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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.					
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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

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 lung function abnormalities in our cohort of over 120 previously deployed military personnel followed in the Center for Deployment Lung Disease at National Jewish Health. 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:** Provide a brief list of keywords (limit to 20 words).

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:** 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.

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?

For this reporting period 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. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

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.

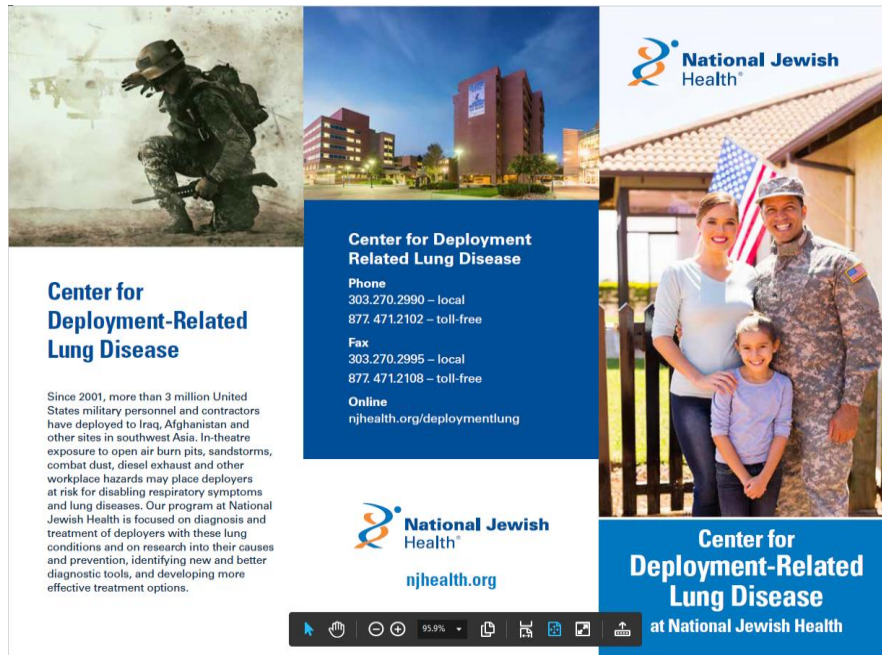
What was accomplished under these goals?

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 Lung 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.
2. We are fully staffed with trained and experienced research coordinators for all aspects of Project 1 deployer study subject recruitment, consenting, testing and data entry.
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 continue to recruit controls for lung clearance index testing (<https://www.nationaljewish.org/clinical-trials/healthy-participants-needed-for-lung-disease-study>).
7. 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.
8. The GLIDE Study Outreach Committee continues to meet monthly to develop and implement plans for study subject recruitment.
9. 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.
10. A GLIDE Study coordinator trained in clinical scheduling schedules research subjects for GLIDE study participation, including for LCI (deployers and controls), deployer chest CT image acquisition, research bronchoscopies and nasal epithelial cell brushings.
11. We continue to successfully consent and enroll deployers and LCI controls who meet our study subject criteria since receiving HRPO approval.
12. 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, Lung Clearance

Index (LCI) testing, quantitative chest CT imaging, nasal epithelial brushing and/or fiberoptic bronchoscopy. We defined “**completed**” as those who completed testing after having been enrolled.

- Number of subjects **screened**/target: **213/250**
- Number of patients **enrolled**/target: **89/250**
- Number of patients **completed**/target: **89/250**

Difficulties encountered

None

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 have replaced one GLIDE Study bronchoscopist who retired with a different pulmonologist who is an experienced bronchoscopist and who has been trained in GLIDE Study research bronchoscopy techniques and approaches.
6. We communicate regularly with laboratory technicians who process study samples to ensure that sample collection yield is maximized.
7. Study bronchoscopists adjusted brushing techniques to enhance bronchial epithelial cell collection from deployer subjects based on feedback from co-investigators.
8. 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 the deployer bronchoalveolar lavage specimens.

Difficulties encountered

1. One of the study occupational pulmonologists has left NJH, though will continue to provide backup as needed for GLIDE Study bronchoscopies. We are training another pulmonologist on GLIDE Study participant evaluations and recruitment.

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. We are scheduling and performing research bronchoscopies on deployer study subjects and have 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 have been delivered to the appropriate labs for storage and analysis of samples.
5. We have trained one of our coordinators in nasal brushing techniques to provide back-up to the lab technicians who have been collecting these samples.

Difficulties encountered

None

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. Our protocol includes an IRB-approved consent form for collection of samples including lung tissue blocks from VATS biopsies done for clinical diagnosis.
2. We have had several meetings with the study pathologist to clarify and finalize protocols for delivery of VATS samples and processing for analysis, and will begin analyzing these samples during the next study period.

Difficulties encountered

None

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

Progress

1. In discussions with study investigators and staff regarding potential GLIDE candidates from our Deployment Lung Disease Registry and Biorepository, we determined which subjects have had a VATS biopsy and meet other requirements for participation in the GLIDE study.
2. As we obtain informed consent from GLIDE study participants, we closely track the number who have had a VATS biopsy, collecting authorization from these subjects so that we can obtain their VATS biopsy tissue for analysis.
3. We have created a new standardized scoring form for the study pathologist to use for interpretation of VATS biopsy samples to assure complete

documentation of histologic findings from all lung compartments (including parenchyma, airways, vascular bed, pleura).

4. We have piloted and finalized the process for requesting and receiving VATS samples from the institutions where the biopsies were performed.
5. We communicated with the USGS to assure that, once they have tested the analysis methods on BAL and brushings, we are ready to send USGS co-investigators stored non-deployer VATS lung biopsy tissue with which to pilot a revised lung tissue digestion process before beginning analysis of GLIDE Study deployer samples.

Difficulties encountered

1. There was a period during the past grant year in which the government was shut down, and we had to wait to send samples to the USGS until employees could return to work.
2. Our co-investigators at the USGS have been optimizing sample analysis methods. We plan to send VATS samples to the USGS co-investigators for analysis along with BAL and airway cell brushing sample analysis.

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, allowing each staff member (3 total) 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.

Difficulties encountered

None.

Subtask 7: Complete LCI measurement from deployers and controls.

Progress

1. We continue to successfully recruit GLIDE Study deployers for LCI testing.
2. We are recruiting LCI control subjects matched with deployer study participants for age, sex, and smoking status.
3. We created and utilize a SAS program that allows us to easily match deployer subjects to controls and that aids in targeted study subject recruitment.
4. We continue to run quality analyses on the LCI machine to assure that they yield comparable results from test to test.
5. We confer regularly with LCI experts in Toronto to assure that testing is meeting the highest quality standards

6. We have begun analyzing preliminary data from LCI testing on deployers and matched controls. We plan to complete control matching before preparing a manuscript to submit for publication.

Difficulties encountered

1. We have had to create a targeted outreach approach to assure that LCI controls are appropriately matched to deployers on demographic variables (sex, smoking, age).
2. The LCI machine malfunctioned during this study period. We were able to identify a problem with the nitrogen level not reaching baseline due to a small leak in a broken piece of the equipment. The GLIDE Study research coordinator was able to troubleshoot the problem in less than a week, ordering a new piece and returning the nitrogen level to baseline.

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 by the two study radiologists for qualitative chest CT image interpretation.
4. We created a method involving a third pulmonary radiologist for chest CT image interpretation to resolve discordant readings between the two radiologists.
5. The REDCap database for chest CT imaging data entry has been finalized.
6. Working with the NJH Imaging Department staff, GLIDE staff developed and utilize a specific CT acquisition form for the GLIDE Study that shows which procedures should be followed for CT imaging for deployer study subjects.

Difficulties encountered

1. Within 6 months, we were able to replace one of the study radiologists who left NJH with another radiologist who has been trained on GLIDE image acquisition and scoring.

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 38 (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 have scored 34 deployer scans and 34 controls matched for age, sex, and smoking status, and data have been archived in our study database.
5. We continue to send deployer scans to NJH radiology technicians to match for controls, load into the database, and prepare for scoring by study radiologists during the next quarter.
6. Study radiologists have begun efforts focused on quantitative analysis for this study population.

Difficulties encountered

None

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

1. None. Though ICP MS analysis of BAL fluid was delayed due to staffing changes at USGS, this issue appears to have been resolved.

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

Progress

1. As mentioned in the progress for subtask 5, we have worked with co-investigators at USGS to pilot a digestion technique using stored VATS lung biopsy samples to assure that analytic methods are optimal. We plan to send GLIDE lung tissue samples (deployer and control) to USGS for analysis.

Difficulties encountered

1. See subtask 5.

Subtask 12: Prepare and submit manuscripts for publication.

Progress

1. We have prepared and submitted a large case series manuscript on clinical findings in consecutively evaluated symptomatic deployers and await reviewer comments.
2. We submitted a manuscript to the Journal of Occupational and Environmental Medicine (JOEM) on the use of a respiratory hazard exposure matrix and Military Occupational Specialty Codes to identify risk factors for respiratory symptoms and diseases in military deployers; the manuscript has been accepted for publication within the next 2 months.
3. Study co-investigators are preparing abstracts for submission and review for presentation at the 2020 American Thoracic Society meeting, and two manuscripts for peer review and publication.

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

During year 2, 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 $\mu\text{g}/\text{cm}^2$, 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 3, we have not conducted any additional studies using the MLE-12 cell line because we have focused on studying responses of primary human airway epithelial cells that we feel are most relevant to human disease (see subtask 2 below). As will be discussed below, studies with primary human airway epithelial cells, conducted in collaboration with Drs. Chu and Day (see below), suggested that higher concentrations of PM induced inflammatory cytokine production. Therefore, we tested the effects of PM from Iraq and Afghanistan in concentrations up to 50 $\mu\text{g}/\text{cm}^2$. These studies revealed that even at these higher concentrations, exposure to PM resulted in a mild increase in cytotoxicity when given as a single stimulus and slightly potentiated epithelial cell cytotoxicity induced by bleomycin (**Fig. 1**).

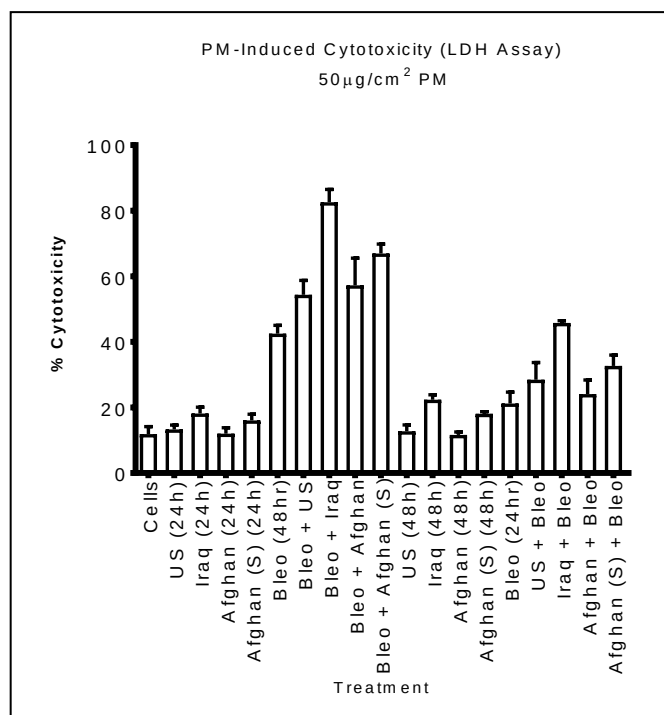


Figure 1. Cytotoxicity assay using LDH release. Note that there is little evidence of cytotoxicity induced by exposure to PM from Iraq or Afghanistan alone whereas exposure to bleomycin alone causes significant cytotoxicity as a single stimulus. There is evidence of enhanced cytotoxicity with combined exposure to PM and bleomycin. Cells = control MLE-12 cells with no added PM or bleomycin. The time of incubation with PM of bleomycin is indicated by 24 h or 48 h.

Having excluded over cytotoxicity as a dominant consequence of exposure to PM, we next explored the possibility that exposure to PM induced apoptosis of MLE-12 epithelial cells. We used caspase 3/7 activation as a marker of activation of the terminal common pathway of apoptosis. Contrary to our predictions however, when given alone at concentrations up to 50 $\mu\text{g}/\text{cm}^2$, PM from Iraq and Afghanistan induced no significant activation of caspase 3/7 (**Fig. 2A**). In contrast, exposure to bleomycin induced marked activation of caspase 3 in a dose-dependent manner that peaked between 14 and 18 hr (**Fig. 2B**). Pre-exposure of cells to PM from Iraq or Afghanistan did not result in potentiation of the apoptosis induced by bleomycin alone (not illustrated).

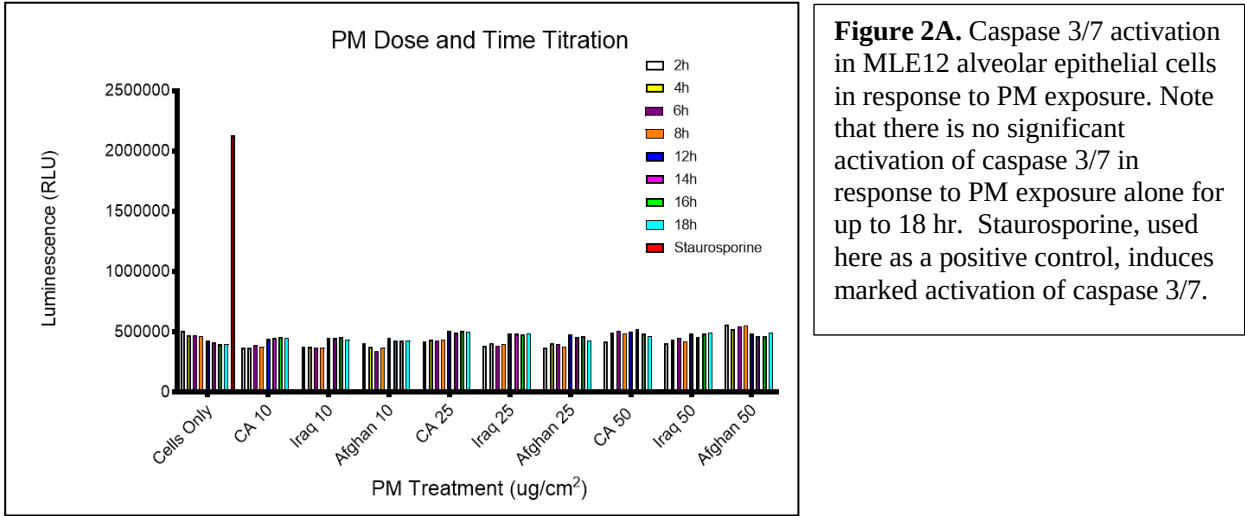


Figure 2A. Caspase 3/7 activation in MLE12 alveolar epithelial cells in response to PM exposure. Note that there is no significant activation of caspase 3/7 in response to PM exposure alone for up to 18 hr. Staurosporine, used here as a positive control, induces marked activation of caspase 3/7.

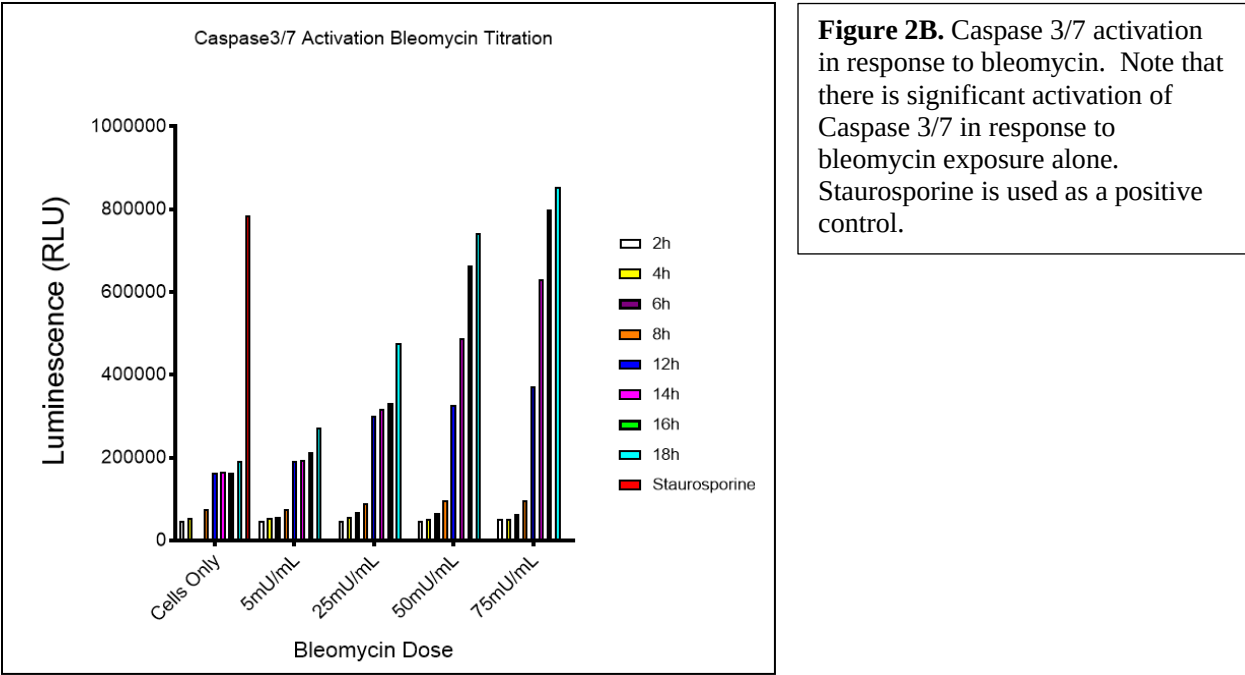


Figure 2B. Caspase 3/7 activation in response to bleomycin. Note that there is significant activation of Caspase 3/7 in response to bleomycin exposure alone. Staurosporine is used as a positive control.

Taken together, the studies examining cytotoxicity and apoptosis indicate that exposure to PM from Iraq or Afghanistan induces minimal evidence of cytotoxicity and does not induce activation of the apoptotic pathway. Accordingly, we decided to focus our efforts on determining more subtle cellular responses of lung epithelial cells to PM exposure that lead to phenotypic alterations in the epithelial cells but do not result in apoptosis or cell death. For these studies, we measured the effects of exposure to PM from Afghanistan on global mRNA expression ('transcriptional profile') of primary human large and small airway epithelial cells and on alveolar epithelial cells. These studies are described in detail below in **Subtask 2**.

We also studied the combined effects of PM exposure and physical injury (scratch wound). We completed a series of experiments quantifying the effects of prior exposure to PM on the ability of lung epithelial cells to heal a wound caused by a scratch injury induced by scratching the monolayer with a pipette tip. For these studies, we used image analysis to measure the area of the wound over time as the primary endpoint. Using this approach, we observed that prior exposure to PM from Iraq caused a slight delay in the closure of the wounds. However, this effect was modest and likely not physiologically important. The results of these studies are summarized in **Figures 3, 4, and 5** below.

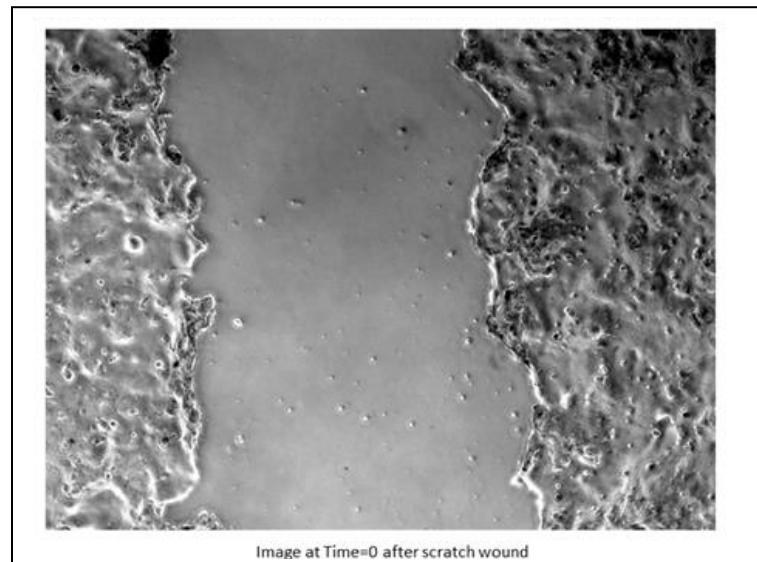


Figure 3. Scratch wound in a monolayer of MLE-12 alveolar-like epithelial cells made using a pipette tip.

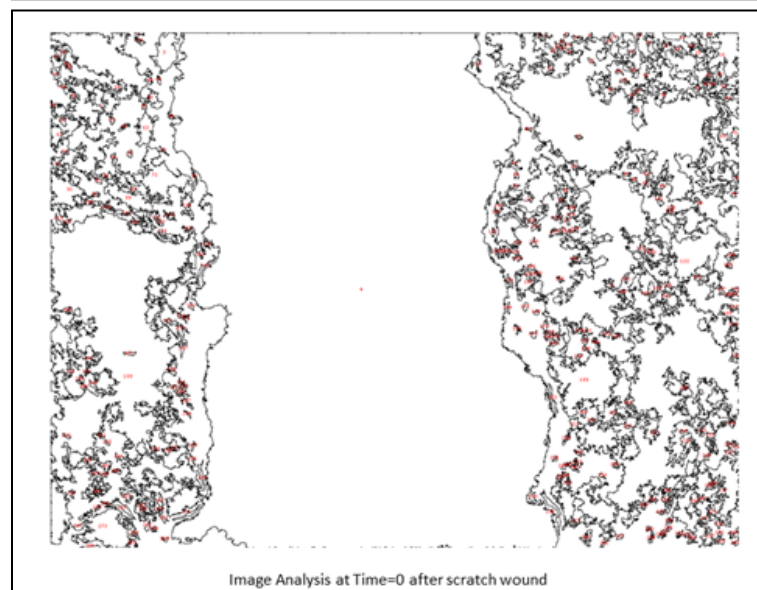
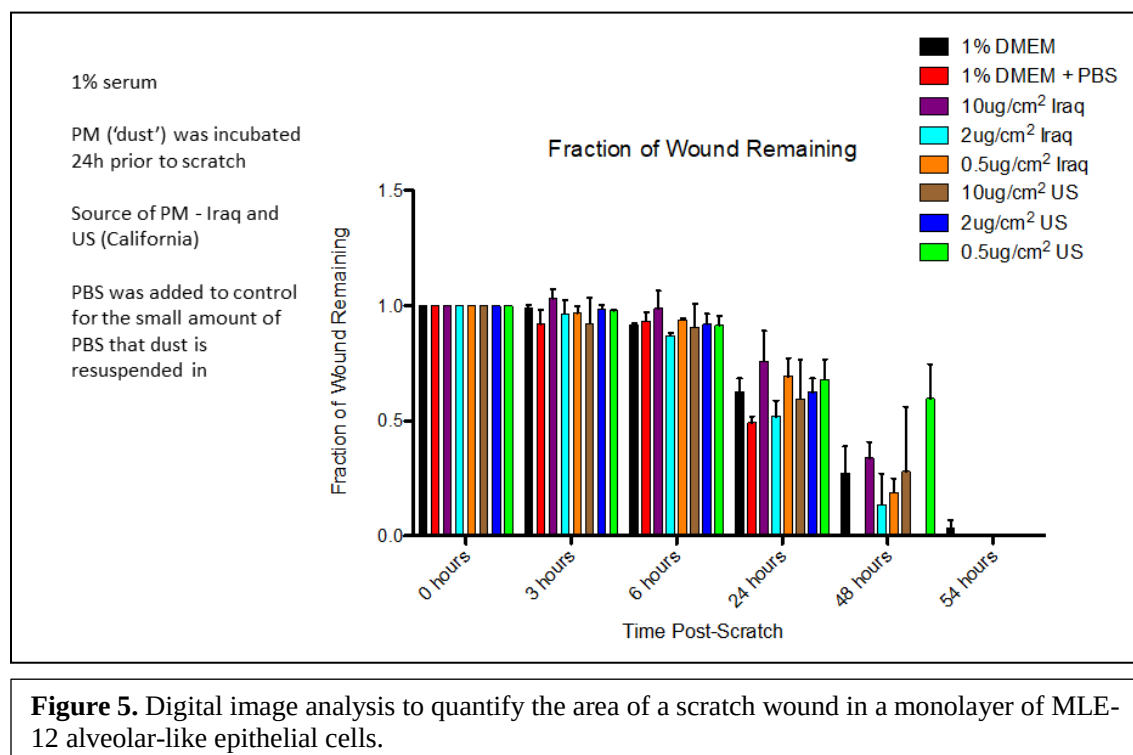


Figure 4. Digital image analysis to quantify the area of a scratch wound in a monolayer of MLE-12 alveolar-like epithelial cells.



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

In year 3, we collaborated with Drs. Chu, Day, Fingerlin, and Seibold to characterize the effects of PM exposure on primary human airway and alveolar epithelial cells. We developed methods using RNA-Seq to determine the changes in global mRNA expression (transcriptional profile) induced by exposure to PM from Iraq and Afghanistan. We used PM from California (China Lake) for comparison, although this PM is not strictly a negative control. We chose 4 hr and 24 hr as our initial time points to capture both early and more delayed alterations in gene expression. These times were chosen based on studies in primary human airway epithelial cells examining the time course of IL-8 protein release in response to exposure to PM that peaked at 24 hr. The timing of IL-8 protein release was used to estimate when the maximal changes in mRNA levels would occur. In previous quarterly reports for Year 3, we provided preliminary data on differential gene expression only in large (tracheal) airway epithelial cells. We have now completed the analysis on the effects of PM exposure on human large and small airway and alveolar type (AT) II epithelial cells from 4 separate donors. Note that for these experiments, for practical reasons (we do not have sufficient lung tissue from non-smokers to isolate all three types from the same donor from one harvest), the large and small airway epithelial cells were from the same donor whereas the alveolar type II cells were from separate donors. The composite data from the three types of epithelial cells (large airway, small airway, and alveolar epithelial cells) will be presented separately.

In brief, these studies demonstrated that exposure of primary airway epithelial cells to PM from Iraq and Afghanistan induced expression of a modest number of genes at 4 hours (283 for large airway cells exposed to Afghanistan PM) that increased significantly to 7643 genes at 24 hr (**Fig. 6**). Using a principal component analysis (PCA), we observed clear distinctions between the transcriptional profiles of cells at 0 and 4 hr after exposure to PM compared to 24 hr (**Fig. 7**).

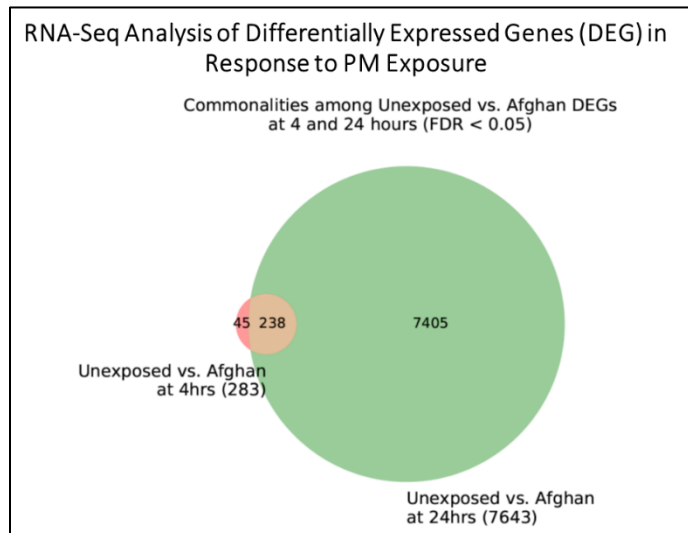


Figure 6. RNA-Seq analysis of changes in mRNA expression of primary human large airway epithelial cells in response to exposure to PM from Afghanistan at 4 and 24 hr.

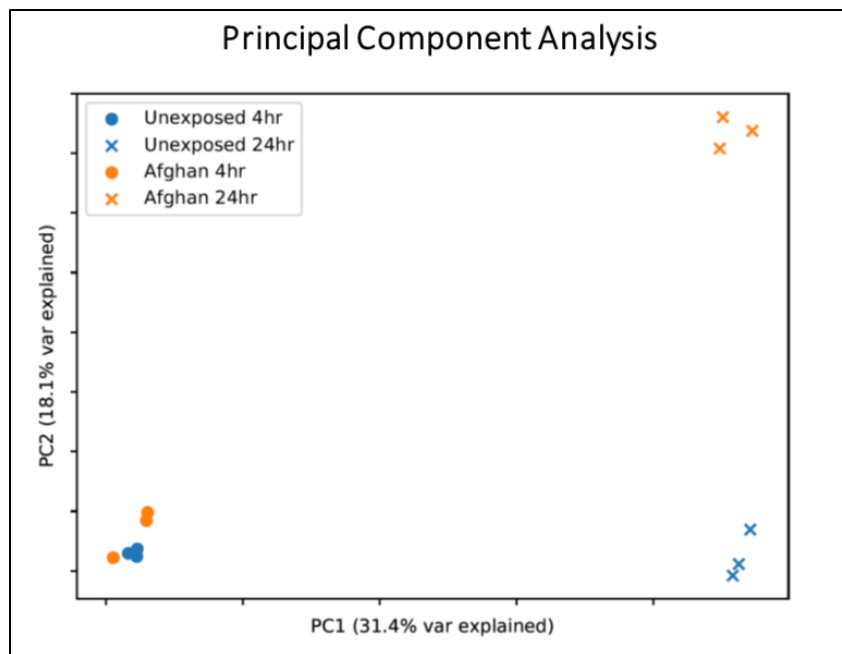
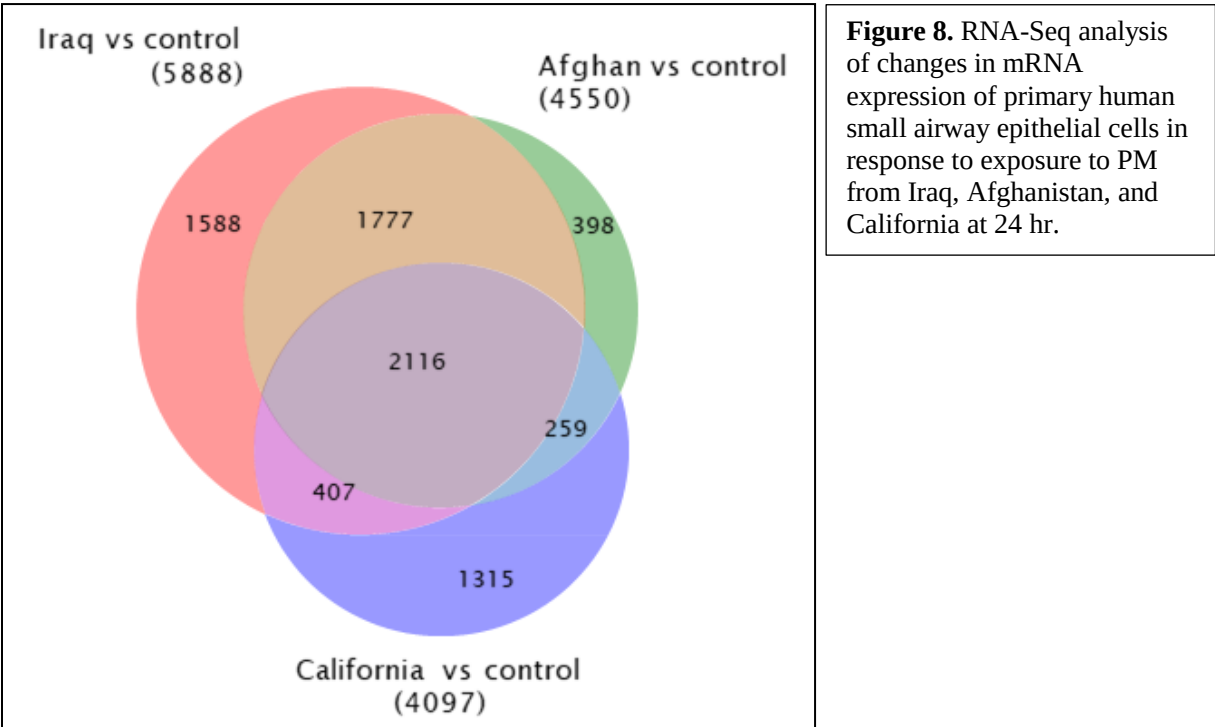


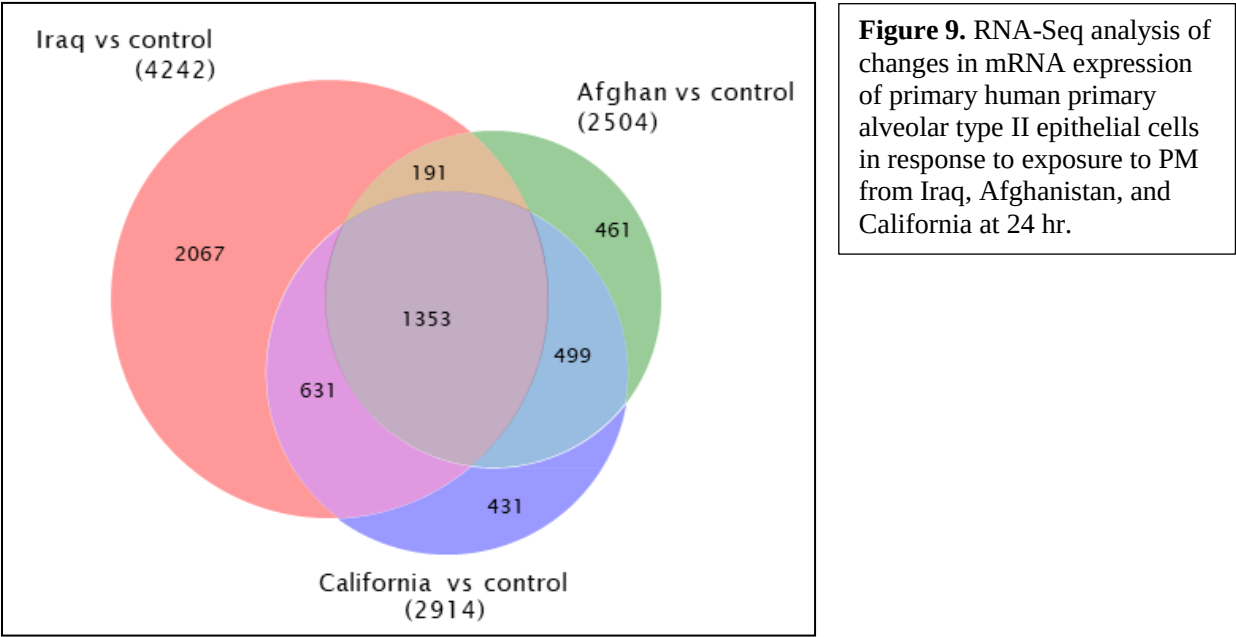
Figure 7. Principal Component Analysis (PCA) of RNA-Seq data from primary human large airway epithelial cells exposed to PM from Afghanistan for 4 and 24 hr. Note the clear separation of the data from the 24 hr exposure time point (upper right quadrant) and the 4 hr time point and unexposed cells (lower left quadrant).

We next examined the effect of exposure to PM from Iraq and Afghanistan on gene expression profiles in primary human small airway epithelial cells. As illustrated in **Fig. 8**, exposure of small airway epithelial cells to PM from Iraq and Afghanistan induced expression of thousands of genes (5888 for Iraq PM and 4554 for Afghanistan PM). Of these, 3893 were in common. Exposure to PM from California also induced significant changes in global gene expression. Of

these, 2016 genes were in common with PM from Iraq and Afghanistan, but 1315 genes were unique (**Fig. 8**).



We next examined the effect of exposure to PM from Iraq and Afghanistan on gene expression profiles in primary human alveolar type (AT) II epithelial cells. As illustrated in **Fig. 9**, exposure of ATII cells to PM from Iraq and Afghanistan induced expression of thousands of genes (4242 for Iraq and 2504 for Afghanistan) in alveolar type II epithelial cells. Of these, 1544 were in common. Exposure to PM from California also induced significant changes in global gene expression. Of these, 1353 genes were in common with PM from Iraq and Afghanistan, and 431 were unique.



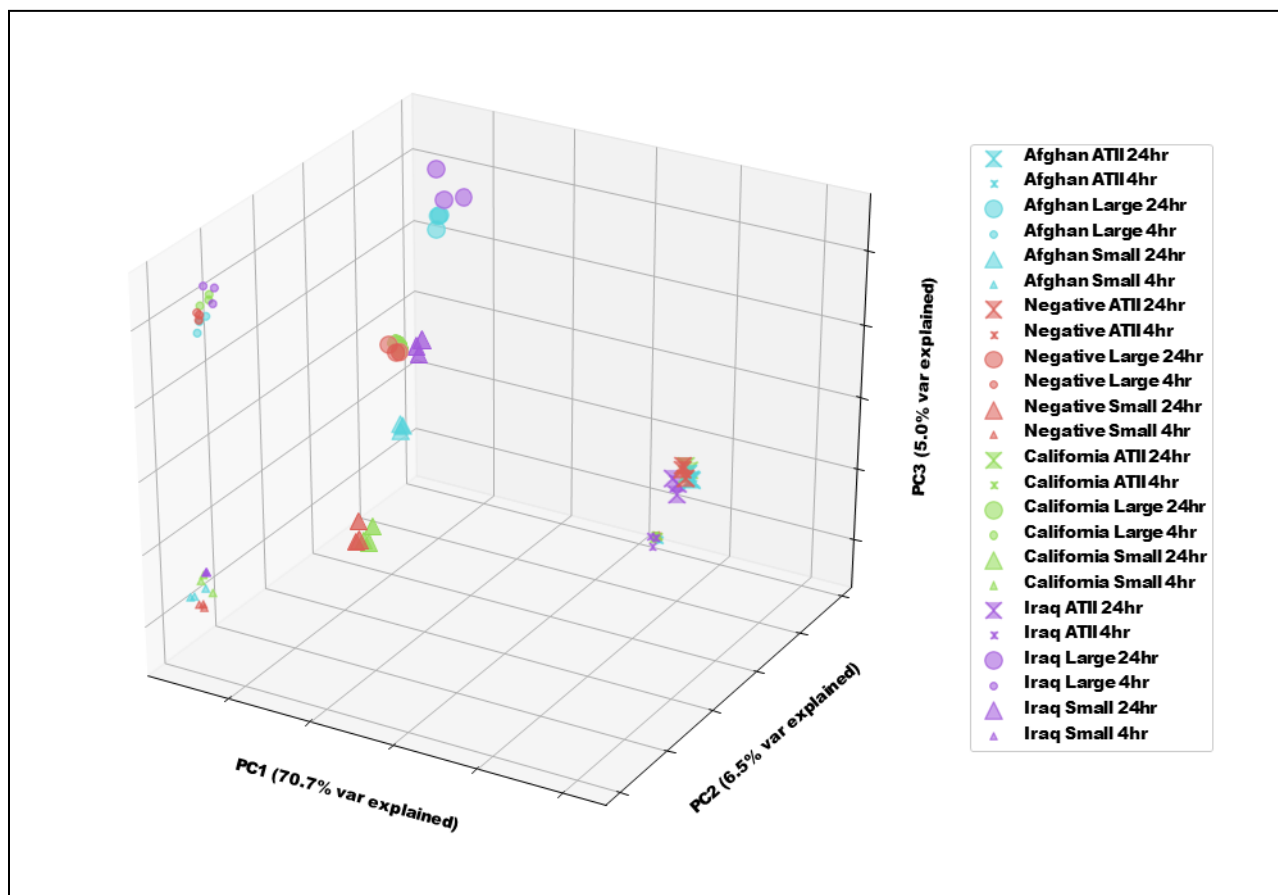


Figure 10. Principal Component Analysis (PCA) of RNA-Seq data from primary human large and small airway epithelial cells and primary human ATII cells exposed to PM from Iraq and Afghanistan for 4 and 24 hr. Note the clear separation of the data from the 24 hr exposure time point for exposure to PM from Iraq and Afghanistan compared to California PM. Also note the clear separation of negative controls from the cells exposed to PM.

To determine the similarities and differences between the transcriptional responses of the large and small airway epithelial cells, we conducted a principal component analysis illustrated in **Fig. 10**. This analysis revealed that the large airway and small airway epithelial cells responded distinctly (note clear clustering of large airway epithelial cells). In addition to cell type specific transcriptional responses, there is evidence of distinct responses to particulate matter from Iraq, Afghanistan, and China Lake (**Fig. 10**). We will be pursuing additional studies to characterize the chemical and physical differences between the particulate matter from Iraq, Afghanistan, and China Lake in collaboration with the US Geological Survey (see below in **Subtask 6**).

We next analyzed the data to look at individual genes that are overexpressed in response to PM exposure. The most highly expressed genes include IL-8, IL-36, Serpin B2, IL-1R1, CXCL1, MUC 16, and ADAM-8 (**Fig. 11**). Using pathway analysis, we determined that many signaling pathways are activated by exposure to PM including intercellular adhesion, matrix metalloproteinase (MMP) signaling, cell adhesion molecule, and acute inflammation. We compared the responses of large airway, small airway, and alveolar epithelial cells to PM exposure.

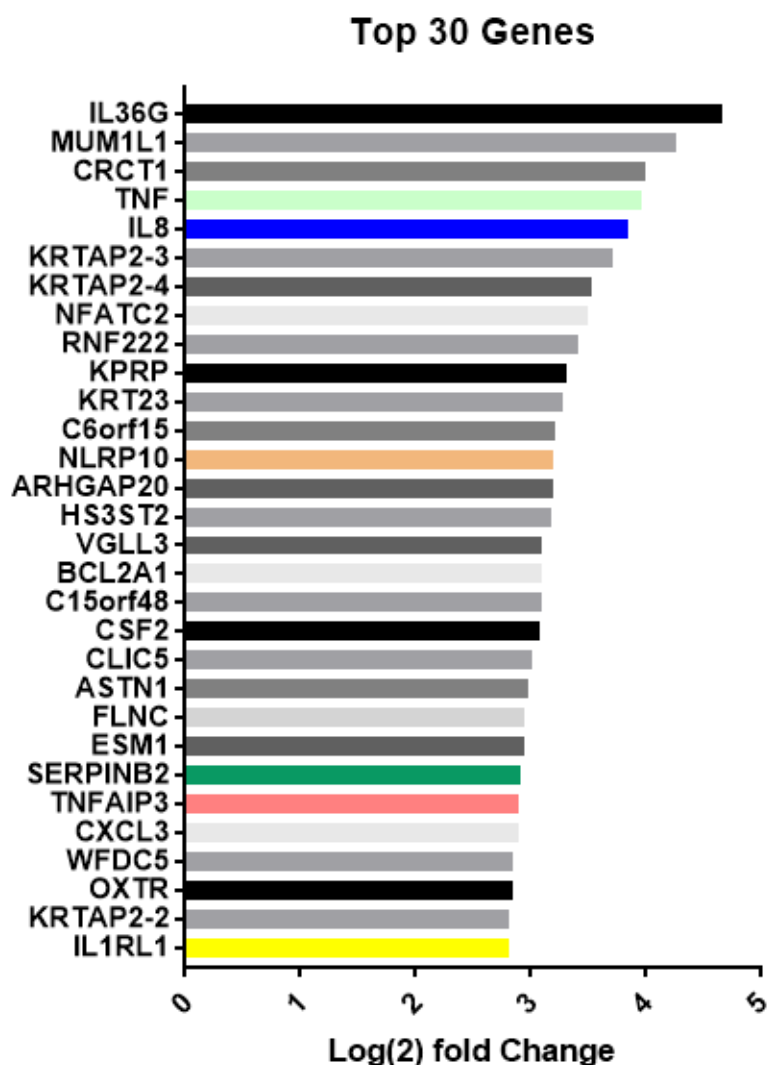


Figure 11. The top 30 genes activated in primary human large airway epithelial cells exposed to PM from Afghanistan for 24 hr as determined by RNA-Seq.

We next undertook a more detailed pathway analysis using a variety of bioinformatics tools including a variety of approaches including GAGE (Generally Applicable Gene set Enrichment for pathway analysis), DAVID, KEGG (Kyoto Encyclopedia of Genes and Genomes), HALLMARK, and IPA (Ingenuity Pathway Analysis). The pathways with the highest representation included IL3-6 and IL-1 signaling, inflammation, inflammasome, leukocyte adhesion, and TNF signaling. Importantly, we observed a strong and unanticipated signal from the pathways driving keratinization and cornification (**Fig. 12**). Genes in these pathways are not expressed in human lung epithelial cells under homeostatic conditions. They are normally expressed in the skin (hence cornification) and GI tract but not lung. These keratin proteins are part of the cytoskeletal system and confer stronger biomechanical properties to the cells. We interpret this as a protective response in the airway epithelial cells to bolster protection from physical and chemical damage from the particulate matter.

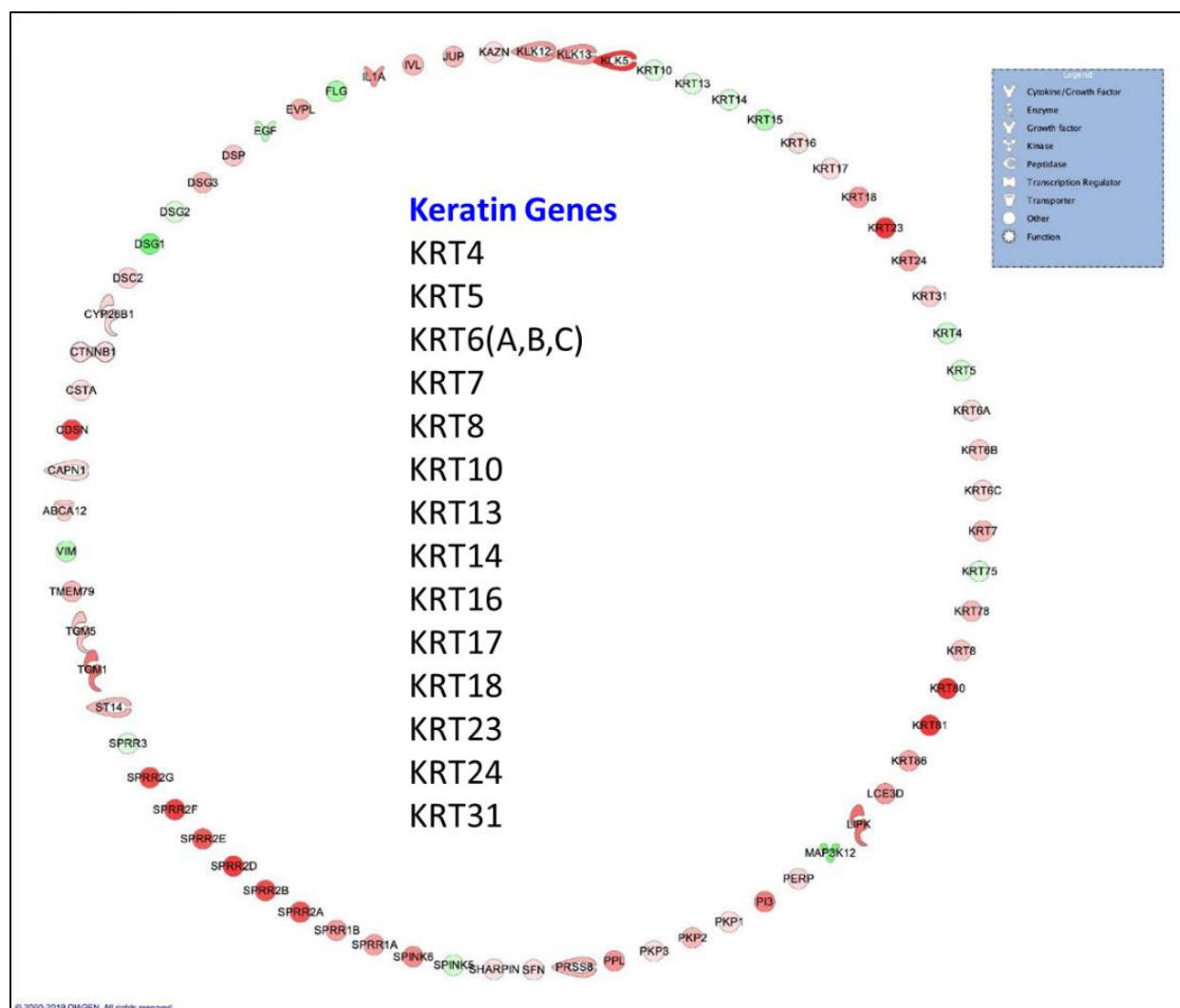


Figure 12. Differential expression of genes in the keratinization and cornification pathways in primary human large airway epithelial cells exposed to PM from Afghanistan for 24 hr as determined by RNA-Seq. Note expression of 17 keratin genes in epithelial cells exposed to PM. These genes are normally not expressed by quiescent human lung epithelial cells.

We next undertook a series of experiments to assess the effects of PM exposure on human alveolar macrophages. We first conducted preliminary experiments with primary cultures of human alveolar macrophage to determine the optimal concentration of particulate matter and the time course of the gene expression using several marker genes including TNF alpha and IL-1 beta by qPCR. Exposure to lipopolysaccharide (LPS) was used as a positive control. These studies demonstrated that there were 2 peaks of gene expression, one at 4 hours (early response genes: **Figs. 13 and 14**) and another at 24 hours (not shown). Accordingly, we then exposed primary human alveolar macrophages to PM (10 $\mu\text{g}/\text{cm}^2$) from Iraq, Afghanistan, and California (10 $\mu\text{g}/\text{cm}^2$) from Iraq for 4 and 24 hr and transcriptomic responses were analyzed by RNAseq followed by bioinformatics analysis including differential gene expression and pathway analysis.

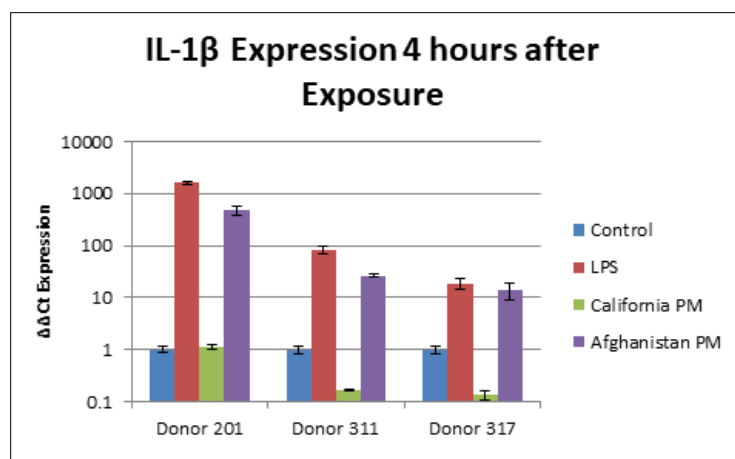


Figure 13. Analysis of IL1- β expression in human primary alveolar macrophages exposed to PM (10 $\mu\text{g}/\text{cm}^2$) from Afghanistan and California *in vitro*. LPS was used as a positive control.

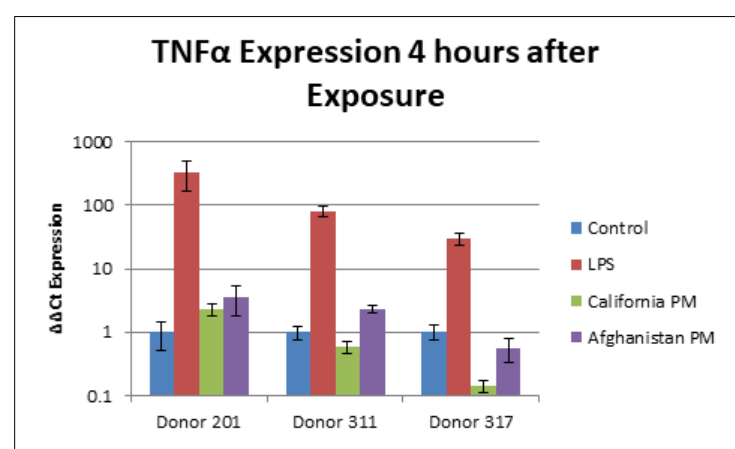


Figure 14. Analysis of TNF α expression in human primary alveolar macrophages exposed to PM (10 $\mu\text{g}/\text{cm}^2$) from Afghanistan and California *in vitro*. LPS was used as a positive control.

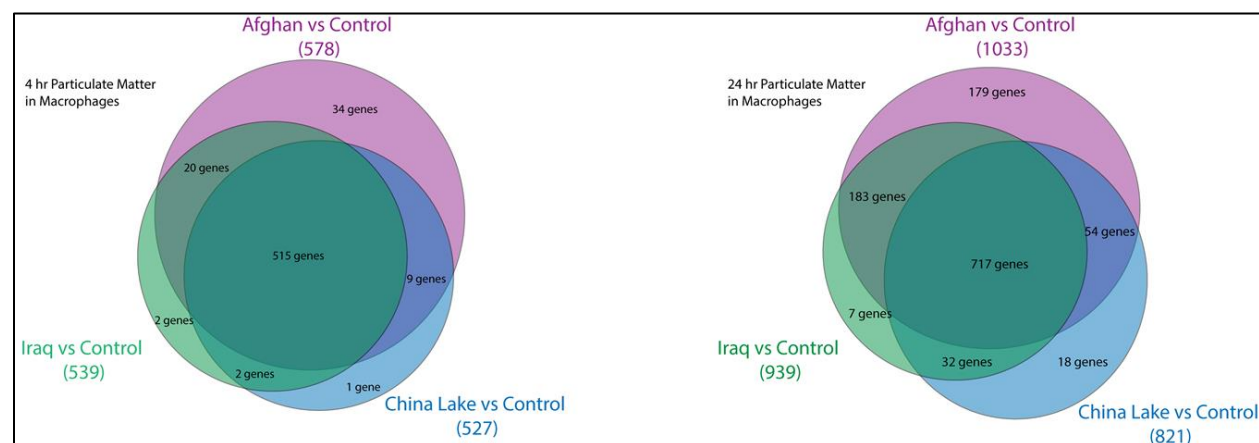


Figure 15. Analysis of differential gene expression in human primary alveolar macrophages exposed to PM (10 $\mu\text{g}/\text{cm}^2$) from Iraq, Afghanistan and California *in vitro*.

As illustrated in **Fig. 15**, exposure of human alveolar macrophages to PM from Iraq, Afghanistan, and China Lake induced differential expression of hundreds of genes at 4 and 24 hr compared to control. Of these genes, 515 were in common at 4 hr and 717 at 24 hr indicating common responses the PM independent of the geographic source of PM. However, at 24 there were 179 unique genes expressed in response to PM from Afghanistan and 9 unique genes

expressed in response to PM from Iraq. We are currently refining this analysis and will focus on both the common and unique gene expression profiles.

Pathway analysis revealed the macrophages responded to PM exposure by expression of a variety of acute inflammatory pathways including rheumatoid arthritis pathway (this is a pathway enriched in inflammatory signaling through IL-1 β and TNF α), TNFR1 and IL-6 signaling, acute phase response signaling (**Fig. 16**).



Figure 16. Pathway analysis of RNAseq using Ingenuity Pathway Analysis tool of differential gene expression in human primary alveolar macrophages exposed to PM (10 $\mu\text{g}/\text{cm}^2$) from Afghanistan *in vitro*.

Using more detailed pathway analysis, we observed a strong signal in the NF κ B pathway that is known to control signaling pathways involved on acute inflammatory responses and is a major target of glucocorticoids. This has important implications for potential therapeutic interventions with inhaled glucocorticoids that are currently first line therapy for asthma. We are currently collating this information and will decide which of these pathways we will investigate in more detail using qPCR of selected genes and western blot analysis to validate these pathways. We will plan to submit a manuscript on these observations this next year.

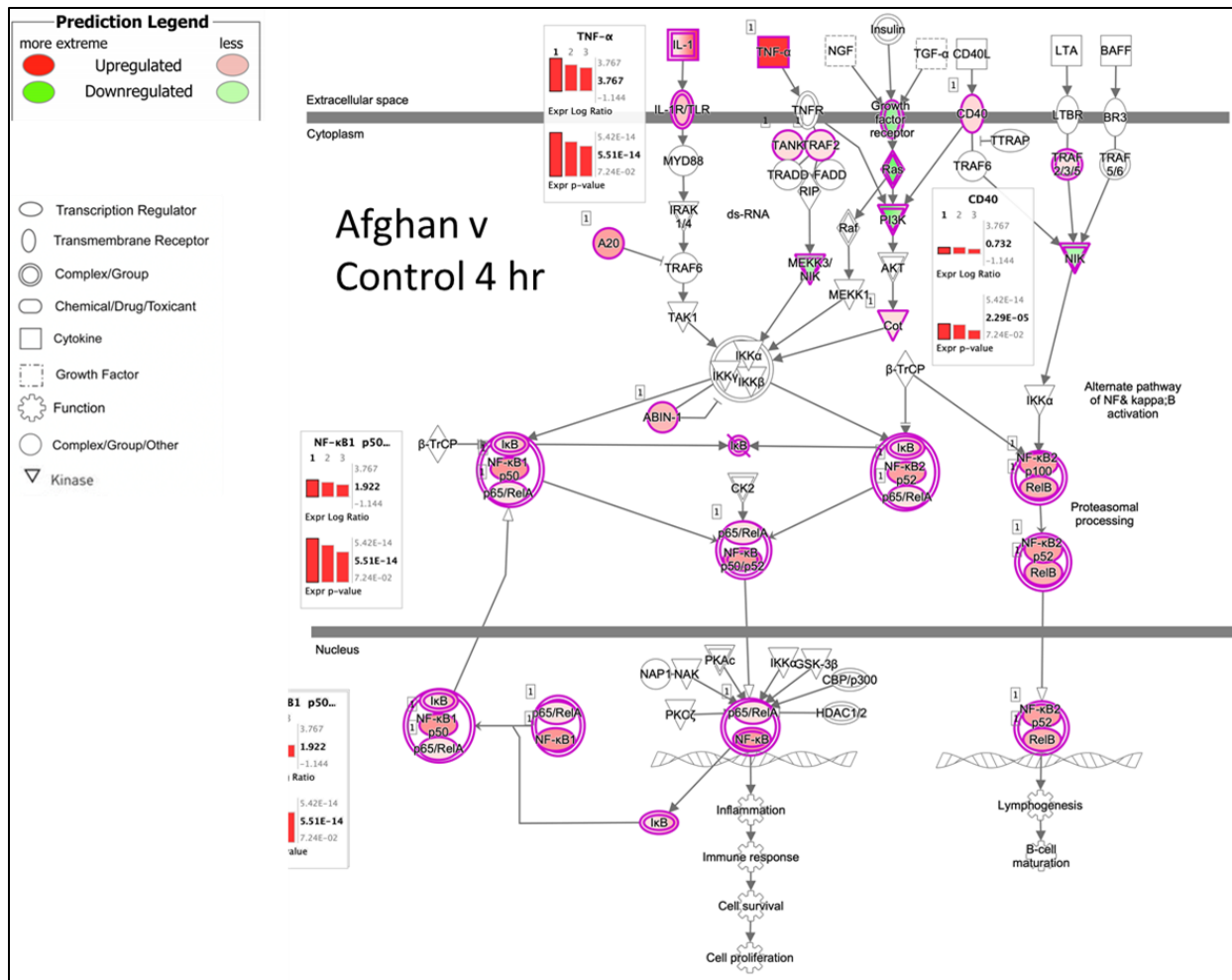


Figure 17. Pathway analysis of RNAseq using Ingenuity Pathway Analysis tool of differential gene expression in human primary alveolar macrophages exposed to PM (10 $\mu\text{g}/\text{cm}^2$) from Afghanistan *in vitro* reveals activation of the NF κ B pathway that is a key controller of inflammation.

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

We have examined the effects of combined exposure on lung epithelial cells to particulate matter and an oxidant stimulus (bleomycin) as described above. We will undertake the studies on the effects of the small molecule WNT inhibitors once we have completed additional studies characterizing the cytotoxicity of PM $\pm$ second stimulus including influenza and physical injury (blast).

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

In the second year, we completed analysis of the initial set of experiments using aerosol exposure to PM from Afghanistan (5 mg/m^3 18 hours per day for 20 days over four weeks) at the NAMRU-Dayton facility. A subset of mice was also exposed to aerosolized silica as a positive control (approved under a different ACURO protocol). Mice were then shipped to National Jewish Health in Denver for analysis. As is illustrated in **(Figure 18)** below, exposure of mice to aerosolized PM from Afghanistan resulted in an acute inflammatory response characterized by increased macrophages (but not neutrophils or lymphocytes) in bronchoalveolar lavage fluid. There was no significant increase in lung collagen content in the mice exposed to aerosolized PM from either California or Afghanistan **(Figure 19)**. In contrast, mice exposed to aerosolized silica had a small but measurable increase in lung collagen. We interpret this to mean that in order to induce pulmonary fibrosis in mice, the amount of PM delivered to the lung must exceed the ability of the lung to clear the PM. High doses of PM over a short period of time delivered directly to the lung induce fibrosis whereas lower concentrations of PM given over a more prolonged period of time by aerosolization do not induce fibrosis when measured at 28 days. Accordingly, we have modified our exposure protocol to increase the concentration of particulate matter to 75 mg/m^3 delivered over 12-14 days and then allow 60 to 90 days for the pulmonary fibrogenic response to develop more fully. We will then measure lung collagen content and histology at the 60 and 90-day time points. These experiments are currently underway.

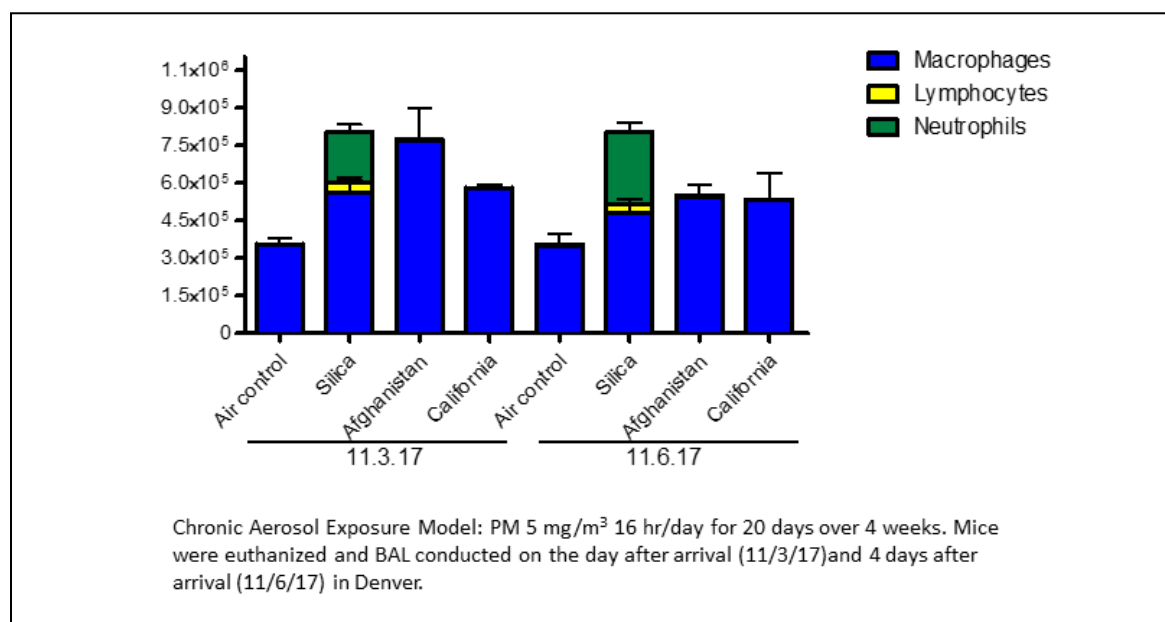


Figure 18. Chronic Aerosol Exposure to PM from Afghanistan, California, and purified silica (5 mg/m^3 for 16 hr/day for 20 days over 4 weeks at the NAMRU Dayton facility. Mice were euthanized and BAL conducted on the day after arrival at NJH (11/3/17) and 4 days after arrival (11/6/17) in Denver.

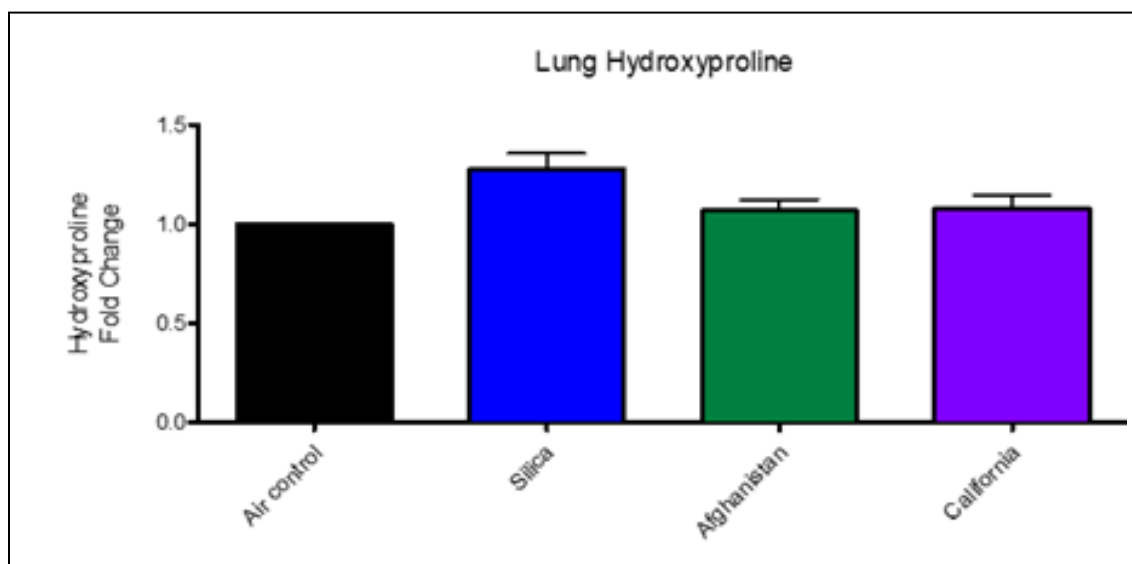


Figure 19. Chronic Aerosol Exposure to PM from Afghanistan, California (China Lake), and purified silica (5 mg/m³ for 16 hr/day for 20 days over 4 weeks at the NAMRU Dayton facility. Mice were euthanized on the day after arrival at NJH and 4 days after arrival in Denver. We observed only small increase in lung collagen content in response to treatment with silica but not with PM from Afghanistan.

Simulated Blast Lung Injury using a Shock Tube

We have set up the blast simulator ('shock tube') in our vivarium (**Fig. 20**) and have calibrated the blast simulator and tested the magnitude and reproducibility of the resulting shock waves (**Fig. 21**). This has been done in collaboration with the manufacturer (Steve Parks from ORA Industries) and monitored closely by our Safety Department who have ensured that operation of this instrument is safe for the operators. We have also fabricated an aluminum strut and nylon mesh 'hammocks' to hold the mice in place during exposure to the blast wave. We tested this configuration and confirmed that the strut and hammock do not disrupt or alter the blast wave and that the hammocks can withstand the force from the blast wave and hold the mice in place. We next tested this system by exposing anesthetized mice (xylazine, ketamine, and acepromazine) to blast overpressure injury. The initial test exposures to overpressure blasts resulted in high (40-50%) mortality in the first few hours. We therefore consulted with our veterinarians to modify our approach. They thought that this was due to cardiovascular collapse from the combination of the anesthetic and the blast wave. We modified the anesthetic combination and decreased the dose of xylazine that is a known cardiac depressant. We also administered saline subcutaneously to the mice just prior to blast exposure to help them maintain intravascular volume. Finally, we ensured that the mice were in a warm environment immediately after the blast exposure using a heat lamp. With these changes, the mortality from the blast exposure decreased to less than 5%. We also tested various positions of the animal relative to the blast wave (right side, left side, dorsal and ventral facing the blast wave) but this did not seem to make any difference to the mortality or to the extent of lung injury as assessed by lung histology and albumin and IgM levels in BAL fluid. We finalized our protocol to right lateral blast wave exposures for consistency. We also tested several types of vinyl/polypropylene membranes in the blast tube and purchased a large amount of the membranes that gave the most consistent blast wave magnitude.



Figure 20. Shock tube manufactured by ORA Industries installed in our vivarium at National Jewish Health. The driving pressure to initiate rupture of the polypropylene-reinforced vinyl membrane is generated using compressed air (Kobalt air compressor). This system is able to achieve a driving pressure of 12-16 psi to rupture the membrane and create a shock wave that approximates IED blast exposures.

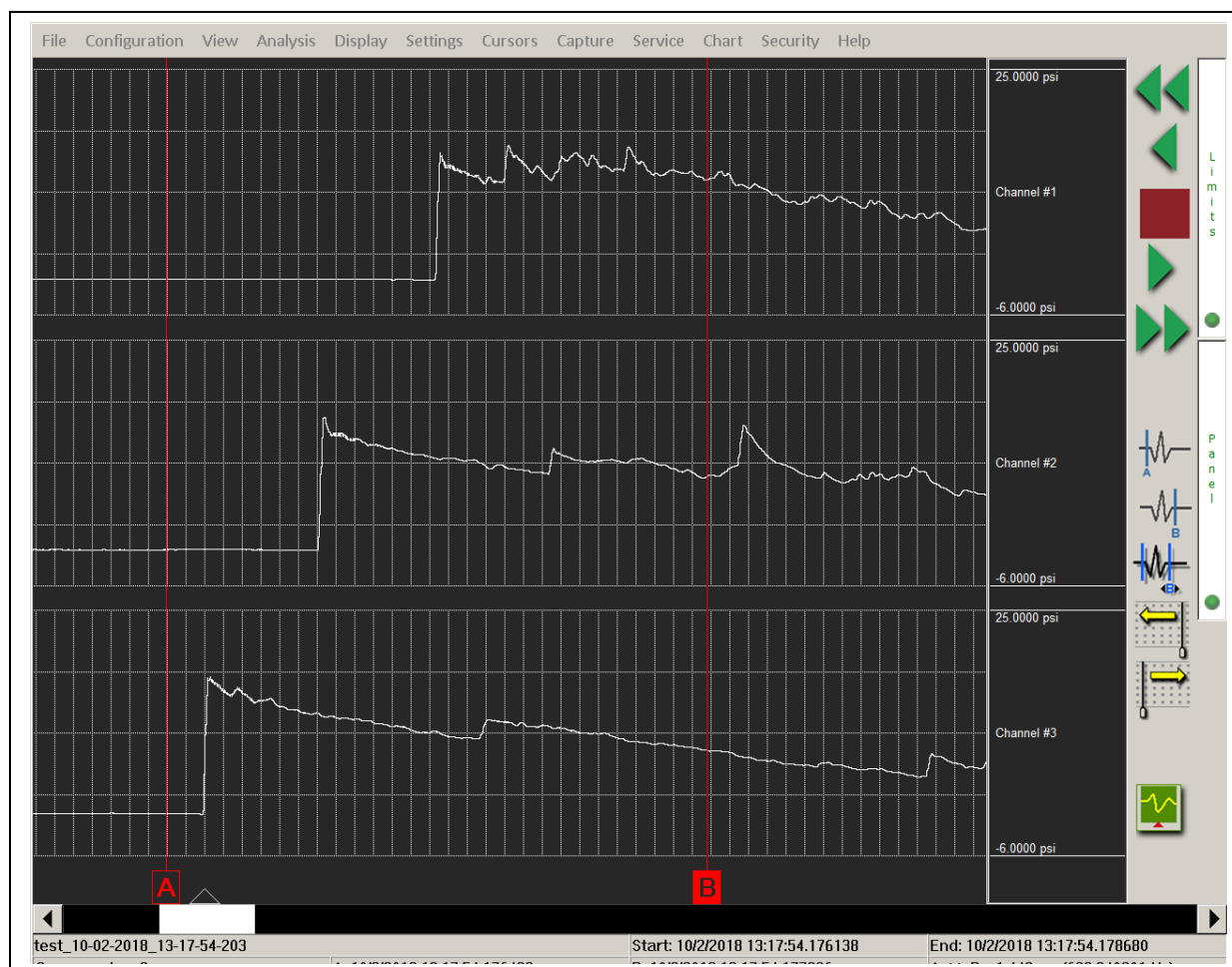


Figure 21. Blast wave profile in the shock tube manufactured by ORA Industries assessed using three pressure transducers spaced longitudinally inside the blast tube. The blast overpressure was generated using compressed air to rupture a nylon composite membrane. This achieved a maximum pressure of 12-15 psi.

Exposure of mice to blast over pressure injury resulted in hemorrhagic lung injury that was characterized by alveolar hemorrhage and inflammation that was most prominent in proximity to air/tissue interfaces such as adjacent to airways (**Fig. 22**). Bronchoalveolar lavage (BAL) analysis revealed that there was an increase in lung permeability as measured by IgM or albumin leak at 24 and 48 hr. (**Fig. 23**).

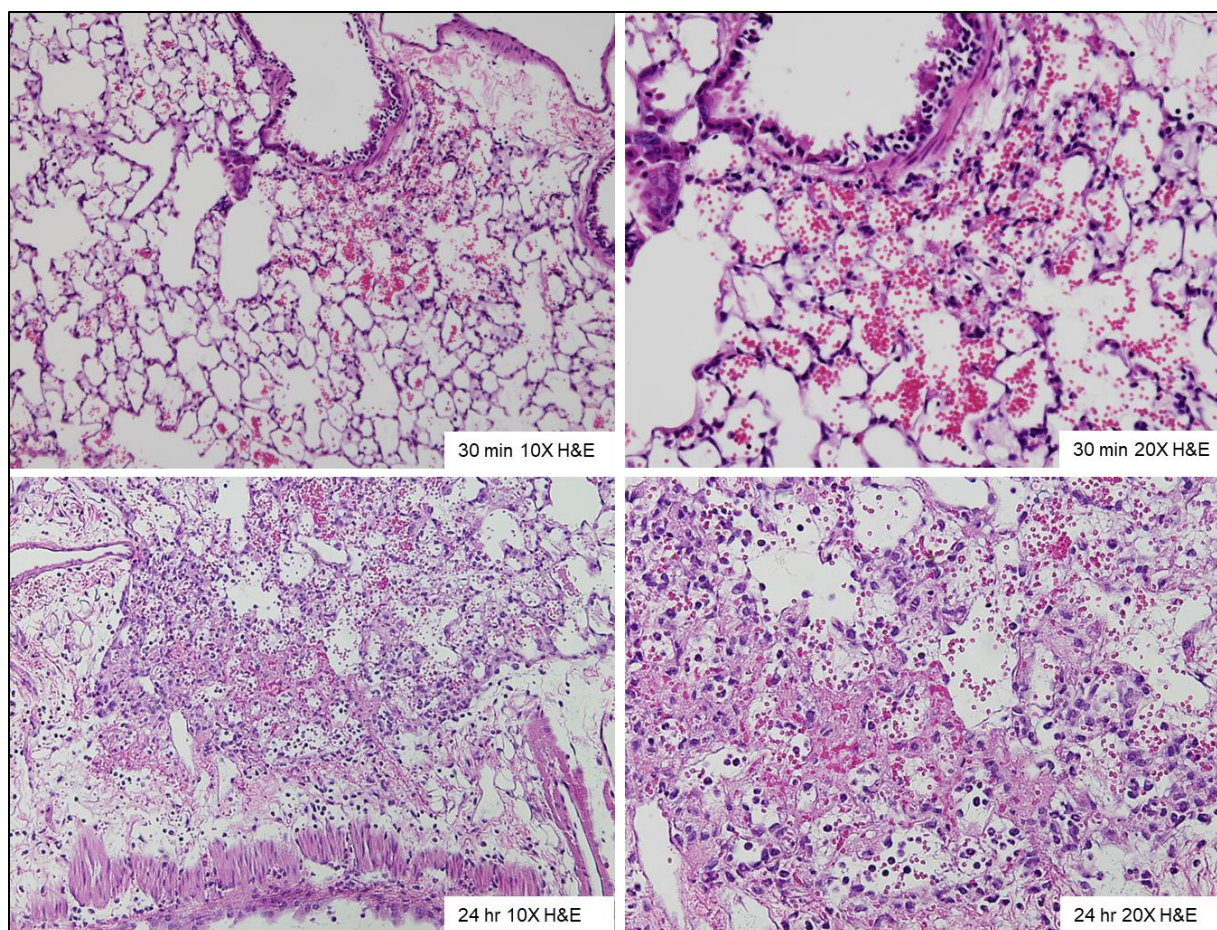


Figure 22. Overpressure blast lung injury after exposure of mice to the blast wave generated by the Shock tube described above. Note evidence of alveolar hemorrhage and inflammation in proximity to air/tissue interfaces such as adjacent to airways.

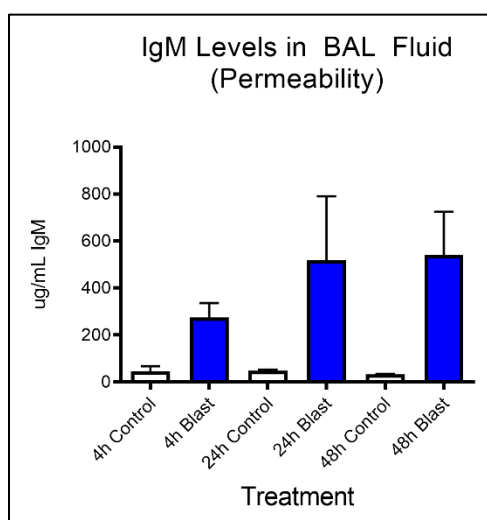


Figure 23. Lung Permeability measured by IgM in BAL fluid after blast overpressure lung injury.

We next developed a model of acute lung injury using H1N1 influenza infection. In CD57Bl/6 mice, influenza infection resulted in systemic illness manifest by weight loss accompanied by acute lung injury with increased epithelial permeability (**Fig. 24**) and histological evidence of lung inflammation and injury (**Fig. 25**). In this model, increased lung permeability (an indicator of epithelial injury) peaks between day 7 and day 10 and then begins to diminish, indicative of epithelial repair.

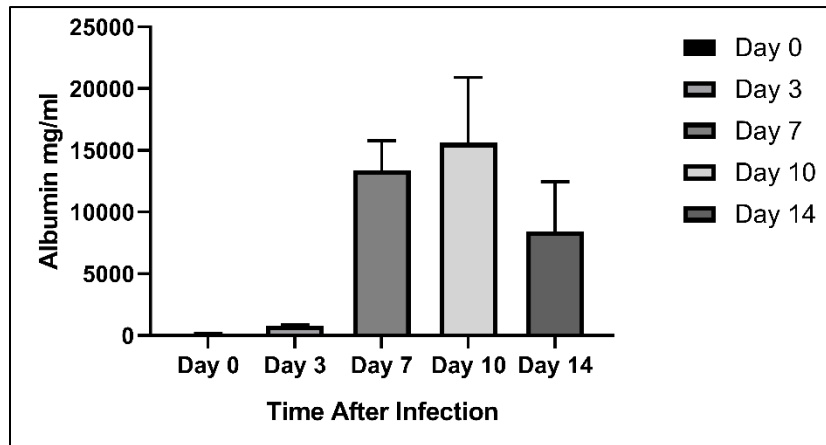


Figure 24. Increase in lung permeability after H1N1 influenza infection as measured by increased albumin levels in BAL fluid peaking between days 7 and 10.

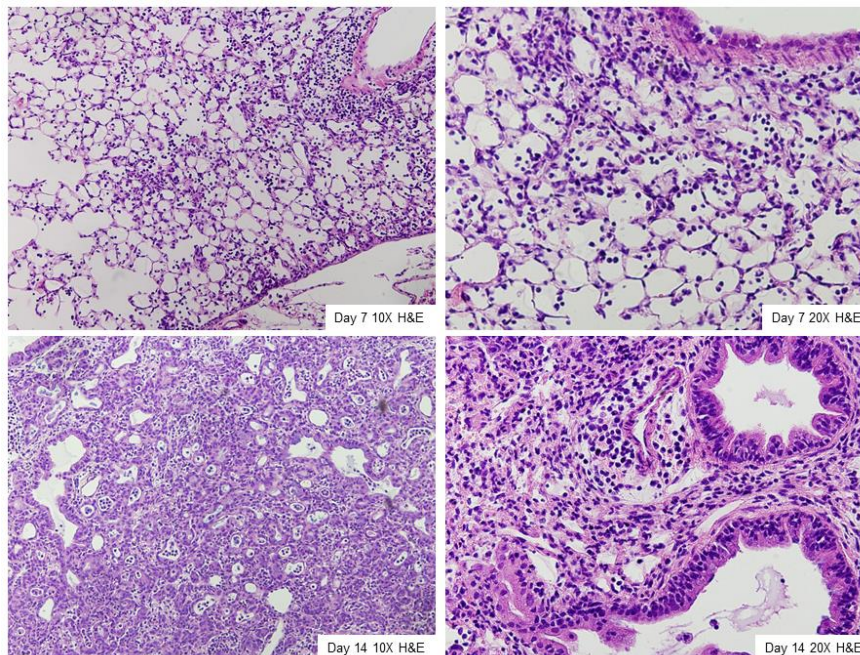


Figure 25. H&E stained histological sections of mouse lung 7 and 14 days after infection with H1N1 influenza. Note evidence of inflammation and epithelial injury at day 7 and evidence of inflammation and epithelial regeneration at day 14.

Difficulties encountered

When given by direct intratracheal installation, the PM from Iraq and Afghanistan induced a robust pulmonary inflammatory response followed by lung fibrosis detectable between 28 and 35 days. By contrast, PM given by aerosolization induced an inflammatory response but did not induce significant fibrosis. This may be 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 have modified our protocol to increase the concentration PM to 75 mg/m³ given over 12 days and then wait 60 to 90 days to analyze lung fibrosis. This is 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 are currently underway and are being done in collaboration with Drs. Wong and Mummy at NAMRU-Dayton.

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 3 we developed a combined exposure model by combining exposure to PM and overpressure blast injury. Mice were first exposed to PM (oropharyngeal instillation) from Afghanistan 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 **Figs. 26-31**, 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 permeability (albumin leak), endothelial injury (serum angiopoietin), and epithelial injury (BAL RAGE). Importantly, all of these parameters were enhanced by subsequent blast overpressure lung injury at both the 24 or 48 hr time points.

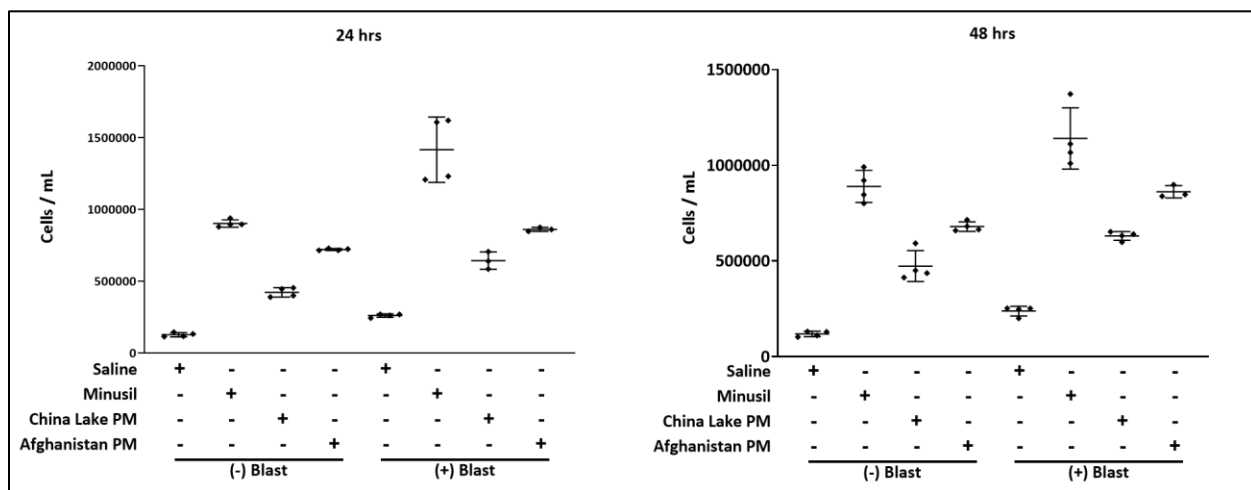


Figure 26. Combined exposure to PM from Afghanistan or China Lake or to purified silica (Minusil) followed by overpressure blast lung injury results in enhanced inflammation as compared to PM instillation alone or to blast injury alone.

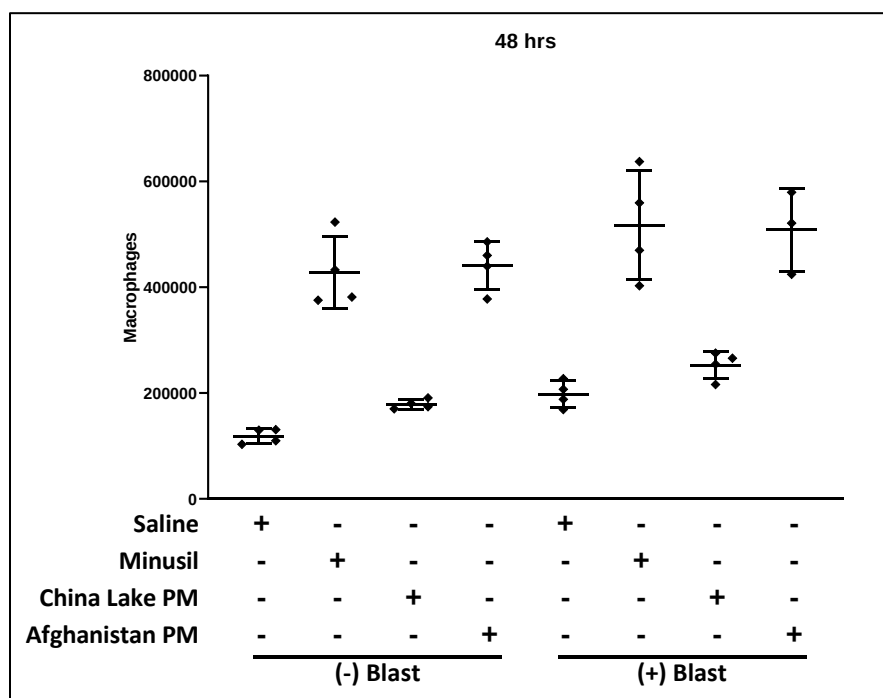


Figure 27. Combined exposure to PM from Afghanistan or China Lake, or to purified silica (Minusil) followed by overpressure blast lung injury results in increased numbers of macrophages in BAL fluid as compared to PM instillation alone or to blast injury alone.

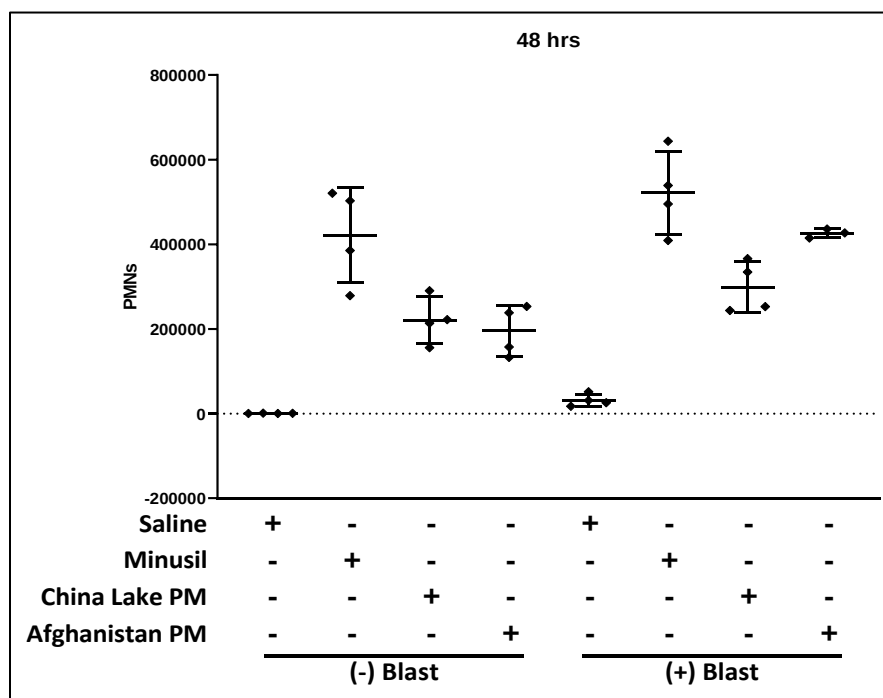


Figure 28. Combined exposure to PM from Afghanistan or China Lake, or to purified silica (Minusil) followed by overpressure blast lung injury results in increased numbers of neutrophils (PMNs) in BAL fluid as compared to PM instillation alone or to blast injury alone.

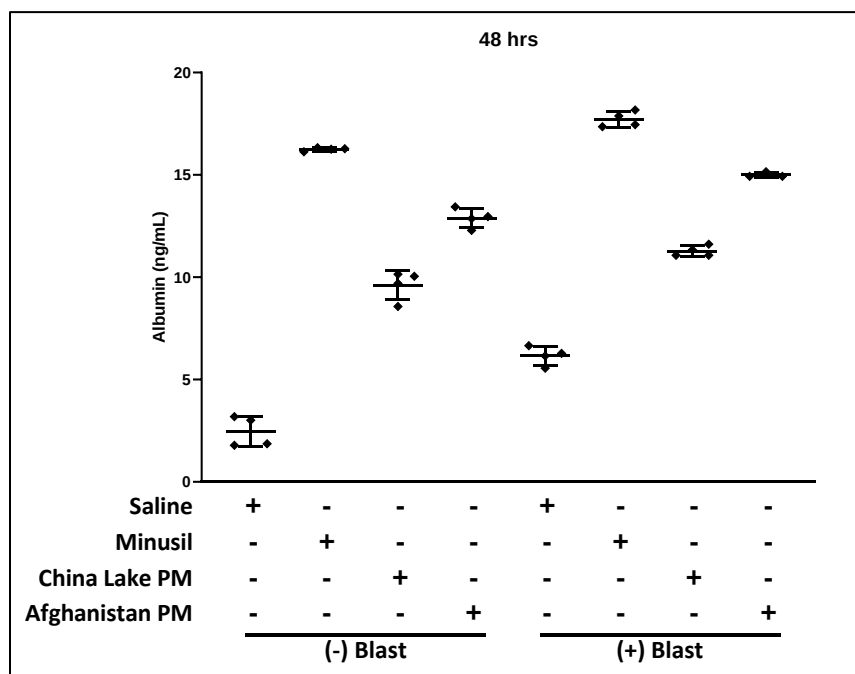


Figure 29. Combined exposure to PM from Afghanistan or China Lake, or to purified silica (Minusil) followed by overpressure blast lung injury results in increased lung permeability as measured by albumin leak into BAL fluid as compared to PM instillation alone or to blast injury alone.

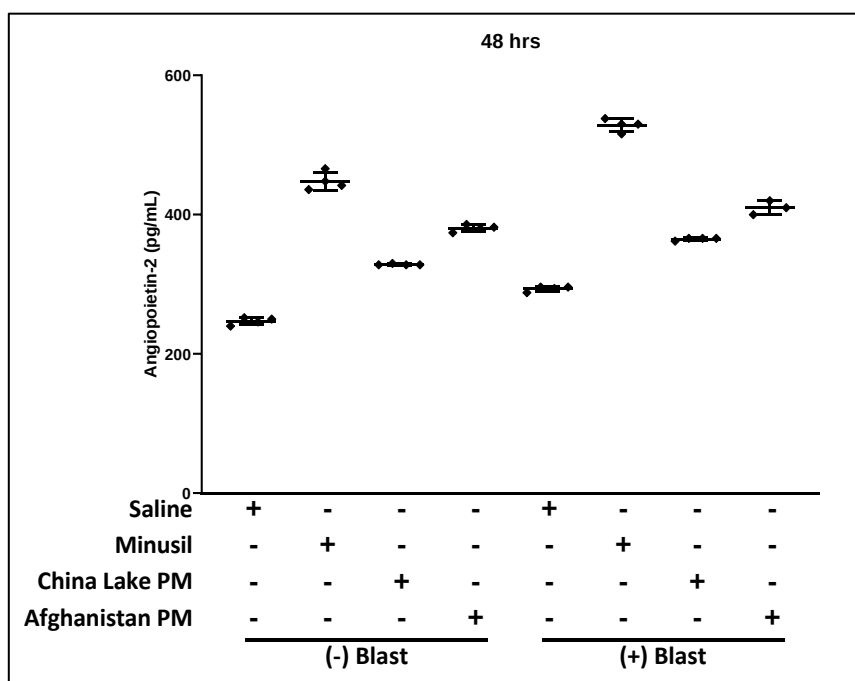


Figure 30. Combined exposure to PM from Afghanistan or China Lake, or to purified silica (Minusil) followed by overpressure blast lung injury results in endothelial injury as measured by serum angiopoietin-2 levels as compared to PM instillation alone or to blast injury alone.

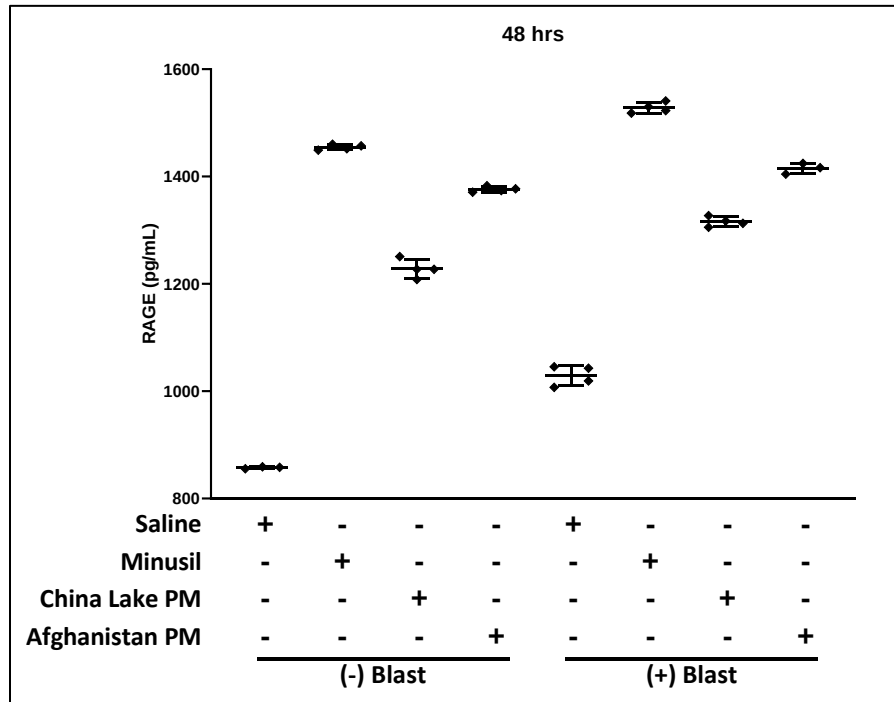


Figure 31. Combined exposure to PM from Afghanistan or China Lake or to purified silica (Minusil) followed by overpressure blast lung injury results in lung epithelial injury as measured by BAL RAGE levels as compared to PM instillation alone or to blast injury alone.

Difficulties encountered

We have not encountered any significant problems with the combined exposures to PM and overpressure blast injury.

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 to collaborate effectively with Drs. Geoff Plumlee, Heather Lowers, Bill Benzel, Kate Campbell-Hay, and David Roth from the US Geological Survey and have progressed in our characterization of the chemical composition and morphology of the particulate matter from Southwest Asia (Afghanistan and Iraq) and California (China Lake), as well as the purified silica (NanoAmor and Minusil). X-ray diffraction was used to identify minerals. Laser diffraction and scanning electron microscopy were used to characterize size and morphology of particles. Inductively coupled plasma optical emission spectroscopy (ICP-OES) was used to characterize the chemical composition. These studies are summarized in **Figures 32 - 37** and in **Tables 1** and **2** below.

Characterization of Aerosolized Dust for Use in Deployment Related Lung Injury Studies

Materials

Three dust samples of aerosolized material either collected from metal pans in exposure chambers or from the high volume aerosol filters were provided to the U.S. Geological Survey

for chemical and morphological characterization. The three original starting materials were top soil from China Lake, California and Bagram Air Force Base, Afghanistan, and SiO₂ from Nanostructured & Amorphous Materials, Inc. (NanoAmor: NA) (Stock # 4856WG) and from US Silica (Minusil).

Bulk Chemistry

A split, 0.25g, of each dust sample provided was submitted for 60 elements inductively coupled plasma optical emission spectroscopy mass spectroscopy (ICP-OES-MS). The samples were fused at 750°C with sodium peroxide. The resultant fused disc is dissolved in dilute nitric acid and the resulting solution analyzed by ICP-OES-MS.

The elements Al, Ca, Fe, K, Mg, P, S, Si, and Ti are given as elemental weight percent (%) whereas the remaining elements are given in parts per million (ppm). Sodium is not analyzed due to fusion with sodium peroxide. Average crustal abundances of the elements are shown as a line in **Fig. 32** for comparison. The X-ray diffraction analysis is summarized in **Table 1**. The elemental abundances are summarized in **Table 2**. S, Ag, Be, Ge, In, Se, Te, are not plotted because below detection for all or most samples.

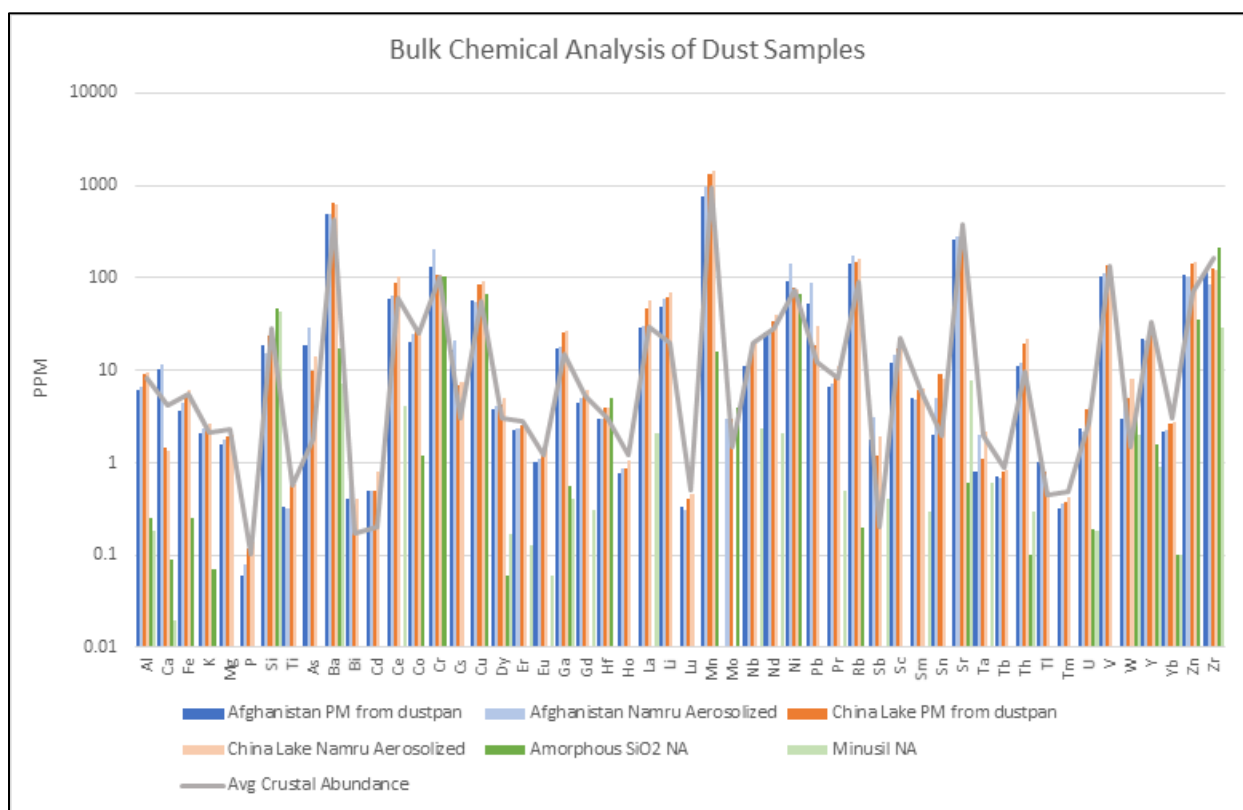


Figure 32. Bulk chemistry of aerosolized particulate matter from Afghanistan, China Lake California, and purified silica (SiO₂) from NanoAmor and Minusil as determined by ICP-OES. The gray line represents the average crustal abundance of the elements (Taylor 1964).

Table 1. X-ray diffraction results. Major indicates greater than 15 wt.%, minor between 5 to 15 wt.%, and trace less than 5 wt.%. Additional phases observed during SEM-EDS analysis are also included.

Mineral Content	Amphibole	Calcite	Chlorite	Mica (including illite)	Plagioclase	Quartz	Notes
NanoAmor (SiO ₂)						Major	Amorphous or nanocrystalline. Trace amounts of iron oxide, titanium dioxide, aluminum oxide, Al-Zr-Si phase, and a Ca-P phase observed with SEM-EDS
Minusil (SiO ₂)						Major	Crystalline silica. Sample appears pure with trace amounts of Ca, Na, and K salts.
China Lake Dust from pan	Minor		Minor	Major	Major	Major	The mica appears to be biotite. Plagioclase and albite both present. Trace amounts of TiO ₂ , monazite, REE epidote, and iron oxide were observed with SEM-EDS
China Lake NAMRU	Minor		Minor	Major	Major	Major	Trace amounts of TiO ₂ , iron oxide and ilmneite observed with SEM-EDS. The mica appears to be biotite. Albite and plagioclase are both present.
Afghanistan Dust from pan	Trace	Major	Minor	Major	Minor	Major	Trace amounts of TiO ₂ , zircon, and clays observed with SEM-EDS.
Afghanistan NAMRU	Trace	Major	Minor	Major	Minor	Major	Trace amounts of FeOx, TiO ₂ , and apatite observed with SEM-EDS.
Bagram AFB Topsoil	Minor	Minor	Major	Major	Major	Major	XRD also suggests trace amounts of talc and kaolinite.

Table 2. The elements Al, Ca, Fe, K, Mg, P, S, Si, and Ti are given as elemental weight percent (%) whereas the remaining elements are given in parts per million (ppm). Sodium is not analyzed due to fusion with sodium peroxide.

Sample	Afghanistan Dust from pan	Afghanistan NAMRU	China Lake Dust from pan	China Lake NAMRU	NanoAmor	Minusil	Avg Crustal Abundance
Al	6.23	6.77	9.12	9.42	0.25	0.18	8.23
Ca	10.4	11.7	1.48	1.33	0.09	0.02	4.15
Fe	3.72	4.42	5.55	6.05	0.25	0.01	5.63
K	2.08	2.31	2.6	2.7	0.07	<0.01	2.09
Mg	1.6	1.8	1.91	2.03	<0.01	<0.01	2.33
P	0.06	0.08	0.12	0.14	<0.01	<0.01	0.105
S	0.3	<0.1	<0.1	<0.1	<0.1	<0.1	0.026
Si	18.6	15.1	24.2	22.1	46.5	43.2	28.15
Ti	0.33	0.32	0.58	0.59	<0.01	<0.01	0.57
Ag	1.	<1	<1	1.	<1	<1	0.07
As	19.	29.	10.	14.	<5	<5	1.8
Ba	483.	497.	642.	622.	17.	7.2	425.
Be	<5	<5	<5	<5	<5	<5	
Bi	0.4	0.6	0.3	0.4	<0.1	<0.1	0.17
Cd	0.5	0.5	0.5	0.8	<0.2	<0.2	0.2
Ce	60.1	63.7	88.7	104.	<0.1	4.1	60.
Co	20.	24.8	27.1	29.1	1.2	<0.5	25.
Cr	132.	206.	108.	110.	105.	<10	100.
Cs	16.9	21.2	6.8	7.6	<0.1	<0.1	3.
Cu	57.	55.	86.	93.	66.	<5	55.
Dy	3.86	4.14	4.29	5.09	0.06	0.17	3.
Er	2.24	2.38	2.59	2.6	<0.05	0.13	2.8
Eu	1.	1.12	1.21	1.4	<0.05	0.06	1.2
Ga	17.5	18.1	25.9	26.6	0.55	0.41	15.
Gd	4.52	4.94	5.35	6.2	<0.05	0.31	5.4
Ge	1.	1.	2.	2.	<1	<1	1.5
Hf	3.	3.	4.	4.	5.	<1	3.
Ho	0.76	0.88	0.88	1.04	<0.05	<0.05	1.2
In	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	
La	28.5	30.5	47.5	56.7	<0.1	2.1	30.
Li	49.	59.	62.	70.	<10	<10	20.
Lu	0.34	0.31	0.41	0.46	<0.05	<0.05	0.5
Mn	767.	955.	1310.	1440.	16.	<10	950.
Mo	<2	3.	3.	<2	4.	<2	1.5
Nb	11.3	12.2	17.4	20.	<0.1	2.4	20.
Nd	26.	27.5	33.9	39.5	<0.1	2.1	28.
Ni	93.	144.	80.	77.	67.	<5	75.
Pb	52.	87.	19.	30.	<5	<5	12.5
Pr	6.51	7.28	9.15	11.	<0.05	0.49	8.2
Rb	143.	176.	146.	159.	0.2	<0.2	90.
Sb	1.8	3.1	1.2	1.9	<0.1	0.4	0.2
Sc	12.	15.	17.	19.	<5	<5	22.

Se	<5	<5	<5	<5	<5	<5	
Sm	5.1	4.9	6.2	6.4	<0.1	0.3	6.
Sn	2.	5.	9.	8.	<1	<1	2.
Sr	262.	279.	211.	168.	0.6	7.9	375.
Ta	0.8	2.	1.1	2.2	<0.5	0.6	2.
Tb	0.72	0.68	0.8	0.84	<0.05	<0.05	0.9
Te	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	
Th	11.	12.1	19.2	21.7	0.1	0.3	9.6
Tl	1.	0.9	0.8	0.5	<0.5	<0.5	0.45
Tm	0.32	0.36	0.38	0.43	<0.05	<0.05	0.48
U	2.39	2.17	3.74	3.99	0.19	0.18	2.7
V	103.	111.	135.	131.	<5	<5	135.
W	3.	5.	5.	8.	3.	2.	1.5
Y	21.9	21.	25.6	26.3	1.6	0.9	33.
Yb	2.2	2.3	2.7	2.8	0.1	0.1	3.
Zn	109.	106.	143.	147.	35.	<5	70.
Zr	118.	86.3	128.	122.	213.	29.2	165.

The SiO₂ material displays a broadened peak over the 20 – 27 degrees 2 θ range typical of amorphous silica. The limited data given by Nanostructured & Amorphous Materials, Inc. indicates a quartz crystallographic structure. Trace amounts of halite, perhaps a contaminant from the collection procedure, were detected (**Figure 32**). Trace amounts of iron oxide, titanium dioxide, aluminum oxide, Al-Zr-Si phase, and a Ca-P phase were observed with scanning electron microscopy and energy dispersive spectroscopy (SEM-EDS).

The China Lake, CA sample is composed of major amounts of quartz, feldspars, and micas and minor amounts of amphibole and chlorite. Trace amounts of TiO₂, monazite, REE epidote, and iron oxide were observed with SEM-EDS.

The Afghanistan topsoil sample contains major amounts of chlorite, mica, feldspar, and quartz with minor amphibole and calcite. The aerosolized Afghanistan sample contains major mica, quartz, and calcite; minor chlorite and feldspar, and trace amphibole. The difference in abundance of the phases between the topsoil and dust sample suggests the amphibole, feldspars, and chlorite are larger than the quartz, calcite, and micas and not as readily aerosolized.

Particle Size Analysis

Approximately 0.1 gram splits of each dust sample were suspended in 15 ml of ultrapure water with 2 drops of 10% by volume of sodium hexametaphosphate solution to deflocculate the material. The suspension was sonicated for 2 minutes, allowed to sit overnight, and then sonicated for another 1 minute prior to analysis to achieve dispersion of the particles. Particle size was determined by laser diffraction on a Mastersizer 2000 coupled to Hydro 2000G (Malvern Instruments Inc., Westborough, Massachusetts). Appropriate refractive indices were selected for NIST glass sphere standards, in-house quartz QC material, and for the analyzed samples. Residuals for particle size models were all <1.

The mean particle sizes based on volume percent and number (of particles) percent is given to better describe the size distribution (**Table 3**). For instance, a couple of large particles will skew

the volume distribution toward a larger mean particle size whereas a number percent distribution better describes the fine particle sizes. **Figures 33 and 34** compare the size distributions of the three aerosolized dust samples based on volume and number percent. It appears the Bagram particle size based on volume percent is dominated by a few large particles as the number of fine particles is greater than the aerosolized sample. This requires further investigation. The number percent distribution (**Figure 33**) and the cumulative number percent (**Figure 34**) show a similar normal distribution of particle size for the China Lake and SiO₂ sample with the Bagram dust having a similar distribution but with slightly finer particle size.

Table 3. Particle size (μm) reported based on volume percent and number percent from laser diffraction particle analysis.

Approx. Particle size (μm)	SiO ₂		China Lake		Bagram Dust		Bagram Topsoil	
Percentile	Vol	Num	Vol	Num	Vol	Num	Vol	Num
10 th	2	1.4	2.8	1.2	3	1	4.5	0.65
25 th	2.8	1.7	3.5	1.6	4.5	1.3	9	0.75
50 th	3	2	5.5	2	6.8	1.9	12	0.95
75 th	5	2.8	9.5	3	13	2.8	65	1.5
90 th	8.2	3.6	16	4	20	4	140	2.3

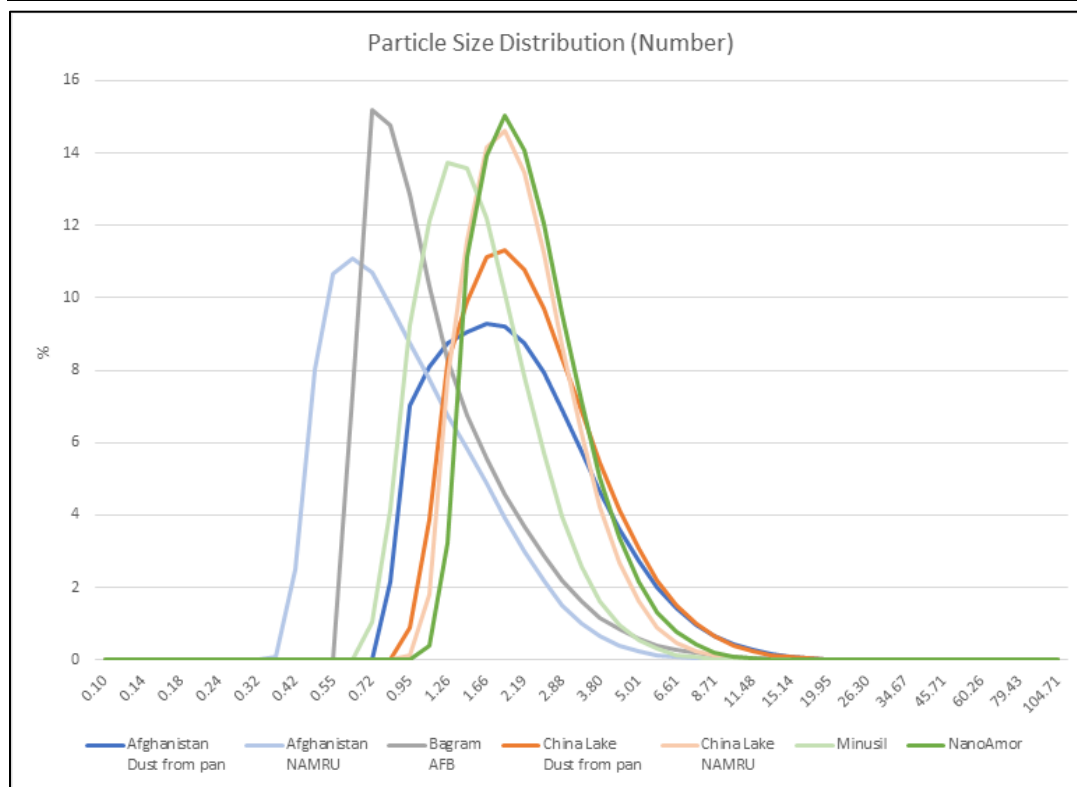


Figure 33. Laser Particle size (μm) distribution based on number of particles analyzed.

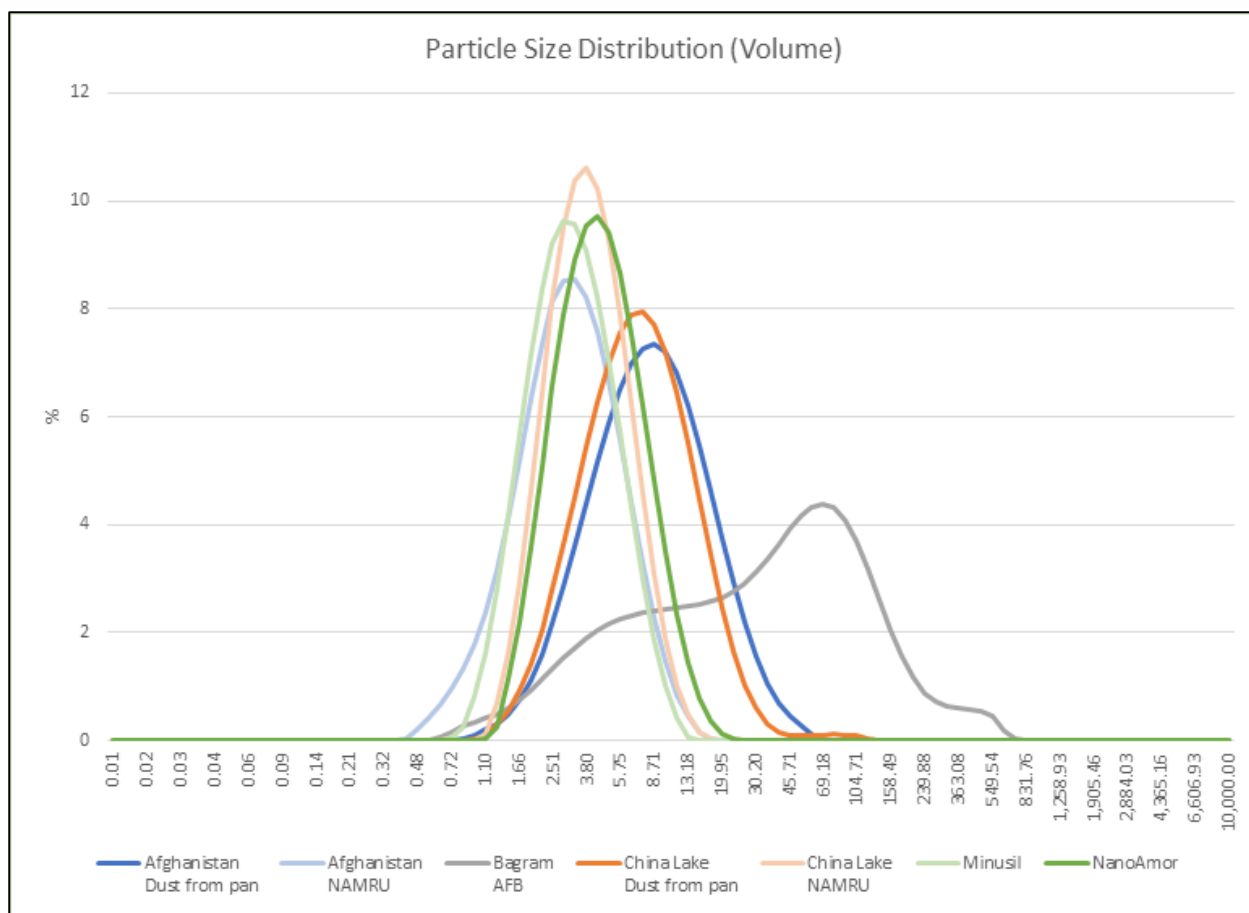


Figure 34. Laser particle size distribution (μm) based on the volume of particles analyzed.

Scanning Electron Microscopy

SiO₂: An automated particle analysis of 5000 particles with SEM-EDS identified Al-rich phases (176), iron oxide (21), calcium-phosphate phase (4), titanium dioxide phase (3), and Zr-rich phases (19) with the remainder being silica. The silica grains display conchoidal fracture as well as angular edges (**Figure 35**). The larger particles appear clean with no surface coatings. Finer particles were observed adhering to some larger particle surfaces.

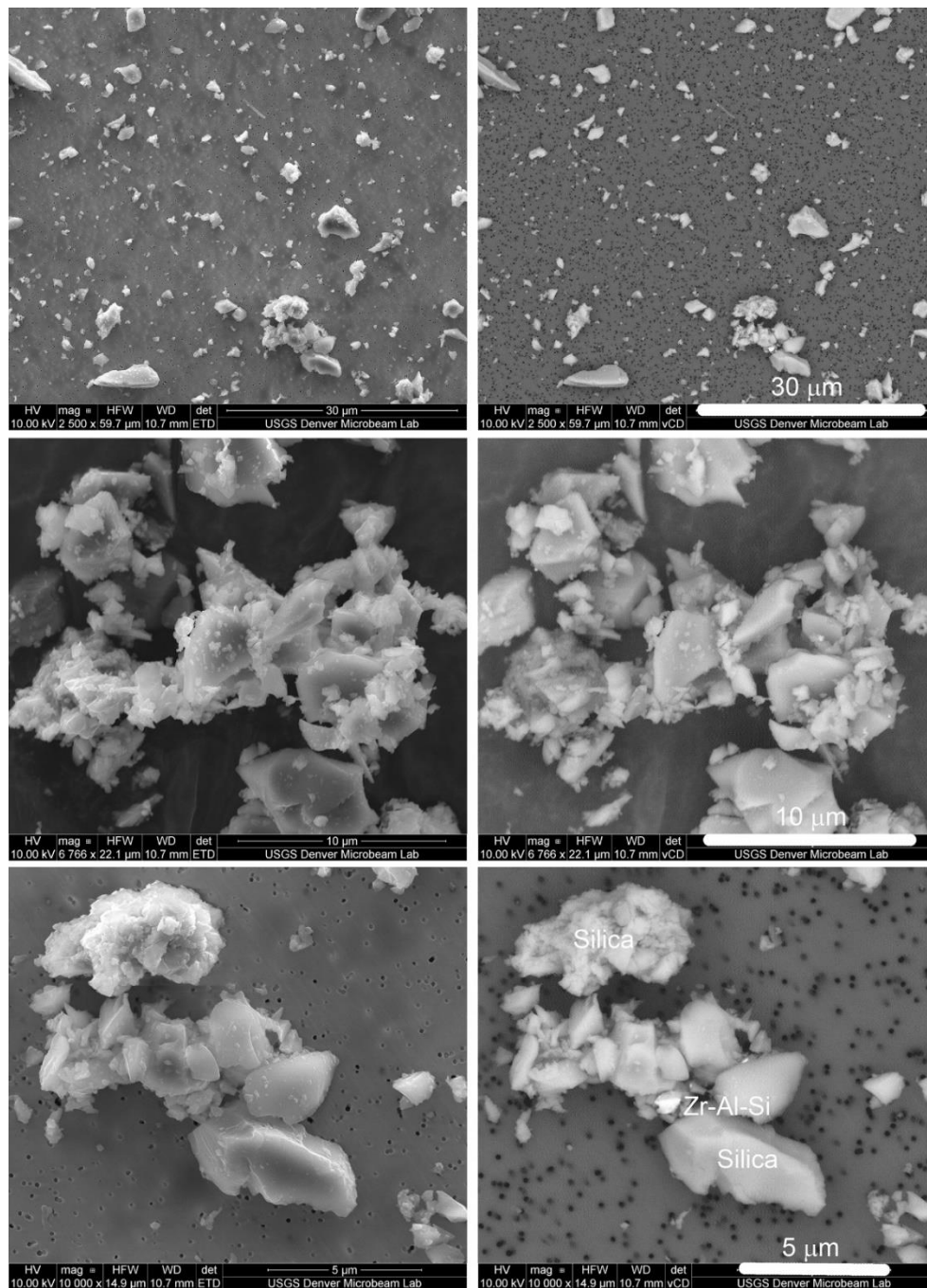


Figure 35. Secondary electron (left) and backscattered electron (right) of the same field of view.

Bagram Dust: Particles have angular edges with some such as the TiO₂ shown in **Figure 36** retaining crystal faces. Many of the particles are coated with clays.

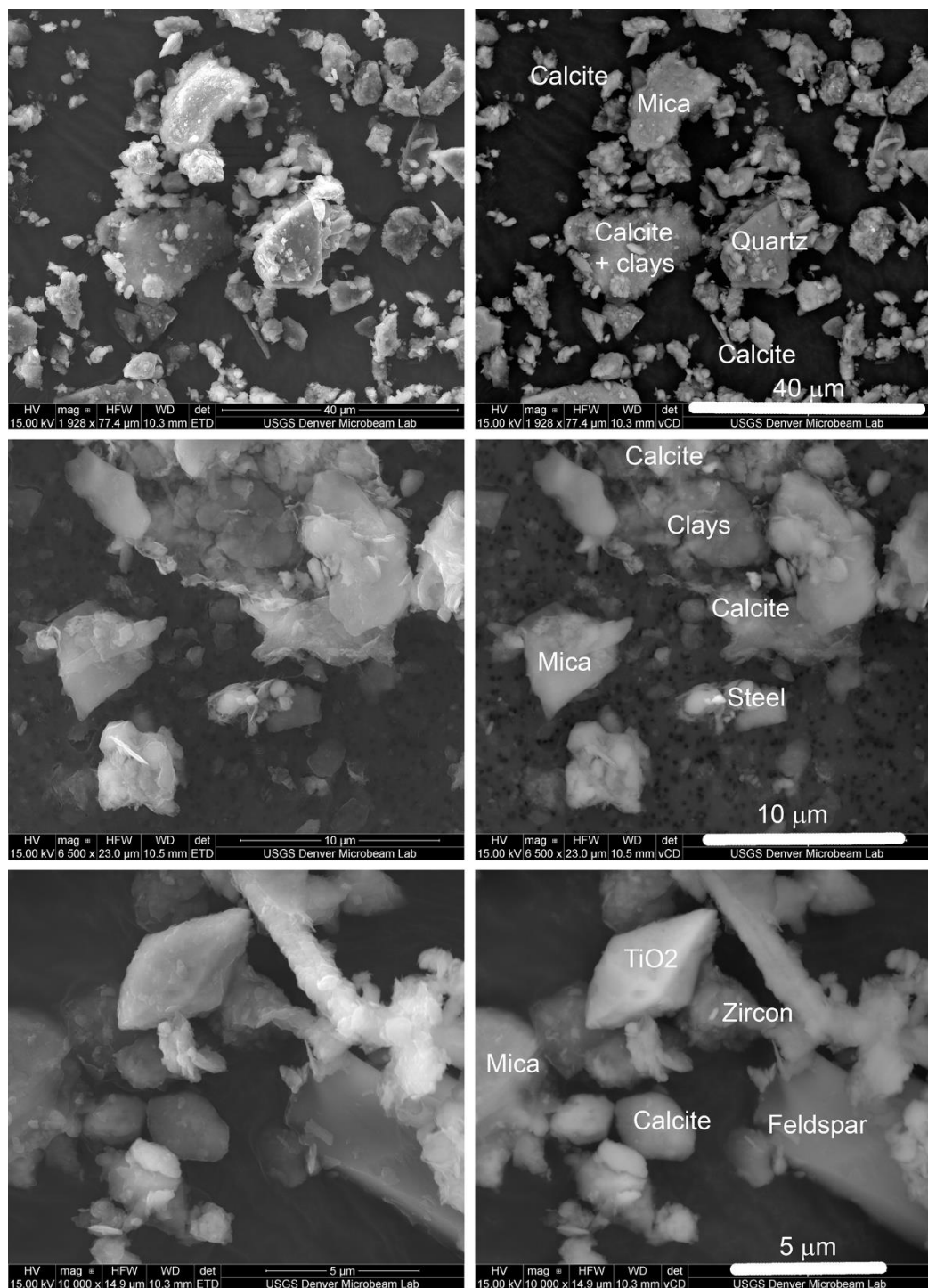


Figure 36. Secondary electron (left) and backscattered electron (right) of the same field of view of Bagram dust.

China Lake Dust: Particles angular to sub-rounded and appear to have less clay coating on larger grains than the Bagram sample (compare **Figures 36 and 37**).

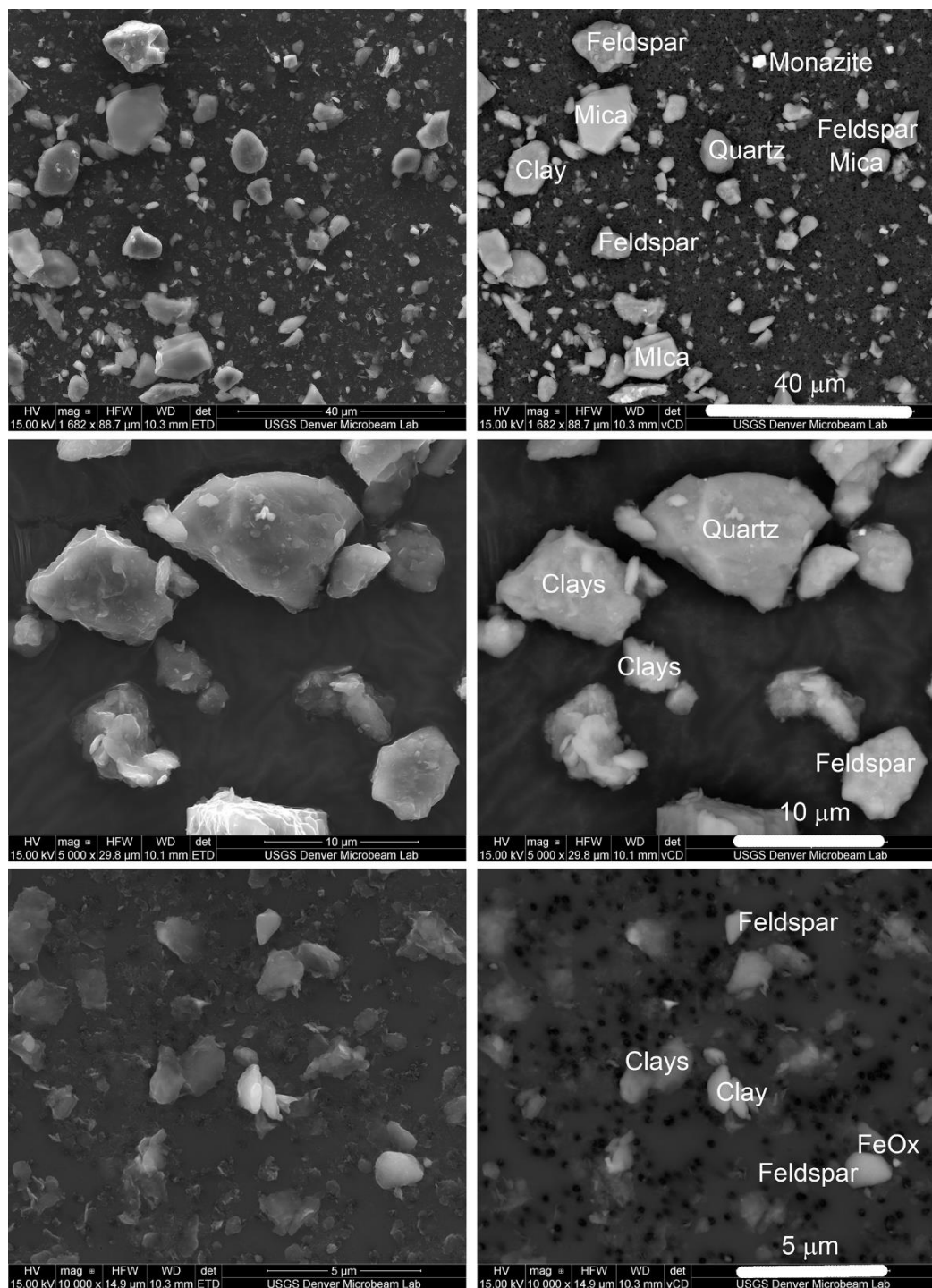


Figure 37. Secondary electron (left) and backscattered electron (right) of the same field of view of China Lake dust.

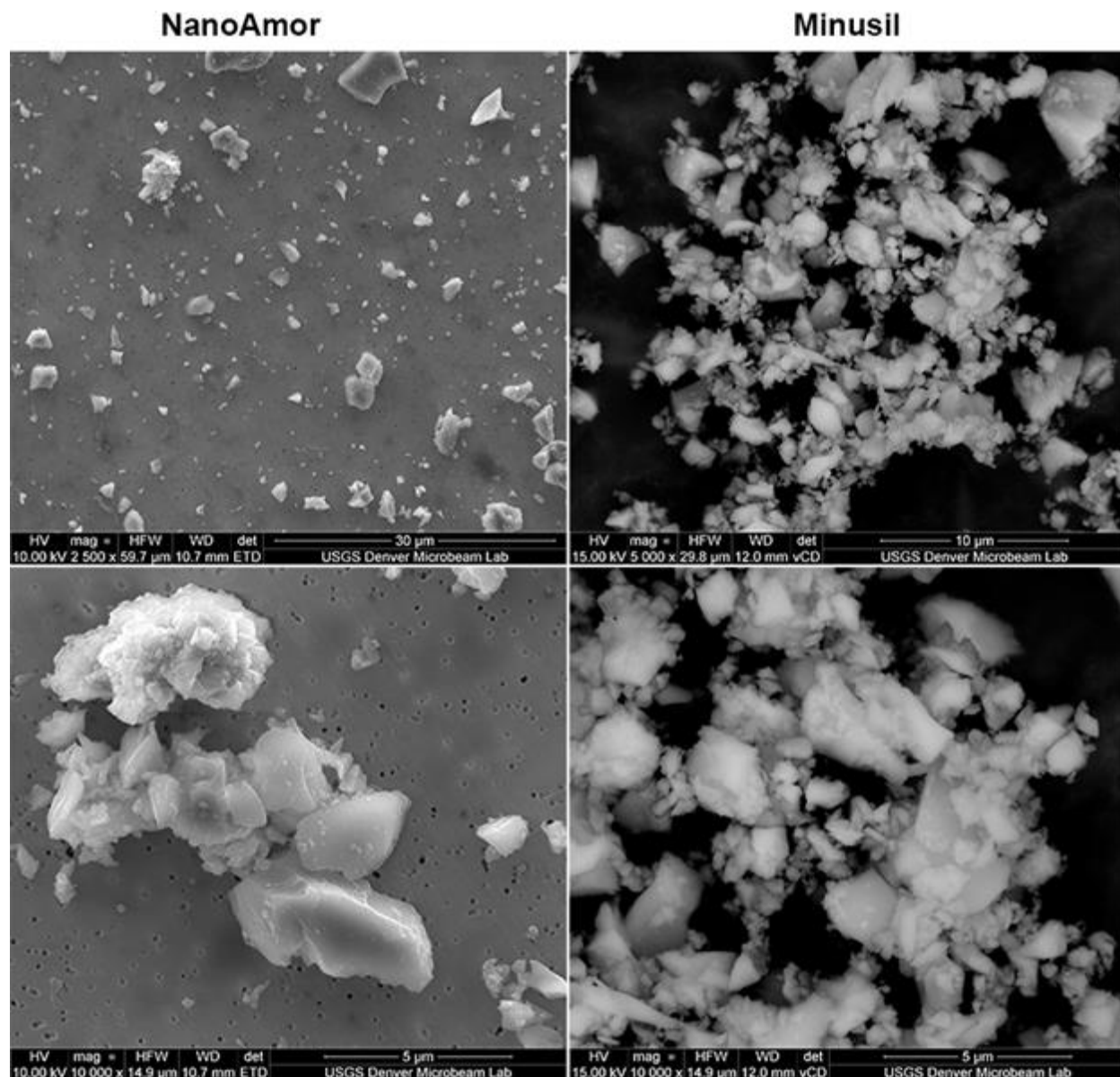


Figure 38. Scanning electron images of the NanoAmor and Minusil samples. The top left image horizontal field width (HFW) is 59.7 μm , HFW of the top right image is 29.8 μm , and the bottom set of images HFW is 14.9 μm .

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

As a proof of concept, we have begun to analyze mouse BAL fluid and lung tissue samples from mice exposed to PM from Afghanistan by oropharyngeal aspiration and these preliminary studies indicate that silicate-containing PM can be detected in BAL fluid and lung tissue. Once these techniques are developed further and are reproducible, we will 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 when we are ready.

Subtask 8. Prepare and submit manuscripts for publication.

Progress

One manuscript has been published (see project 3 – Berman R et al, *Toxicol Sci* 2018). Three abstracts were submitted and presented at the Military Health System Research Symposium in August 2019 in Orlando, FL.

The abstract from project 2 entitled: Exposure to Airborne Particulate Matter from Southwest Asia Induces Inflammatory, Proteolytic, and Keratinization Pathway Gene Expression in Primary Human Airway Epithelial Cells. Downey G et al. was selected for an oral presentation in a mini-symposium at the Military Health System Research Symposium in August.

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. Two abstracts reporting the results of the studies to date with PM exposure in mice and PM exposure to lung epithelial cells were presented as a posters and one abstract was presented as an oral presentation in a mini Symposium at the Military Health System Research Symposium Orlando, FL in August 2018.

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. This will include statistical analysis.
2. Complete mineralogic and ultrastructural analysis of a second aliquot of aerosolized PM from Afghanistan with the US Geological Survey.

3. Continue to refine techniques to analyze BAL fluid and lung tissue mineral and metal content with the US Geological Survey comparing methods of sample preparation (hydrogen peroxide, bleach) and then begin analysis of human VATS lung biopsy samples.
4. Complete the analysis of the experiment using increased concentration (75 mg/m³) of aerosolized silica at the NAMRU Dayton facility and assess whether waiting longer (72 days) after completion of inhalation is needed to develop enhanced lung injury and fibrosis in mouse models.
5. Complete experiments of blast overpressure lung injury in mice with or without prior exposure to PM.
6. Complete experiments of influenza infection induced lung injury in mice with or without prior exposure to PM.

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*.

1. Done

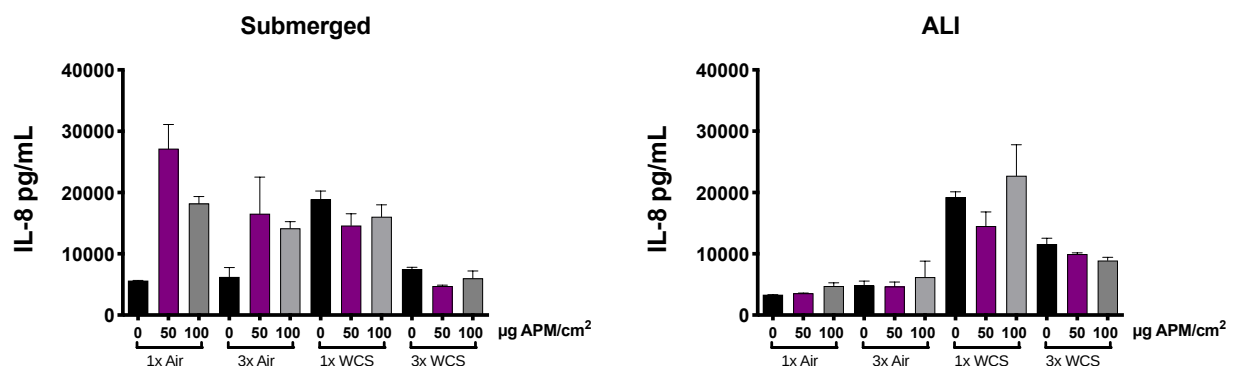
Subtask 2. Determine combined effects of PM and allergic stimuli and cigarette exposure on bronchial epithelial cells *in vitro*.

Progress

1. We are continuing to collect deployed subject bronchial bushings to see if responses to Afghan PM are similar or not with our responses from controls reported in last progress report. We continue to work on different exposure paradigms.

Single vs. Repeated Smoke Exposure

Experimental overview: Bronchial epithelial cells from a healthy subject were cultured on transwells under submerged or air-liquid interface (ALI) conditions. Cells were exposed to air or whole cigarette smoke (WCS, 1 cigarette, 10 minutes/exposure) in a single or repeated manner. APM was added immediately after the last WCS exposure.



Results:

1. No effect of APM on IL-8 production in WCS conditions, either culture system
2. ALI: No effect of APM after either exposure
3. Both: Significant cell death occurs after repeated smoking

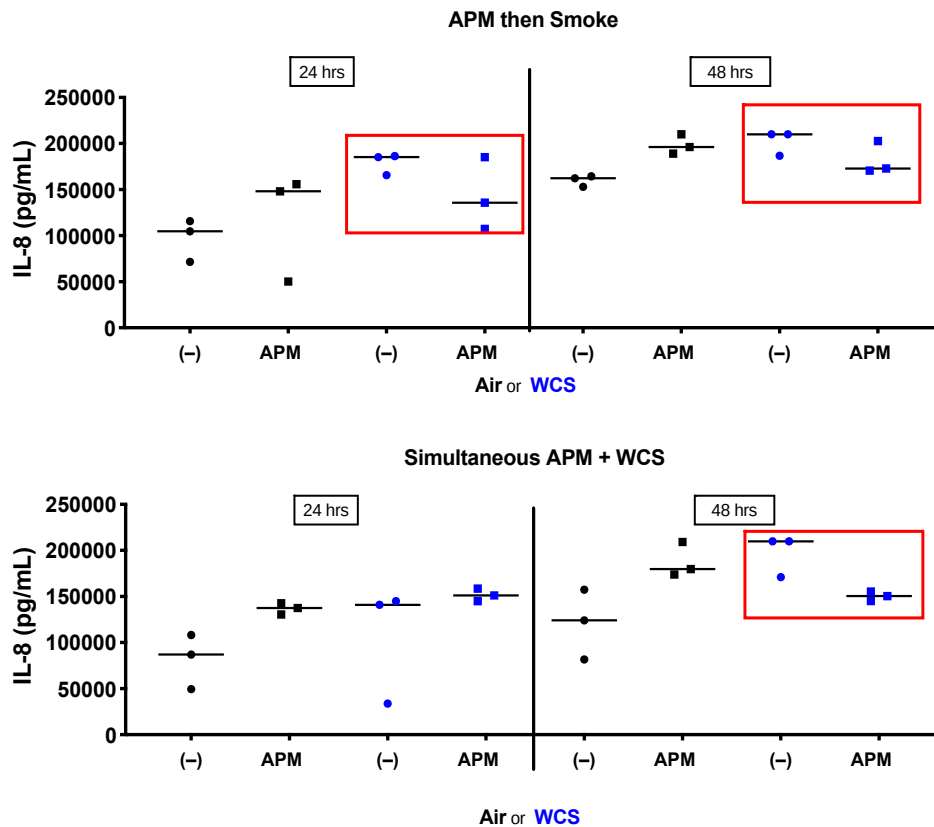
Single Smoke Exposure, Shorter Duration to Reduce Toxicity

Experimental overview: Cells from the same subject cultured on transwells in submerged conditions (no ALI) were exposed to WCS for a single 2-minute exposure. Plate #1 was treated

with APM 24 hrs prior to WCS and Plate #2 was treated with APM immediately before WCS exposure (a 'simultaneous' treatment).

Results:

1. APM induces IL-8 in air exposed cultures and reduces IL-8 after WCS
2. No noticeable cell death after 2 minutes of WCS exposure



Future Directions

1. Investigate mechanisms of APM reducing WCS induced inflammation
2. Investigate APM induced induction of negative regulators (time course)

Difficulties encountered:

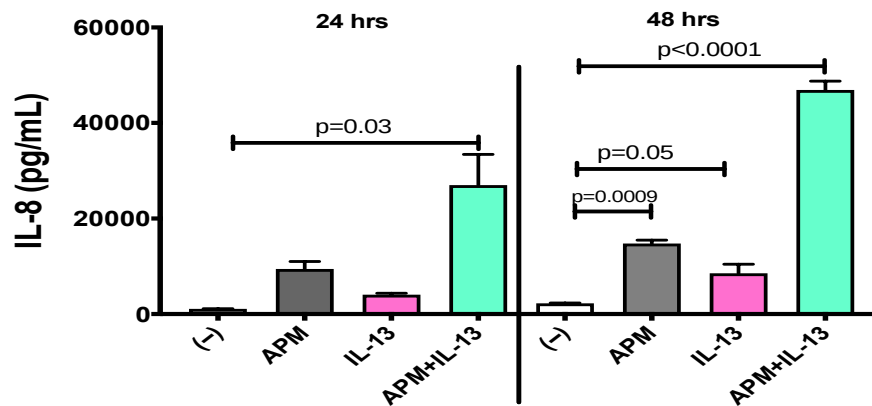
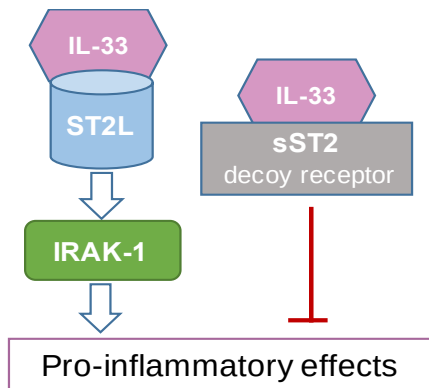
None

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).

1. We have run both a whole mouse APM exposure at NAMRU and oropharyngeal aspiration (NJH) models during this year. We are still optimizing APM dose and exposure times to optimize model.

Progress

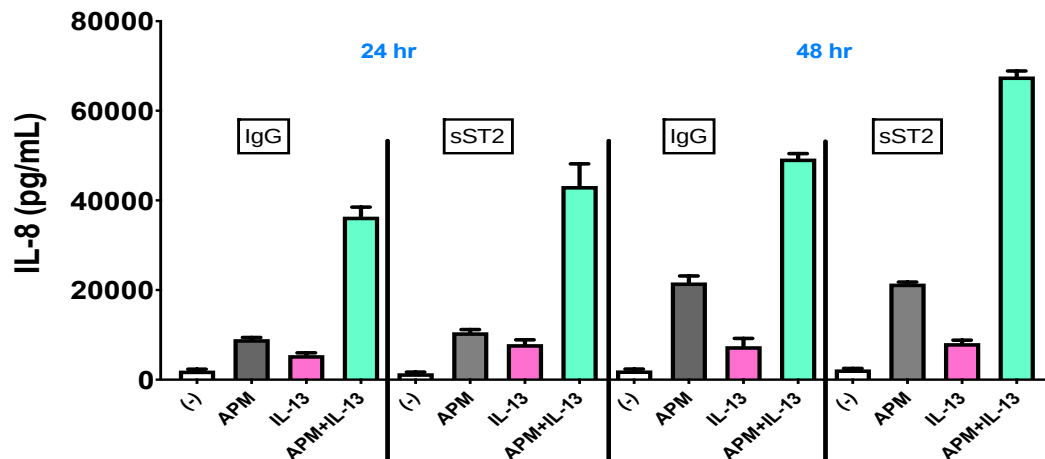
1. Investigate the IL-33/ST2 pathway in IL-13 and APM induced inflammation using an ST2 decoy receptor.



We have previously shown that APM and IL-13 stimulate the release of the chemokine IL-8 from human bronchial epithelial cells at both 24 and 48 hours.

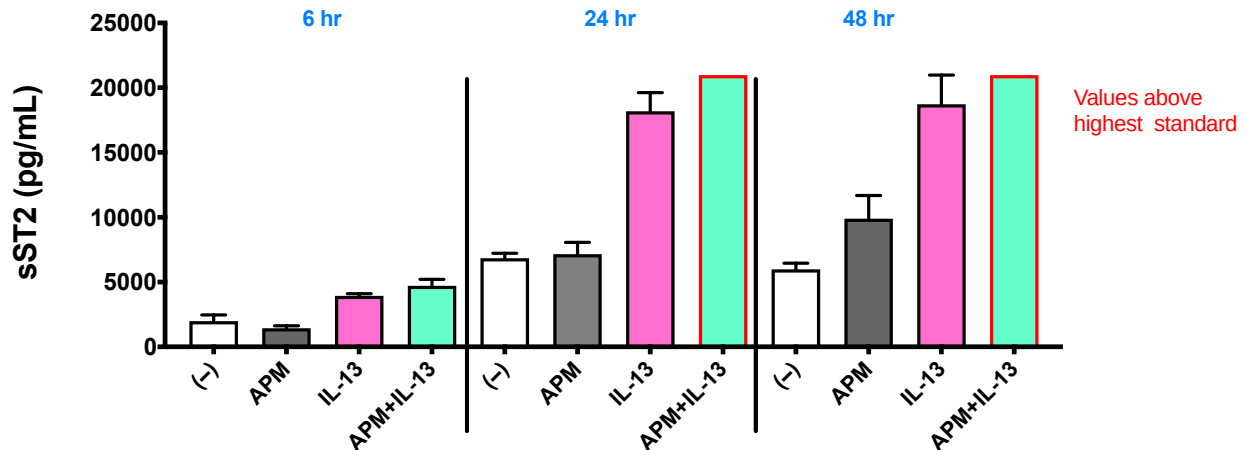
We wanted to test whether this was due to IL-33 release that signals through the ST2L resulting in the elevated levels of IL-8 release from bronchial epithelial cells.

To test this we used a decoy receptor known as soluble ST2 (sST2) or IgG (used as a control) to see if sST2 would block the APM and IL-13 stimulation of IL-8 release



Unexpectedly we did not see an attenuation of the APM or IL-13-mediated IL-8 release by the epithelial cells with the addition of sST2 at either 24 or 48 hours. In fact, we saw a small increase in IL-8 release at 48 hours with sST2 treatment in the APM/IL-13 treatment group.

To investigate this further, we checked whether the APM may promote the release of the endogenous sST2 in our culture system.

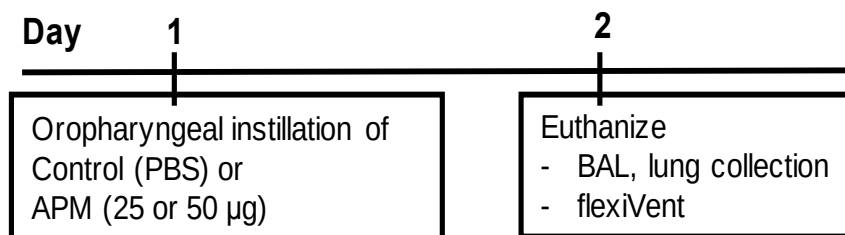


We saw that IL-13 and to a lesser extent the APM was increasing the levels of the endogenous sST2 in a time dependent manner into the culture media and may explain why there was no effect of adding exogenous sST2 to this culture system. It is interesting to note that the combination of APM and IL-13 greatly increased the release of endogenous sST2. This may be a defensive mechanism the epithelium deploys to limit airway inflammation and something that may be defective in deployers that develop respiratory disease.

Future directions:

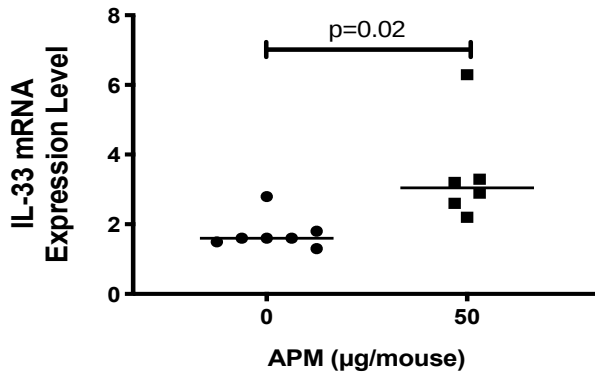
1. Measure sST2-unbound IL-33
2. Improve controls: add IL-33 and sST2 to non-APM exposed cultures to verify if sST2 is binding/neutralizing IL-33
3. Test whether this system is altered in airway epithelial cells from deployers with asthma symptoms.

In vivo studies investigating ST2/IL-33 role in APM induced airway inflammation

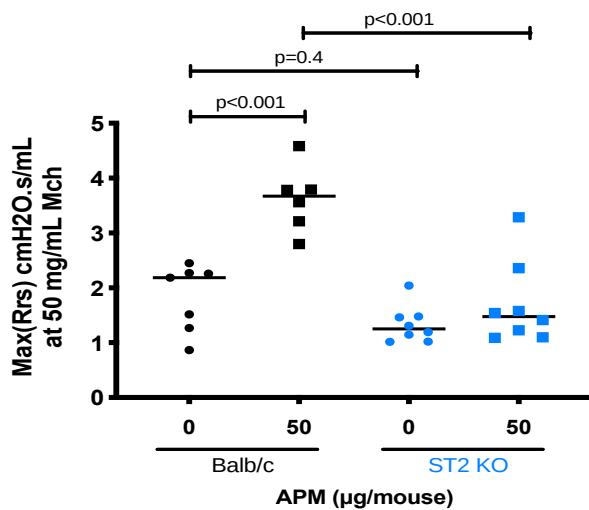


Mouse model system

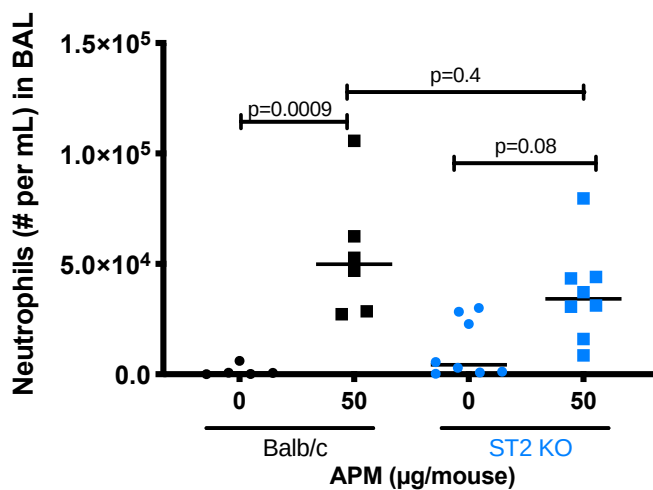
of APM exposure using ST2 KO mice.



Wild type mice instilled with APM have increased lung gene expression for IL-33.



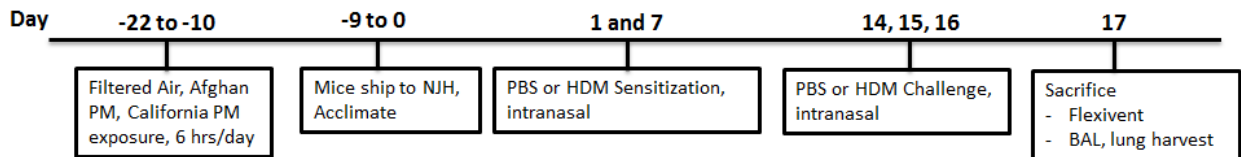
Airway hyperreactivity is increased by APM instillation in wild type mice but attenuated in the ST2 KO mice. This helps support a role of IL-33 release by APM as a possible mechanism by which APM exposure promotes asthma phenotype development in deployers.



Neutrophilic inflammation is not affected in the ST2 KO mice which suggests the airway reactivity stimulated by APM is not dependent upon neutrophilic inflammation.

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

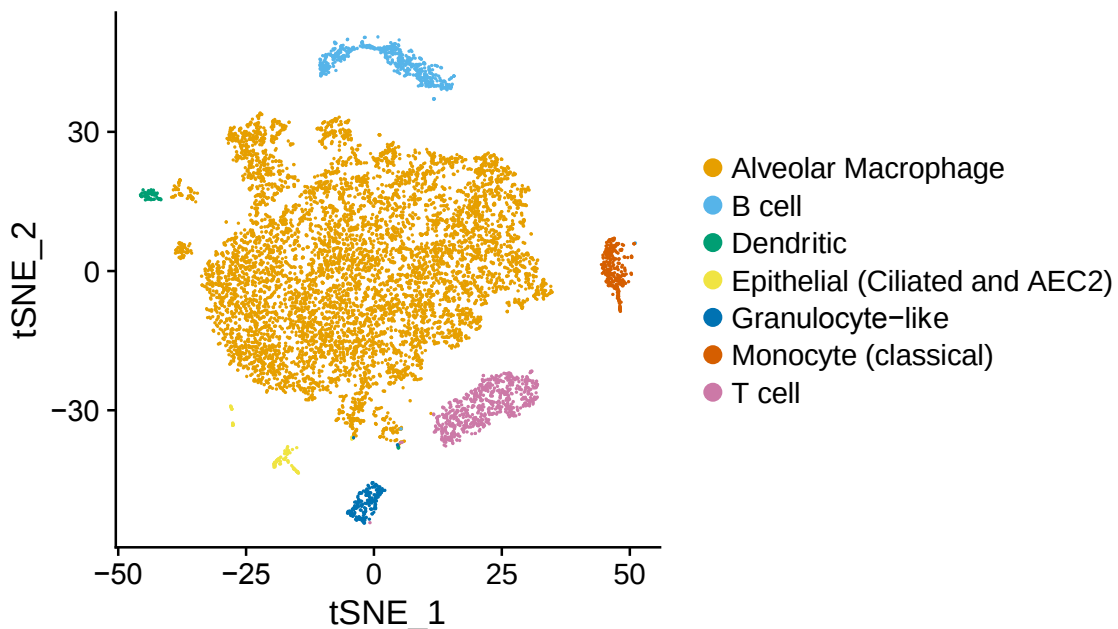
C57 mice:
N = 7 to 8/group



Mice 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.

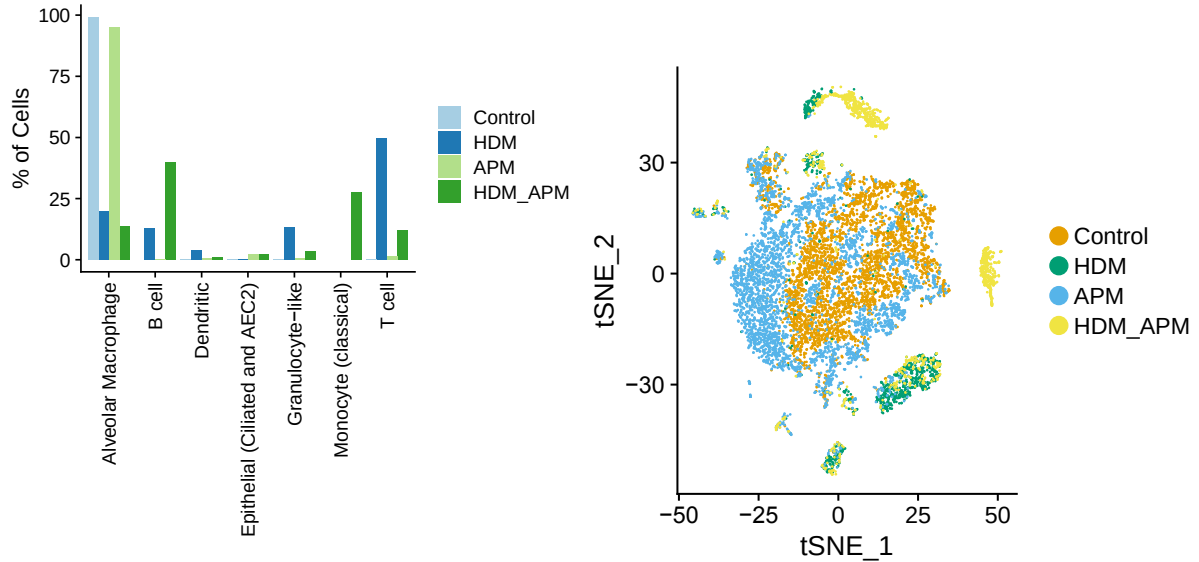
Single cell RNA sequencing on BAL cells collected from mice from these studies.

Unsupervised clustering on cell types based on gene expression induced by all conditions



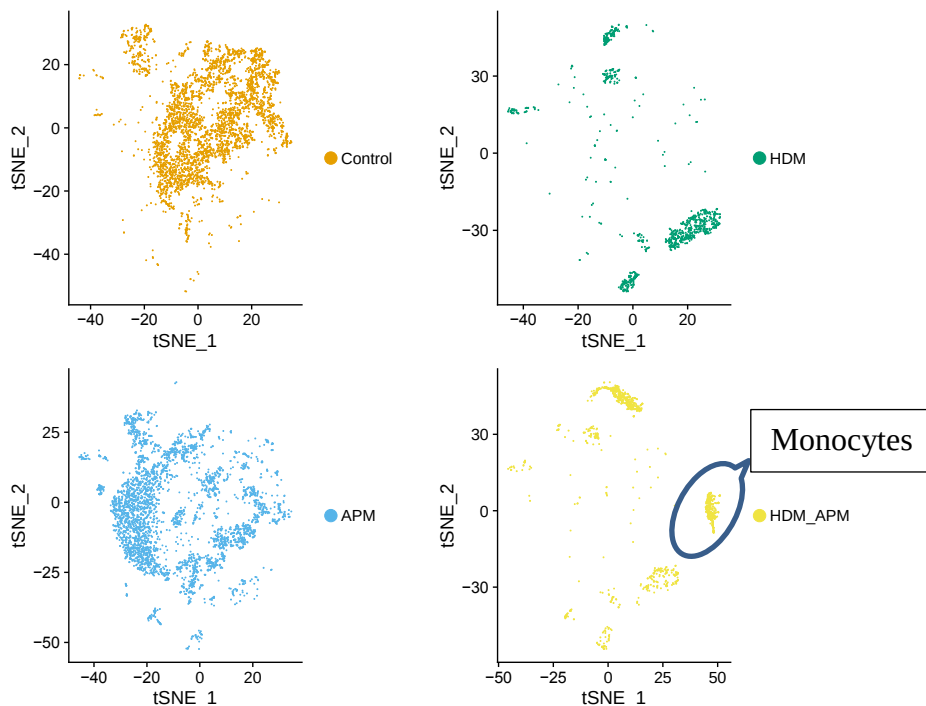
BAL cells from NAMRU exposure conditions clustered based on gene expression.

BAL cells pattern changes with treatment groups. Controls are vehicle treated, house dust mite (HDM) sensitized only group, Afghanistan particulate matter (APM) only group, and Combination of HDM and APM group. We see changes in B cell, monocyte, and T cell population in the combined group.



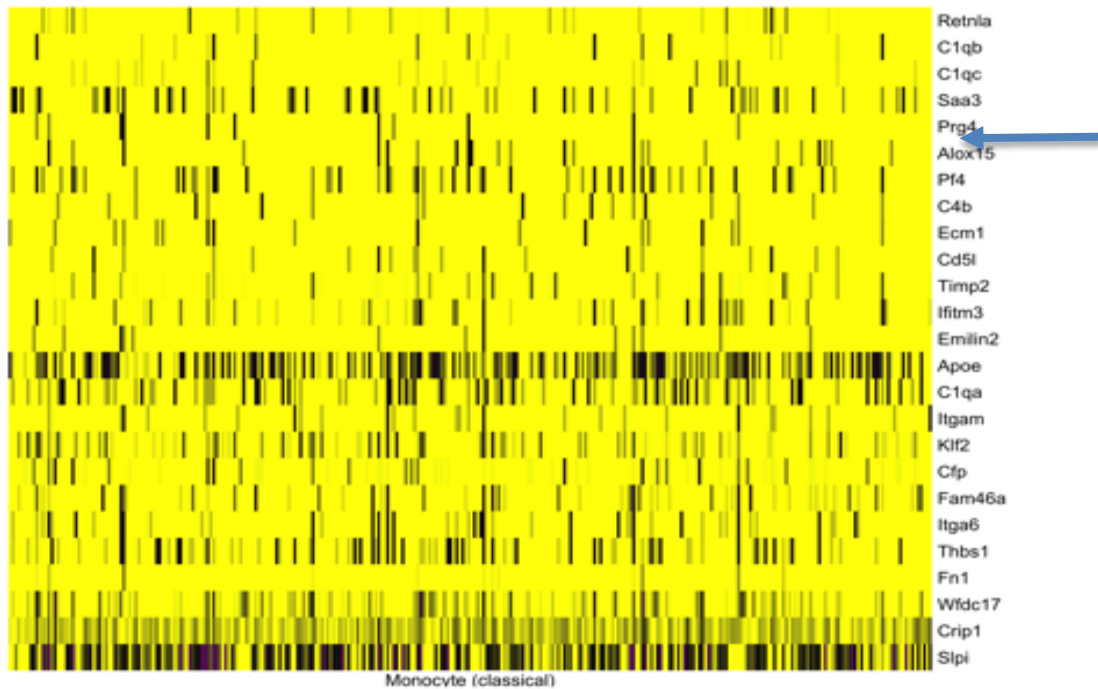
Proportion of different cell types across treatments

BAL cells pattern changes with treatment groups. Change noted in macrophage population gene signature with APM treatment. Another notable change was the appearance of a unique monocyte population in the HDM and APM combination group.



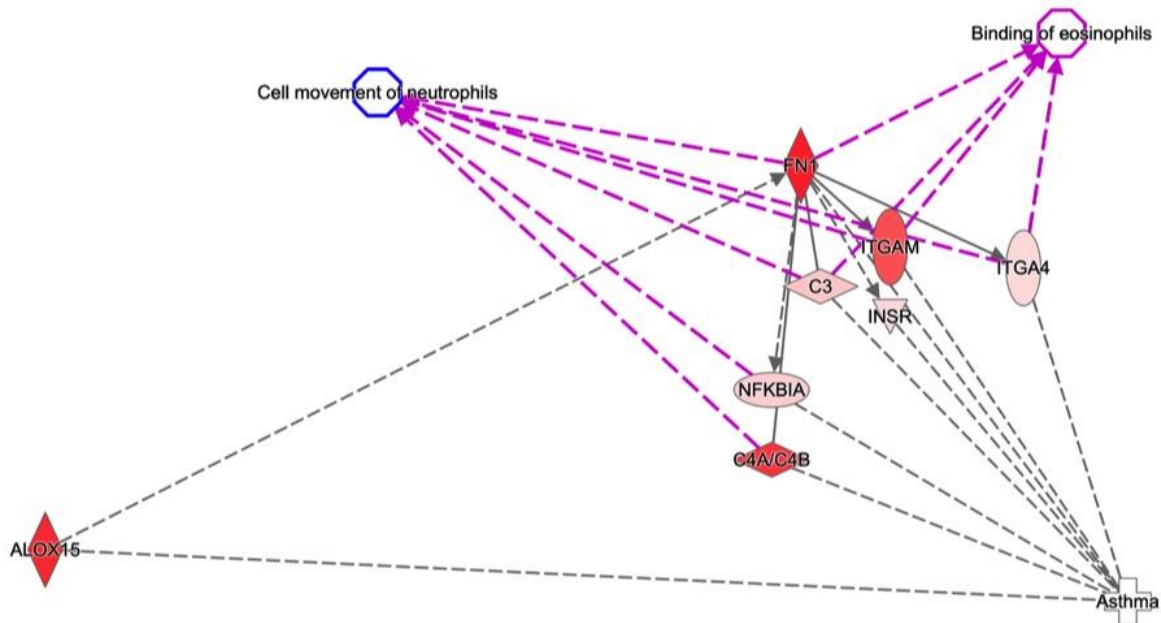
Clustering of cells based on treatment reveals distinct population changes

Gene list associated with monocyte cluster. The gene Alox15 is a 15-lipoxygenase that has been previously implicated in severe asthma.



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).



Gene pathway analysis connecting Alox15 with asthma.

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

Progress

1. Further refine the APM and house dust mite exposure model.
2. Pending establishment of cigarette smoke exposure model in mice exposed to APM and/or house dust mite.

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.

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, Rafael 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 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)

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. Exposure conditions for cigarette smoke, *in vitro*

|

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 plan to continue studying the interactions between PM, allergic stimuli and cigarette smoke exposures *in vitro* using both human lung epithelial cells and mouse lung epithelial cells (Subtasks 2 and 5). Optimized cigarette smoke exposures for *in vitro* studies.
3. We are accruing human bronchial epithelial cell brushings from deployed subjects for subtasks 2, 3, 5 and 6.
4. BAL single cell RNAseq data from the APM, HDM and combined exposure groups for possible mechanistic leads on how these two exposures interact with each other in modulating asthma responses. Identified BAL cell specific gene responses to APM and HDM. Found monocyte cell population and 15-lipoxygenase gene expression that may be contributing to enhanced airway reactivity and asthma development in deployers. Validate 15-lipoxygenase target in mouse tissue from NAMRU study.

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. Through Project 1, in the past year, this study has continued to recruit and enroll subjects who fulfill the requirements for deployed subjects. Deployed subjects have been categorized by Dr. Rose and her consent team into groups as to keep the sample types (asthmatic, DDLD, or asthma + DDLD) blinded in the gene expression study analysis. From these recruited subjects in Project 1, we continue to collect and isolate RNA and DNA samples from nasal and bronchial brushings collected from deployed subjects to fulfill Subtask 1. To date, we have successfully collected samples from all of the recruited subjects as listed below:

Table 1: Samples Collected from Deployed Subjects (as of 9/29/2019)

	Total # Nasal Samples	Total # Bronchial Samples	Number of Paired Nasal/Bronchial Samples
Group 1	8	7	7
Group 2	4	3	3
Group 3	23	15	15

2. In the past reporting year, we have continued to utilize our SOPs, data queries, and established guidelines for control and asthmatic non-deployed individuals from the NJH Biobank. We have continued to collect and isolate RNA and DNA samples from nasal and bronchial brushes collected from these patients for transcriptome analysis. To date, we have successfully collected RNA and DNA samples from all of the subjects as listed below:

Table 2: Samples Collected from Non-Deployed Subjects (as of 9/29/2019)

	Total # Nasal Samples	Total # Bronchial Samples	Number of Paired Nasal/Bronchial Samples
Controls	20	19	17
Asthmatics	36	26	26

3. Our SOPs are in place and minimal adjustment was needed to current protocols and processing guidelines. Collection of ongoing samples from the deployed subjects and

non-deployed subjects continues according to outlined guidelines and processing protocols.

4. In the past reporting year, we have achieved and collected our target number of bronchial brushes (paired with nasal brushes from the same donor) from non-deployed asthmatic subjects (n=10). We have consulted with the NJH Biobank data management team to confirm whether any follow-up diagnoses or medical visits at NJH were made on the non-deployed asthmatic subjects. Using the data available in quarter 2, we concluded that of the samples collected from 27 donors (n = 21 paired nasal/bronchial samples) 14 of these subjects still qualify and pass the study qualification guidelines established in the beginning of the study (Figure 1). Of the donors that we have collected samples for, 9 either fall outside of our age range of 18-65 years old, or have potentially confounding disease diagnoses including COPD, ILD, or NTM infection. These subjects are still recognized as “asthmatic”, and will require further discussion to determine if they should be included or excluded from further analysis in this study. We have continued to collect paired samples from non-deployed asthmatics at the NJH biobank to account for subjects who may be disqualified from the study due to confounding factors. In the next reporting period we will conduct the same re-analysis of the donor history for both asthmatic and control non-deployers to assess the inclusion or exclusion from our study and subsequent analysis.

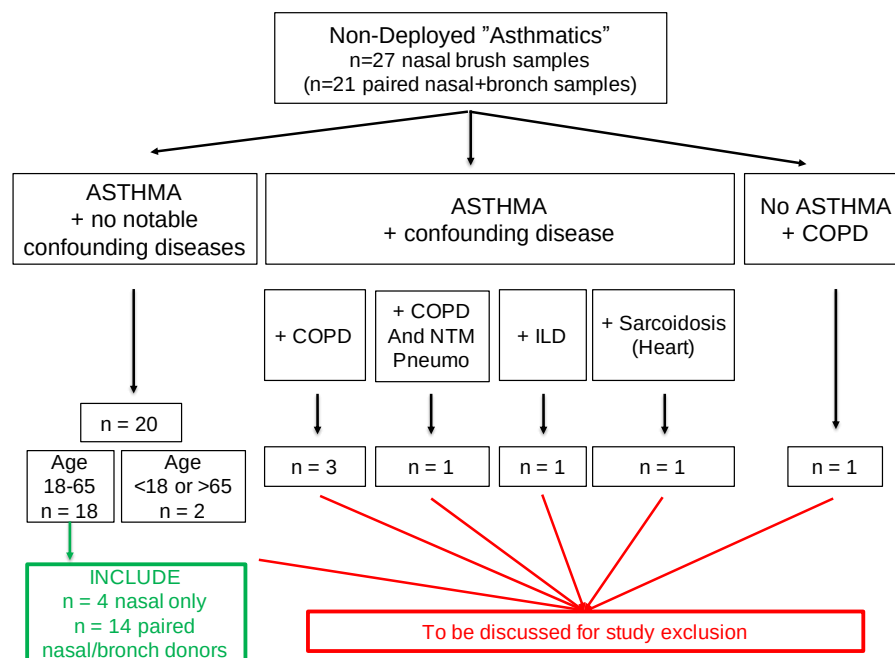


Figure 1: Schematic of current non-deployed asthmatics the study has collected nasal and bronchial brush samples from, follow-up diagnoses made for asthma and potentially confounding diseases, and decision for future subject inclusion in study

5. The recruitment of non-deployed controls has been difficult as few subjects recruited through the biobank meet the criteria for controls as set by our queries. In the past quarter, we have started collecting samples from other research cohorts, consented

through the biobank, that match our non-deployed control criteria. In the past 3 months, this sample pool has nearly doubled our pool of samples (total of 9 collected in past quarter), and gives us confidence that we will be able to hit our target number of nasal and paired bronchial samples within the next year.

6. As part of the sample collection and RNA isolation protocol, we have begun to assess RNA quality for subsequent library preparation for RNAseq analysis. We 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. We have completed RIN assessments on 70% of our extracted RNA samples and found that the average RIN values for nasal RNA samples is 7.8 and for bronchial RNA samples is 8.1. These values indicate that our isolated RNA is of good to very good quality with low levels of RNA degradation, and thus we are confident that we are banking high quality RNA for our gene expression studies, ensuring the most accurate and high quality data to be produced for analysis.

Difficulties Encountered

1. The recruitment of deployers in Group 2 has been minimal and based on the disease status of these patients (which is currently blinded to Project 4 personnel), could have an impact on our power of analysis if enough subjects aren't able to be recruited. This has been discussed with the other project leads in this study, as well as the project patient coordinators and will be monitored as we proceed through ongoing patient recruitment.
2. Recruitment of deployers has been slower than anticipated in Project 1. Following discussion with all personnel, we have established a tentative timeline for the end of 2019, and the end of 2020, to complete recruitment so that Project 4 data analysis may be completed in a timely manner. Currently, we are planning how to begin whole transcriptome analysis on the subset of subjects we have already recruited and collected samples from. This will allow us to begin collecting data from the nasal and bronchial RNA samples across the non-deployed and deployer cohorts and begin sample analysis for Subtask 2.

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

Progress

1. We have recognized the need to control for gender, race, and admixture in differential expression analyses. This is difficult to perform with DESeq2, therefore we have installed and set up code to run the limma package for differential expression controlling for confounders. Secondly, to better identify subgroups of patients with different disease biology we are working on the use of “grade of membership models” pioneering in admixture analyses and recently applied to gene expression data using the CountClust R package. We have installed this code and we are working with its application in some of our datasets in preparation for its use on the GLIDE subject RNA-seq data.
2. We continue to work on and have generated a consolidated pipeline to run all the code we have generated reported here and in prior reports, for accomplishing the first level RNA-seq analysis of: (1) demultiplexing, (2) RNAseqQC, (3) GSNAP mapping, (4) HTSEQ

gene expression quantification, (5) Kalisto transcript variant expression, (6) DESEQ2 normalization and differential expression analysis.

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. We have continued to work on the genetics and have established pipelines for analysis. We have established the fastQTL program code on our cluster. We anticipate using fastQTL for the eQTL analysis due to its speed of analysis. We have also established code in our pipeline to run QTLtools functions to identify independent hits for each of the eGenes identified. Lastly, we are implementing ASC software to determine the allelic fold changes associated with each of the eQTLs.
2. We have progressed in the development of methods for the analysis of the genome-wide genetic data. Specifically, we have established the analytical methods to QC DNA variant data, impute new genetic variants to the dataset from 1000 genomes, determine genetic ancestry, and we have also established code on our cluster to run the matrix eQTL program. We are working to build an automated pipeline of the QC, DNA variant calls, imputation, and eQTL analysis methods. We are also working on methods to call genetic variants from RNA-seq data using GATK software. This pipeline will be used on the *in vivo* airway brushing eQTL analysis in this project.
3. For genotyping of donors for genetic analysis, we have begun working with the sequencing core and their genome-wide genotyping protocols we will use for our analysis in this study. We have established sample preparation guidelines for required concentrations and quality of DNA, as well as appropriate sample submission protocols and paperwork necessary for the project. Through other cohort projects, we have utilized these services and are currently establishing data storage, QC, and analysis protocols that will be able to be applied to the samples in the GLIDE study.

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.

1. Following discussion about the pros and cons of digesting organic tissue for ICP-MS with bleach vs hydrogen peroxide, it was decided that hydrogen peroxide (H₂O₂) is the ideal chemical to use to digest the organic material from the airway brush samples collected in this study, as well as to sterilize the samples. As analytical grade hydrogen peroxide degrades over time, most manufacturers include heavy metal stabilizers in the buffer for prolonged shelf stability. We have eliminated two manufacturers that include the heavy metal Tin in their preparations as it will interfere with the mass spectrometry analysis in this study. We tested a third manufacturer which includes a proprietary stabilizer of which we tested to determine if the buffer will be able to be used for the samples in this study.
 - a. Validation studies were completed with this form of hydrogen peroxide as a 1:1 solution with our samples. Analytical chemists at the USGS conducted the same preparation of this blank sample as our actual biological samples will including

preparation and clean-up procedures. It was determined that the proprietary components of the chosen hydrogen peroxide sample does contain tin as a stabilizer.

- b. A face-to-face meeting was held to discuss this data and how to proceed with analysis. We believe that the stabilizing components may be able to be subtracted out from the samples during the analysis, and it was agreed that a blank sample of the hydrogen peroxide will be submitted with each sample to best account for its components in each ICP-MS batch analyzed.
2. It was necessary to determine if the sample solutions being used in this study were compatible with the sample processing and ICP-MS analysis being conducted by the USGS. Blank buffers were submitted from each project to represent those being used for both the cell fractions of the airway brushings, the supernatants collected from the airway brushings, and the solution used to collect the BAL samples from the deployed subjects (Project 1, 2, and 3). All blank solutions were analyzed for any contaminating heavy metals or components contained in the buffers themselves that may interfere with or need to be accounted for in the biological sample digestates.
 - a. Data analysis identified that the saline solutions and hydrogen peroxide solutions being used to collect the cell sample supernatants for Project 4 are relatively “clean” and will be relatively straight forward to subtract out the minimal contaminants in these solutions during sample analysis.
 - b. However, analysis identified that the buffer currently being used to store cell samples for ICP-MS analysis in Project 4 is particularly challenging as it contains high levels of multiple components (Fe, K, Mg, Mn, Na, P, S, Sr) which will be difficult to correct for in sample analysis.
 - i. Moving forward, cell samples will be collected in 18.2 milliQ water as it was determined to be to best and cleanest buffer to use for this process following discussion between Project 4 personnel and the USGS scientists.
 - c. It was discussed that in general, Cl interferes with the detection of As, Se, and V and additional digestion of samples will be necessary if these are elements of interest moving forward.
3. Following the results from the blank sample preparation and analysis, a subset of biological samples for ICP-MS analysis were submitted to the USGS for analysis. We decided to submit a subset of samples from each project to determine if any of the samples have detectable components, and which samples those are. Samples were submitted as follows:
 - a. Project 1: Human BAL supernatants
 - i. To determine if detectable levels of measured components are present in these samples, 5 BAL samples were submitted from recruited deployers in the study.
 - b. Project 2 and 3: Mouse BAL samples
 - i. To determine if detectable levels of measured components are present in these samples, 3 BAL samples from mice that underwent intratracheal instillation of Iraq dust were submitted for analysis. These samples have the highest chance of detection as they were treated with the Iraq dust sample of interest in this study.
 - c. Project 4: Nasal brush cells and supernatants, and *in vitro* study supernatants

- i. To determine if detectable levels of measured components are present in these samples, cell suspensions and supernatants collected from the nasal brushes of 1 deployed subject and 1 non-deployed subject were submitted for analysis. As a positive control, the supernatants from our *in vitro* airway epithelial cell stimulations (which contain the Iraq dust used for stimulations) were also submitted for analysis.
4. Semi-quantitative analysis of the Iraq dust in culture media from the *in vitro* cell stimulations submitted by Project 4 indicated higher levels of various metals and elements compared to the control samples. This data provides insight that upon elimination of the more challenging sample buffers, and the use of a more ICP-MS-friendly buffer in its place, the analysis and subsequent threshold of detection will be better for future sample analysis using airway donor epithelial and supernatant samples.
5. Following analysis identifying buffers and buffer components that are unsuitable for ICP-MS analysis, further discussion determined that the ideal buffers to use for storage of samples and downstream analysis include PBS+EDTA+EGTA for nasal and bronchial cell supernatants. Further discussion identified that milliQ water would be the best buffer for nasal and bronchial cell lysates for storage and downstream analysis. During the past 3 reporting periods, we have collected all samples in these new, modified buffers for ICP-MS analysis.
6. Additional communication between all personnel has also established SOPs for processing of frozen samples at National Jewish, and transport of decontaminated samples to USGS personnel for delivery to the USGS facility in Boulder, CO. This protocol for all subsequent samples will minimize batch variation between analysis runs, and will keep the flow-through of processing-transport-analysis as consistent as possible for all samples over the duration of the study.
7. To assess the content of the nasal cell suspensions and the supernatants from the nasal samples in their new storage and analysis buffers, we submitted a new round of paired samples to the USGS for processing and ICP-MS analysis. Submitted samples were analyzed by USGS personnel, and analysis parameters were decided upon for how to account for the volume of buffers, H₂O₂, and sample, as well as how to blank for these buffer components. Data analysis found that even the highest abundant components were below the level of detection once the sample collection buffers were accounted for. Further analysis methods are being considered to see if any are more sensitive, and deployer samples will be submitted for analysis in the next reporting period to determine if those samples contain detectable levels of the components being measured in the ICP-MS analysis.

Difficulties Encountered

1. Storage of cell samples from nasal and bronchial brushes have been stored in culture media until this point. However, this media type proved quite difficult for ICP-MS analysis. There are still more than enough donors left to recruit to meet our number of subject samples for analysis, and Project 4 is now harvesting these samples in a USGS-preferred buffer (milliQ water) moving forward.
 - a. Furthermore, samples currently stored in the culture media buffer may be able to be isolated upon thawing and placed into the correct buffer. Future trials will need to be completed to validate this process.

- Overall, the list of sample buffers being used are challenging matrices for analysis, however, further samples and data analysis methods are being developed and discussed to adapt to these samples as we move forward.
- 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. Additionally, the cell lysates we have provided for analysis also have ICP-MS data values below the limit of detection. We have discussed that we will submit three additional deployer samples for analysis to determine if deployer samples have detectable limits of any of the ICP-MS measured components, and 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.

Progress

- In the past year, we have utilized our optimized and finalized culture methods for growing nasal cells from the donor brushes. Cells are grown using a feeder cell layer to culture cells directly from the brushes, followed by a feeder-free growth passage in expansion media from Stem Cell Technologies. These culture protocols optimize both the growth of healthy and expandable cells for further stimulation experiments, as well as optimize/minimize the cost of reagents necessary for harvesting and storage of the samples.
- Expansion, freezing, reviving from storage, and successful differentiation has been validated using our culture methods. Ongoing experiments have utilized these methods and derive well-differentiated mucociliary ALI cultures, and we are confident in a high success rate of differentiation at air-liquid interface of nasal AEC cultured from the nasal brushes from both deployed and non-deployed subjects in our study in current and future experiments.

Cell Expansion Progress

- Through Project 1, this study has continued to recruit and enroll subjects who fulfill the requirements for deployed subjects. Deployed subjects have been categorized by Dr. Rose and her consent team into groups as to keep the sample types (asthmatic, DDLD, or asthma + DDLD) blinded in the gene expression study analysis. From these recruited subjects in Project 1, we continue to collect and culture airway epithelial cells from the nasal and bronchial brushings collected from deployed subjects to fulfill Subtask 5. Thus far we have collected samples from all of following subjects:

Table 3: Nasal Samples Cultured from Deployed Subjects (as of 9/29/2019)

	Total # Nasal Samples	Successfully Expanded Nasal Samples
Group 1	8	8 (100% success)
Group 2	4	4 (100% success)
Group 3	18	17 (94.4% success)

2. We have also continued to collect and expand nasal airway epithelial cells from non-deployers as per our established SOPs. Samples collected from non-deployed subjects consented through NJH Biobank have been successfully collected from the subjects as listed below:

Table 4: Nasal Samples Cultured from Non-Deployed Subjects (as of 9/29/2019)

	Total # Nasal Samples	Successfully Expanded Nasal Samples
Controls	19	17 (89.4% success)
Asthmatics	34	29 (85.2% success)

***In Vitro* Stimulation/Exposure Progress**

1. Following preliminary experiments, we found that stimulation with Iraq dust PM at 10ug/cm² resulted in a distinct, though notably lower response in nasal airway epithelial cells compared to tracheal airway epithelial cells. As the objective in this project is to study nasal airway epithelial cells, to get a more robust response, while maintaining a physiologically relevant stimulation, subsequent experiments have utilized a stimulation concentration of 20ug/cm² of Iraq dust in the basal cell and ALI culture stimulations.
2. In preparation of the first manuscript for Project 4, we are establishing the role of the epithelium and the individual cell types in the response to Iraq dust APM. In order to do this, we set out to first analyze the responses of the airway epithelium by comparing the response of epithelial cells collected from anatomically distinct regions of the airway (tracheal versus nasal sites). Based on previous preliminary findings, we anticipated the response of cells from both anatomical locations to produce similar responses to dust stimulation. Basal cells from tracheal (n=9), and nasal airway epithelial cells from non-deployed asthmatics collected in this study (n=10) were stimulated with Iraq dust APM (20ug/cm²) for 24hours. Samples were harvested for RNAseq gene expression studies and cytotoxicity assays.
 - a. Cytotoxicity:
 - i. Minimal to nearly no cytotoxicity was seen in basal cells grown from both the tracheal airway epithelium (<2%) and nasal airway epithelium (<1%) in response to 24 hours stimulation with Iraq dust (20ug/cm²).
 - b. Gene expression response:
 - i. Utilizing stimulations of basal cells from both upper (nasal) and lower (tracheal) airways, we performed 24 hour stimulations with Iraq dust at 20ug/cm² to test the transcriptional response of the airway cells to the increased concentration of dust. We found that the response of both upper and lower airway cells was distinct and conserved in all samples as seen in the MDS plot in Figure 2 (left panel). This conserved response was characterized by >1300 DEGs in lower airway cells, and >1900 DEGs in upper (nasal) airway cells. Gene ontology categories were similar to those from preliminary studies including epidermal differentiation, and TGF-B signaling.

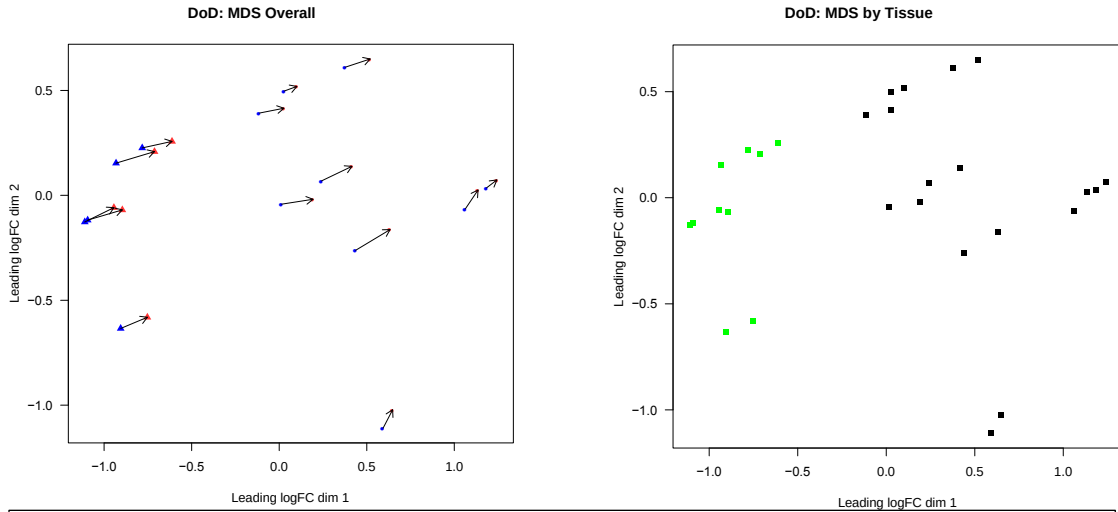


Figure 2: MDS plots of upper (nasal) and lower (tracheal) airway basal cells following stimulation with Iraq dust PM (Left Panel) blue = control stimulations; red = dust stimulated, (Right Panel) Green = tracheal, Black = Nasal samples

- ii. MDS plots show that while the global response is conserved between tissue types, nasal and tracheal basal cells cluster separately which suggests a partial gene expression profile that is distinct to each tissue type in response to dust (Figure 2 – right panel). However, nasal and tracheal basal cells share a high level of similarity in significantly induced genes ($FDR < 0.05$, $LFC > 0.3$) with 54% of nasal DEGs and 65% of tracheal DEGs being similar in upregulation to desert dust (Figure 3).

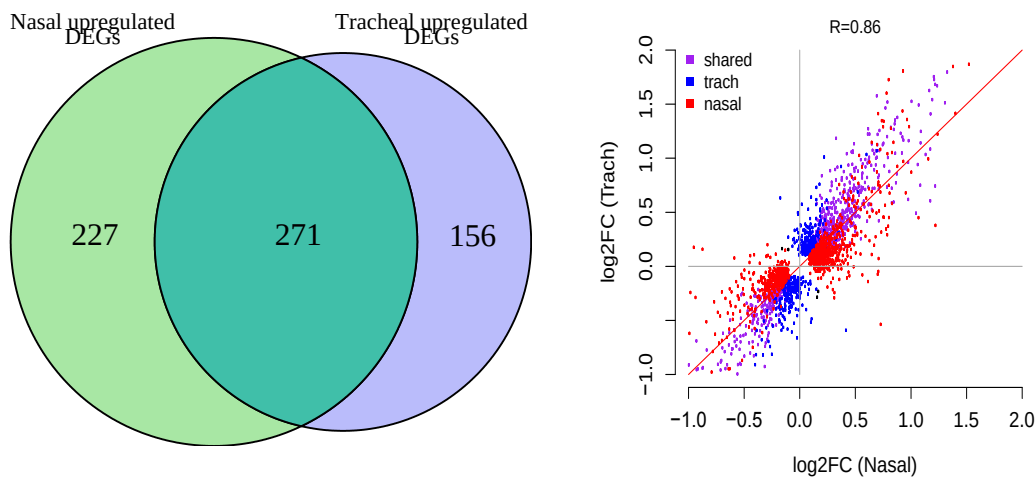


Figure 3: (Left panel) Venn diagram illustrating overlap of significantly induced genes airway epithelial nasal (left) and tracheal (right) basal cells in response to Iraq desert dust (20ug/cm²) for 24hours. Genes in diagram include DEGs with $FDR < 0.05$ and $LFC > 0.3$ (Right panel) Dot plot illustrating all differentially expressed genes specific to nasal (red), tracheal (blue), and shared between nasal and tracheal (purple) basal cells following 24hr stimulation with Iraq dust (20ug/cm²) indicates a high correlation ($r=0.86$) of log₂ fold change values of shared DEGs

- iii. Moreover, the log2 fold change in shared DEGs between both nasal and tracheal basal airway cell stimulations are highly correlated ($r=0.86$), suggesting that not only are similar transcriptional changes occurring in cells from both anatomical locations, but that they are up- or down-regulating those signals in a similar manner (Figure 3).
- iv. Top enriched gene ontology categories were similar between basal cells from anatomically distinct locations (tracheal vs nasal) and include epidermal differentiation, detoxification/AHR signaling pathways, and IL-1 and NFkB inflammatory signaling pathways (Figure 4; left panel).
- v. Further inquiry into the epidermal differentiation GO term led to the investigation of the Epidermal Differentiation Complex (EDC) which is a large genomic region that is largely responsible for driving epidermal differentiation. Notably, both nasal and tracheal basal cells significantly upregulated >20 genes located within this EDC region in response to dust. Upregulated genes included 6 *S100* family genes, 8 *SPRR* family genes, various genes involved in the cornification process, and 2 transcription factors (*KLF4* and *GRHL3*) known to drive the EDC complex of genes (Figure 4; right panel).
- vi. These data confirm that nasal and tracheal airway basal cells respond in a highly similar manner to Iraq desert dust, and supports the use of cells isolated from the nasal airway epithelium as a good model for lower airway disease and epithelial responses to Iraq dust.

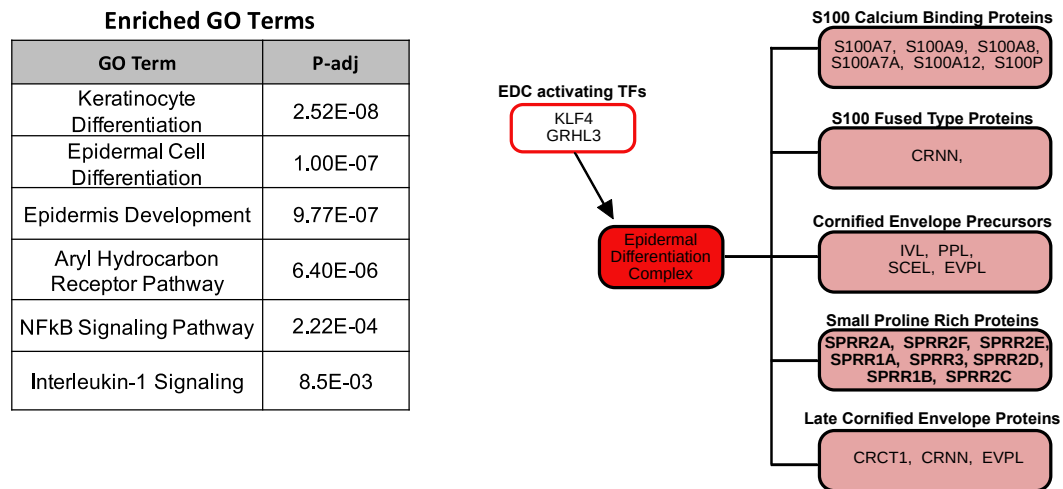


Figure 4: (Right Panel) Top shared gene ontology pathway hits from top induced DEGs in response to Iraq dust in airway basal cells.
(Left panel) Schematic diagram of some of the transcription factors which drive the EDC, as well as gene families, and specific genes, in the EDC which are upregulated by Iraq dust stimulation in both nasal and tracheal basal airway epithelial cells (FDR <0.05)

2. To expand on the preliminary experiments described above and for experimental analysis for the manuscript in preparation, we have used the same donors (nasal) used for the basal cell stimulations above, and differentiated them at air-liquid interface.
 - a. Cytotoxicity: Iraq dust PM stimulation (20ug/cm²) resulted in very low cytotoxicity induced in differentiated ALI cultures from the nasal cultures (<2% change from control stimulations).
 - b. Transcriptomic response:
 - i. Gene expression analysis identified that differentiated nasal epithelial cultures (n=10) responded in highly similar manner with >4,500 DEGs in response Iraq dust PM (20ug/cm²) stimulation (Figure 5).

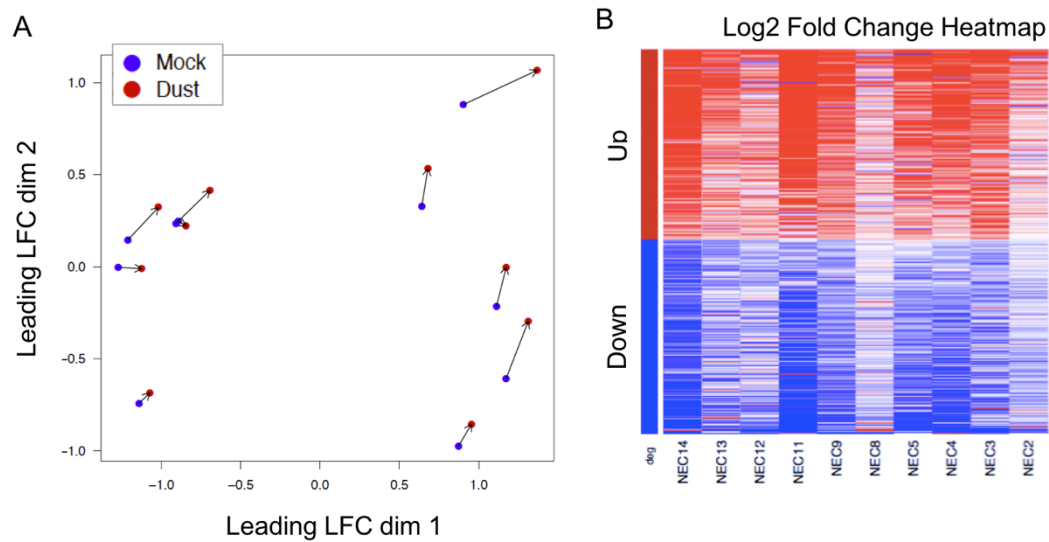


Figure 5: A: MDS plot of differentiated nasal airway epithelial cultures (n=10) following stimulation with Iraq dust PM for 24hours (20ug/cm²). **B:** Heatmap showing the log2 fold change values of the >4,500 DEGs (FDR < 0.05) in differentiated nasal cultures in response to Iraq dust PM.

- ii. Stimulation with dust significantly upregulated the expression of 12 *S100* and *SPRR* family genes in differentiated nasal ALI cultures, which are involved in epidermal differentiation and inflammation in the airway (Figure 6A).
- iii. Iraq dust exposure dramatically induced a strong inflammatory signaling pathway as indicated by the increase in gene expression of the inflammatory mediator *CXCL8* (Figure 6B; p-value = 4.8E-04), over 15 inflammatory cytokines, and chemokines, and the upregulation of over a dozen genes in the NFkB signaling GO term (Figure 6C; p-value = 6.25E-18).
- iv. Nasal ALI cultures also upregulated the expression a variety of inflammatory IL-1 signaling family members in response to Iraq dust. Such inflammatory signaling members include *IL1A*, *IL1B*, *IL36g*, and *IL33* (Figure 6D).
- v. Additionally, the most significantly down-regulated gene ontologies include focal adhesion complexes and cadherin binding.

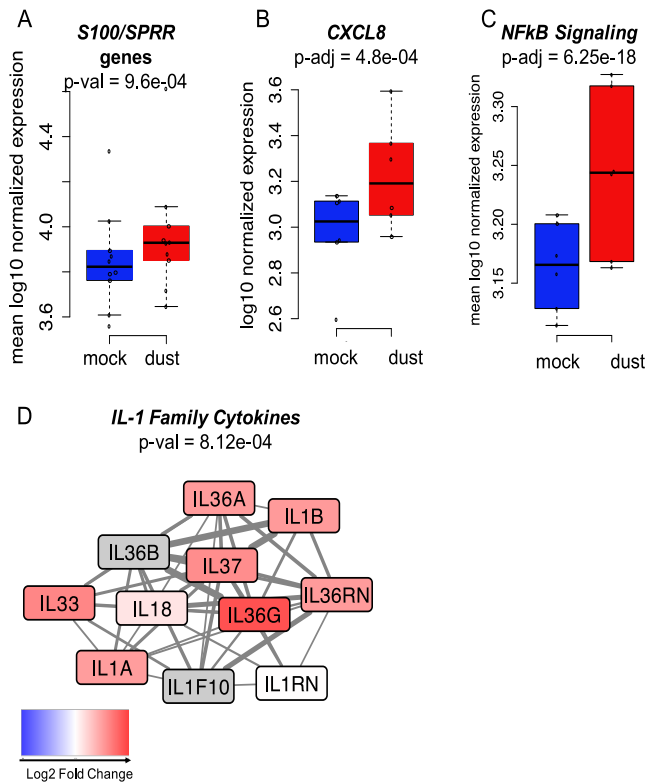


Figure 6:

Boxplots (A-C) and network diagrams (D) illustrating the upregulation of inflammatory mediators and pathways by mucociliary nasal cultures in response to dust including S100/SPRR family (A), CXCL8 (B), NFkB signaling GO terms (C), and the IL-1 family of inflammatory cytokines (D).

- vi. Together, these data suggest a remodeling of the airway epithelium, and we are currently following up with additional *in vitro* experiments to further explore this biology and the transformation of the differentiated airway epithelium.
- c. Single cell RNAseq Analysis:
 - i. To further investigate the response of the airway epithelium, we have also recently conducted experiments using single cell RNA sequencing, which will allow us to analyze the gene expression of each cell type present in the airway epithelium (ciliated, secretory, and basal). Additional bulk and single cell RNAseq analysis is currently in progress to further investigate the inflammatory, differentiation, and detoxification pathways at a tissue wide, and a single cell level to fully understand and characterize this response to acute Iraq dust PM stimulation.
- d. Investigating inflammatory drivers of epithelial dust-response in nasal ALI cultures
 - i. To understand the inflammatory signals important in driving the dust response in the nasal AEC ALI cultures, we conducted independent stimulations of the same nasal donors differentiated at ALI. Stimulations were conducted with the IL1-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. This data is currently being sequenced and analyzed, and we will report our findings and analysis in the next reporting period.
- e. Chronic desert dust exposure of mucociliary epithelium

- i. To assess the impact on airway epithelial remodeling during chronic, repeated exposure to desert dust, we conducted daily stimulations over a 7d exposure model. Data analysis identified >3,000 DEGs compared to control cultures.
 - ii. Analysis indicated inflammatory pathways, including the IL-1 family of cytokines, as well as other inflammatory signals are still induced following long term stimulation with desert dust.
 - iii. Current experiments aim to further examine this transcriptome data, as well as validate the cell-type expression using IHC and RNAscope on histological samples collected from the stimulations.
- 3. The stated objective in Project 4 Aim 3 is to investigate the role of dust exposure and how it affects the IL-13 response in the airway epithelium. To explore this aim using these sample nasal donors differentiated at ALI, we also performed stimulations using 24hour mock or Iraq dust stimulations (20ug/cm²) followed by 24hr IL-13 stimulation.
 - a. Cytotoxicity: Regardless of the stimulation with either mock or Iraq dust PM conditions prior to IL-13 treatment, cultures from all donors (nasal n=6) resulted in low cytotoxicity induced in differentiated ALI cultures (average response of <2% change from control stimulations).
 - b. Transcriptomic response:
 - i. Gene expression analysis identified >3,000 DEGs in dust+IL13 vs mock+IL13 stimulation conditions of differentiated ALI cultures. Notably, the vast majority of these dysregulated genes are those driven by the dust response seen in the dust vs mock stimulations described above. Interestingly, a small subset of the DEGs upregulated by pre-exposure to dust are novel and seemingly only upregulated in the dust+IL13 stimulations. Current studies aim to repeat these findings.
 - ii. Further investigation of the dust only response was completed using interaction analysis to identify whether the pre-exposure to dust significantly altered the IL-13 response. We determined that there were no significant DEGs in the interaction analysis, indicating that pre-exposure of dust did not significantly alter the IL-13 response in a 24hr dust + 24hr IL13 stimulation model. We are currently discussing alterations to our stimulation model, exposure timelines, and order of stimulations to mimic that of the disease that may be seen in deployed asthmatics and DDLN subjects.

Difficulties Encountered:

1. Prior studies utilizing tracheal airway epithelial cultures implemented a 48hr stimulation with Iraq dust particulate matter. The studies here, utilizing nasal airway epithelial cells, used a 24hr stimulation protocol prior to gene expression analyses. It is possible that stimulation of the nasal airway epithelium results in a less robust response to the Iraq dust samples compared to the tracheal airway epithelial cultures, as described above (no DEGS with FDR < 0.05). However, it is also a possibility that the longer stimulation of 48hrs (compared to 24hours used here) is important in the development of the responses mounted by the epithelium to inhaled dust particulate matter. Further investigation will

be conducted using the nasal airway epithelial cells to determine if the 24hr or the 48hr stimulation model is most appropriate for this study.

- a. Experiments established the use of Iraq dust at 20ug/cm² for 24 hours for cell stimulations and that this concentration elicits a robust transcriptional response, while resulting in negligible cytotoxicity. This concentration is currently being used in all current and future experiments outlined in this grant for Project 4.
2. The recruitment of deployers in Group 2 has been minimal and based on the disease status of these patients (which is currently blinded to Project 4 personnel), could have an impact on our power of analysis in our *in vitro* stimulations if enough subjects aren't able to be recruited. This has been discussed with the other project leads in this study as well as the project patient coordinators and will be monitored as we proceed through ongoing patient recruitment. Alteration to the subject groups being stimulated in subtask 5 may be re-assessed in the future and will be further discussed as the project progresses.

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. In experiments described above, we have conducted stimulations of PM and PM+IL13 on non-deployed subjects (n=6) to be included in this study. Ongoing stimulations and alteration to this stimulation model are currently being discussed to continue this stimulation progress for the total study donors in the next year (n=50/group x 3 groups).
2. For next-generation library preparation, the use of the new whole transcriptome KAPA HyperPrep library kits has been validated on our Beckman Coulter Biomek instrument used to make sequencing libraries in a more consistent and high-throughput manner. Using this library kit, we obtain a greater number of genes detected and counted compared to that from the targeted AmpliSeq library kits. Furthermore, the use of the KAPA HyperPrep kits allows for more robust data acquisition from a lower input of RNA in the reaction. While our nasal and bronchial RNA sample extraction method is robust and yields high concentrations of good quality RNA, this low input allows for the construction and analysis of samples that have lower RNA yields due to sample and donor variability.
3. 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.
4. Recent experiments described above have been utilizing control samples from non-deployed subjects to develop the baseline response to Iraq dust PM. These samples have been collected and expanded in an identical manner to those collected from the deployers. As we have begun to culture these samples, the majority of donor cells expand well in culture and successfully differentiate into basal, secretory, and ciliated airway epithelial

cells. This gives us confidence in the success of further culture and differentiation of the cells from both the non-deployers as well as the deployers, which will be used in the near future for Iraq dust stimulations of the entire GLIDE sample cohort.

Subtask 7. Prepare and submit manuscripts for publication.

Progress

Preparation of the first manuscript for Project 4 is currently underway. The goal is to submit this manuscript laying out the groundwork for how the differentiated airway epithelium, and the individual cell types that compose it, respond to Iraq dust particulate matter

1. Tentative title: “Airway basal cells coordinate response to Iraq dust APM in the airway epithelium”
 - a. Projected submission date: Winter 2019

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. Sample decontamination and digestion methods using hydrogen peroxide, guidelines for sample preparation, and sample delivery to the USGS have been established.
5. 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.
6. 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.
7. All individual analytical methods in the differential gene expression workflow are established.
8. Analytic methods for eQTL and RNAseq variant calling methods development have been established.
9. Preliminary PM dose response stimulations have been developed and established under acute and chronic conditions. Such treatments are not toxic to the airway epithelial cultures grown at air-liquid interface.
10. Experimental stimulation methods have been established for the exposure of AEC to dust, followed by IL-13 to study the ‘two-hit hypothesis’ in our model, and additional models are being optimized.

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

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. “Training” activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. “Professional development” activities result in increased knowledge or skill in one’s area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Project 1

The Project team has participated in a number of professional development activities. Several team members attended the 2019 annual American Thoracic Society international conference in Dallas, TX and presented at data on clinical findings in deployers with dynamic airway collapse/tracheobronchomalacia and eosinophilic syndromes. We also participated in a symposium, presenting research findings on the utility of military occupational specialty codes for characterizing deployment inhalational exposures. The Project 1 team continues to present cases with deployment-related lung disease for discussion and management at a bi-monthly Deployment Lung Disease clinical case conference at NJH.

Project 2

Dr. Downey attended the 2018 American Thoracic Society annual international conference in San Diego, CA and participated in the symposium on deployment-related lung disease. Dr. Downey attended and presented a poster and delivered an oral presentation at the Military Health System Research Symposium (MHSRS) Orlando, FL in August 2019.

Project 3

Drs. Chu and Day are supervising 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 attended and presented a poster at the Military Health System Research Symposium (MHSRS) Orlando, FL in August 2019.

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 was supervising and training Ms. Katie Li, a post-baccalaureate who will be applying to medical school, 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. Drs. Seibold and Everman attended the 2019 American Thoracic Society annual international conference in Dallas, Texas and presented a poster on the dust-induced transcriptional response of the airway epithelium. Furthermore, Dr.

Everman had a poster at the 2019 Military Health System Research Symposium (MHSRS) in Orlando, FL on the airway epithelial response to desert dust.

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.

Drs. Rose and Downey participated in a workshop titled ‘Respiratory Health and Service in Southwest Asia and Afghanistan’ that took place at the 2018 annual meeting of the American Thoracic Society in San Diego, CA. The ATS Workshop report was published in Annals of ATS in August 2019.

Project 2

An abstract reporting the initial studies with PM exposure in mice was presented as a poster presentation and in an oral presentation in a symposium at the Military Health System Research Symposium (MHSRS) in Orlando, FL in August 2019.

Project 3

Presented Abstract at the Society for Toxicology meeting at San Antonio, Texas 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 discussed with academic and industry research professionals at both the 2019 ATS conference, and the 2019 MHSRS conference.

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

If this is the final report, state “Nothing to Report.”

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 plan to 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.

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.

Subtask 7. Complete LCI measurements from deployers and controls.

Plans for the next year. We plan to continue to recruit and test study subjects, and anticipate completing this LCI subtask during the next year.

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

Plans for the next year. We plan to obtain the remaining CT scans up to our target number and will proceed with quantitative analysis of images from both deployers and controls.

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 continue to analyze our data and submit manuscripts for peer review and publication. Our case series, follow up LCI study, MOS paper, and analysis of imaging findings on expiratory dynamic airway collapse as well as asthma phenotypes in deployers will be completed this year.

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, chemical, and infectious stimuli.

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 implement methods 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 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 chemical injury with bleomycin and on the impact of combined exposure to particulate matter and bleomycin.

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 blast over pressure lung injury and examine the combined effects of prior exposure to particulate matter on subsequent blast overpressure 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 develop methods to measure particulate matter and mineral content of the lungs from the animal models of PM exposure and begin to study the surgical lung biopsies from deployers.

Subtask 8. Prepare and submit manuscripts for publication.

Plans for the next year. We have published 2 manuscripts in this last year and will submit a third in the spring.

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 studies comparing deployers with asthma epithelial cell responses to deployers without asthma in this *in vitro* model system.

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

Plan for the next year: Continue studies looking at a role for ST2/IL-33 pathway in APM's ability to enhance house dust mite airway hyperreactivity.

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

Plan for the next year: Continue studies comparing deployers with asthma epithelial cell responses to combined cigarette smoke, APM and IL-13 in our *in vitro* model system.

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: Continue to collect nasal and bronchial brushes from deployer recruitment led by Project 1, and of non-deployed subjects through the NJH biobank. 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: First batch of *in vivo* samples will be prepped and analyzed for gene expression studies. Further analysis will continue upon further sample collection, and is dependent on recruitment by Project 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.

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: Perform ICP-MS analysis on subset of deployer samples; 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 and will begin to conduct stimulations on deployer nasal AEC samples. We will continue to analyze transcriptomic responses under these stimulation conditions, and begin to acquire genotype information for genetic variant determination.

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.

Subtask 7. Prepare and submit manuscripts for publication.

Plan for the next year: Current experiments and a manuscript in preparation aim to set the groundwork for the baseline response of the AEC to Iraq dust PM, we are hoping to submit this manuscript in Winter 2019

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

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

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

- 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 implemented a noninvasive assessment of small airways injury disease using lung clearance index (LCI) and have applied this technique to screen over 50 previously employed military personnel.
- We have identified quantitative methods to analyze chest CT scans from previously deployed military personnel and will use 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 dictated transcript homework 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?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

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?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

PROJECT 1.

Nothing to Report.

PROJECT 2.

Nothing to Report.

PROJECT 3.

Nothing to Report.

PROJECT 4.

Nothing to Report.

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/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. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

PROJECT 1.

We have made modifications in our approach to acquisition of BAL and nasal and bronchial epithelial cells in control study subjects that will improve reliability and clinical phenotyping. This modification will not result in significant changes in objectives or scope of the project.

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 are currently examining the effects of higher concentrations of silica and particulate matter (up to 70 mg/m³) given over 2 weeks followed by a period of up to 72 days to see if there is delayed development of 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.

Nothing to Report.

PROJECT 4.

Nothing to Report.

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

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

PROJECT 1.

Nothing to Report.

PROJECT 2.

Nothing to Report.

PROJECT 3.

Nothing to Report.

PROJECT 4.

Nothing to Report.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Due to initial delay in obtaining IRB and HRPO approval for GLIDE study subject recruitment, we have underspent in those parts of the grant budget that cover costs for study subject reimbursements, deployer bronchoscopies with BALs and nasal brushings, and chest CT scans. Since IRB and HRPO approval, we have been actively recruiting and hope to make up for the initial delay.

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: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”

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

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

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, **Kreffft SD**, Mallon T, Miller RF, Morris MJ, Proctor SP, Redlich CA, **Rose CS**, Rull RP, Saers J, Schneiderman AI, Smith NL, Yiallourous 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.
- **Books or other non-periodical, one-time publications.** *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*
 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
 - **Other publications, conference papers, and presentations.** *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to Report.
 - **Website(s) or other Internet site(s)**
List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report.

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

Nothing to Report.

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

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report.

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *biospecimen collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change.”

Example:

Name: Mary Smith
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID): 1234567
Nearest person month worked: 5

Contribution to Project: Ms. Smith has performed work in the area of combined error-control and constrained coding.
Funding Support: The Ford Foundation (Complete only if the funding support is provided from other than this award).

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
 Project Role: Project Leader Project 1
 Researcher Identifier (e.g. ORCID ID): N/A
 Nearest person month worked: 2 (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: 12 (annually)
 Contribution to Project: No change

Name: E Brigitte Gottschall
Project Role: Pulmonologist and occupational medicine physician
and clinical researcher, Project 1

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

Name: Lauren Zell-Baran
Project Role: Research Staff, Project 1
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 7 (annually)
Contribution to Project: No change

Name: James Crooks
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: Silpa Krefft, MD, MPH
Project Role: Co-Investigator, Project 1
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 3 (annually)
Contribution to Project: No change

Name: Matthew Strand
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: Scoring for HRCT scan interpretations including
quantitative dynamic expiratory imaging; imaging
support for GLIDE research case conference

Name: Claudia Onofrei,
Project Role: Co-I, Project 1
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1 (annually)
Contribution to Project: Subject research bronchoscopies and clinical data acquisition.

Name: William Janssen
Project Role: Co-Investigator, Project 2
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1 (annually)
Contribution to Project: No change

Name: Meghan Nelson
Project Role: Lab Researcher, Project 2
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 2 (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: 8 (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: 3 (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,
Project Role:	
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1 (annually)
Contribution to Project:	
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:	Shaan Gellatly, PhD
Project Role:	Post Doctoral Research Associate, Project 3
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1 (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:	12 (annually)
Contribution to Project:	Ms. Berman's work has been focused on investigating the role of Afghanistan particulate matter in regulating airway inflammation and airway obstruction (hyperresponsiveness) related to asthma. She is currently working on generating data for finishing two manuscripts. In addition, she is involved in studying the effects of Afghanistan particulate matter on cigarette smoke-mediated airway inflammation using the human airway epithelial cell air-liquid interface culture model.

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:	Meghan Cromie, PhD
Project Role:	Research Associate, 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:	4 (annually)
Contribution to Project:	No change

Name:	Nathan Jackson
Project Role:	Lead Bioinformatics Analyst, Project 4
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1 (annually)
Contribution to Project:	No change

Name:	Katie Li
Project Role:	Bioinformatics Analyst Trainee, Project 4
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	7 (annually)
Contribution to Project:	No change

Name:	Cydne 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: Geoffrey Plumlee
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: Less than 1
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: Nochange

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
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
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?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Dr. Downey (Project PI) was awarded UH3TR002445, Use of the Src Family Kinase Inhibitor Saracatinib in the Treatment of Pulmonary Fibrosis, which is a continuation of previous project, UG3TR002445 of the same title. Dr. Downey will continue to provide 1.2 calendar months of effort to this project, which will have no effect on his continued work on this project. Dr. Max Seibold (Project 4) was awarded a multi-PI project, R01HL117004 entitled, The Airway Functional Genomics of Bronchodilator Drug Response in Minority Children with Asthma with effort of 1.2 calendar months; Multi-PI project, U01HL138626, Natural History of Viral Induced Airway Dysfunction and Asthma in Minority Children, with budgeted effort of 1.8 calendar months. In addition, Dr. Seibold is receiving support on project, PO1HL107202, Exploring the Biology of Persistent Type 2 Airway Niches in Asthma, which is a continuation of project P01HL107202, Innate and Adaptive Immune Responses in Th2-High Asthma at 0.9 calendar months. Due to the completion of several projects Dr. Seibold was collaborating on, the addition of these newly funded projects will have no effect on his performance on project 4 of this research. Dr. Noah Zaitlen (Project 4) is receiving funding support from project R01CA227466, Genetics of Immune Related Adverse Events and Response to Immunotherapy (ZIV), in the amount of 0.24 calendar months annually. In addition, he is receiving support in the amount of 1.2 calendar months on the project, Improved Methods for Inference of Genotype-Specific Response to Environmental Toxins (AYROLES). The addition of these new projects will not affect the effort Dr. Zaitlen performs on this project. Dr. Karen Mumy (NAMRU-D) was awarded funding from the U.S. Navy PMA-273 Program Office entitled, The Potential Effect of Aviation Relevant Chemicals on Physiological Episodes at 1.2 calendar months. The addition of this research will not affect her work on this project.

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:

Organization Name:

Location of Organization: (if foreign location list country)

Partner's contribution to the project (identify one or more)

- Financial support;
- In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);
- Facilities (e.g., project staff use the partner's facilities for project activities);
- Collaboration (e.g., partner's staff work with project staff on the project);
- Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and
- Other.

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: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.

Please see attached Quad chart.

9. **APPENDICES:** Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a *curriculum vitae*, patent applications, study questionnaires, and surveys, etc.

Mechanisms and Treatment of Deployment-Related Lung Injury: Repair of the Injured Epithelium

PR150109 Year 3 Quarter 3 Report

W81XWH-16-2-0018

PI: Gregory P Downey, MD

Org: National Jewish Health

Award Amount: \$9,558,794

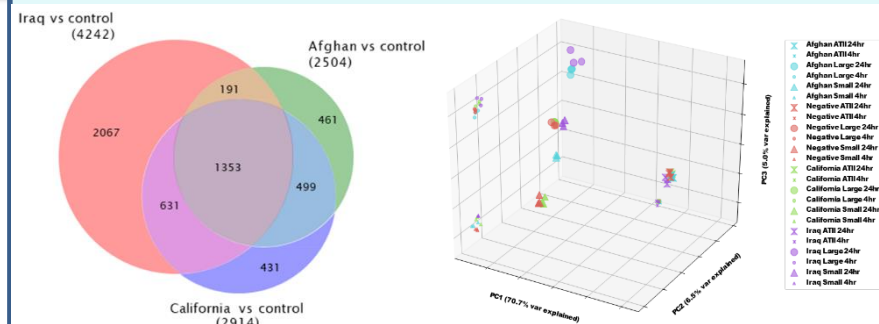


Study/Product Aim(s)

- Establish clinical infrastructure and processes for recruitment of study subjects (deployers and controls) from NJH Deployment Lung Clinic.
- Establish *in vitro* and animal models of exposure of alveolar epithelial cells to airborne PM and combined effects of physical, chemical, and infectious stimuli.
- Establish *in vitro* and animal model of exposure of bronchial epithelial cells to airborne PM.
- 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.

Approach

We will employ a combined approach collecting physiological and radiological studies on previously deployed cohort of military personnel, cellular and molecular analysis of biological samples (epithelial cells) from this cohort, and preclinical *in vitro* and animal models of epithelial injury induced by exposure to particulate matter (PM) plus physical, chemical and biological agents.



Transcriptional alterations in primary human large and small airway epithelial cells induced by PM from Iraq and Afghanistan compared to China Lake

Accomplishments: We established the NJH Deployment Lung Center, obtained IRB/HRPO approval and continue to actively recruit. We are analyzing CT scans of deployers. We have established *in vitro* models and are assessing effects of PM on respiratory epithelial cells using RNAseq. We have obtained IACUC and ACURO approval for animal experiments and are studying the effects of instilled or aerosolized PM from Iraq and Afghanistan on lung injury and comparing the effects of a second stimulus such as blast overpressure lung injury and influenza infection.

Timeline and Cost

Activities	CY	17	18	19	20	21
Establish clinical cohort of deployers and controls at NJH						
Establish <i>in vitro</i> and animal models of PM induced alveolar epithelial injury						
Establish <i>in vitro</i> and animal models of PM induced bronchial epithelial injury						
Determine molecular biomarkers of deployment related lung diseases						
Estimated Budget (\$K)		\$2000	\$2000	\$2000	\$2000	\$2000

Goals/Milestones

CY16/17 Goals – Established Deployment Lung Disease Clinic and Preclinical Models of Deployment lung disease

- ✓ NJH IRB and HRPO approval; NJH IACUC and ACURO approval

CY17/18 Goals – HRPO approval; Recruit deployers and controls and begin physiological testing of human subjects; Begin experiments on animal models with inhalational exposure to PM; Molecular testing of respiratory epithelia

- ✓ Recruitment of Deployers underway, animal models and *in vitro* experiments underway; molecular testing of respiratory epithelia using RNASeq underway

CY18/19 Goals – Recruit deployer subjects and controls; *in vitro* and animal experiments; molecular testing of respiratory epithelia

- ✓ Recruitment of deployers, animal models and *in vitro* experiments underway; focus on characterizing effects of PM on respiratory epithelia and macrophages using RNASeq

CY19/20 Goals – Recruit deployer subjects and controls; *in vitro* and animal experiments; molecular testing of respiratory epithelia

CY20/21 Goal – Complete recruitment of subjects; analyze physiological and imaging data; complete animal and *in vitro* studies; analyze and publish data

Comments/Challenges/Issues/Concerns

Budget Expenditure to Date

Projected Expenditure: \$5,712,400

Actual Expenditure: \$4,561,634

Updated: (10/15/2019)