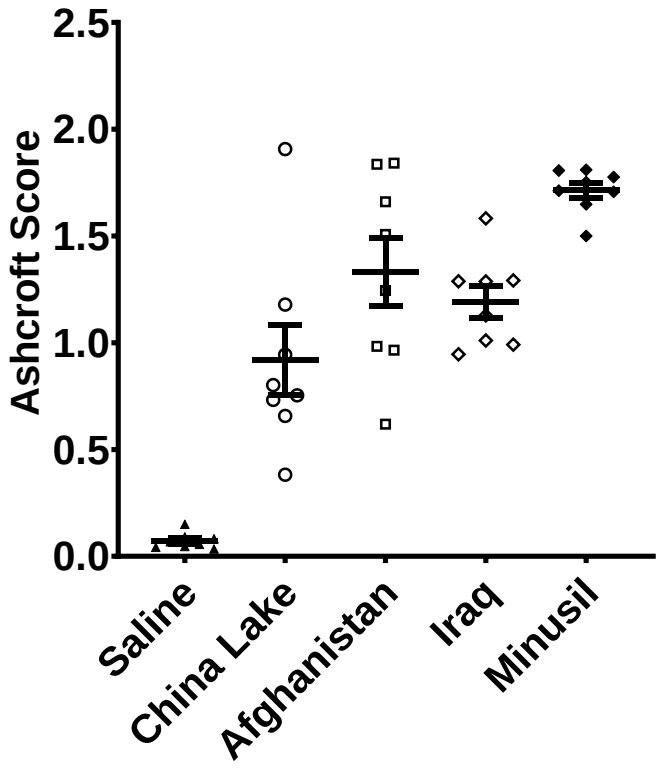


Figure 13. Respiratory exposure to PM from Afghanistan, Iraq, or China Lake or to crystalline silica resulted in pulmonary fibrosis as assessed by quantitative histological analysis (Ashcroft score).

To begin to dissect the mechanisms involved in these inflammatory and fibrogenic responses in the lung to exposure to silicate containing particulate matter, we used quantitative PCR arrays (QIAGEN) to assess changes in the mRNA expression of fibrogenic genes in the mouse lungs. These results are illustrated in **Figure 14**. From these results it is apparent that particulate matter exposure results in enhanced expression of a variety of pro-inflammatory and proteolytic genes including CCL-3, TNFα, IL-1α, IL-1β, MMP-2, MMP-3, MMP-13, MMP-14, TIMP-1, CCL-11 as well as profibrotic genes TGF-β1, Col3a1, and CTGF.

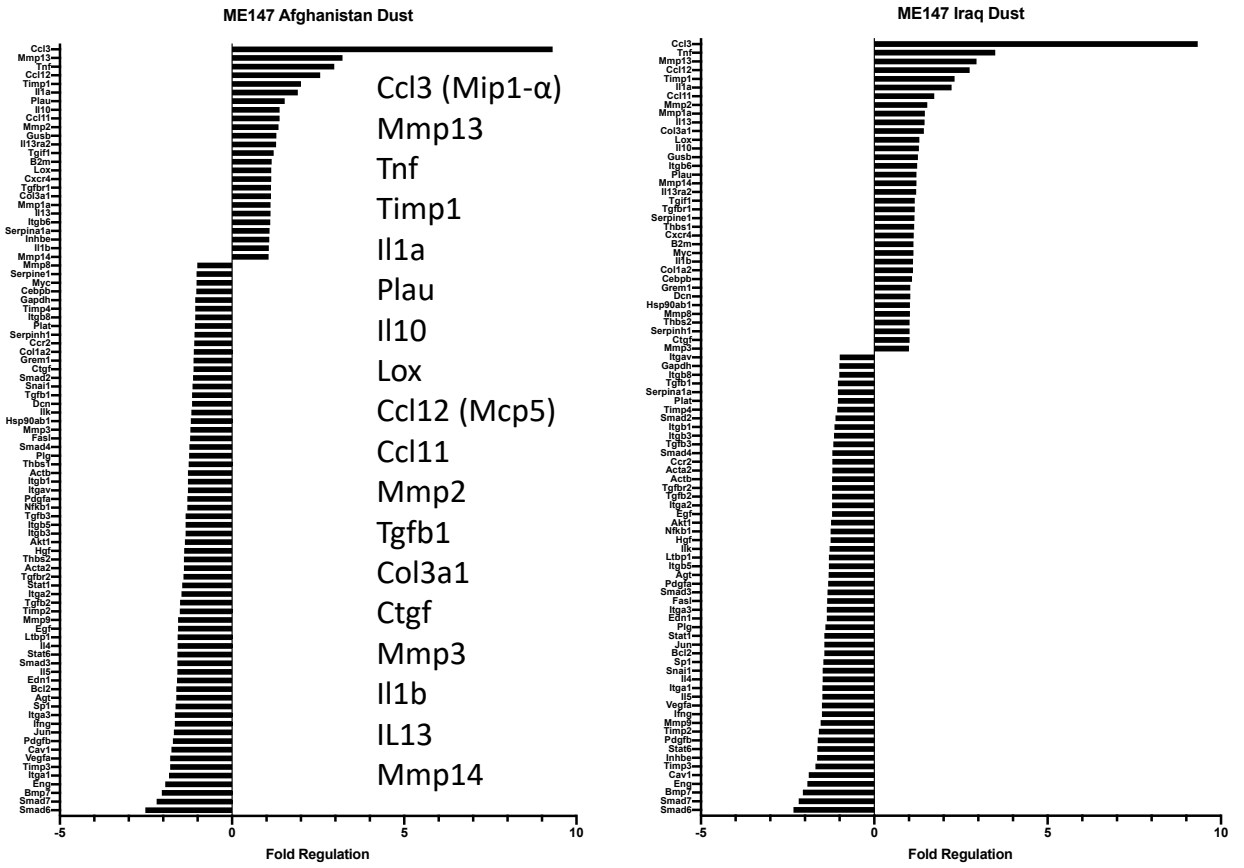


Figure 14. Gene Expression Alterations in Mouse Lungs in Response to Multiple Dose Instillation of PM from Iraq and Afghanistan. QIAGEN qPCR arrays.

Blast Overpressure Lung Injury using a Shock Tube

As outlined in the reports from Year 3, we have developed a robust model of blast over-pressure acute lung injury that results in pulmonary hemorrhage, injury, and inflammation (**Figure 15**) and an increase in lung permeability (**Figure 16**).

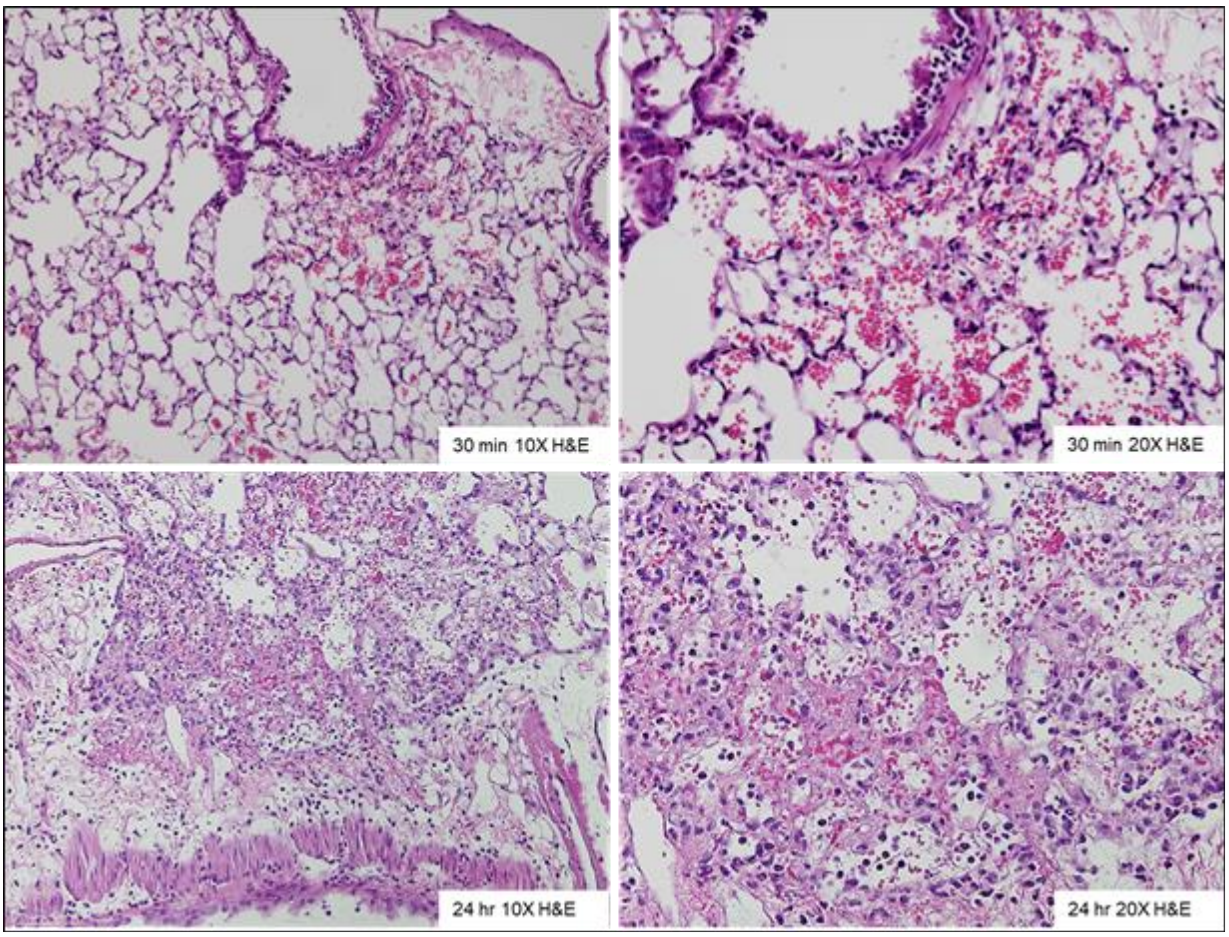


Figure 15. Overpressure blast lung injury after exposure of mice to the blast wave generated by the Shock tube described above. Note evidence of alveolar hemorrhage and inflammation in proximity to air/tissue interfaces such as adjacent to airways.

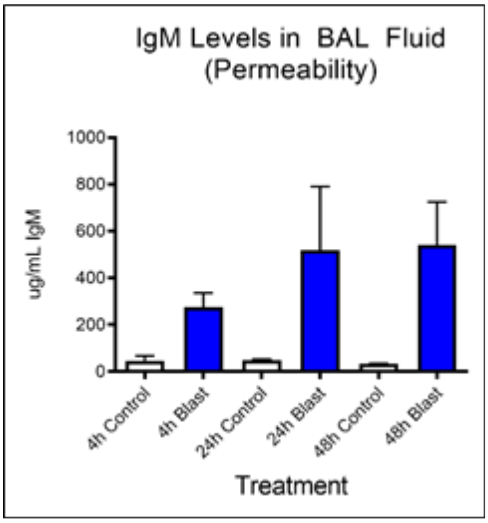


Figure 16. Increased lung epithelial permeability measured by IgM in BAL fluid after blast overpressure lung injury.

In Year 4, we have studied the effects of combined exposure to PM from Afghanistan, China Lake or to purified structured silica (Minusil) prior to blast overpressure lung injury (the '2-hit model). We completed two large experiments (n=7-8 for every group) the results of which are summarized below. For these experiments, mice were first exposed to PM (oropharyngeal aspiration) from Afghanistan, Iraq, or China Lake, or to purified silica (Minusil - used here as a positive control) using the multiple dose (10 mg/kg every second day for 5 doses over 10 days) followed by blast overpressure injury 24 hr later. As illustrated in **Figures 17-23**, oropharyngeal instillation of PM from Afghanistan or China Lake, or of purified silica (Minusil) using the multiple dose protocol resulted in pulmonary inflammation (increased number of total cells including macrophages and neutrophils in BAL fluid), increased lung permeability (albumin leak), endothelial injury (serum angiopoietin), and epithelial injury (BAL RAGE). Importantly, all of these parameters were amplified by subsequent blast overpressure lung injury at both the 24 or 48 hr time points. This is evidence of an additive effect on lung injury of prior exposure to particulate matter followed by blast overpressure lung injury. This data is strongly supportive of our 2-hit hypothesis.

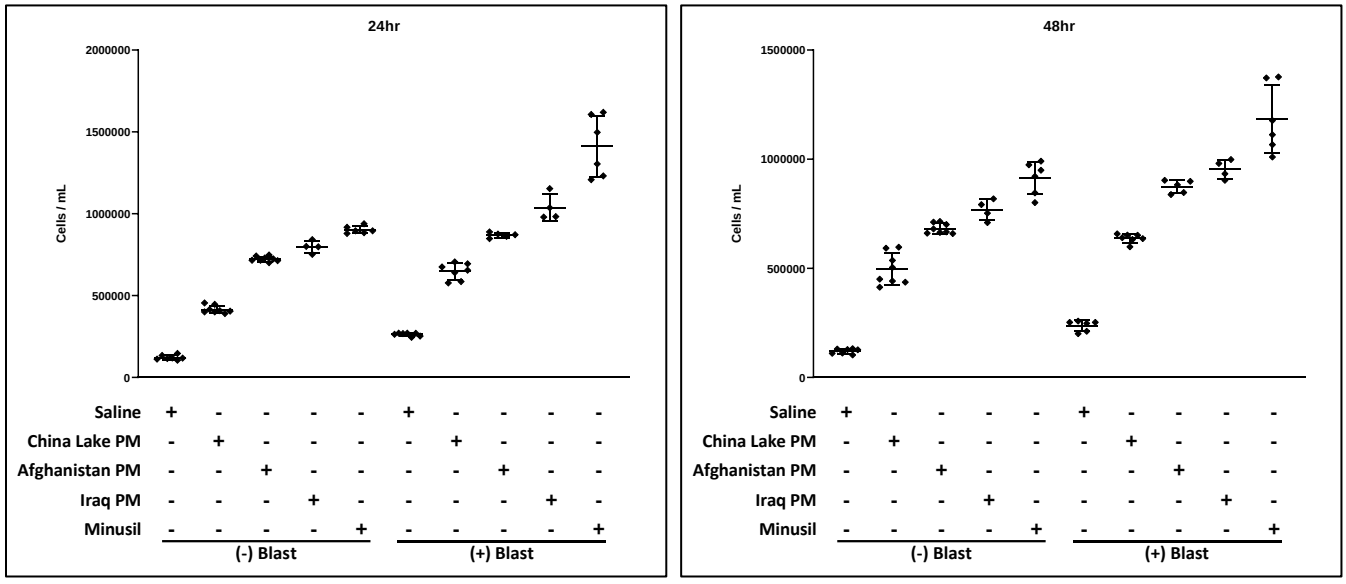


Figure 17. Combined exposure to PM from Afghanistan, Iraq, or China Lake, or to purified silica (Minusil) followed by overpressure blast lung injury results in increased numbers of total cells in BAL fluid as compared to PM instillation alone or to blast injury alone.

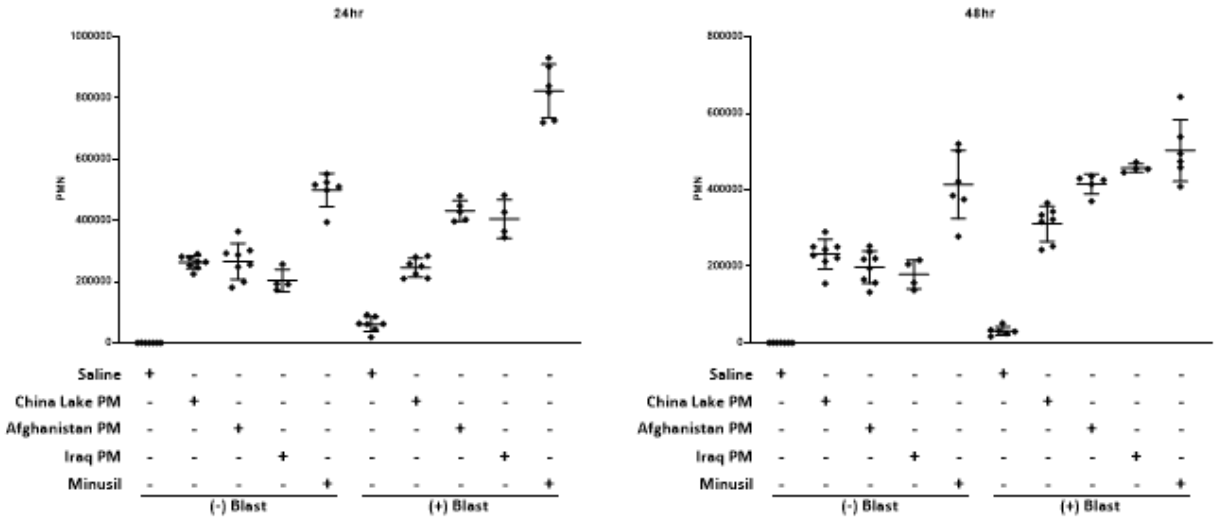


Figure 18. Combined exposure to PM from Afghanistan. Iraq, or China Lake, or to purified silica (Minusil) followed by overpressure blast lung injury results in increased numbers of neutrophils (PMN) in BAL fluid as compared to PM instillation alone or to blast injury alone.

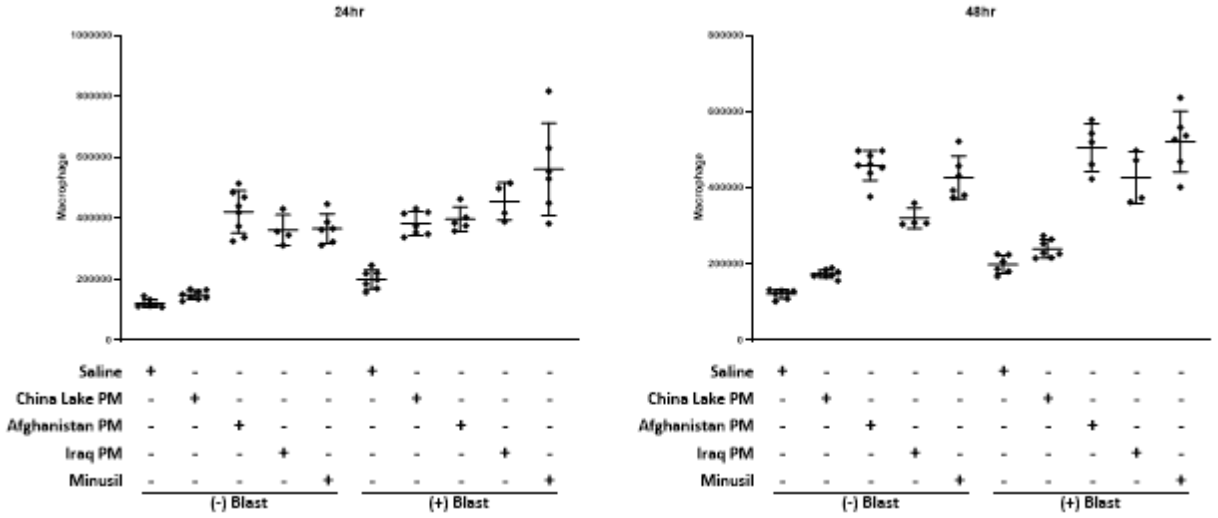


Figure 19. Combined exposure to PM from Afghanistan, Iraq, or China Lake, or to purified silica (Minusil) followed by overpressure blast lung injury results in increased numbers of macrophages in BAL fluid as compared to PM instillation alone or to blast injury alone.

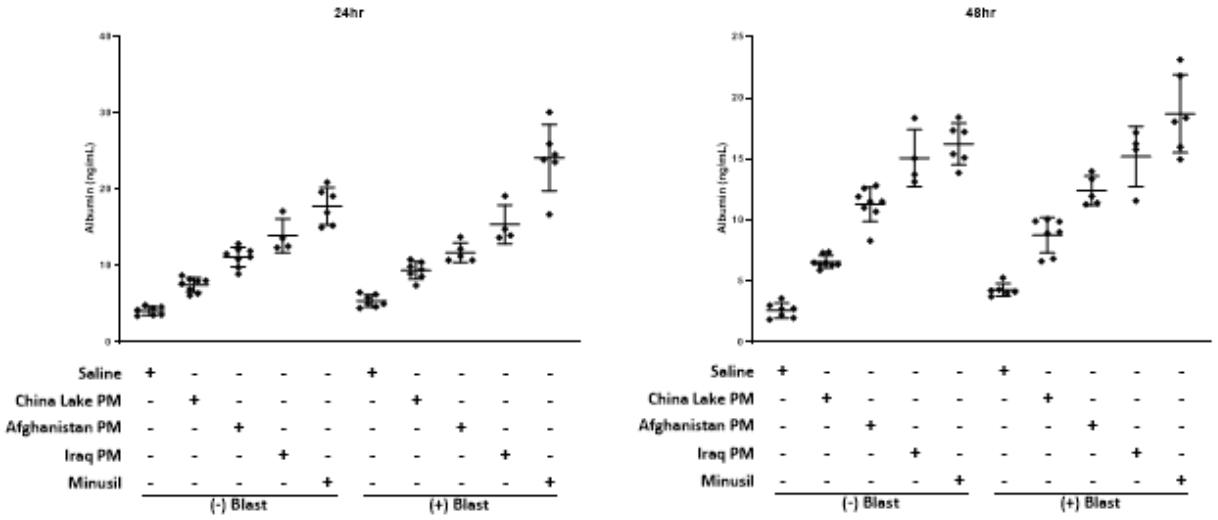


Figure 20. Combined exposure to PM from Afghanistan, Iraq, or China Lake, or to purified silica (Minusil) followed by overpressure blast lung injury results in increased albumin levels in BAL fluid (lung permeability) as compared to PM instillation alone or to blast injury alone.

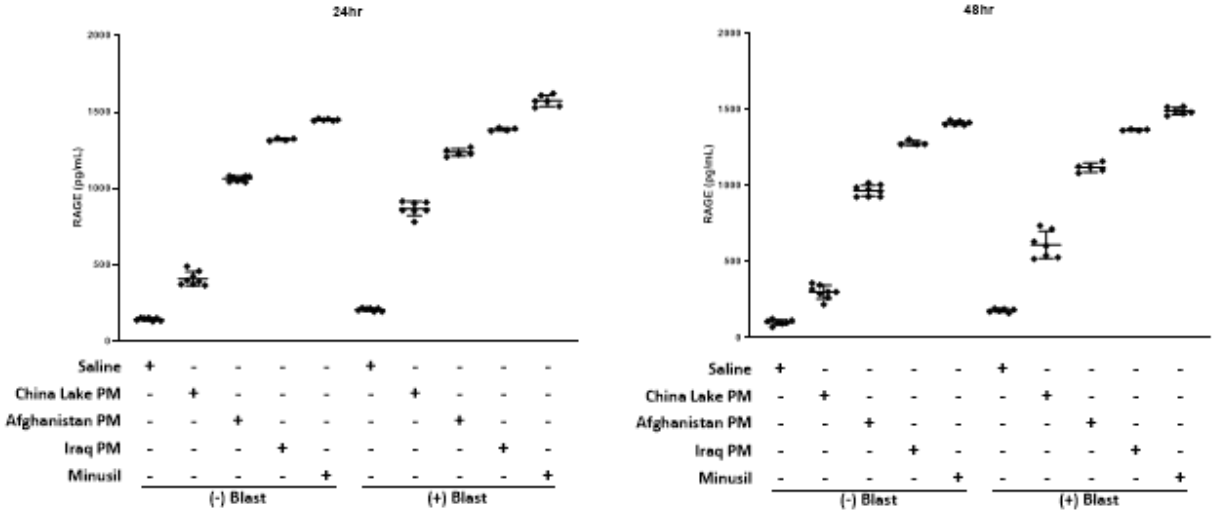


Figure 21. Combined exposure to PM from Afghanistan, Iraq, or China Lake, or to purified silica (Minusil) followed by overpressure blast lung injury results in increased RAGE levels (epithelial injury) in BAL fluid as compared to PM instillation alone or to blast injury alone.

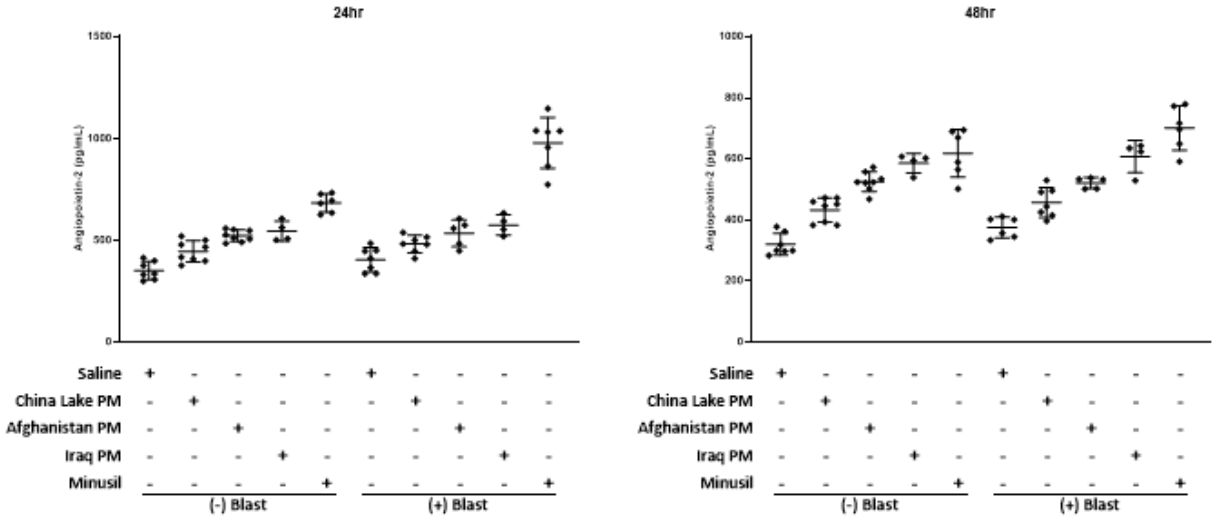


Figure 22. Combined exposure to PM from Afghanistan or China Lake, or to purified silica (Minusil) followed by overpressure blast lung injury results in enhanced endothelial injury as measured by serum angiopoietin-2 levels as compared to PM instillation alone or to blast injury alone.

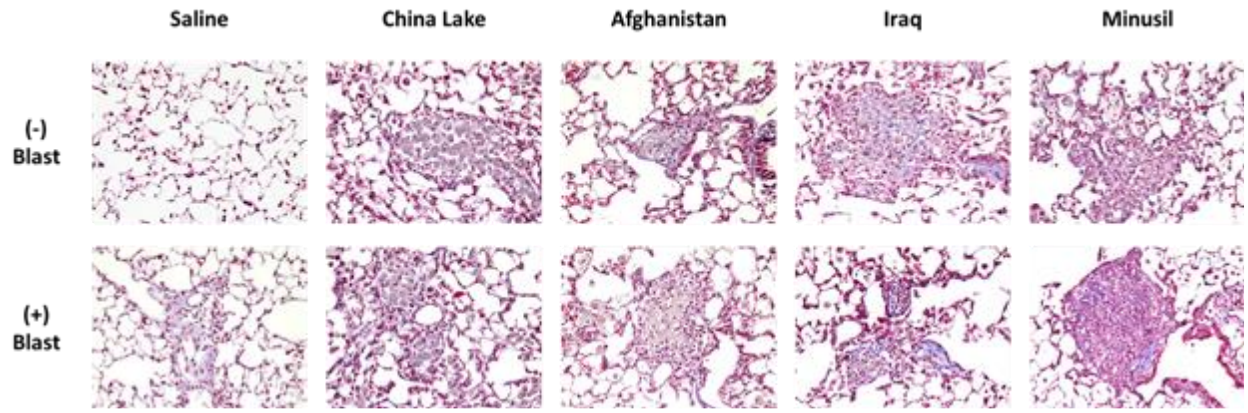


Figure 23. Histological appearance of lungs from mice with combined exposure to PM from Afghanistan or China Lake, or to purified silica (Minusil) followed by overpressure blast lung injury. Note the presence of macrophages containing PM and the appearance of granuloma-like structures in mice exposed to PM from Afghanistan and Iraq and to purified silica (Minusil).

Difficulties encountered

When given directly into the lung, either by intratracheal installation or oropharyngeal aspiration, the PM from Iraq and Afghanistan induced a robust pulmonary inflammatory response followed by lung fibrosis detectable between 28 and 35 days after the last dose. By contrast, PM given by aerosolization induced an inflammatory response but did not induce significant fibrosis as judged by histological examination. This is likely due in part to the efficient filtering of aerosolized PM by the nasopharynx of mice. In addition, the lungs of mice have a very rapid clearance rate for particulate matter when given below a threshold dose. Both factors likely contribute to the lack of fibrosis induced by aerosol exposures to PM, even purified silica. As noted above, we modified our aerosolization protocol to increase the concentration PM to 75 mg/m^3 given over 12 days and then waited 60 days to analyze lung fibrosis. This time frame was based on published literature using aerosolized silica exposure in mice where aerosolized silica induced fibrosis that was measurable only at higher concentrations and when the analysis was delayed 60 to 90 days after silica exposure. These experiments were done in collaboration with Drs. Wong and Mumy at NAMRU-Dayton and have been completed. We observed biochemical evidence of increased lung collagen (hydroxyproline) but no histological evidence of pulmonary fibrosis using trichrome and picrosirius red staining.

Subtask 5. Determine combined effects of PM and physical, chemical, and infectious stimuli in mouse models. Test effects of small molecules on acute lung injury.

Progress

In the last quarter of Year 4 we have begun to test the effects of small molecule inhibitors of the WNT β-catenin pathway (ICG-001) on lung injury from blast overpressure lung injury. We are doing these experiments in collaboration with Dr. Michael Kahn at City of Hope Medical Center in California who is the discoverer of the ICG-001 compound. We decided to study the effects of ICG-001 in the blast overpressure lung injury because this model is brief (longest time point 72 hours) and thus the ICG-001 can be given by IP injection twice daily for 3 days as opposed to administration by mini-osmotic pump would be required for longer studies. In the design of this experiment, we chose to administer the first dose of the ICG-001 1 hour after the blast exposure to realistically reflect what might happen on the battlefield where soldiers are exposed to an IED blast and medics could hopefully reach them within an hour to administer the medication. As illustrated in **Figure 24.** blast overpressure lung injury was attenuated by ICG-001 50 mg/kg with the first dose administered 1 hr after the blast exposure and continued twice daily for 48 hours. There was some variability between animals in this first experiment and we are currently

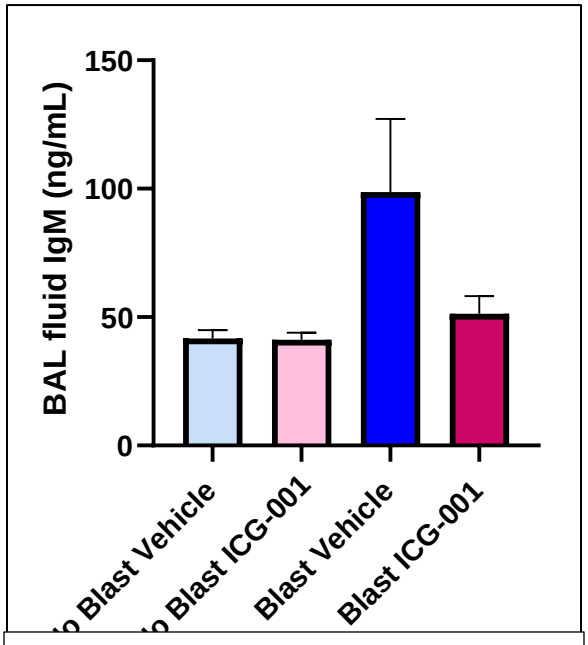


Figure 24. Lung permeability as measured by [IgM] in BAL fluid for mice exposed to blast over pressure injury (Blast) with or without treatment with ICG-001. Mean±SEM n=5-6 animals per group.

optimizing the experimental protocol to minimize any inter-animal variability. Our plans for the next year are to optimize and then repeat this experiment and extend the time out to 72 hours. We then plan to study the effects of ICG-001 on pulmonary inflammation and fibrosis induced by exposure to particulate matter from Iraq and Afghanistan. These experiments will extend to 35 days after the last particulate matter exposure. For the studies we will need to administer the ICG-001 by implantable subcutaneous osmotic pumps.

Difficulties encountered
We continue to experience some variability in the peak blast pressure between individual membranes used to generate the blast overpressure. We have purchased a new lot of nylon membranes and had them precision cut using laser technology at a commercial site. We hope that this will minimize the variability between membranes. We also purchased Mylar membranes and are currently testing them for reproducibility of blast pressure.

Subtask 6. Develop and refine techniques to analyze metal and mineral content of VATS lung biopsies focusing on the distal lung parenchyma and alveolar areas using LA-ICP-MS, and FE-SEM.

Progress
We have continued our collaboration with Drs. Geoff Plumlee, Heather Lowers, Bill Benzel, Kate Campbell-Hay, and David Roth from the US Geological Survey and have made progress in characterization of the particle number and elemental composition of the lungs of mice that had particulate matter from Afghanistan and Iraq and California (China Lake) instilled by oropharyngeal instillation. For this analysis, we used inductively coupled plasma mass spectrometry (ICP-MS). These studies are summarized below in **Table 1** and **Figure 25**. Notably, the concentrations of some of the elements are very low in concentration and below the levels in the blanks (**Figure 25**). These studies indicate that silicate-containing PM can be detected in BAL fluid and lung tissue although the levels present for some metals are close to the limits of detection.

Slice_thickness_(um)	0	40	40	40	40	40	40	40	40	40	40	40	40
Slice_area_(cm2)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Slice_volume_(cm3)		1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Filter_(g)	0.0373	0.0381	0.0392	0.0414	0.0420	0.0422	0.0377	0.0382	0.0389	0.0401	0.0408	0.0424	0.0408
Tissue+Filter_(g)	0.0373	0.0456	0.0464	0.0555	0.0496	0.0502	0.0542	0.0459	0.0475	0.0471	0.0553	0.0503	0.0506
Filter_+_Tissue_(g)_after_xylene_and_dry_overnight	0.0367	0.0376	0.0388	0.0409	0.0415	0.0419	0.0372	0.0380	0.0386	0.0397	0.0402	0.0422	0.0404
Weight_of_tissue_(g)	-0.0006	0.0001	0.0002	0.0001	0.0001	0.0003	0.0001	0.0004	0.0003	0.0002	0.0000	0.0004	0.0002
Suspension_volume_(mL)	25	25	25	25	25	25	25	25	25	25	25	25	25
Aliquote_volume_(uL)	25000	25000	25000	25000	25000	25000	25000	25000	25000	25000	25000	25000	25000
Fraction_of_suspension_	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Active_area_filter_(mm2)	181	181	181	181	181	181	181	181	181	181	181	181	181
Field area (um)	48	48	48	48	48	48	48	48	48	48	48	48	48
Field height (um)	42	42	42	42	42	42	42	42	42	42	42	42	42
Field area (um)	2025	2025	2025	2025	2025	2025	2025	2025	2025	2025	2025	2025	2025
# of fields completed	56	18	20	7	12	26	22	26	44	34	19	55	54
Fraction of filter examined	0.0006	0.0002	0.0002	0.0001	0.0001	0.0003	0.0002	0.0003	0.0005	0.0004	0.0002	0.0006	0.0006
Total # particles	1089	973	913	976	314	986	745	919	827	913	957	352	470
Total #geologic material	692.0000	845.0000	780.0000	743.0000	197.0000	745.0000	422.0000	660.0000	579.0000	605.0000	831.0000	137.0000	156.0000
Totalparticles/cm3	#DIV/0!	4.8E+06	4.1E+06	1.2E+07	2.3E+06	3.4E+06	3.0E+06	3.2E+06	1.7E+06	2.4E+06	4.5E+06	5.7E+05	7.8E+05
Ag_phase	1	0	0	0	0	0	0	0	0	0	0	0	0
Al_oxide	41	1	1	6	0	18	2	3	6	15	2	5	9
Al_oxide_mix	15	2	2	0	0	3	0	0	1	17	5	0	2
Al_P_phase	0	1	0	0	1	0	1	0	0	0	3	0	0
Al_S_phase	0	0	0	1	0	1	0	0	0	0	0	0	0
Aluminosilicate_Al_gt_Si	15	3	3	7	2	10	0	1	3	29	231	3	3
Aluminosilicate_Al_lt_Si	68	137	146	186	26	168	67	97	84	116	430	23	13
Au_phase	2	0	0	0	0	0	0	0	2	2	1	0	0
Ba_phase	1	1	0	0	0	0	0	1	0	0	0	1	0
C_Cl	35	11	6	13	12	45	33	45	45	34	8	24	24
Ca_Al_oxide	0	0	1	0	0	0	0	0	0	3	0	0	0
Ca_Al_silicate	11	5	4	3	2	1	2	5	2	61	13	0	0
Ca_Mg_Al_Si	1	0	0	1	0	1	0	1	0	22	0	0	0
Ca_Mg_Fe_Al_silicate	11	2	0	0	0	2	1	0	1	2	0	0	0
Ca_Mg_Fe_silicate	9	2	0	0	1	3	1	3	2	4	2	0	0
Ca_Mg_phase	0	0	2	0	1	1	2	2	0	1	0	0	0
Ca_Mg_silicate	9	0	0	0	0	3	0	2	2	2	0	1	0
Ca_Mg_Ti_Al_Si	0	0	0	0	0	0	0	0	0	3	0	0	0
Ca_P_phase	0	0	0	1	1	2	8	0	1	0	0	0	0
Ca_P_phase_mix	1	0	1	0	1	1	2	1	0	1	0	0	0
Ca_phase	13	3	5	2	0	1	7	8	5	24	2	11	27
Ca_phase_mix	5	2	1	0	0	0	0	1	0	8	0	0	2
Ca_silicate	5	0	0	0	0	0	0	2	5	8	1	2	0
Ca_Ti_oxide	1	0	0	0	0	0	0	0	0	0	0	0	0
CaCl2	18	0	1	2	10	24	32	50	11	24	4	24	3
Carbonaceous	138	28	45	165	10	83	197	66	97	96	69	91	153
Carbonaceous_No O	11	2	7	4	6	1	6	2	8	7	0	11	8
Cr_phase	4	2	0	0	0	2	6	3	2	2	3	3	2
Cu_phase	0	0	1	0	2	0	0	0	0	1	0	0	0
Fe_aluminosilicate	4	9	16	5	4	2	3	5	12	6	0	0	1
Fe_Cr	16	1	0	0	0	0	0	2	4	2	0	2	9
Fe_Ni	9	0	1	0	1	2	0	4	3	5	4	3	18
Fe_Ni_Cr	2	2	0	0	0	0	0	0	2	0	0	0	6
Fe_oxide	53	23	15	12	2	32	15	30	32	44	7	22	51
Fe_oxide_mix	32	50	42	29	18	24	20	38	23	41	14	16	5
Fe_silicate	6	3	2	1	1	3	1	4	2	1	1	1	0
Fe_Ti_oxide	0	0	0	0	0	1	0	0	0	3	0	0	0
Fe_Ti_oxide_mix	0	1	1	0	0	0	0	0	1	0	0	0	0
Feldspar_	1	0	0	0	0	0	0	0	0	0	0	0	0
Feldspar_K	15	25	13	11	3	73	50	45	50	13	6	8	5
Feldspar_Na	12	10	6	4	5	17	16	19	17	14	1	5	3
Feldspar_NaK	2	4	3	1	0	3	2	3	6	1	1	2	1
Feldspar_Plag	26	2	1	0	0	5	6	3	13	5	3	1	3
K_aluminosilicate	27	149	94	70	37	78	45	92	87	36	11	13	11
Mafic	9	7	7	3	3	3	1	6	2	18	0	0	0
Mg_aluminosilicate	5	85	128	128	19	105	65	76	42	13	2	3	2
Mg_Fe_aluminosilicate	46	238	251	190	49	119	70	144	127	39	9	10	6
Mg_Fe_phase	3	1	0	0	0	0	5	4	3	7	6	2	7
Mg_Fe_Silicate	8	1	0	2	0	3	0	3	5	1	4	0	1
Mg_phase	0	1	0	1	0	0	2	3	1	1	1	0	0
Mg_silicate	1	15	11	9	3	7	8	7	4	5	0	2	4
Mn_phase	0	1	0	0	0	0	0	0	0	0	0	0	0
Na_K_Ca_S_Cl	5	1	5	0	2	0	6	2	5	2	3	0	2
Na_P_phase	0	0	0	0	1	0	0	0	0	0	0	0	0
NaCl	1	0	1	0	0	0	0	6	0	0	0	0	2
Ni_phase	7	0	1	0	0	1	0	1	1	1	0	0	6
P_phase	1	0	0	0	0	0	0	0	0	0	0	0	0
REE	0	0	0	0	0	0	0	0	1	0	0	0	0
S_phase	0	0	1	0	0	0	0	0	0	0	0	0	0
Silica	226	59	45	70	24	79	35	60	58	69	30	31	54
Silica_mix	101	24	16	20	3	30	12	30	17	17	5	9	2
Sn_phase	1	0	0	0	51	4	1	1	5	2	2	14	12
Ti_Fe_oxide	0	4	0	1	0	0	1	1	1	3	8	0	2
Ti_Fe_oxide_mix	1	6	0	1	0	0	0	0	1	2	0	0	0
Ti_oxide	29	25	8	10	6	13	6	13	12	42	28	7	9
Ti_oxide_mix	23	22	18	13	6	10	6	19	12	33	36	2	2
Titanite	0	2	0	2	0	2	2	2	1	3	0	0	0
Zr_phase	2	0	1	2	1	0	0	3	0	2	1	0	0

Table 1. ICP-MS results from lungs of mice instilled with PM from Iraq, Afghanistan, or China Lake.

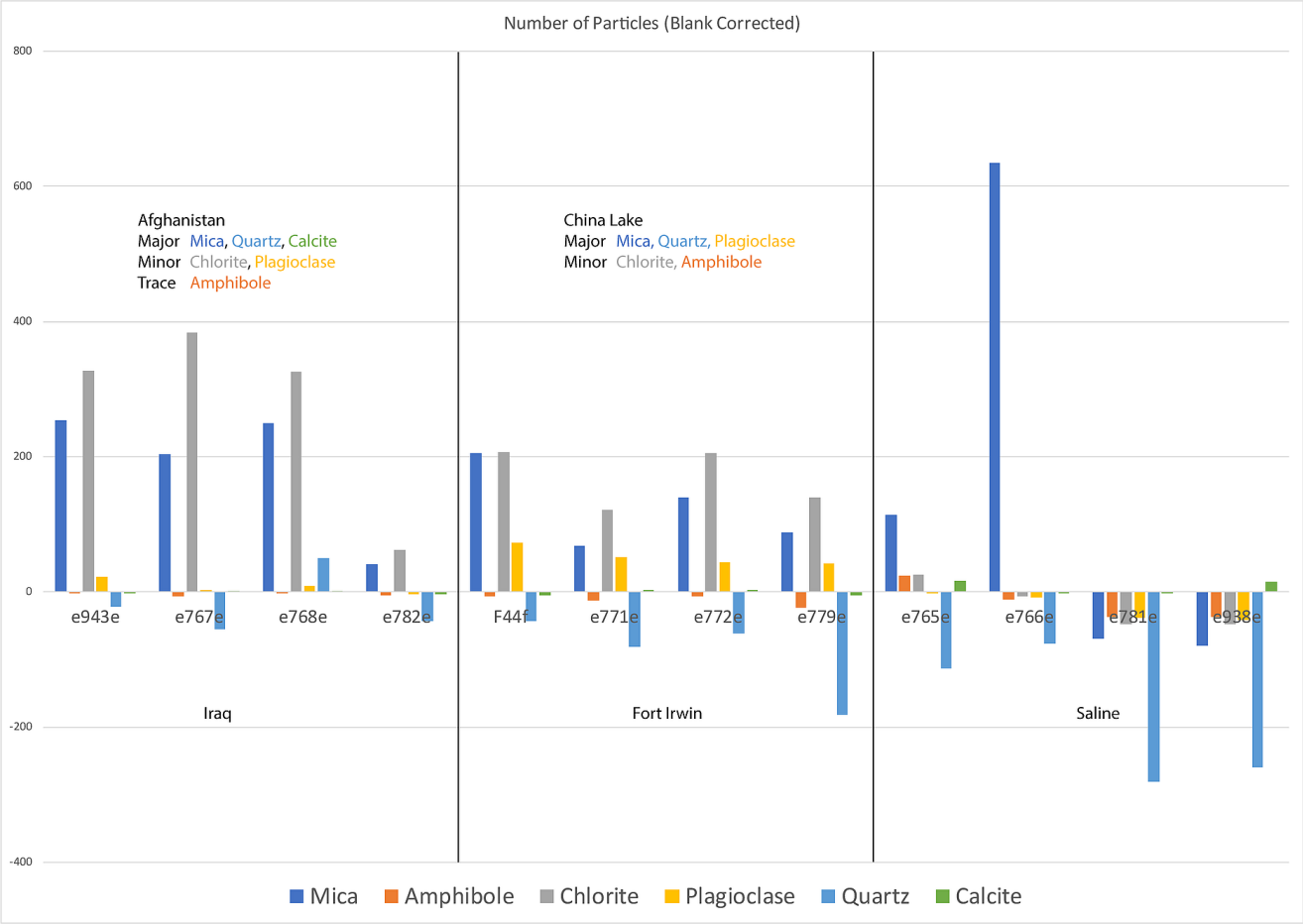


Figure 25. ICP-MS results from lungs of mice instilled with PM from Iraq, Afghanistan, or China Lake.
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Difficulties encountered.
We found that the concentrations of some of the elements in the lung are very low and in fact below the levels measured in the blanks (**Figure 25**).

Subtask 7. Complete analysis of VATS lung biopsies focusing on the distal lung parenchyma and alveolar areas using LA-ICP-MS, and FE-SEM.

Progress
We have just completed the analysis of mouse lung samples demonstrating feasibility to measure PM content in lung tissues. In Year 5 we plan to analyze the PM mineral content of VATS lung biopsy samples stored at the University of Colorado Hospital. We have approval from the University of Colorado IRB (CoMIRB) from HRPO to do this analysis in Year 5.

Subtask 8. Prepare and submit manuscripts for publication.

Progress
Three manuscripts have been published (see project 3 – Berman R et al, *Toxicol Sci* 2018). Three abstracts were submitted and accepted for presentation at the Military Health System Research Symposium in August 2020 in Orlando, FL. Unfortunately, this symposium was cancelled because of COVID-19. The abstracts were published on line.

Milestones Achieved:

1. ('2-hit') *In vitro* epithelial cell culture model protocols have been completed characterizing the combined effects of PM and bleomycin on lung epithelial cell toxicity.
2. We have completed the molecular analysis of the effects of PM exposure on primary human lung epithelial cells (large airway, small airway, and alveolar type II cells) using RNA-Seq. We are currently completing advanced pathway analysis (Ingenuity Pathway Analysis).
3. *In vivo* murine model protocols reveal robust small airways and parenchymal inflammation and fibrosis by PM from Iraq and Afghanistan when delivered by direct i.t. instillation or by oropharyngeal aspiration.
4. We have completed the initial inhalational exposure experiments in which mice were exposed to aerosolized particulate matter from Afghanistan and from California in the NAMRU-Dayton facility. We have completed the analysis of these experiments and in contrast to i.t. installation, aerosol exposure to PM induces mild inflammation but no detectable fibrosis. A modified protocol in which the concentration of the PM was increased to 75 mg/m³ given over 12 days resulted in only mild fibrosis when analyzed at 72 days. We are in the process of conducting a third set of experiments with the higher dose protocol using purified silica (Minusil) as a positive control. Seventy-two days will be the end point for this next set of experiments.
5. We have begun to test the effects of a WNT/β-catenin inhibitor ICG-001 on epithelial injury in vitro and on lung injury in vivo in the blast overpressure lung injury model.
6. Two abstracts reporting the results of the studies to date with PM exposure in mice and PM exposure to lung epithelial cells were accepted as posters and one abstract was accepted as an oral presentation in a mini Symposium at the Military Health System Research Symposium Orlando, FL in August 2020. The symposium was cancelled but the abstracts are available online.

Goals for next reporting period to accomplish goals and objectives:

1. We will complete the pathway analysis of RNA-seq of human large airway, small airway, and alveolar type II cells including bioinformatic statistical analysis and include this data in a manuscript to be submitted for publication.
2. Complete experiments characterizing the effects of ICG-001 on blast overpressure lung injury in mice.
3. Complete experiments of influenza infection induced lung injury in mice with or without prior exposure to PM.
4. Complete experiments of characterizing the effects of ICG-001 on influenza infection induced lung injury in mice.
5. Analyze the mineral and metal content of human VATS lung biopsy samples with the US Geological Survey.

PROJECT 3. Impact of Cigarette Smoke on PM-induced Airway Epithelial Injury and Exacerbation of Asthma and Bronchiolitis in Deployed Military Personnel.

Major Task 3: Establish *in vitro* bronchial epithelial cell and animal model exposure to airborne PM.

1. We have accomplished the development of National Jewish Health Human Live Cell Core protocols and procedures. We have our local National Jewish Health IACUC, USAMRMC Animal Care and Use Review Office (ACURO) approval for mouse experiments.

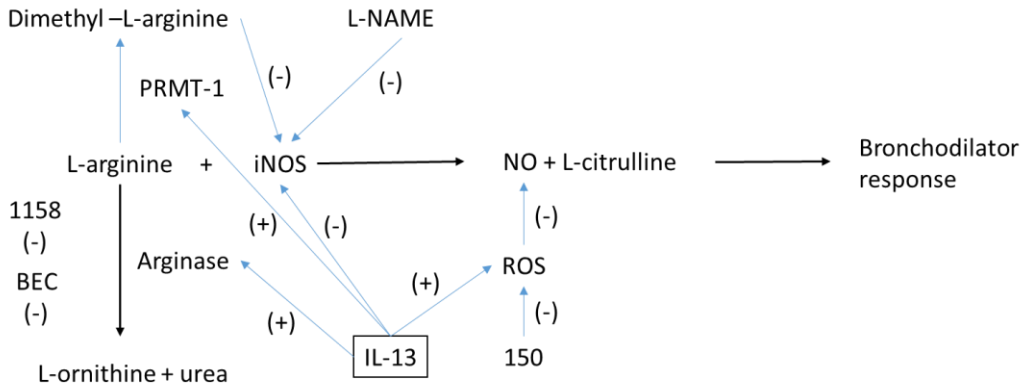
Subtask 1. Develop and refine methods to study the effects of PM, allergic stimuli, and cigarette exposure on bronchial epithelial cells *in vitro.*

Progress
Done

Subtask 2. Determine combined effects of PM and allergic stimuli and cigarette exposure on bronchial epithelial cells *in vitro.*

Progress

1. We have published our results with lung epithelial cell exposure to PM and allergic stimuli showing that Afghanistan PM enhances pro-inflammatory effects in IL-13 treated epithelial cells via Toll receptor 2 signaling (Berman et al. Toxicol Sci 166:345-353, 2018). We have also previously reported responses of epithelial cells with cigarette smoke which was similar to that observed with PM. These finding have moved the focus of this task toward a more mechanism approach to better understand the interactions of PM with allergic stimuli.
2. Afghanistan PM (APM) induces iNOS in macrophages along with increased levels of nitric oxide than is inhibited by IL-13 exposure. The mechanism behind the IL-13-mediated inhibition of nitric oxide, a known endogenous bronchodilator, is unknown. We hypothesize that this could partially account for APM-mediated increase in airway hyperreactivity we see in our allergic animal models. We have tested a number of potential pathways to better understand the molecular mechanisms behind this observation. A number of studies were performed in J744 macrophage cell lines using pharmacological inhibitors to define the increased production of nitric oxide upon APM exposure and the ability of IL-13 to ablate this endogenous bronchodilator.



Schematic of several possible mechanisms by which IL-13 may mediate the inhibition of nitric oxide (NO) formation and pharmacological approaches to test possible mechanisms.

- Verification that APM increase in nitrite and nitrate (NOx) was produced by increased iNOS activity through inhibition with iNOS inhibitor, LNAME. We also verified that APM increased apoprotein levels of iNOS in the J744 cells and that IL-13 had little effect on this increased apoprotein expression.

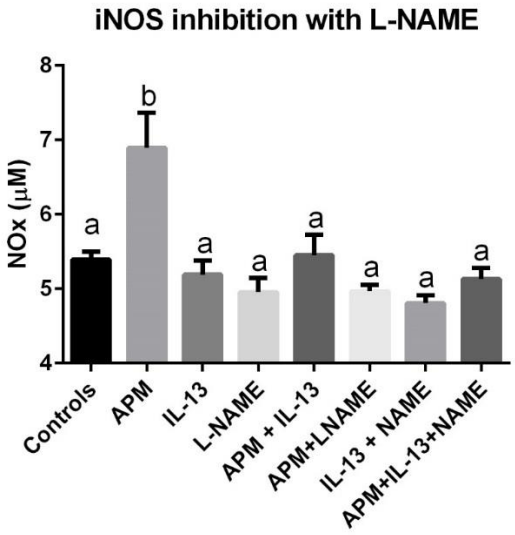
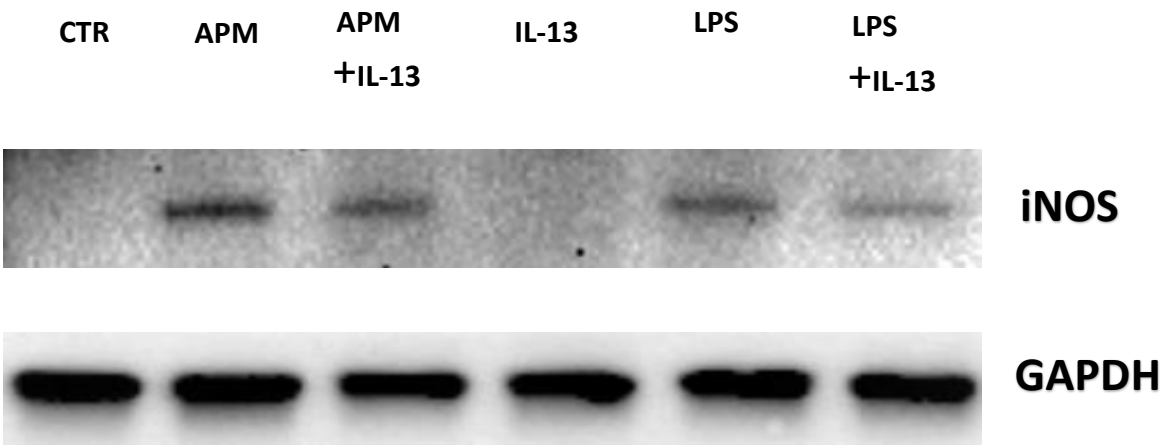
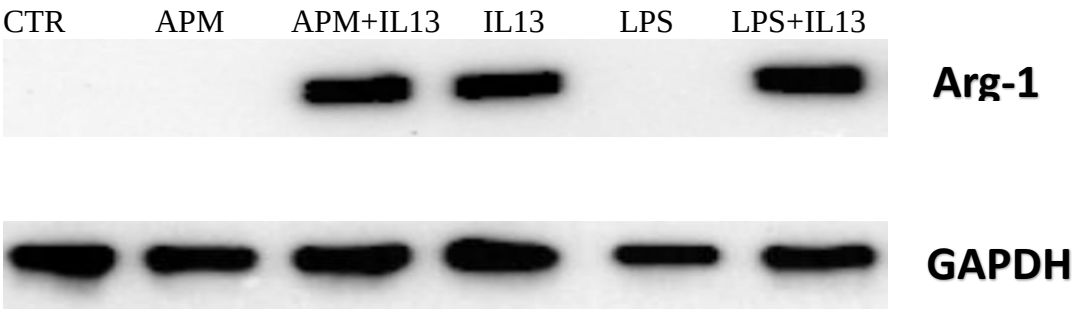


Figure 1. J744 cells were treated with IL-13 overnight and then exposed to APM with and without L-NAME (1mM) and nitric oxide production assessed 24 hours later measuring nitrite and nitrate levels (NOx) in the culture media. IL-13 was as effect as the known NOS inhibitor, LNAME, on blocking NO production in these cells.

- Verification that APM increases the expression of iNOS in J744 and the effect of IL-13 on iNOS protein expression by western blot.



- One possibility that may account for these observed effect is the well-known ability for IL-13 induced expression of arginase to decrease L-arginine availability for iNOS activity.



- We used two arginase inhibitors to test this hypothesis (CB-1158 and BEC).

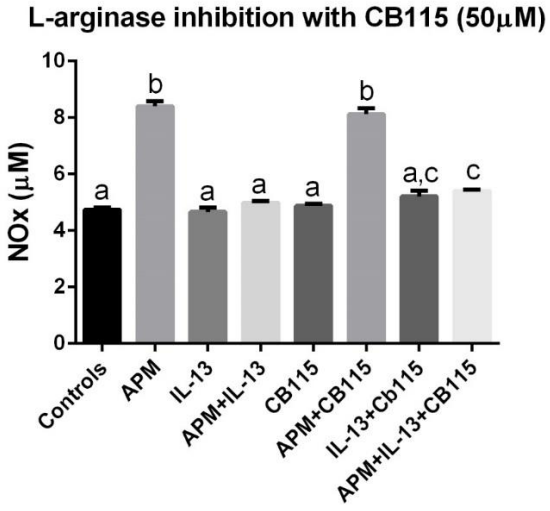


Figure 2. J744 cells were treated with IL-13 overnight and then exposed to APM with and without CB1158 (50uM) and nitric oxide production assessed 24 hours later measuring nitrite and nitrate levels (NOx) in the culture media. The combination of APM+IL-13+CB1158 did not reverse the IL-13 inhibition of APM-mediated increase in NO. This data suggested that the IL-13 effect is not through increasing arginase activity.

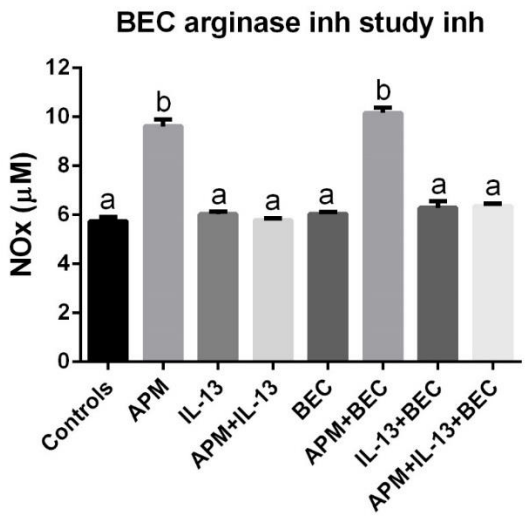


Figure 3. J744 cells were treated with IL-13 overnight and then exposed to APM with and without BEC (50uM) and nitric oxide production assessed 24 hours later measuring nitrite and nitrate levels (NOx) in the culture media. The combination of APM+IL-13+BEC did not reverse the IL-13 inhibition of APM-mediated increase in NO. This data suggested that the IL-13 effect is not through increasing arginase activity and decreasing arginine availability.

- Another possible mechanism for IL-13 inhibition of APM-mediated NO production could be through increased activity of protein methyltransferases (PRMT). Increase in PRMT can lead to the accumulation of dimethyl-L-arginine that is a known endogenous inhibitor of NOS in the same manner as L-NAME. To test this possibility, we used a well characterized PRMT-1 inhibitor furamidine (25uM).

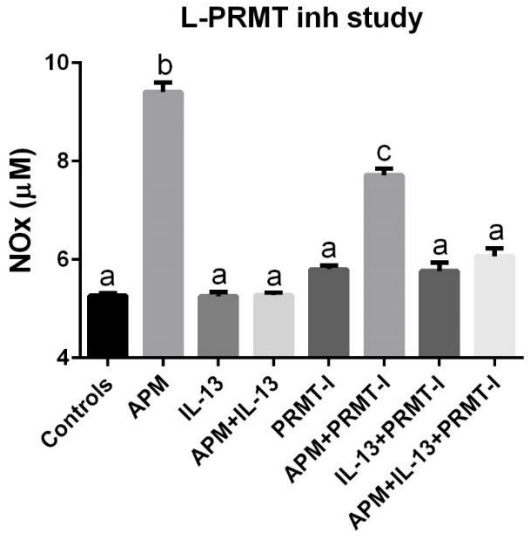


Figure 4. J744 cells were treated with IL-13 overnight and then exposed to APM with and without furamidine (25uM) and nitric oxide production assessed 24 hours later measuring nitrite and nitrate levels (NOx) in the culture media. The combination of APM+IL-13+furmadine did not reverse the IL-13 inhibition of APM-mediated increase in NO. This data suggested that the IL-13 effect is not through increasing PRMT activity and increasing dimethyl-L-arginine levels.

- Another possible mechanism for IL-13 inhibition of APM-mediated NO production could be through increased production of reactive oxygen species (ROS) that rapidly reacts with NO to produce peroxynitrite, a well-known bronchoconstrictor. To test this possibility, we used a well characterized ROS scavenger AEOL10150 (50uM).

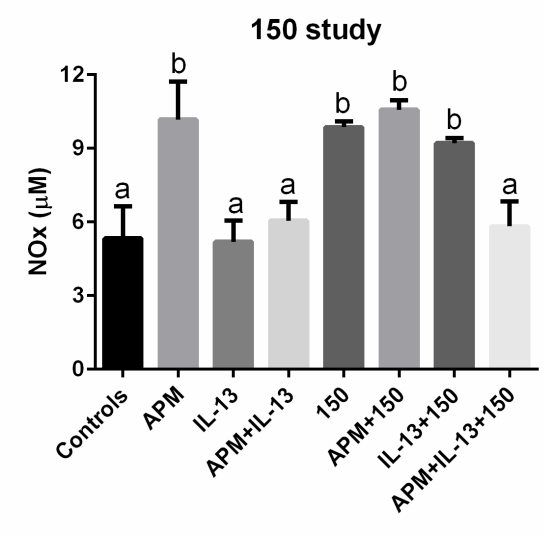


Figure 5. J744 cells were treated with IL-13 overnight and then exposed to APM with and without AEOL10150 (50uM) and nitric oxide production assessed 24 hours later measuring nitrite and nitrate levels (NOx) in the culture media. The combination of APM+IL-13+AEOL10150 did not reverse the IL-13 inhibition of APM-mediated increase in NO. This data suggested that the IL-13 effect is not through increasing ROS activity and decreasing NO levels.

Table of pharmacological inhibitors

Name	Concentration	Use	Figure
L-NAME	1 mM	NOS inhibitor	1
CB-1158	50uM	Arginase inhibitor	2
BEC	50uM	Arginase inhibitor	3
Furamidine	25 uM	PRMT-1 inhibitor	4
AEOL10150	50uM	Reactive oxygen species (ROS) scavenger	5

Results:

1. Verified the ability of APM to induce iNOS protein and activity in macrophage cells and that IL-13 treatment blocks this effect.
2. We tested a number of potential pathways that IL-13 may mediate inhibition of APM-mediated NO production.
3. All the tested pathways did not reverse the IL-13 effect on APM-mediated NO production.

Future Directions:

1. There are still a few pathways to test to identify the molecular mechanism behind IL-13's effect on APM-mediated NO production. We will look at the possibility that IL-13 is effecting cofactors needed for iNOS to produce NO. One possibility is that IL-13 is limiting the availability of tetrahydrobiopterin causing iNOS to become uncoupled. It has been reported that uncoupling of iNOS leads to the increased production of ROS that could be involved in airway hyperreactivity.

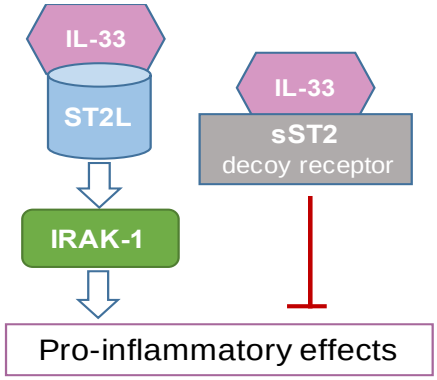
2. Change the model system to precision cut lung slices from mice and humans. This system will allow us to couple molecular changes to airway hyperresponsiveness in the same system and also compare rodent to human effects.

Difficulties encountered:
None

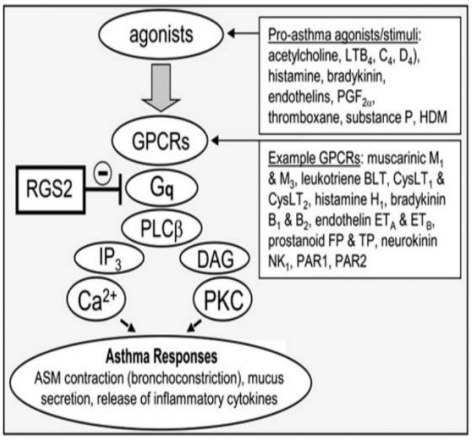
Subtask 3. Develop and refine methods to study the combined effects of PM and allergic stimuli and cigarette exposure on mouse models of airways hyperresponsiveness (asthma).

Progress:

1. We have refined our animal models that has led to a recent accepted manuscript, Berman et al IL-33/ST2 signaling modulates Afghanistan particulate matter-induced airway reactivity in mice. Toxicol Appl Pharmacol On line, 2020.
    - This paper details the investigation the IL-33/ST2 pathway in IL-13 and APM induced inflammation using an ST2 decoy receptor.



    - This paper also tested the effects of using a ST2 decoy receptor as a possible treatment for APM-mediated airway hyperresponsiveness.
    - This paper also describes a role for RGS2 in the downstream pathway associated with APM-mediated airway hyperresponsiveness.

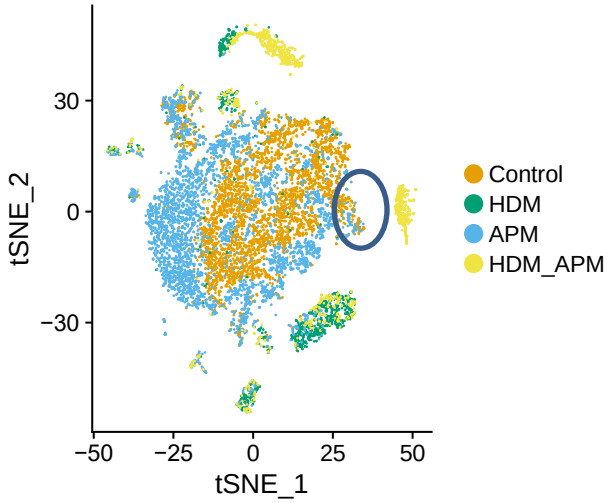


Future directions:

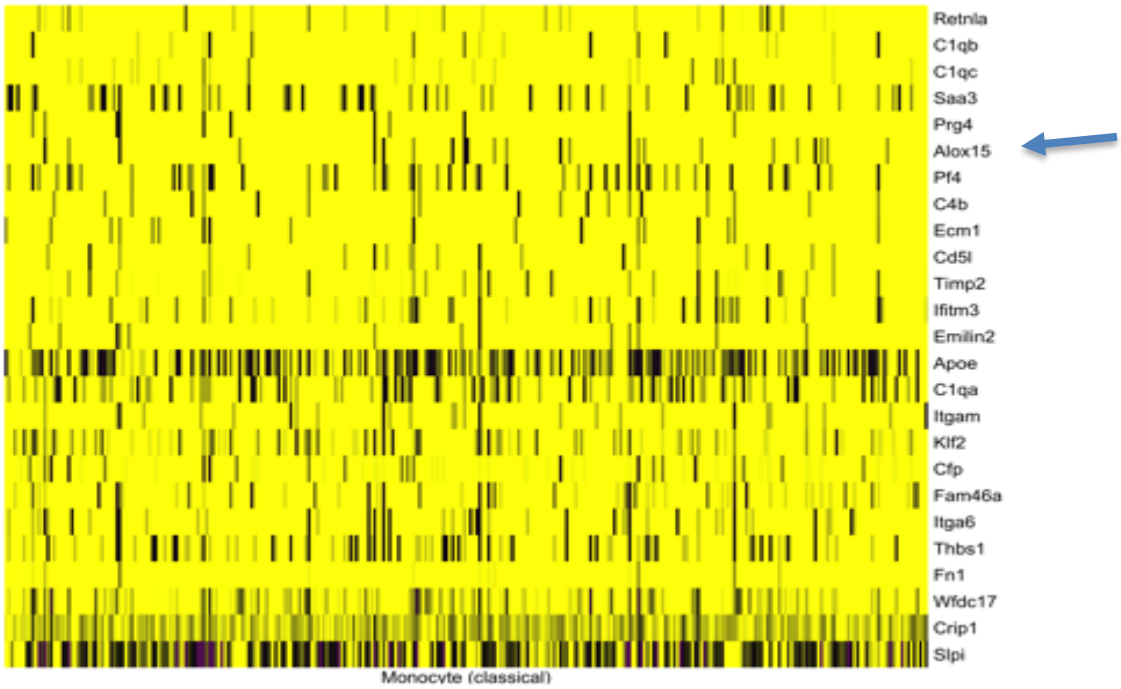
1. Test whether this system is altered in airway epithelial cells from deployers with asthma symptoms.
2. Test this pathway in human precision cut lung slices.

Afghanistan and California APM inhalation exposure at the NAMRU Dayton facility in combination with house dust mite sensitization

1. We have completed our studies comparing whole mouse APM inhalation exposures in house dust mite sensitization model and have submitted the manuscript for review. In this manuscript, we describe mice that were exposed to Afghanistan PM (APM) by whole body inhalation exposure (6 hr/day for 2 weeks) then sensitized with house dust mite (HDM) antigen and airway reactivity was assessed at day 17 by methacholine challenge. Interestingly, the APM exposure alone had no effect on airway reactivity. The mice that received the combination showed the greatest increased airway resistance (Rrs) at the higher methacholine challenge doses.
2. We performed single cell RNAseq on BAL cells isolated from these animals and found a unique monocyte and B cell population only observed in the APM/HDM interaction group.



3. The monocyte population had a unique gene signature with upregulation of ALOX15 (15-lipoxygenase) gene that has been shown to be upregulated in asthmatics.



4. Heatmap associated with the monocyte cluster in the APM+HDM treatment group. Alox15 gene expression associated with pathways controlling neutrophil influx and eosinophil function through expression of adhesion molecules (fibronectin and integrins).
5. We went on to validate ALOX-15 expression in monocytes from mouse lung sections in the interaction group

Future directions:

1. Test whether this system is altered in airway epithelial cells from deployers with asthma symptoms.
2. Test this pathway in human precision cut lung slices.

Difficulties encountered:

None

Subtask 4. Determine combined effects of PM and allergic stimuli and cigarette exposure on mouse models of airways hyperresponsiveness (asthma).

Progress

1. We have decided to focus the last year of effort on further studying the molecular mechanisms of APM/HDM interactions, since cigarette smoke produced a particulate response similar to the APM.

Subtask 5. Develop and refine techniques to analyze metal and mineral content of bronchial epithelial cells from brushings using laser capture microdissection, LA-ICP-MS, and FE-SEM.

Progress

1. We are working with the US Geological Survey to refine the system further. See project 2 for update on particulate characterization

Subtask 6. Complete analysis of bronchial epithelial cells isolated from bronchial brushing using LA-ICP-MS, and FE-SEM.

Progress

1. Methodology is currently being pursued. See project 2 for update

Subtask 7.

Progress

1. We have published one manuscript to date: Berman et al. Afghanistan particulate matter enhances pro-inflammatory responses in IL-13-exposed human airway epithelium via TLR2 signaling. *Toxicol. Sci*. 166(2), 345-353, 2018.
2. Society of Toxicology Poster (2018): Afghanistan particulate matter increases mouse airway hyperresponsiveness partially through IL-33 signaling. Reena Berman; Katie Kopf, Rafuel Alam; Greg Downey; Brian J. Day, PhD*; and Hong Wei Chu, MD* **(***Co-senior authors)
3. Society of Toxicology abstract (2019): Single Cell RNAseq Reveals a Unique Monocyte Population in BAL Cells of Mice Challenged with Particulate Matter and Allergen. Reena Reena Berman; Elysia Min; Jie Huang, MD; Katrina Kopf, DVM, MS; Gregory P. Downey, MD; Brian A. Wong, PhD; Kent Riemondy; Harry A. Smith, MPH;
   Hong Wei Chu*, MD; Brian J. Day*, PhD **(***Co-senior authors)
4. Published second manuscript: Berman et al. IL-33 /ST2 signaling modulates Afghanistan particulate matter-induced airway reactivity in mice. *Toxicol. Appl Pharmacol*, On-line, 2020.

Milestones achieved:

1. Collection of bronchial brushing airway epithelial cell culture model data of APM and allergic response.
2. Two *in vivo* animal exposure protocol development for APM exposure by whole body inhalation and by oropharyngeal aspiration.
3. *In vivo* interaction between Afghanistan APM and house dust mite sensitization on airway hyperreactivity.
4. Molecular mechanisms involved in APM interaction with HDM allergic sensitization.
5. Possible therapeutic intervention using ST2 soluble receptor.

Major activities:

1. Examine human bronchial epithelial cells from deployers with and without asthma exposed to APM *in vitro* in regards to IL-13 and IL-33 expression (ongoing).
2. We are accruing human bronchial epithelial cell brushings from deployed subjects for subtasks 2, 3, 5 and 6.
3. Setting up more integrated model system using precision cut lung slices that have all the lung cell types in proper anatomical locations.

4. Using the precision cut lung slices to validate earlier findings between APM and allergic asthma.
5. Using precision cut lung slices from mouse and humans to validate findings in our rodent models to humans.

PROJECT 4. Omics' Analysis of Airway Epithelium in Deployment-Related Lung Diseases.

Major Task 4: Establish 'omics' approaches to analyze the transcriptome and genetics of *in vivo* and *in vitro* airway epithelium from subjects with deployment-related lung diseases to determine mechanisms of these diseases, the molecular effects of PM exposures, and to identify minimally invasive biomarkers for these diseases and exposures.

Subtask 1. Establish methods to identify biomarkers and disease mechanisms for deployment lung diseases (asthma and DDLD) using whole-genome molecular analyses of *in vivo* airway epithelium.

Progress

1. During this past reporting year, we have continued to recruit deployed subjects into the GLIDE study. In order increase the number of deployers consented for this study, Project 1 personnel have implemented a new strategy in consenting potential deployers. Previously, consent was contingent upon knowledge of a diagnosis (either asthma, DDLD, or asthma+DDLD) prior to contact for consent and further scheduling of a subsequent visit to NJH. In this reporting period (Year 4), deployed subjects who meet all study criteria, have been approached for consent for immediate nasal brush or consent and to schedule a return appointment for a paired nasal and bronchial brush collection, if possible, at their first visit to NJH. Follow-up diagnostics are then completed and subjects are placed into appropriate categories following testing results. This has increased the number of patients who have been recruited into the study over the past year. Deployed subjects have been categorized by Dr. Rose and her consent team into groups as to keep the sample types (asthma, DDLD, or asthma + DDLD) blinded in the gene expression study analysis. In the last year, we have successfully collected RNA and DNA from nasal and bronchial brushes collected from recruited deployers as listed below:

   Table 1: Samples Collected from Deployed Subjects: 9/30/2019 – 9/29/2020

	Total # Nasal Samples	Total # Bronchial Samples	Number of Paired Nasal/Bronchial Samples
Group 1	2	0	0
Group 2	0	0	0
Group 3	4	2	2
Group TBD	4	2	2

2. Within the past year, we have also continued to collect and isolate RNA and DNA samples from brushed cells collected from non-deployed subjects consented through NJH Biobank. Additionally, we have collaborated with the Asthma Characterization Protocol (ACP) study at NJH, a study working towards building a cohort of highly characterized asthmatic and control subjects for research purposes. Furthermore, we have collaborated with a number of other studies at NJH which has allowed for the collection of numerous asthma and healthy control patients sample to be collected, using the same stringent qualification criteria as the GLIDE study, for our non-deployer patient groups. As such, we have established that these patients in these studies get dual consented into the

biobank, to be used in the GLIDE study. These patient recruitment options have dramatically increased the number of both controls and asthmatic subjects into our study. We continued to successfully collect RNA and DNA samples from the subjects as listed below:

Table 2: Samples from Non-Deployed Subjects: 9/30/2019 – 9/29/2020

	Total # Nasal Samples	Total # Bronchial Samples	Number of Paired Nasal/Bronchial Samples
Controls	6	2	2
Asthmatics	34	2	2

3. During this past year, we have consulted with the NJH Biobank data management team as well as the GLIDE deployer subject diagnoses, in order to confirm whether any follow-up diagnoses or medical visits at NJH were made on the non-deployed or deployed subjects. Confounding comorbidities have resulted in exclusion from our study.
    a. As a result of these confirmatory checks, we excluded nearly half of our control subjects, as they had follow-up diagnoses which resulted in disqualification from the control group
        i. These subjects were excluded due to lung cancer, ILD, IPF, COPD, sarcoidosis, and recent respiratory infections.
        ii. Several subjects switched categories from control to asthmatic, after follow-up diagnoses identified them as asthmatics
    b. We excluded 9 subjects from the asthmatic group, due to confounding diseases including COPD and recent bacterial respiratory infections.
    c. We continue to complete these checks on all non-deployed subjects collected moving forward following a minimum of 30 days post sample collection to allow for collection of follow-up diagnostics to enter the electronic medical record system.
    d. Furthermore, several GLIDE deployers switched groups (largely out of Group 2 and into Group 1 or 3) following additional test results and biopsy confirmation of disease status.

4. We have completed collection of the number of targeted samples for both paired nasal and bronchial (n=10), and nasal only (n=50) samples from our non-deployed asthmatic group.

5. We are making good progress in recruitment of the non-deployed control nasal only samples (n=17) and are only a couple paired samples short of our goal of 20 subjects. As the new access to samples through the ACP cohort continues, patients continue to be consented for sample collection from both the NJH biobank and the ACP/biobank study.

6. Recruitment of deployers has been at 1-2 per month, up until COVID-19 halted recruitment and collection of samples in March for safety of clinicians and research personnel. Currently, we are working to implement a system to re-instate patient

recruitment and sample collection from both deployed and non-deployed subjects. In order to do this, the following guidelines will be followed:

a. Patients must obtain a negative SARS-CoV-2 PCR test <4 days prior to sample collection
    i. These samples will be processed under current BSL2 safety protocols
b. If no PCR test can be performed prior to brush acquisition:
    i. We have begun the process of applying for biosafety approvals to process fresh nasal and bronchial brush samples from untested patients under BSL2+ safety conditions and lab protocols
    ii. After samples have been processed fresh, the sample will then be tested in the Seibold lab for SARS-CoV-2 by qPCR. Following a negative test of the collected samples, BSL2+-initiated cultures will proceed under BSL2 protocols. If patient tests positive, BSL2+ cultures will be discarded and patient will be ineligible to participate in the study.

7. To begin constructing RNA-seq libraries, we have completed quality control (QC) on all samples collected for the GLIDE study through September 2020. We quantified all samples using a Bioanalyzer to record sample concentration for accuracy in library preparation (**Figure 1**). We also measured the RNA integrity number (RIN) value of our extracted samples in which a higher number (1 poor – 10 perfect) indicates the quality score and degradation level of an RNA sample (**Figure 2**). All samples, regardless of RIN, will be included in the construction of RNAseq libraries. The RNA RIN values in our study indicate that our isolated RNA is of good to very good quality with low levels of RNA degradation.

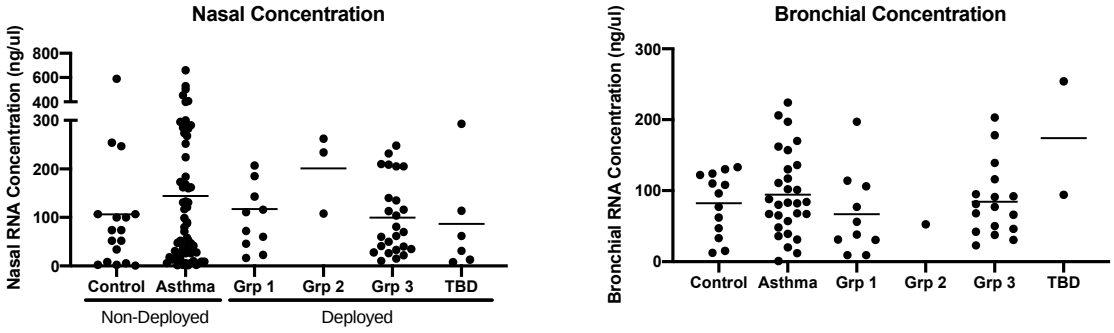


Figure 1: Bioanalyze quantitation of all in vivo nasal and bronchial brush samples from non-deployed and deployed subject for the GLIDE study through 9/2020. Data is reported as ng/ul and media value bars are shown for each sample group.

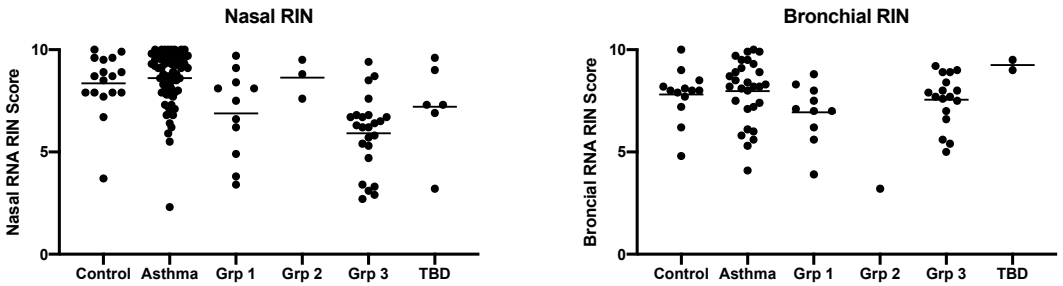


Figure 2: RNA integrity number (RIN) score of all in vivo nasal and bronchial brush samples from non-deployed and deployed subject for the GLIDE study through 9/2020. Data is reported as ng/ul and media value bars are shown for each sample group.

8. Of the >200 RNA samples from nasal and bronchial brushes from this study, we have begun to construct sequencing layouts and the samples are currently in the queue for preparation by our automated Biomek FXp liquid handling robotic system. Each plate includes samples from all groups in the study to avoid batch effects, and library preparation and sequencing results and QC will be completed in Quarter 1 of Year 5.
    a. Thus far, we have completed library prep and sequencing of 28 samples, all of which passed QC, and are pending further analysis upon the completion of the other samples.

Difficulties Encountered:

1. We had multiple deployers on the schedule to approach for consent, and 4 paired nasal and bronchial sample collection appointments scheduled prior to the outbreak of COVID-19 in the US. Due to increased safety measures put into place, we have postponed these research procedures. However, we are optimistic about our new approach to consenting deployers, as described above, and expect to reach our recruitment goals quickly when it is safe to resume research bronchoscopies.
    a. COVID-19 halted sample collection for 6 months, however, we are working on re-initiating recruitment and collection procedures to fulfill the targeted number of subjects, while maintaining the safety of our clinicians and lab personnel. In the meantime, we are proceeding with sample analysis, and will add in any additional samples collected in Year 5 as they are recruited and collected.

2. Re-query of non-deployed controls resulted in removal of nearly half our consented control subjects due to confounding diagnoses that resulted from their medical visit. However, the ACP coordinators we are working with have agreed to increase the rate of recruitment of healthy control subjects, and we are confident this will aid in reaching our recruitment goals quickly once recruitment, and nasal and bronchial brushes are allowed to be collected in a safe manner following the COVID-19 outbreak.

Subtask 2. Complete analysis of biomarkers and disease mechanisms for deployment lung diseases (asthma and DDLD) using whole-genome molecular analyses of *in vivo* airway epithelium.

Progress

1. We continue to work on "grade of membership models" analysis of our datasets which we believe will help us to deconvolute mechanisms operating across different cell types in the airway brushings collected from the deployers.

2. Additionally, our lab submitted a manuscript describing the single cell transcriptome profile of 8 distinct cell populations, and their developmental intermediates, found within the in vivo human tracheal airway epithelium (Goldfarmuren and Jackson, et al BioRxiv 2019; In Revision Nature Comm). Using these datasets, we are implementing the use of these gene sets and cell type-specific markers to further investigate, understand, and deconvolute bulk RNAseq data sets. These analyses will give us a much clearer understanding of the responses we see in the nasal and bronchial brushes collected in Subtask 1.

Subtask 3. Establish methods to determine the role of genetics and airway trace metal/PM exposures on *in vivo* airway molecular changes in deployment lung diseases.

Progress

1. Methods have previously been established for eQTL analyses and interpretation and continue to be implemented and refined across numerous studies in our lab. These methods will be implemented upon the completion of Project 4 Subtask 1 which is defined by the timeline of Project 1 recruitment.

Subtask 4. Complete analysis to the determine genetics factors and airway mineral and metal/PM exposures that result in molecular changes in the *in vivo* airway and predispose to deployment lung diseases.

1. Previously, we have discussed sample analysis and data from prior cell suspensions and supernatants from nasal and bronchial brushes that were analyzed. As data from these samples from non-deployers showed no quantifiable measurement of elements by ICP-MS, we decided to re-submit one more batch of samples from deployers, to determine if there are quantifiable elements in samples from deployed subjects. This batch was blinded and included samples from non-deployers to control for batch effects.
    a. There were measurable levels of the elements Ca, Mg, Fe, K, P, and S in at least half of all samples measured. However, in many of these measurements the measurement error was greater than or equal to the detected level of the element.
    b. The elements Al and SiO_2 were detected in <3 of the 16 samples analyzed
    c. While there were detectable levels of Mn, Sr, and Cu in most samples, again many of the levels were found to be less than or equal to the measurement error of that element assay.
    d. Rb, Ba, Zn, and Pb were found in low but detectable levels, but only in supernatant samples; they were absent in cell lysate samples.
    e. There were several elements that were not detected, or below the limit of quantitation, including As, B, Be, Bi, Br, Cd, Co, Cr, Ga, Li, Mo, Ni, Sb, Se, Sn, Tl, and V
    f. Rare earth elements were undetectable, with the exception of Ce and La

2. Overall, it was evident that supernatant samples, as compared to cell lysates, were more likely to produce detectable/measurable levels of certain elements by ICP-MS analysis.

3. Currently, we are working to combine this round of analysis, with the first several rounds of non-deployer analyses, to determine if there are similarities between nasal and bronchial samples, or if there are differences between elements detected between deployer and non-deployer samples.

4. For the genotyping of DNA from *in vivo* brush samples for genetic determinants of response in this subtask, and for eQTL response analysis in subtask 5, we have completed QC quantitation of all samples collected through early 2020 (Figure 3). We found that 97% of all nasal and 96% of all bronchial samples pass the QC threshold for the amount of DNA necessary for genotyping analysis as set out by previous established guidelines with our collaborators.

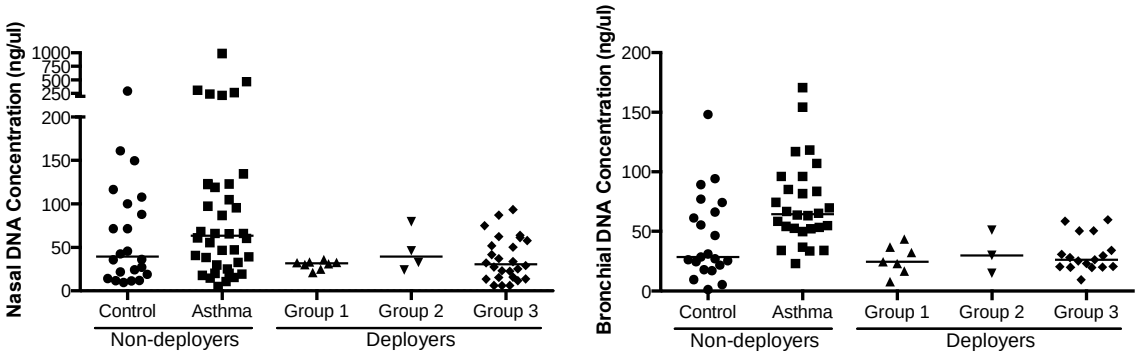


Figure 3: Quantitation of all in vivo brush samples collected in the GLIDE study for DNA genotyping analysis through 3/2020. Data is reported as ng/ul and media value bars are shown for each sample group.

5. We have contacted collaborators who perform genotyping arrays, and whose labs are back up and running following the COVID-19 shut-down. We are currently working on making sure our samples meet the specs necessary (concentration, plate format, diluent, etc), and are working to schedule sample shipment, assay selection, and timing of completing this data acquisition by December 2020.

Difficulties Encountered

1. From our current data, we have found that both the nasal and bronchial supernatant sample volumes are too low and result in data below the limit of detection by ICP-MS analysis. Additionally, the cell lysates we have provided for analysis also have ICP-MS data values below the limit of detection. Following analysis of new samples described above, we will re-assess this aim upon data analysis from those samples in the next reporting period.

Subtask 5. Establish methods for isolation and culture of nasal epithelial cells, measurement of the transcriptional response to PM, how PM modifies the transcriptional response to IL-13, and the genetic determinants of these responses.

Cell Expansion Progress

1. Within the past year, we have continued to collect and expand nasal airway epithelial cells as per our established SOPs. Deployed subjects have been categorized by Dr. Rose and her consent team into groups as to keep the samples types (asthmatic, DDLD, or asthma + DDLD) blinded in the *in vitro* stimulation and exposure study experiments. Within this reporting period, we have successfully collected samples from the recruited subjects as listed below:

   Table 3: Nasal Samples Cultured from Deployed Subjects: 9/30/2019 – 9/29/2020

	# Nasal Samples	Successfully Expanded Nasal Samples
Group 1	0	0
Group 2	2	2
Group 3	4	4
Group TBD	4	4

2. Within the past year, we have continued to collect and expand nasal airway epithelial cells as per our established SOPs. Consent of non-deployed healthy and asthmatic subjects in conjunction with the ACP/biobank study has increased the number of consented subjects, with great success. Samples collected have been successfully cultured as listed below:

   Table 4: Nasal Samples Cultured from Non-Deployed Subjects: 9/30/2019 – 9/29/2020

	# Nasal Samples	Successfully Expanded Nasal Samples
Controls	6	4
Asthmatics	34	33

3. In concordance with Subtask 1, we have achieved our targeted number of expanded nasal brushes for non-deployed asthmatics (n=50), and are nearly at the halfway point (n=21) for culture expansion of non-deployed controls.

4. Following discussion with all project leads, for *in vitro* stimulations we will combine deployers in groups diagnosed with asthma, and those diagnosed with both asthma and DDLD, into a single category to study deployers with asthma and their response to Iraq sourced PM for Subtask 5. As of this progress report, in total, we have successfully expanded cells from 15 subjects for this aim.

5. Due to COVID-19 safety precautions, all non-emergent procedures at National Jewish, including nasal and bronchial brush collections for research studies were put on hold in March 2020
   a. As of mid-August, we have received permission to re-initiate collection of nasal brush samples for RNA and DNA isolation.

b. We will be allowed to proceed with cell culture of nasal and bronchial brushes under the 2 following events; either:
    i. Patient has a negative SARS-CoV-2 diagnostic test result by nasal brush test within 2 days of the sample collection, OR
    ii. Following extraction of RNA from the brush collected for RNA/DNA lysates, patient sample tests negative by qPCR by Project 4 personnel using CDC-based primer/probe assay for SARS-CoV-2 in the research lab
c. Several deployers have already been scheduled for brush collection by Aim 1 personnel, and the biobank will be collecting samples from COVID-19 negative patients in the near future, as they are currently implementing safety protocols for collection and processing.

In Vitro Stimulation/Exposure Progress:

1. Iraq PM composition:

a. Prior investigation identified that PM from Iraq was composed of >6 bacterial phyla and contained measurable amounts of endotoxin (LPS). Novel methods have been developed which allow for not only the identification of bacterial components, but also allow for the identification of fungal, viral, plant, and animal DNA as well. Current analyses are underway on the Iraq PM sample to identify the source of all the DNA in the PM sample for better understanding of the components that make up respirable PM from Camp Victory, Iraq.
b. Novel methods have been developed by our collaborators which allow for not only the identification of bacterial components (16s rDNA), but also allow for the identification of fungal, viral, plant, and animal DNA (18s rDNA), as well. Further analyses have been conducted within this reporting period and have found the following:
    i. Iraq PM (aka Camp Victory Particulate Matter; CVPM) contained the highest number of 16s and 18s reads, containing notably higher DNA composition than other samples tested, including Afghanistan PM (Bagram PM; BPM) and Ft. Irwin California PM (FIPM) as seen in the left panel of **Figure 4.**
    ii. Iraq PM had the highest microbiome alpha diversity (by all 3 indices measured: Observed, Chao1, and Shannon); left panel **Figure 5**
    iii. DNA from the Iraq PM sample contained >90% prokaryotic DNA, 5.8% eukaryotic DNA, which did not vary from the ratio found in Afghanistan PM (BPM) or the blank control. Notably, Iraq PM was unique as it contained DNA identified as 3% archaea, which were not identified in any other sample tested.
    iv. All 3 PM samples are distinctly different in microbiome beta diversity measures, demonstrating that samples are unique from one another (right panel **Figure 5**).

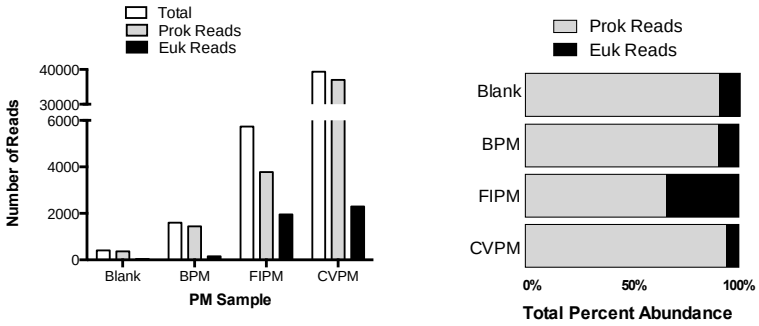


Figure 4: (Left Panel) Total prokaryotic and eukaryotic sequencing reads from blank PBS, BPM (Afghanistan PM), FIPM (Ft. Irwin CA PM), and CVPM (Camp Victory Iraq PM). (Right Panel) Percent of total reads that belong to prokaryotes or eukaryotes in each sample analyzed.

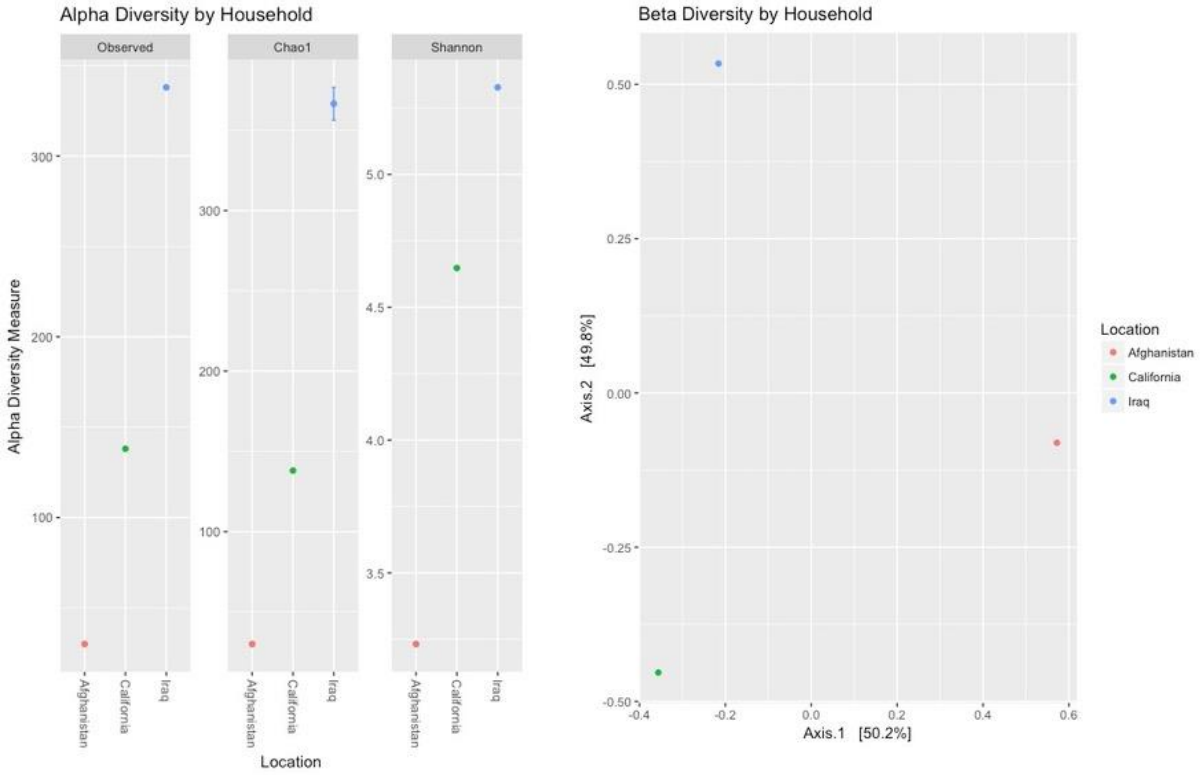


Figure 5: (Left Panel) Alpha diversity plots of the microbiome between Iraq, Afghanistan, and California PM samples based on observed (bacterial richness/number of taxa observed), Chao1 (bacterial abundance evenness), and Shannon (abundance and observed distribution) indices. (Right panel) Beta diversity plot demonstrates distinct microbiome composition between all 3 PM samples.

2. Investigating inflammatory drivers of epithelial PM-response in nasal ALI cultures (update)
    a. To understand the inflammatory signals important in driving the dust response in the nasal AEC ALI cultures, we conducted independent stimulations of nasal epithelial cells differentiated at ALI. Stimulations were conducted with the IL-1 family cytokines IL1A, IL1B, and IL36G to investigate the overlap of the single cytokine-treated AEC response with that initiated from the 24hour dust exposure.
    b. Stimulations with each cytokine resulted in a dramatic transcriptome shift in differentiated nasal airway epithelial cells (n=5 donors). IL1A and IL1B stimulation resulted in a consistent shift in the transcriptome as illustrated by MDS (Figure 6), and altered the expression of 7,057 and 9,358 DEGs, respectively. Additionally, we found that co-stimulation with IL1A and IL1B together for 24hours, resulted in a similar shift, significantly altering the expression of 9,662 genes.
    c. Stimulation with IL36G for 24hours resulted in a notable shift in the transcriptome, though less so than with IL1A and IL1B, and resulted in

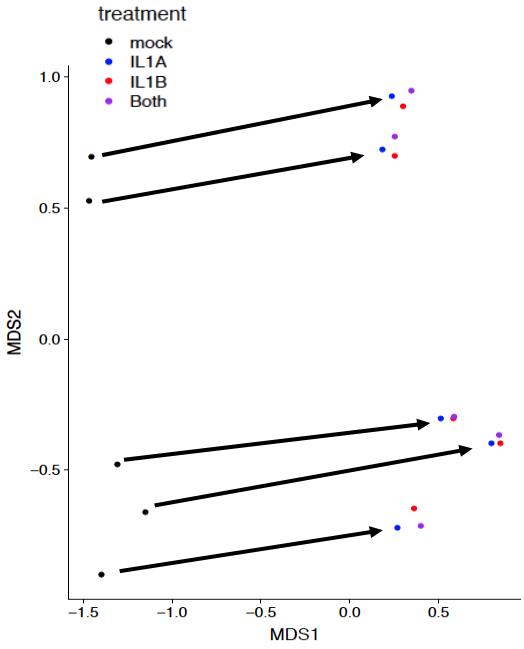


Figure 6:
MDS plot of differentiated nasal airway epithelial cells cultured at ALI following 24hours of mock (black), IL1A (blue), IL1B (red), or both IL1A and IL1B (purple) stimulation; n=5 donors.

1,317 DEGs (n=5 donors).

6. We found that the overlap in shared DEGs between Iraq PM and IL-1 cytokines were largely enriched for a variety of pro-inflammatory signals, stress response signals, and further upregulation of IL-1 cytokine signals (Figure 7).

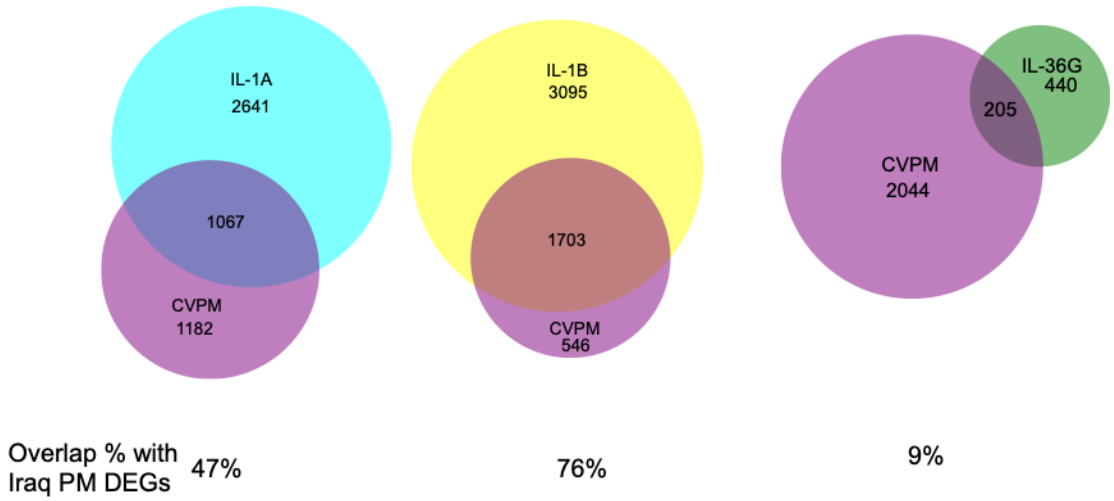


Figure 7: Venn diagrams showing the overlap in upregulated differentially expressed genes between 24hour Iraq PM and IL1A, IL1B, or IL36G stimulations (n=5 donors)

7. We have been working to identify the role of IL-1 inflammatory signaling in the PM response, and so we have looked into the overlap of differentially expressed genes between the Iraq PM stimulations and the IL-1 stimulations. (Figure 8).
    a. Notably, Iraq PM stimulations had a distinct overlap in DEGs with IL-1B (bue box; 618 genes), or IL-1A and IL-1B (purple box; 890 genes), suggesting the important role of IL-1 signaling in the Iraq PM response.
        i. These signals were enriched in a variety of pro-inflammatory signals, and stress response signals
    b. While distinctly lower, there was a unique overlap in response between Iraq PM and IL-36G response as well (green boxes)
    c. Interestingly, there was a shared response between all 3 IL-1 cytokines and Iraq PM response genes (red box; 177 genes)
        i. These shared responses defined a core PM response, likely driven by IL-1 cytokines, that were characterized by inflammatory mediator S100, SPRR, VNN, and SAA genes, inflammatory driving genes including CXCL8, IL1B, IL36G genes, and multiple mitochondrial stress related NDUF and COX genes.

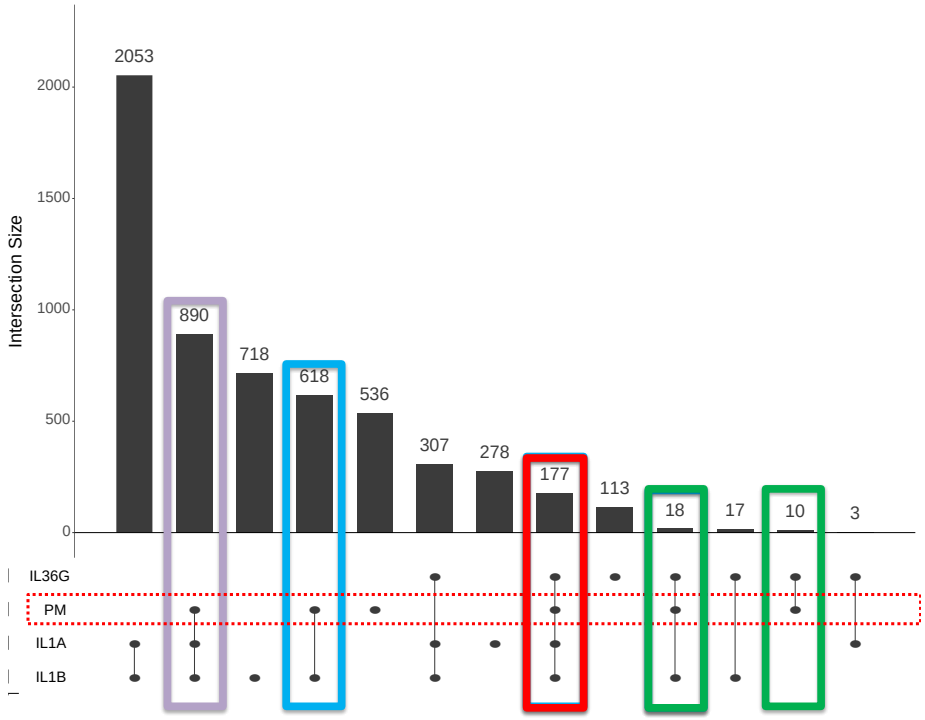


Figure 8: Upset plot of the significantly upregulated differentially expressed genes (DEGs; FDR<0.05) in each of our stimulation conditions (Iraq PM, IL-1A, IL-1B, IL-36G). Plot identifies genes that are unique or shared between each condition or set of conditions.

8. Further experiments and stimulation models are needed in order validate our two-hit hypothesis model, and to further understand the response of the airway epithelium to Camp Victory Iraq PM. However, in order to conduct such experiments Project 4 did not have an adequate amount of sample to conduct stimulations and requested additional Iraq PM sample. Thus, we obtained a new vial of PM from Project 2 personnel (named "V2 PM"). The sample was irradiated to ensure sterility for a total dose of 2.5MRad prior to stimulations. Nasal cells from the same donors cultured and stimulated previously with the original Iraq PM were cultured and differentiated at ALI for stimulations with V2 PM. Cultures were stimulated at the same concentration (20ug/cm2) with the V2 PM sample for 24 hours prior to harvest. Whole transcriptome analysis was completed in order to compare response between the original aliquot and new PM sample.
    a. Stimulation of differentiated nasal epithelial cells with the V2 PM sample resulted in a noteably lower number of differentially expressed genes (DEGs) as compared to the original Iraq PM sample (V2 PM = 151; original Iraq PM = >4500). Additionally, of the genes that were dysregulated in each sample, there was very little overlap between the 2 PM samples.
    b. Following discussion with Project 2 personnel and follow-up the source and labeling of the tube Project 4 was given, it was determined that the sample indicated as Iraq, was in fact from the aerosolized Ft. Irwin

California PM sample. While this error paused our progress in initiating further experiments, it also provided us with an RNA-seq dataset to compare nasal epithelial responses between different PM sources. Interestingly, this data had little overlap, and the response to Ft. Irwin California PM is severely muted as compared to the response initiated by Iraq PM (Figure 9). This brings further justification to studying PM from Iraq and understanding its components and how it contributes to dysregulation of the airway epithelium upon exposure.

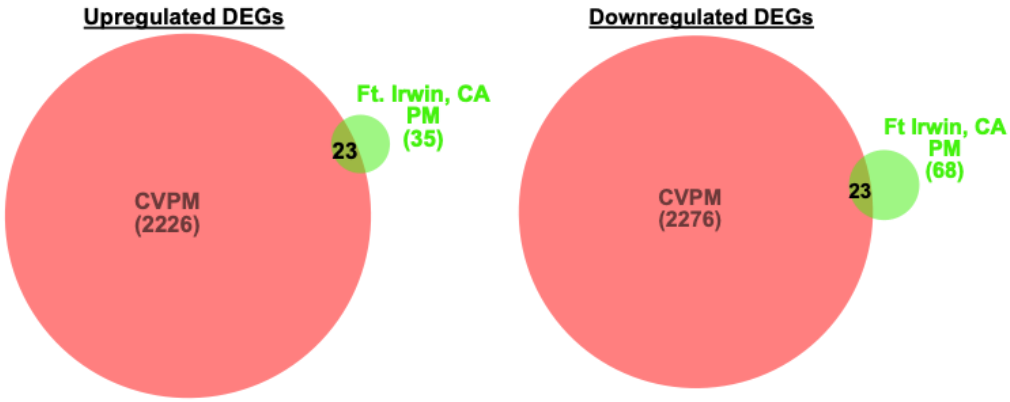


Figure 9: Venn diagrams showing the overlap in upregulated (left) and downregulated (right) differentially expressed genes between 24hour stimulation with Iraq PM and Ft. Irwin California PM (n=5 donors).

9. Further experiments and stimulation models are needed in order validate our two-hit hypothesis model, and to further understand the response of the airway epithelium to Camp Victory Iraq PM. However, in order to conduct such experiments Project 4 did not have an adequate amount of sample to conduct stimulations and requested additional Iraq PM sample. Thus, we obtained a new vial of PM from Project 2 personnel (named "Iraq V3 PM"). The sample was irradiated to ensure sterility for a total dose of 2.5MRad prior to stimulations, and will be used for subsequent stimulations and compared to the response seen with our original Iraq PM vial supplied to Project 4.

10. To confirm our model for our 'two-hit' hypothesis, we performed a pilot experiment using a 96 hr stimulation model. We first performed either mock or Iraq V3 PM stimulations for 72 hours. Following initial 72 hr stims, we continued mock or Iraq PM stimulation for 24 hours, and added in IL-13 exposure for the final 24 hours (Figure 10). This model aimed to measure, 1: the remodeling response of Iraq PM following chronic exposure, and 2: if this remodeling effect by chronic Iraq PM exposure alters the airway epithelial response to the potent asthma-associated cytokine IL-13 (Figure 4).
    a. From this experiment, we will be conducting RNAseq to assess the transcriptional responses initiated by this chronic exposure.

b. We generated libraries for all samples from this experiment, and upon the Sequencing Core resuming operations, submitted for sequencing analysis.
c. Gene expression analysis was conducted to confirm that this 4d (96 hr) exposure model with Iraq PM initiated a response within the differentiated mucociliary epithelial cultures in vitro. Our data indicated that extended repeated stimulation with Iraq source PM results in a significant response, with 1380 genes differentially upregulated (FDR <0.05) and 2623 genes significantly downregulated.
d. The 24 hr IL13 response produced an equally robust response with 1317 upregulated and 1322 downregulated genes following stimulation (FDR <0.05).
e. To identify whether pre-exposure to PM altered the IL13 response, we performed interaction analysis and found 10 genes that had a significantly altered response to IL13 when pre-exposed to Iraq PM. Furthermore, though not significant, we found that pre-exposure to PM resulted in higher log fold change in inflammatory markers including CXCL8 and IL36G, as well as a more exaggerated response of mucins including MUC5B and MUC5AC.
    i. Currently, we are testing new methods of identifying these changes to better understand the changing IL13 response following PM exposure of the epithelium

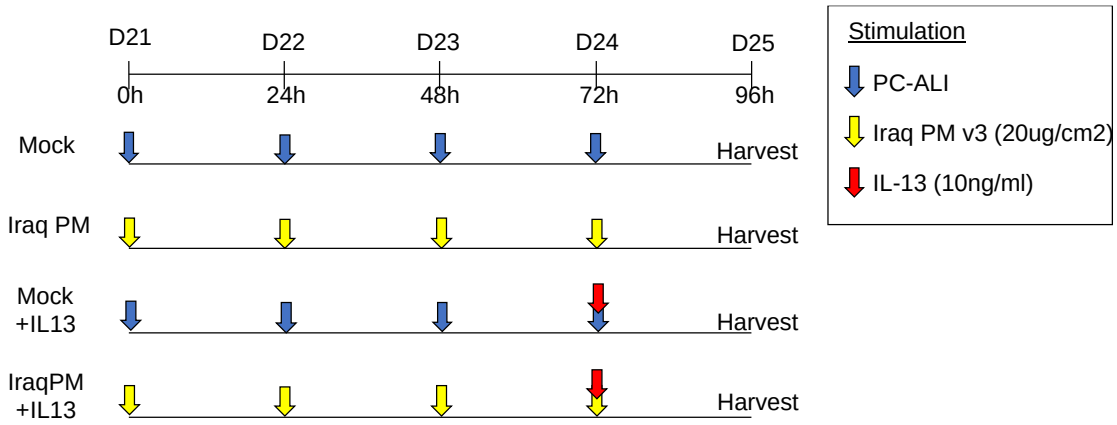


Figure 10: Timeline of chronic PM exposure followed by IL-13 stimulation to investigate 'two-hit' hypothesis for Project 4 Subtask 5.

11. To perform these experiments on the entire cohort *in vitro*, we plan to begin these stimulations using the model outlined above. Unfortunately, due to COVID-19, there is a significant shortage in the material used to make our transwell inserts for *in vitr*o mucociliary differentiation, as it is a key material in the making of PPE masks, this has put out initiation of this aim on hold for the past 2 months.
    a. However, in the last of week of September we found and secured the number of transwell inserts needed for these experiments, and plan to begin and complete all stimulations laid out in this study (nasal cultures from 150 donors), during Quarter 1 of Year 5.

Subtask 6. Complete analysis of the transcriptional response to PM, how PM modifies the transcriptional response to IL-13, and the genetic determinants of these responses.

Progress

1. Analytical methods continue to be improved upon for the analysis of the PM and PM+IL13 response in the airway epithelium. In order to further understand our gene expression results from the above studies, our bioinformatic analyses continue to utilize interaction analysis models using DESeq2 and MetaSoft to further understand the interaction and heterogeneity of responses between tissue types (tracheal vs nasal) and between stimulation conditions (PM vs IL13 vs PM+IL13). Implementation of these new methods has been done in our preliminary stimulations as listed above in Subtask 5 and ongoing optimization will give us a better understanding of the transcriptional response to PM in the larger subject cohort.

2. Using our data sets from single cell sequencing of the *in vivo* human tracheal airway epithelium, which describe the transcriptome of multiple cells types in the airway, we have begun to implement the datasets in interpretation of *in vitro* bulk RNAseq data, as will be collected in Subtask 5. We have reported this use in a recently accepted manuscript studying the in vitro response of the airway epithelium to organic components found in air pollution (Montgomery et al, AJRCMB 2020), and are confident it will add a great insight into the cell-type specific responses initiated by Iraq PM stimulation in our model.

3. We plan to begin in vitro stimulations by late October, and have outlined a schedule for in vitro stimulations of the current existing nasal samples we have in our cohort stocks, to be completed as we continue to recruit and process subjects for Subtask 1. This schedule will allow us to perform 96hr exposures + 24 hr IL13 stimulations on 5-10 donors per week. Following this timeline, we plan to have all stimulations completed by early 2021.

4. Furthermore, we plan to extract samples, prepare libraries, and perform RNAseq in real time, as to have all *in vitro* stimulation data sequenced by the Quarter 1 of Year 5.

Subtask 7. Prepare and submit manuscripts for publication.

Progress

1. Although the ATS 2020 conference has been cancelled due to COVID-19, Project 4 submitted an abstract, which was accepted for a poster presentation, to the international American Thoracic Society (ATS) conference to be held in May 2020 entitled, “Desert dust exposure induces IL-1 family-mediated inflammation and remodeling of the mucociliary airway epithelium”.

2. The first manuscript for Project 4 is planned to be submitted later November. The goal is to submit this manuscript laying out the groundwork for further composition of the Iraq PM, and how the differentiated airway epithelium and the individual cell types that compose it, respond to Iraq dust particulate matter.

Tentative title: “Desert PM exposure induces IL-1 family-mediated inflammation and remodeling of the mucociliary airway epithelium”

a. Projected submission date: November 2020

Milestones Achieved:

1. Criteria and search queries have been developed, and implemented for the recruitment of deployers and the collection of age/gender/smoking history matched non-deployers for our project. Search queries and sample collection for non-deployed subjects through the National Jewish Health Biobank are currently being utilized. Samples are being collected from subjects who meet the asthma or control status, age, and comorbidity guidelines set by our search parameters.
2. SOPs for airway brush sample collection and processing are in place and being implemented across different labs and research groups for sample culturing, RNA/DNA extraction, and sample storage methods.
3. Collection of the paired bronchial/nasal brushes from non-deployed asthmatics has been achieved (n=10). Verification of these subjects has been conducted and we have confirmed that we have ample subjects with paired nasal/bronchial brush samples that meet our study guidelines for RNAseq analysis.
4. Collection of nasal brush only samples from non-deployed asthmatics has been achieved (n=50), and confirmed by patient history follow-up.
5. Sample decontamination and digestion methods using hydrogen peroxide, guidelines for sample preparation, and sample delivery to the USGS have been established.
6. Buffer samples and reagents have been validated by the USGS and determined to not contain high levels of contaminating elements or metals that may interfere with the ICP-MS machinery or the analysis of the biological samples collected for this study.
7. RNAseq robotic protocols are complete and validated for construction of RNAseq libraries for comparison of gene expression from *in vitro* exposures and *in vivo* studies.
8. All individual analytical methods in the differential gene expression workflow are established.
9. All analytic methods for eQTL and RNAseq variant calling methods development have been established.
10. PM dose response stimulations have been developed and established under acute(24hr) and chronic (7day and 4day) conditions. Such treatments are not toxic to the airway epithelial cultures grown at air-liquid interface and induce significant inflammatory, stress, and remodeling gene expression responses.
11. A chronic PM exposure model, followed by an acute treatment with IL13, has been established as a working model to determine what effect pre-exposure of PM has on the inflammatory IL-13 response in the airway epithelium

What opportunities for training and professional development has the project provided?

Project 1

The Project 1 team has participated in a number of professional development activities over the past grant year, though opportunities were constrained by the COVID-19 pandemic. Several team members (including Ms. Zell-Baran and Dr. Krefft, along with study co-authors) were planning to present GLIDE Study data in person at the 2020 annual American Thoracic Society International Conference in Philadelphia, PA and at the Military Health System Research Symposium (MHSRS) in Kissimmee, FL but instead interacted and shared this data electronically. Ms. Zell-Baran and Dr. Krefft presented their research findings at a National Jewish Health Department of Medicine Research Symposium in February 2020. The Project 1 team continues to present clinical cases who have undergone evaluation for deployment-related lung disease for discussion and management at a bi-monthly Deployment Lung Disease clinical case conference at NJH (including review of chest imaging with a pulmonary radiologist and lung histology with a pulmonary pathologist), in partnership with clinicians and researchers at the Eastern Colorado Veterans Administration Health Care System (ECVAHCS). Dr. Rose continues to provide primary research mentorship for Dr. Krefft who is the recipient of a Career Development Award at the ECVAHCS. Dr. Rose was an invited member of the National Academies of Sciences, Engineering and Medicine Committee on the Respiratory Health Effects of Airborne Hazards Exposures in the Southwest Asia Theater of Military Operations, with a final report published in September 2020.

Project 2

The project 2 team has participated in many developmental activities in the last year. With members of the project team 1, members of project team 2 including Dr. Gregory Downey and Bill Janssen were planning to attend the 2020 American Thoracic Society annual international conference in Philadelphia PA and the Military Health System Research Symposium (MHSRS) in Kissimmee, FL. However, both conferences were canceled because of the COVID-19 pandemic. Members of the team attended the virtual (Zoom) sessions by the American Thoracic Society in August 2020. Dr. Downey presented the data from the mouse models of particulate matter exposure and human lung epithelial cell in vitro exposure models to the citywide pulmonary research in progress meeting as well as to the National Jewish health research meetings.

Project 3
Drs. Chu and Day successfully supervised the training of a Ph.D. graduate student, Reena Berman, through the Toxicology graduate program in the School of Pharmacy at the University of Colorado. Ms. Berman is working on the studies proposed for Project 3. Ms. Berman received her PhD in Toxicology in June 2020. She is currently employed by Novartis Pharmaceutics.

Project 4
Drs. Seibold and Everman have provided training to lab members in the processing and banking of brush samples for this project. Dr. Jackson has supervised and trained Dr. Peter DeFord and Blake Williams, both new Bioinformatic Analysts, in the analysis of whole transcriptome sequencing data and general bioinformatic analysis. Dr. Everman is taking a supervisory role in the cell biological aspects of Project 4 under the direction of Dr. Seibold and is thus presenting results of Project 4 for discussion in monthly meetings. Dr. Everman had an abstract accepted for the 2020 American Thoracic Society annual international conference on the dust-induced transcriptional response of the airway epithelium.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Project 1.
An Outreach Committee meets regularly and has developed several tools for enhancing public understanding of deployment-related lung disease as well as for recruiting potential study subjects for participation in the GLIDE Study. As detailed above under "Accomplishments," these tools include a website and brochure as well as two publications accepted this year.

Project 2
Two abstracts reporting the results of studies with PM exposure to human lung epithelial cells were accepted as poster presentations and one was accepted as an oral presentation for the Military Health System Research Symposium (MHSRS) in Orlando, FL in August 2020. As noted above, the symposium was canceled but the abstracts and research talk summaries were posted online. The key findings from this research project were also presented at the university of Colorado citywide pulmonary research conference and at the National Jewish health research conference.

Project 3
Presented Abstract at the Society for Toxicology virtual meeting in March 2019 on Afghanistan PM enhancing pro-inflammatory responses in UL-13 exposed human airway epithelium by TLR2 signaling.

Project 4
The aims that are outlined by Project 4 and data that has been collected as a result of the Seibold lab's research efforts have been presented internally at multiple laboratory meetings. Additionally, these findings have been discussed and analyzed with departmental faculty and research investigators at meetings held by the Center for Genes, Environment, and Health at National Jewish Health. Furthermore, the project outlined by Project 4 and its effects on deployed military members, and the greater scientific field, was published online as an accepted abstract from the virtually held 2020 ATS conference.

What do you plan to do during the next reporting period to accomplish the goals?
"

PROJECT 1. Exposure Characterization and Identification of Noninvasive Methods for Diagnosis of Deployment-Related Lung Disease

Major Task 1: Continue recruitment of study subjects (deployers and controls) from NJH Deployment Lung Clinic.

As outlined in the Statement of Work, the following subtasks under Major Task 1 will be undertaken during the next reporting period to accomplish our goals:

Subtask 1. Consent patients from our NJH Deployment Clinic.

> **Plans for the next year.** We will continue to offer the opportunity to participate in the GLIDE Study to all symptomatic deployers who undergo clinical evaluation in our Center of Excellence and who meet study inclusion criteria.

Subtask 3. Collect, analyze, and archive bronchial (B) and nasal (N) brushings through live cell core.

> **Plans for the next year.** We plan to obtain and analyze these samples using our revised control sample protocols, with modifications in sample acquisition based on COVID-19 pandemic-related requirements for protecting study staff and participating subjects.

Subtask 5. Collect, analyze, and archive VATS biopsies from deployers and controls.

> **Plans for the next year.** We are collecting and analyzing archived biopsy specimens, and will continue to do so through the next year. We plan to begin data analysis using results from the standardized pathology scoring system for VATS biopsies in 2021.

Subtask 7. Complete LCI measurements from deployers and controls.

> **Plans for the next year.** We have completed control enrollment for LCI and will continue to enroll deployer subjects. We have completed data analysis and submitted a manuscript of LCI findings in deployers compared to healthy controls for review and publication.

Subtask 9. Complete CT scans and quantitative analysis from deployers and controls.

> **Plans for the next year.** We plan to expand our sample size for deployer chest CT images in October/November 2020, then proceed with analysis of data from independent radiologists' interpretations along with quantitative airway imaging analysis.

Subtask 11. Complete analysis of VATS lung biopsies using LA-ICP-MS and FE-SEM.

> **Plans for the next year.** We plan to work with USGS to complete the majority of these analyses during the next year.

Subtask 12. Prepare and submit manuscripts for publication.

> **Plans for the next year.** We plan to complete data acquisition for chest CT imaging and lung pathology findings in 2020, then analyze study data and submit manuscripts for peer review and publication 2021.

PROJECT 2. Acute Lung Injury in Deployed Military Personnel: Basic Mechanisms and Novel Therapeutic Approaches

Major Task 2: Establish *in vitro* and animal models of exposure of alveolar epithelial cells to airborne PM and combined effects of physical, chemical, and infectious stimuli.

Subtask 1. Develop and refine *in vitro* cell culture models using cell lines to study combined effects of PM and physical, chemical, and infectious stimuli on alveolar epithelial cells.

> **Plans for the next year**. We plan to continue to refine and complete the analysis of the experiments using *in vitro* cell culture models of exposure of lung epithelial cells to airborne PM and combined effects of physical (scratch wound and electrical wound models), chemical, and infectious stimuli (influenza infection).

Subtask 2. Develop and refine *in vitro* cell culture models using primary human alveolar epithelial cells to study combined effects of PM and physical, chemical, and infectious stimuli on alveolar epithelial cells.

> **Plans for the next year.** We will complete the experiments examining the effects of viral infection with influenza virus *in vitro* using cultured lung epithelial cells. We will complete the analysis of transcriptional responses using RNAseq of primary human lung epithelial cells and alveolar macrophages exposed to PM from Afghanistan, Iraq, and China Lake.

Subtask 3: Determine combined effects of PM and physical, chemical, and infectious stimuli on alveolar epithelial cells *in vitro*. Test effects of small molecule modulators of the WNT pathway on epithelial injury.

> **Plans for the next year.** We will test whether small molecule modulators of the WNT pathway (ICG-001) can prevent the injury to lung epithelial cells. Since particulate

matter alone did not cause significant injury to the epithelial cells, we will focus first on the effects of ICG-001on physical injury to the epithelial cells using the scratch wound and the electrical wound with the ECIS system. Next we will study the effects of ICG-001 on the effects of combined exposure to particulate matter and scratch wound. We will examine the effects of ICG-001 on epithelial injury induced by influenza viral infection.

Subtask 4: Develop and refine animal (mouse) models of PM exposure and the effects of physical, chemical, and infectious stimuli on acute lung injury.

Plans for the next year. We plan to complete the experiments quantifying lung injury induced by influenza infection and examine the combined effects of prior exposure to particulate matter on subsequent influenza induced lung injury.

Subtask 7. Complete analysis of VATS lung biopsies focusing on the distal lung parenchyma and alveolar areas using LA-ICP-MS, and FE-SEM.

Plans for the next year. Working with the USGS, we will continue to refine methods to measure particulate matter and mineral content of the lungs from the animal models of PM exposure and then use these methods to quantify the concentration of PM in surgical lung biopsies from deployers.

Subtask 8. Prepare and submit manuscripts for publication.

Plans for the next year. We have published 3 manuscripts and will submit a fourth manuscript for publication during Year 5.

PROJECT 3. Impact of Cigarette Smoke on PM-induced Airway Epithelial Injury and Exacerbation of Asthma and Bronchiolitis in Deployed Military Personnel.

Major Task 3: Establish *in vitro* and animal model of exposure of bronchial epithelial cells to airborne PM.

1. Examine differences in bronchial epithelial cell responses between non-deployed and deployed subjects in the IL-13 *in vitro* model.

Plan for the next year: Continue work on defining the molecular mechanism of IL-13 inhibition of NO production in macrophages and in mouse and human precision cut lung slices.

2. Examine mechanism of APM's ability to enhance house dust mite airway hyperreactivity *in vivo*.

Plan for the next year: Move studies into the precision cut lung slices so we can begin to compare rodent responses with human responses.

3. Examine the effects of cigarette smoke and APM in IL-13 *in vitro* model.

> **Plan for the next year:** Focus last year on better understanding the mechanisms of interaction between APM and HDM models and not pursue the cigarette smoke since early studies suggest similar effects of cigarette smoke particulates as seen with APM.

4. Compare PM properties between deployer's VATS biopsy samples and our Afghanistan PM samples.

> **Plan for the next year:** Continue working on standard operating procedures and methods for this task with the USGS team.

PROJECT 4. Omics' Analysis of Airway Epithelium in Deployment-Related Lung Diseases.

Major Task 4: Establish 'omics' approaches to analyze the transcriptome and genetics of *in vivo* and *in vitro* airway epithelium from subjects with deployment-related lung diseases to determine mechanisms of these diseases, the molecular effects of PM exposures, and to identify minimally invasive biomarkers for these diseases and exposures.

Subtask 1. Establish methods to identify biomarkers and disease mechanisms for deployment lung diseases (asthma and DDLD) using whole-genome molecular analyses of *in vivo* airway epithelium.

> **Plan for the next year:** To fulfill our target numbers for remaining groups, we will continue to collect nasal and bronchial brushes from deployer recruitment led by Project 1, and of non-deployed subjects through the NJH biobank. We will also continue to monitor quality of biomolecule isolation from brushes for gene expression studies of the *in vivo* airway epithelium.

Subtask 2. Complete analysis of biomarkers and disease mechanisms for deployment lung diseases (asthma and DDLD) using whole-genome molecular analyses of *in vivo* airway epithelium.

> **Plan for the next year:** Library construction and RNA-sequencing of all of the RNA samples currently collected and extracted (n=160) from both deployed and non-deployed subjects will be completed in the next reporting period. QC will be performed to ensure the quality level of the data, and analyses will be initiated.

Subtask 3. Establish methods to determine the role of genetics and airway trace metal/PM exposures on *in vivo* airway molecular changes in deployment lung diseases.

> **Plan for the next year**: Continue to validate and improve upon pipelines constructed for these analyses, begin utilizing pipelines on generated data from Subtask 2.

Subtask 4. Complete analysis to determine genetics factors and airway mineral and metal/PM exposures that result in molecular changes in the *in vivo* airway and predispose to deployment lung diseases.

> **Plan for the next year:** We will combine several of the ICP-MS datasets that have been collected, to determine if detectable levels of elements in cell lysate and supernatant samples from deployers and non-deployer nasal and bronchial samples

Subtask 5. Establish methods for isolation and culture of nasal epithelial cells, measurement of the transcriptional response to PM, how PM modifies the transcriptional response to IL-13, and the genetic determinants of these responses.

> **Plan for the next year:** In addition to ongoing sample collection from subject recruitment in Project 1, we will continue to culture and conduct stimulations on non-deployer samples. In the next reporting period we will complete stimulations on all currently isolated and expanded nasal AEC samples. We will extract all RNA samples, and begin to obtain RNA-sequencing data and will continue to analyze transcriptomic responses under these stimulation conditions.

Subtask 6. Complete analysis of the transcriptional response to PM, how PM modifies the transcriptional response to IL-13, and the genetic determinants of these responses.

> **Plan for the next year:** Analysis of transcriptomic responses will begin in the next year, but is dependent on Subtask 5, and more so the recruitment of subjects led by Project 1. Genotyping of subjects will be initiated, and data will be obtained from all DNA samples currently collected and extracted in our cohort.

Subtask 7. Prepare and submit manuscripts for publication.

> **Plan for the next year:** Current experiments have resulted in the initiation of 2 separate manuscripts which aim to: 1) set the groundwork for the acute baseline response of the AEC to Iraq dust PM (submission goal – Winter 2020), and 2) provide characterization of the AEC response to chronic, repeated exposure to Iraq PM, the resultant remodeling of the AEC, and how it affects subsequent responses to inflammatory stimuli (submission goal – Fall 2021).

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

- We have a busy Deployment Lung Clinical Center and have evaluated over 200 patients, many of whom are participants in the GLIDE Study.
- We have described the spectrum of respiratory diseases that can affect military men and women who have deployed to Southwest Asia and Afghanistan, including asthma, bronchiolitis (inflammation of the small airways), vocal cord dysfunction, and expiratory central airways collapse.
- We have implemented a noninvasive assessment of small airways injury disease using lung clearance index (LCI) testing (a simple breathing test that takes around 30 minutes to perform) and have applied this technique to screen over 70 previously deployed military personnel and over 100 healthy controls.
- We have identified quantitative methods to analyze chest CT scans from previously deployed military personnel with persistent respiratory and are using this approach to assess lung injury in patients who agree to participate in the study.
- We have developed novel *in vitro* and preclinical animal models of epithelial injury and fibrosis in response to exposure to airborne particulate matter.
- We have developed detailed transcript pathway analysis to examine the effects of exposure to particulate matter on altered epithelial cells *in vitro* and have begun to examine the effects of combined exposures to particulate matter and mechanical trauma, chemical injury, allergens, and infection.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

PROJECT 1.
Nothing to Report.

PROJECT 2.
Nothing to Report.

PROJECT 3.
Nothing to Report.

PROJECT 4.
Nothing to Report.

What was the impact on technology transfer?

PROJECT 1.
Nothing to Report.

PROJECT 2.
Nothing to Report.

PROJECT 3.
Nothing to Report.

PROJECT 4.
Nothing to Report.

What was the impact on society beyond science and technology?

PROJECT 1.
Nothing to Report.

PROJECT 2.
Nothing to Report.

PROJECT 3.
Nothing to Report.

PROJECT 4.
Nothing to Report.

5. CHANGES/PROBLEMS:

:

Changes in approach and reasons for change

PROJECT 1.
Nothing to Report.

PROJECT 2.
The one issue that has arisen for Project 2 is that delivery of even moderate to high concentrations of silicate containing particulate matter even of purified silica by aerosol delivery using the whole body exposure chambers results in only small amounts of lung injury and fibrosis. We have done a large experiment examining the effects of higher concentrations of silica and particulate matter 70 mg/m^3) given over 2 weeks followed by a period of 60 days to see if there is delayed development of fibrosis. As reported above, we saw no histological evidence of pulmonary fibrosis. We think that part of the issue is the efficient filtration system of the nasopharynx of rodents that filters out much of the aerosolized particulate material that therefore does not gain access to the lung. We have developed a method of oropharyngeal aspiration that results in significant amounts of lung injury and fibrosis. This will be a method of choice for most of the remainder of the experiments instead of the aerosol delivery.

PROJECT 3.
We are moving toward using precision cut lung slices (PCLS) since this system has numerous advantages over epithelial or macrophage cell culture. The big advantages of the PCLS is that: 1) it contains all the lung cells types in a preserved anatomical structure; 2) allow us to perform airway hyperresponsiveness studies in slices in situ; and 3) allow direct comparison of lung slice responses between mouse and man.

PROJECT 4.
Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

PROJECT 1.
Collection of deployer BAL, BEC and NEC samples was temporarily halted due to the COVID-19 pandemic while additional institutional safety policies were implemented. We are currently awaiting completion of a fully equipped BSL2Plus laboratory to assure that all study subject samples can be processed and analyzed safely by GLIDE Study investigators.

PROJECT 2.
Nothing to Report.

PROJECT 3.
Some delays in the project were caused by the recent COVID-19 crisis that affected laboratory access and access to clinical research subjects.

PROJECT 4.
Nothing to Report.

Changes that had a significant impact on expenditures

Due to initial delays in obtaining IRB and HRPO approval for GLIDE study subject recruitment, we underspent in the first years of funding for grant budget covering costs for study subject reimbursement, and for deployer bronchoscopies with BALs and nasal brushings. With IRB and HRPO approval, we were actively recruiting and had made substantial progress in meeting targets for recruitment and sample acquisition. With recognition of the COVID-19 pandemic in March 2020, a number of study subjects who had agreed to undergo study bronchoscopies and nasal brushings were postponed pending implementation of pandemic-required policies and precautions, leading to another decrease in anticipated expenditures.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/ or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects
Nothing to Report.

Significant changes in use or care of vertebrate animals.
Nothing to Report.

Significant changes in use of biohazards and/or select agents
Nothing to Report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

  Journal publications.

1. **Berman R**, **Downey GP**, **Dakhama A**, **Day BJ**, **Chu HW**. Afghanistan particulate matter enhances pro-inflammatory responses in IL-13-exposed human airway epithelium via TLR2 signaling. *Toxicol. Sci*. 2018 166(2):345-353. doi: 10.1093/toxsci/kfy217. PMID:30169750.

2. Garshick E, Abraham JH, Baird CP, Ciminera P, **Downey GP**, Falvo MJ, Hart JE, Jackson DA, Jerrett M, Kuschner W, Helmer DA, Jones KD, **Krefft SD**, Mallon T, Miller RF, Morris MJ, Proctor SP, Redlich CA, **Rose CS**, Rull RP, Saers J, Schneiderman AI, Smith NL, Yiallouros P, Blanc PD. Respiratory Health after Military Service in Southwest Asia and Afghanistan. An Official American Thoracic Society Workshop Report. *Ann Am Thorac Soc*. 2019 Aug;16(8):e1-e16. doi: 10.1513/AnnalsATS.201904-344WS.

3. **Zell-Baran LM**, **Meehan R**, **Wolff J**, **Strand M, Krefft SD, Gottschall EB**, Macedonia TV, Gross JE, Sanders OL, Pepper GC, **Rose CS**. Military Occupational Specialty Codes. Journal of Occupational and Environmental Medicine 2019; 61: 1036-1040.

4. **Krefft SD**, **Wolff J**, **Zell-Baran L**, **Strand M**, **Gottschall EB**, **Meehan R**, **Rose CS**. Respiratory Diseases in Post-9/11 Military Personnel Following Southwest Asia Deployment. J Occup Environ Med 2020.

5. **Berman R, Kopf KW, Min E, Huang J, Downey GP, Alam R, Chu HW, and Day BJ**. IL-33/ST2 signaling modulates Afghanistan particulate matter-induced airway reactivity in mice. *Toxicol. Appl. Pharmacol*. On line, 2020.

- **Books or other non-periodical, one-time publications.**
-

1. Everman JL, NJ Jackson, B Saef, K Li, C Rios, MT Montgomery, CS Rose, BJ Day, H Chu, GP Downey, MA Seibold (2019) Mechanisms of military deployment-related lung diseases: Desert dust exposure induces epidermal differentiation of both human airway epithelial stem cells and the mucociliary airway epithelium. ATS 2019 Abstract; AJRCCM

2. Everman JL, NJ Jackson, B Saef, K Li, C Rios, MT Montgomery, CS Rose, BJ Day, H Chu, GP Downey, MA Seibold (2019) Desert dust exposure induces epidermal

differentiation of both human airway epithelial stem cells and the mucociliary airway epithelium. MHSRS 2019 Poster Abstract

3. **Zell-Baran L, Rose CS, Lynch DA**, Charbonnier J, **Oh AS, Wolff J**, Kluiber A, Wilson C, and **Humphries SM**. Quantitative Airway Wall Thickening by Pi10 On Chest Imaging is Increased in Symptomatic Military Deployers Compared to Controls, and is Inversely Related to Forced Expiratory Volume in One Second. American Journal of Respiratory and Critical Care Medicine 2020;201:A4344. Abstract.

4. **Krefft SD, Oh AS, Wolff J, Zell-Baran L**, and **Rose CS**. CT Imaging to Define Expiratory Central Airway Collapse (ECAC) and Examination of Risk Factors for ECAC in Personnel Deployed to Iraq and Afghanistan." American Journal of Respiratory and Critical Care Medicine 2020;201:A4336. Abstract.

- **Other publications, conference papers, and presentations**.
  Nothing to Report.

- **Website(s) or other Internet site(s)**

- **Technologies or techniques**

- **Inventions, patent applications, and/or licenses**

  Nothing to Report.

- **Other Products**

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Gregory P. Downey, MD
Project Role:	Principal Investigator, Project Leader Project 2
Researcher Identifier (e.g. ORCID ID):	0000-0003-3253-5862
Nearest person month worked:	2 (annually)
Contribution to Project:	No change

Name:	Cecile Rose, MD, MPH
Project Role:	Project Leader Project 1
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1 (annually)
Contribution to Project:	No change

Name:	Richard Meehan, MD
Project Role:	Co-Investigator, Project 1
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1 (annually)
Contribution to Project:	No change

Name:	Stephen Humphries, PhD
Project Role:	Co-investigator, Project 1
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1 (annually)
Contribution to Project:	No change

Name: Richard Kraus, PA
Project Role: Physician Assistant, Project 1
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1 (annually)
Contribution to Project: No change

Name: Jenna (Marcotte) Wolff
Project Role: Clinical Research Coordinator, Project 1
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 6 (annually)
Contribution to Project: No change

Name: Lauren Zell-Baran, MPH
Project Role: Research Staff, Project 1
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 2 (annually)
Contribution to Project: No change

Name: James Crooks, PhD
Project Role: Biostatistician, Project 1
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1 (annually)
Contribution to Project: No change

Name: Matthew Strand, PhD
Project Role: Biostatistician, Project 1
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1 (annually)
Contribution to Project: No change

Name: Carlyne D. Cool, M.D.
Project Role: Pulmonary Pathologist, Project 1
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1 (annually)
Contribution to Project: No change

Name: Andrea Oh, M.D.
Project Role: Co-I, Project 1
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1 (annually)
Contribution to Project: No change

Name: Claudia Onofrei, M.D.
Project Role: Co-I, Project 1
Researcher Identifier (e.g. ORCID ID): N/A

Nearest person month worked:	1 (annually)
Contribution to Project:	No change

Name:	Katrina Kopf
Project Role:	Director of small animal physiology core lab, Project 2
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2 (annually)
Contribution to Project:	No change

Name:	Helen Roybal
Project Role:	Lab Researcher, Project 2
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	9 (annually)
Contribution to Project:	No change

Name:	Keriann Beke
Project Role:	Lab Researcher, Project 2
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	5 (annually)
Contribution to Project:	No change

Name:	Daniel Foster
Project Role:	Lab Researcher, Project 2
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3 (annually)
Contribution to Project:	No change

Name:	Paul Reynolds, PhD
Project Role:	Senior Researcher, Project 2
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2 (annually)
Contribution to Project:	No change

Name:	Bradley Richards
Project Role:	Lab Researcher, Project 2
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1 (annually)
Contribution to Project:	Bradley Richards will be responsible for conducting the studies involving blast over pressure lung injury to mice, testing the effects of combined blast overpressure lung injury with exposure to particulate matter, and testing the effects of ICG-001 on blast over pressure lung injury and on lung injury and fibrosis induced by PM exposure.

Name:	Brian Day, PhD
Project Role:	Co-Project Leader Project 3
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2 (annually)
Contribution to Project:	No change

Name:	Hong Wei Chu, PhD
Project Role:	Co-Project Leader Project 3 and Co-Investigator Project 4
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2 (annually)
Contribution to Project:	No change

Name:	Jie Huang
Project Role:	Lab Researcher, Project 3
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6 (annually)
Contribution to Project:	No change

Name:	Reena Berman
Project Role:	Graduate Student Researcher, Project 3
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	10 (annually)
Contribution to Project:	No change

Name:	Nicole Pavelka
Project Role:	Research Associate, Project 3
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2 (annually)
Contribution to Project:	No Change

Name:	Christina Lisk
Project Role:	Research Fellow, Project 3
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1 (annually)
Contribution to Project:	Ms. Lisk is responsible for performing airway epithelial cell cultures and human and mouse precision-cut lung slice experiments to study the impact of particulate matter on airway hyperresponsiveness and inflammation in the context of an asthma condition.

Name:	Max Seibold, PhD
Project Role:	Project Leader Project 4
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1 (annually)

Contribution to Project: No change

Name: Noah Zaitlen, PhD
Project Role: Co-investigator, University of California, Los Angeles, Project 4
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1 (annually)
Contribution to Project: No change

Name: Jamie Everman, PhD
Project Role: Lead Cell Biology Post-doc, Project 4
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 5 (annually)
Contribution to Project: No change

Name: Nathan Jackson, PhD
Project Role: Lead Bioinformatics Analyst, Project 4
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 2 (annually)
Contribution to Project: No change

Name: Cydney Rios
Project Role: Laboratory Manager, Project 4
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1 (annually)
Contribution to Project: No change

Name: Peter DeFord, PhD
Project Role: Bioinformatics Analyst, Project 4
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1 (annually)
Contribution to Project: No Change

Name: Geoffrey Plumlee, PhD
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: < 1 month (annually)
Contribution to Project: No change.

Name: Heather Lowers
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 3 months (annually)
Contribution to Project: No change

Name:	Bill Benzel
Project Role:	Researcher
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1 month (annually)
Contribution to Project:	No change

Name:	Kate Campbell
Project Role:	Researcher
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1 month (annually)
Contribution to Project:	No change

Name:	David Roth
Project Role:	Researcher
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1 month (annually)
Contribution to Project:	No Change

Name:	Karen Mumy, PhD
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1 annually
Contribution to Project:	No change

Name:	Brian Wong, PhD
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1 annually
Contribution to Project:	No change

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Dr. Gregory Downey, project PI, was awarded project BMS IPF Cohort, entitled, “Identification of Disease Progression Biomarkers in the National Jewish Health IPF cohort”. The addition of this new project will not adversely affect his performance on this research project. Dr. Downey will continue to provide 20% effort or 2.4 personnel months on this project. Additionally, project W81XWH-16-2-0029, entitled, “Role of Matrix Metalloproteinase-3 in Deployment-Related Pulmonary Fibrosis” has concluded. Dr. Cecile Rose was awarded project AFC820-59, entitled, “Elucidating the individual and combined toxicity of coal”. The addition of the new project will not adversely affect her performance on this research project. Dr. Rose will continue to provide 10% effort or 1.2 personnel months on this current project. Dr. Hong Wei Chu was awarded, R01AI150082 entitled, “Role of immunoproteasome in airway viral infection” and R01AI152504 entitled, “Parkin in Mitochondrial dysfunction and Airway inflammation of Obese Asthma”. The addition of these two awards will not adversely affect his performance on this project. Dr. Chu will continue to provide 15% or 1.8 personnel months on this project. Dr. Chu’s

research project R01AI106287 entitled, “IRAK-M in Lung Defense against Rhinovirus Infection”, concluded in November 2019. Dr. Max Seibold was awarded, R01AI152504 entitled, “Parkin in Mitochondrial dysfunction and Airway inflammation of Obese Asthma”, project PHL107202C (Co-I) entitled, “Exploring the Biology of Persistent Type 2 Airway Niches”, and project UAI151958A-01S entitled, “Experimental and Computational Analysis of the Human Epidemiology and Response to SARS-CoV-2 (HEROS) Cohort”. The addition of these projects will not adversely affect his performance on this research project. Dr. Seibold will continue to provide 15% or 1.8 personnel months on this project.

What other organizations were involved as partners?

Organization Name: NAMRU Dayton
Location of Organization: (if foreign location list country) Dayton, Ohio
Partner’s contribution to the project (identify one or more) Collaboration on inhalational toxicology

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

Please see attached Quad chart.

9. APPENDICES: