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14. ABSTRACT

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

15. SUBJECT TERMS-
None listed

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1. **INTRODUCTION:** 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.

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

Progress

- In December 2016, we hired a Clinical Research Coordinator who has been trained on the GLIDE (**G**ROUP on **L**UNG **I**NJURY from **DE**PLOYMENT) study protocol and aims. She has completed CITI training and REDCap database user training for study IRB requirements including subject recruitment and consenting. She has become proficient in the use of the REDCap database and data entry. She completed spirometry and LCI training in Jan/Feb 2017, and is NIOSH-certified for spirometry testing.
- We developed a GLIDE study flow sheet for human data and samples; a process for study subject consenting and scheduling; and obtained National Jewish Health (NJH) IRB approval for the GLIDE Study in December 2016. We submitted the study to HRPO in March 2017 and are still awaiting approval. We continue to enroll patients in our existing Deployment Lung Disease Registry and Biorepository (not using funds from the current grant), and will contact these patients to explore their interest in participating as study subjects in GLIDE when the final HRPO approval has been obtained.
- A comprehensive REDCap database for the clinical components of this study has been developed, including military and other occupational exposure data, medical and smoking histories, symptoms, and the available spectrum of lung function testing including body plethysmographic PFTs, methacholine challenge and cardiopulmonary exercise testing.
- We completed processes for linking the database with the National Jewish Electronic Health Record (Allscripts), and have pilot tested the procedures for downloading all pulmonary function test results from the EMR into REDCap.
- The GLIDE Study Outreach Committee has been assembled, met four times, and identified a multifaceted approach to study subject recruitment.
- We developed a Deployment-Related Lung Disease brochure for outreach and study subject recruitment.
- We updated the National Jewish Health website to include information on, and links to, the GLIDE study.
- We created clinical research flowsheets for scheduling and testing both local and out of state deployers who consent to participate in the GLIDE study.
- The Project 1 lead and coordinator met with the NJH clinical administrative leadership to assure that our GLIDE Study efforts intersect efficiently with institutional clinical intake and scheduling processes.
- We developed and disseminated a Deployment Lung Disease fact sheet for use by NJH Call Center staff to assure that deployers seeking evaluation are directed to the Deployment Lung Center for scheduling. We met with Call Center staff to educate them on our clinical research efforts and to enlist their help in the intake and scheduling processes.

- A clinical questionnaire has been created that is specific for deployers who seek evaluation at NJH.
- We prepared a Clinical Information Packet that will be sent to deployers who scheduled clinical evaluation at NJH. This packet provides links to the website and information on the GLIDE Study for their consideration.
- As we prepare for study subject recruitment and testing (pending HRPO approval), a second GLIDE Study coordinator was hired in August 2017. She has completed NJH orientation, CITI training and REDCap database user training. She is scheduled for spirometry training with NIOSH certification, and is becoming familiar with the protocols and procedures that are central to the GLIDE Study. She will work with the other study coordinator to complete questionnaires on study subjects; do data entry; assist with outreach and recruitment; obtain study samples; perform LCI and spirometry testing; help coordinate scheduling; and work closely with GLIDE co-investigators and study staff.

Subtask 2. Establish protocols for bronchoscopies and bronchoalveolar lavage (BAL) and collection of nasal and bronchial brushings. Develop live cell core protocols and procedures.

Progress

- We established a protocol with all necessary consents for bronchoscopies and bronchoalveolar lavage (BAL) and for collection of nasal and bronchial brushings for deployer study subjects.
- We obtained permission for use of the NJH live cell core through IRB approved protocols and procedures for GLIDE study controls.
- We have developed standard operating procedures and protocols for all bronchoscopies conducted at National Jewish Health including bronchoalveolar lavage, bronchial epithelial brushing, and nasal epithelial brushing methods so that samples collected from patients with asthma and deployers will be directly comparable.
- We established standardized methods for culture of bronchial and nasal epithelial cells and for cryopreservation of cultured epithelial cells

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

Progress

- GLIDE Study staff and investigators have developed and finalized standardized methods for collecting and analyzing these samples. Once HRPO approval for GLIDE is obtained, we will begin collecting samples from deployers.

Subtask 4. Establish protocols for collection of samples including lung blocks and cells from VATS biopsies done for clinical diagnosis.

Progress

- We have an IRB-approved consent form for collection of samples including lung blocks and cells from VATS biopsies done for clinical diagnosis.

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

Progress

We continue to collect and analyze clinical VATS biopsies on symptomatic deployers seen in our clinical center of excellence. We have met with our colleagues at the US Geological Survey and finalized methods to analyze VATS lung biopsies. We await HRPO approval in order to accession these clinical samples with informed consent for GLIDE Study purposes.

Subtask 6. Establish LCI using Multiple Breath Washout technique:

Progress

- We developed a Procedures Manual for LCI measurements.
- In October 2016, we sent two GLIDE study members to Toronto for comprehensive training in LCI testing and quality assurance.
- We established a dedicated LCI testing space in the Clinical Translational Research Center at NJH where we will also perform GLIDE study subject consenting.
- We performed LCI testing for a different research study that enabled the GLIDE Study coordinator and other staff to gain additional proficiency in LCI performance and quality.

Subtask 7: Complete LCI measurement from deployers and controls.

Progress

- We continue to perform LCI tests on non-GLIDE Study participants. We await HRPO approval to enroll deployers and controls with informed consent for GLIDE Study purposes.

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

Progress

- Our protocol includes an IRB-approved consent form for chest CT quantitative pulmonary and textural analysis.
- We have established a dataset of CT regions of interest for airway wall thickening, centrilobular nodularity, and air trapping, and tested several classifier configurations in preparation for a deep learning approach to CT classification of signs of large and small airway injury.
- We have prepared a research CT acquisition form for use in the NJH Imaging Department
- A process for assuring that Imaging staff are familiar with the GLIDE CT acquisition protocol and paperwork has been developed through several meetings and communications.
- We have developed a scoring sheet for characterizing imaging findings from deployer/control CT scans, and will finish pilot testing the scoring sheet by September 2017.
- Pending the final version of the CT scoring sheet, we will build this portion of the REDCap database and begin data acquisition and data entry.

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

Progress

- With support from an experienced statistician/database expert, we have queried the control CT scan database to establish demographic linkages (including age, smoking status and gender) with deployer study subjects.
- A process for independent scoring of deployer and control CT scans by two cardiothoracic radiologists has been developed, with a plan for consensus reads to resolve discrepancies as they arise.
- To assure accurate data entry of CT scoring information, Dr. Richards has completed REDCap database user training and will assist in this data documentation.
- We await HRPO approval for obtaining and analyzing chest CT scans with quantitative textural analysis on deployers.

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

Progress

- We have established methods for elemental and PM profile analyses of lung tissue in collaboration with the US Geological Survey.

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

- We have established murine (MLE-12) and human (BEAS2B) lung epithelial cell culture conditions on ECM coated tissue culture plastic and in Transwell semipermeable inserts.
- We have established conditions whereby exposure of lung epithelial cells to particulate matter (PM) reproducibly induces production of inflammatory cytokines (IL-1, IL-6, TNF-α) and at higher concentrations, cell death.
- We have developed a reproducible scratch model (physical force) of epithelial injury and repair using MLE-12 cells with quantification by transmitted light microscopy and image analysis.
- We have established reproducible models of PM, chemical/oxidant (bleomycin) and influenza virus-induced epithelial injury using MLE-12 cells. For these studies, we have changed from using trypan blue exclusion to LDH release by ELISA using a Cytotoxocity assay kit (Sigma) as this is much more reproducible and accounts for differences in rates of cell division.

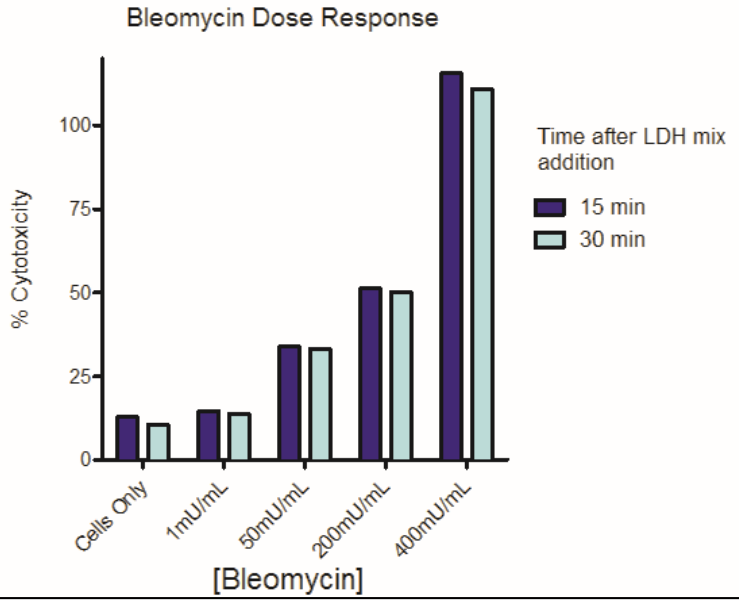
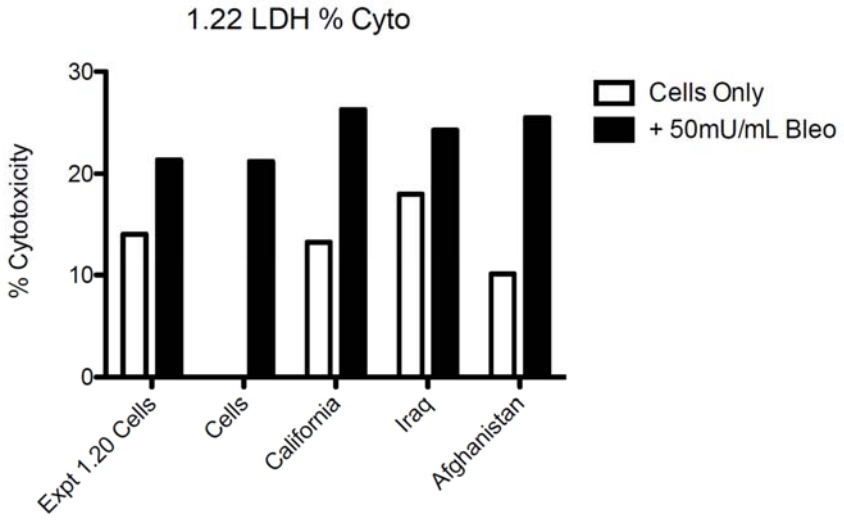


Fig. 1. Quantification of bleomycin-induced epithelial cytotoxicity using LDH release assay (Sigma). Note the dose-response relationship in the cytotoxicity the cytotoxicity induced by bleomycin.

Using this method, we have demonstrated enhanced cytotoxicity of epithelial cells with the



combination of PM and bleomycin exposure (**Fig. 2**)

Fig. 2. Quantification of bleomycin-induced epithelial injury and effect of prior exposure to particulate matter (PM). Note that prior exposure to PM from Iraq or Afghanistan enhances the cytotoxicity of bleomycin. In this experiment, the effect of prior exposure to PM from California also potentiated the cytotoxicity of bleomycin.

- We have begun to implement assays to quantify apoptotic cell death of lung epithelial cells. We have tested several methods and decided on the caspase 3 assay. In our preliminary studies, we have observed that bleomycin induces apoptotic cell death of the cultured MLE-12 epithelial cells in a dose dependent manner.
- We have established conditions to study the effects of a second injurious stimulus ('second hit') after priming the cells with prior exposure to particulate matter (PM). Our initial experiments confirm our preliminary data that pretreatment with PM from Iraq or Afghanistan (5- 10 $\mu g/cm^2$) significantly delay wound closure in the scratch model.

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. These experiments will require human alveolar epithelial cells from 10 explanted donor lungs from the International Institute for the Advancement of Medicine.

Progress

- We have established the system and specific techniques to culture human primary alveolar epithelial cells from explanted donor lungs not used for transplant (obtained from Colorado Donor Alliance and from the International Institute for the Advancement of Medicine. These cultured epithelial cells form monolayers in an air liquid interface. In the next year, we will study the effects of injurious stimuli on the primary cells after we have completed the studies with the epithelial cell lines.
- **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 will plan to start these studies after completion of Subtask 2.

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

- We obtained local (National Jewish Health) IACUC approval for the proposed animal experiments on 01-AUG-2016 and USAMRMC/ACURO Animal Care and Use Review Office approval on 14-NOV-2016.
- We completed a titration of the new lot of bleomycin using a single i.t. dose. We found the optimal dose to be 2 U/kg and we will use this dose for all future experiments.
- We obtained new samples of PM from Afghanistan from Drs. Karen Mumy and Brian Wong at NAMRU-Dayton and have tested them in our mouse model. There are much larger quantities of this source of PM available to us (~2 Kg) that will be necessary to complete the aerosolization studies (several weeks of daily exposure). This sample of PM was generated from sterilized topsoil/sand collected in Afghanistan that was aerosolized using the Wright dust feeder in the inhalational toxicology facility at NAMRU-Dayton. The respirable fraction (~ 5 μm) was collected on filters, eluted and sent to us to test.
- We completed initial testing of the effects of particulate matter (PM) from Afghanistan and compared this to the effects of PM from Iraq. We have used both single dose and

multiple dose (5 instillations every other day) exposures. In the multiple dose exposure model (5 doses given over 10 days) we observed that instillation of the PM from Iraq or Afghanistan induced an increase in lung collagen content measured by lung hydroxyproline (**Fig. 2**) and by histochemical analysis using H&E, trichrome (**Fig. 3**) and picrosirius red staining. We also observed inflammation centered around the alveolar ducts with PM from both Iraq and Afghanistan.

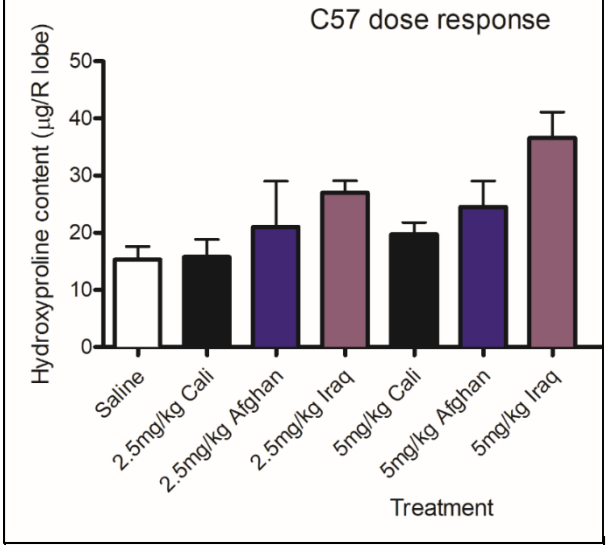


Fig. 2. Pulmonary fibrosis as measured by an increase in lung collagen content induced by multiple dose (5) instillation of PM from Iraq and and Afghanistan as measured by hydroxyproline. PM from California (Cali) was used as a control.

- We also studied the effects of PM from the desert area of California (Fort Irwin) as a control. These PM induced much less inflammation and fibrosis as compared to the PM from Iraq and Afghanistan.
- We are planning to conduct a formal dose response up to 10 mg/kg for the PM from Iraq and Afghanistan to ensure that we are using an optimal dose. We have obtained approval from the NJH IACUC for this increase of PM dose and submitted an amendment to ACURO in Aug 2017.
- We have sent samples of the PM from Afghanistan to the USGS for characterization of the size of the particles and their chemical composition.

Difficulties encountered. The DoD funding to NAMRU Dayton was delayed until January 2017. They have modified their caging to fit mice and tested the aerosolization of sand/top soil from Afghanistan. They submitted and received approval from their local WPAFB IACUC in June 2017 and from ACURO in July 2017. They are now set up for the inhalational exposures to PM, which will begin in the fall of 2017.

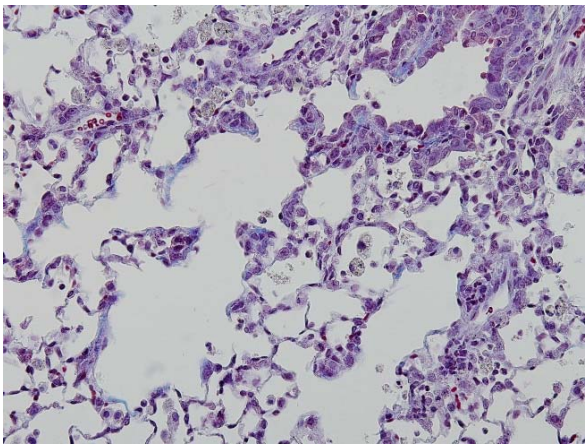


Fig. 3. Instillation of multiple doses of PM from Afghanistan induces inflammation centered on the terminal bronchioles and alveolar ducts. Collagen deposition can be detected in these areas – stains blue (Trichrome 20X magnification)

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

- We have started to develop influenza culture techniques beginning with titration of titers by plaque forming assay using MDCK cells.
- We have conducted preliminary experiments in mice using intranasal instillation of H1N1-PR8-IAV and demonstrated that we are able to reproducibly infect C57Bl/6 mice with influenzae and that this induces a robust viral pneumonia with diffuse pulmonary inflammation.
-

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

Progress

- We have received approval from the University of Colorado IRB (the samples are stored at the University of Colorado Hospital) but do not yet have final approval from HRPO.

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 above, we have not yet started these studies because we are awaiting HRPO approval.

Subtask 8. Prepare and submit manuscripts for publication.

Progress

No manuscripts have been submitted to date.

Milestones Achieved:

1. Local (National Jewish Health) IACUC Approval 01-AUG-2016
2. USAMRC/ACURO approval 14-NOV-2016
3. *In vitro* epithelial cell culture model protocols in development
4. *In vivo* murine model protocols in development
5. NJH IRB Approval Dec-2016. Awaiting HRPO approval

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.

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

We have accomplished the development of live cell core protocols and procedures. We have our local National Jewish Health IACUC and USAMRMC Animal Care and Use Review Office Approval for mouse experiments

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

Progress

- We have developed and refined methods to study effects of PM allergic stimuli and cigarette smoke exposure on bronchial epithelial cells *in vitro*.
- Switching dust from Iraqi dust to Afghanistan dust to be comparable with dust to be used in animal models of dust exposure.

- **Method development of culturing asthmatic epithelial cells**

  - We examined whether airborne particulate matter (APM) from Iraq and cigarette smoke promote asthmatic airway epithelial cell inflammatory response and injury. Nasal brushing cells from a patient with asthma were cultured at air-liquid interface and then stimulated with APM (50μg/cm^2), cigarette smoke (CSE 1%) or combination of both. After 24 and 48 hr of treatment, we measured pro-inflammatory cytokine IL-8 (ELISA kit), transepithelial electrical resistance (TEER) to indicate cell barrier function, and E-cadherin to indicate the integrity of cell junctions. We found that APM, CS and particularly the combination of both increased the production of IL-8 (**Figure 1**). Additionally, APM and CS reduced the TEER at 24 hr and E-cadherin at 48 hr. Our initial experiment suggests the pro-inflammatory and injurious effects of APM and CS on upper airway epithelial cells from an asthmatic subject. We will repeat this experiment in more asthmatics as well in normal subjects using the banked nasal and bronchial epithelial cells from brushings. *These studies helped us establish our protocols for sampling epithelial cells obtained from human brushings, air-liquid interface culturing of primary ells from brushing, and endpoint measurements of inflammation and injury.*

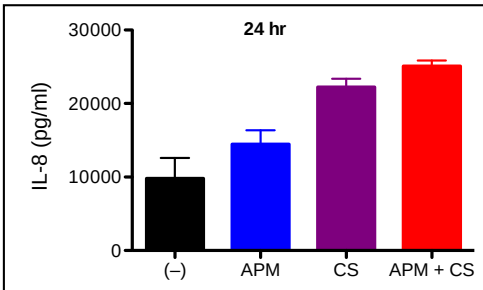


Figure 1. Primary nasal epithelial cells from asthmatic subject exposed for 24 h to airborne particulate matter (APM, 50μg/cm^2) or cigarette smoke (CS, 1%) stimulated IL-8 release.

- **Method development of culturing normal human bronchial epithelial cells**
  - We tested the cytotoxic effects of APM from Iraq in the absence or presence of cigarette smoke exposure in primary normal human brushed bronchial epithelial cells. We used the submerged cell culture system to determine the sensitivity of injured airway epithelium (basal cells) to APM and cigarette smoke exposure. Briefly, cells under submerged culture conditions were exposed to APM from Iraq (10 µg/cm^2) with or without cigarette smoke exposure. At 6, 24 and 48 hours post treatment, cell supernatants were collected to measure lactate dehydrogenase (LDH) as a measure of cytotoxicity. LDH levels were significantly higher in cells exposed to both PM and cigarette smoke at all the three time points than non-treated cells or cells treated with PM or cigarette smoke alone. As our data using the intact well-differentiated airway epithelial cells did not show any significant cytotoxic effects of PM and cigarette smoke, our results suggest that in injured airways, exposure to PM particularly in the presence of cigarette smoke exposure could be detrimental to airway functions.

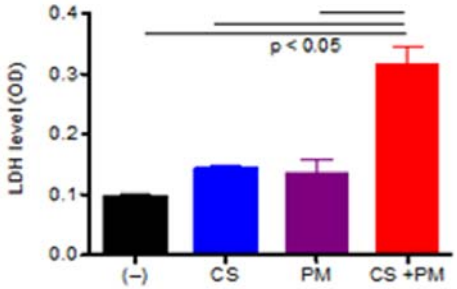


Figure 2. Primary bronchial epithelial cells from control subject exposed for 24 h to Iraq particulate matter (PM, 10µg/cm^2) or cigarette smoke (CS, 1%) or combination and lactate dehydrogenase (LDH) release was measured as marker of cytotoxicity. (n=4 replicates/group)

  - Using the above cell culture system, we also determined if oxidative stress may serve as a mechanism whereby PM induces airway epithelial pro-inflammatory (e.g., IL-8) responses. Using N-Acetyl-L-cysteine (NAC), an inhibitor of reactive oxygen species (ROS), we found that NAC was not able to inhibit PM-induced IL-8 production although it suppressed cigarette smoke-induced IL-8. These data suggest that in injured airway epithelium, PM does not appear to induce pro-inflammatory response through the oxidative stress pathway.
- **Effect of particulate matter (PM) from Afghanistan on airway epithelial cell morphometry, cytotoxicity and inflammation.**
  - Due to a potential change in the source of PM from Iraq to Afghanistan for our animal exposures, our human data would be in line with the animal studies to further reveal the impact of PM on airway structural changes (e.g., remodeling) and inflammation. We

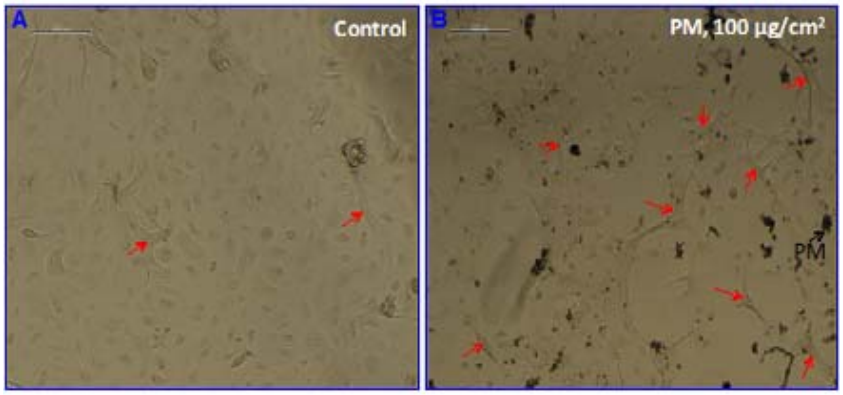


Figure 3. Exposure to particulate matter (PM, 100 ug/cm2) from Afghanistan for 72 hours reduced the density of cultured primary human nasal epithelial cells. Notably as compared to the control (A), fibroblast-like cells (red arrows) were increased following PM exposure (B).

tested if particulate matter (PM) from Afghanistan affects human primary airway epithelial cell morphology, cytotoxicity and inflammation.

- PM effects on cell morphometry. Briefly, we obtained primary nasal epithelial cells from a healthy subject, and cultured the cells under the submerged condition in the presence or absence of PM from Afghanistan at 5, 10, 15, 50 and 100 μg/cm^2. At 24, 48, 72 and 96 hours after PM treatment, cells were observed under a phase contrast microscope to examine cell morphological changes. When cells were exposed to lowers doses (5 to 15 μg/cm^2) of PM, no significant changes in cell morphology were observed. However, at higher PM doses (50 and 100 μg/cm^2), nasal epithelial cells demonstrated two major morphological changes. First, the cell density was reduced by about 50% (**Figure 3**) especially at 72 and 96 hours post PM exposure, suggesting the cytotoxic effect of PM. Second, there are more fibroblast-like cells following PM exposure, indicating the enhancing effects of PM on epithelial-mesenchymal transition (EMT). As EMT is involved in tissue repair and fibrosis, our data suggest that extended exposure to PM may result in airway fibrosis, remodeling and airway obstruction. We will confirm this idea by measuring markers of EMT such as vimentin and α-smooth muscle actin.
- PM dose and time response on cell cytotoxicity. Using the same methodology described above, we examined changes in cytotoxicity in human nasal cell cultures exposed to PM from Afghanistan at 5, 10, 15, 50 and 100 μg/cm^2. Cytotoxicity was assessed by measuring the release of lactate dehydrogenase (LDH) at 24, 48, 72 and 96 hours after PM treatment (**Figure 4**). An increase in LDH release compared with control is reflective of cytotoxicity. Human nasal epithelial cells well tolerated PM exposures up to 100ug/cm2. There was some loss in LDH activity at 100 ug/cm2, which may be due to the PM at this concentration inhibiting the LDH enzyme activity. We plan to check this possibility by using another method to measure cytotoxicity such as a live cell dye assay, calcein AM.

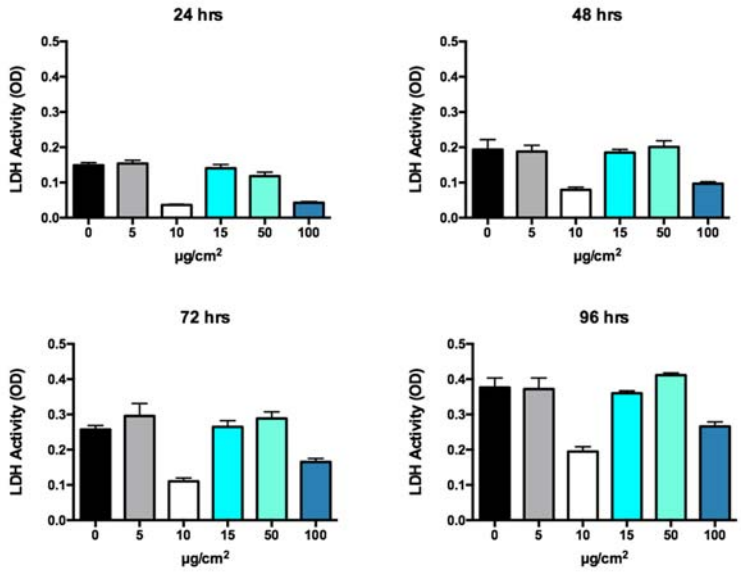


Figure 4. Cultured primary human nasal epithelial cells can tolerate increasing levels of particulate matter (PM, up to 100 μg/cm^2) from Afghanistan for 96 hours without evidence of cytotoxicity as measured by lactate dehydrogenase (LDH) release.

- PM dose and time response on cell inflammatory response. We measured the pro-inflammatory cytokine IL-8 in PM-exposed cells. We examined changes in IL-8 release in human nasal cell cultures exposed to PM from Afghanistan at 5, 10, 15, 50 and 100 µg/cm^2. IL-8 release was assessed by ELISA at 24, 48, 72 and 96 hours after PM treatment (**Figure 5**). PM increased IL-8 in a dose-dependent manner. Interstingly, we did not observe any significant cytotoxic effects of PM, as indicated by changes in LDH release. Our data futher suggest the active role of PM in airway epithelial remodeling and inflamamtion.

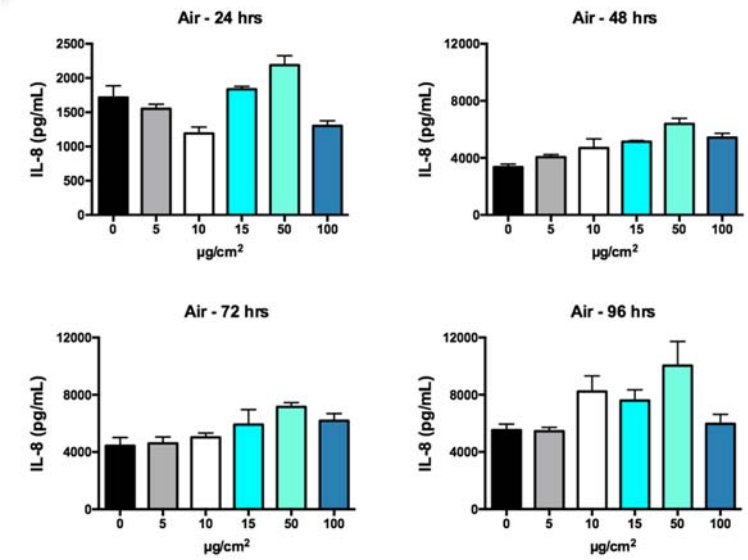


Figure 5. Cultured primary human nasal epithelial cells exposed to increasing levels of particulate matter (PM, up to 100 µg/cm^2) from Afghanistan show dose response increase in inflammation as measured by IL-8 release.

- Effects of PM and cigarette smoke exposures on cell inflammatory responses. We measured the pro-inflammatory cytokine IL-8 in PM-exposed cells in combination with cigarette smoke. We examined changes in IL-8 release in human nasal cell cultures exposed to PM from Afghanistan at 5, 10, 15, 50 and 100 µg/cm^2 in the presence of absence of cigarette smoke extract (10%). IL-8 release was assessed by ELISA at 24, 48, 72 and 96 hours after PM treatment in the presence or absence of cigarette smoke extract (10%) (**Figure 6**).

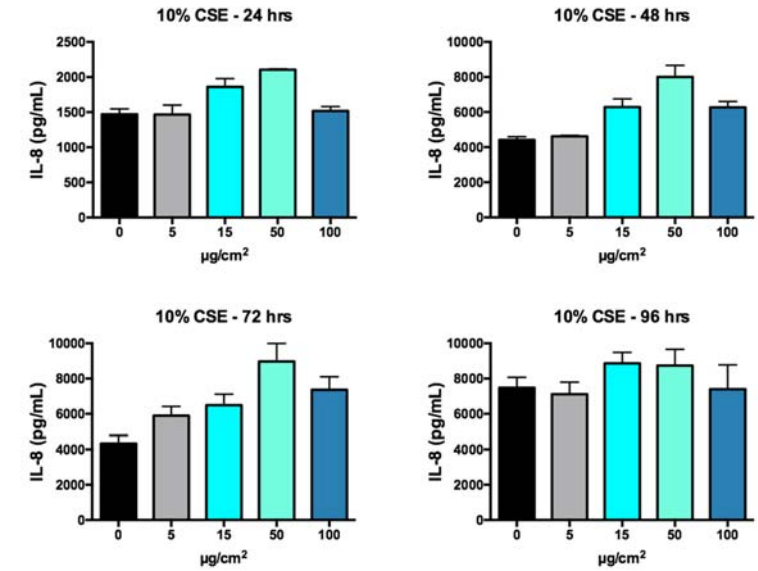


Figure 6. Cultured primary human nasal epithelial cells exposed to increasing levels of particulate matter (PM, up to 100 µg/cm^2) from Afghanistan show dose response increase in inflammation as measured by IL-8 release that is enhanced by cigarette smoke extract (CSE).

Again we see a dose response effect of the PM on Il-8 release that is maximal at 72 hours and that this dose response is enhance by cigarette smoke exposure. We still see a decrease in IL-8 release at the 100 ug/cm2 PM concentrations that may be due to toxicity. These studies suggest we limit our PM exposures to 50 ug/cm2 and 72 hour exposures.

- **Effect of particulate matter (PM) from Afghanistan on asthma phenotype (IL-13) airway epithelial cell inflammation.**
  - We have been investigating using an IL-13 treatment of airway epithelial cells to produce an asthma inflammatory phenotype model that could be used to investigate mechanistic interactions with PM exposures with or without IL-13 or cigarette smoke exposures. The model uses primary brush bronchial airway epithelial cells that are expanded on feeder cells. These cells are grown in air-liquid interface (ALI) until tight junctions occur (resistance measurement >50 ohms). ALI was achieved on day 12 where a group of cells were also treated with IL-13 (10ng/ml) and on day 14 treated with PM (5-50ug/cm2) and/or whole cigarette smoke (WCS) vapor (1 hr). Cell apical fluids were measured for changes in IL-8, LDH and TGFb2 96 hours after treatments.
  - <u>PM dose and time response on cell cytotoxicity.</u> We examined changes in cytotoxicity in human bronchial cell cultures treated with IL-13 (10ng/ml) and then exposed to PM from Afghanistan at 5,15, and 50 µg/cm^2 with or with WCS vapor exposure. Cytotoxicity was assessed by measuring the release of lactate dehydrogenase (LDH) at 96 hours after PM or WCS treatments (**Figure 7**). Again we see very little evidence of cytotoxicity with PM levels up to 50ug/cm2. The WCS exposure was associated with a small increase in cell cytotoxicity that was not affected by PM, but was decreased by IL-13 treatment. This data suggests that an asthma airway epithelial cell phenotype may be resistant to cigarette smoke-induced injury.

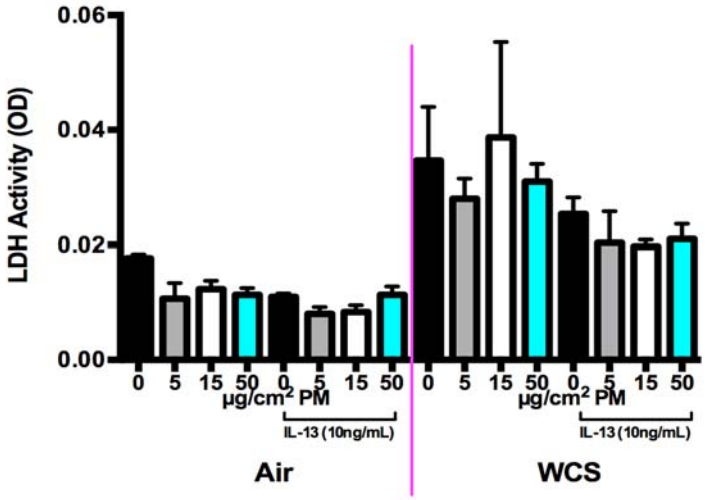


Figure 7. Cultured primary human bronchial epithelial cells can tolerate increasing levels of particulate matter (PM, up to 50 µg/cm^2) from Afghanistan for 96 hours without evidence of cytotoxicity as measured by lactate dehydrogenase (LDH) release. Interestingly, WCS increased LDH release that was decreased with IL-13 treatment.

- Effects of PM and cigarette smoke exposures on cell inflammatory responses. We measured the pro-inflammatory cytokine IL-8 in IL-13 (10ng/ml) treated bronchial epithelial cells exposed in combination with PM from Afghanistan at 5,15, and 50 µg/cm^2 with or with WCS vapor exposure. IL-8 release was assessed by ELISA at 96 hours after PM or WCS treatments (**Figure 8**). Cells exposed to PM at 96 hours had no increase in IL-8 release and IL-13 pre-treatment decreased this response. Interestingly, WCS exposure increased IL-8 release, which was not affected by PM exposure, and IL-13 pre-treatment enhanced this response in the PM treatment groups. These data suggest there are complex mechanisms and interactions between disease phenotypes, PM exposures and cigarette smoke.

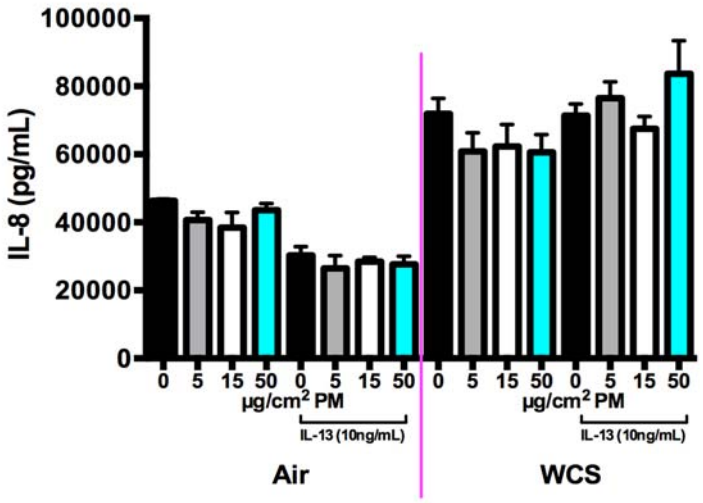


Figure 8. Cultured primary human bronchial epithelial cells exposed to increasing levels of particulate matter (PM, up to 50 µg/cm^2) from Afghanistan is enhanced by whole cigarette smoke. Interestingly, IL-13 treatment decreased PM inflammatory response but not in the WCS group.

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

Progress

- Pending HRPO approval so we can start recruiting deployer subjects for bronchial brushing

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

Progress

- We are in the process of setting this system up and are also working on the logistical challenges associated with timing PM exposures at NAMRU for projects in Tasks 2 and 3 animal studies which are pending IACUC and ACURO approvals.

- Method development of mouse cigarette smoke exposure models to be used in conjunction with Afghanistan PM exposures. We have developed whole animal cigarette smoke exposures using a TE-10 smoke exposure system (Teague Enterprise). Adult mice were exposed to cigarette smoke from Kentucky reference cigarettes (3R4F, Kentucky University) for 3h/day for 15 days. A FTC method of puffing cigarettes for 2 s once a minute at a volume of 35 cm^3 was utilized to generate side stream and mainstream CS. The TE-10 smoking system generated an environmental tobacco smoke by mixing mainstream and side stream smoke. The cigarette smoke was diluted with air to maintain a total suspended particulate matter average of 80 mg/m^3 and carbon monoxide levels below 300 ppm. Mice exposed to cigarette smoke for 3days/week for 15 days were examined for changes in lung inflammation by measuring cytokine levels (mouse analog of human IL-8, KC) in bronchoalveolar lavage fluid (BALF). Lung damage assessment was determined by measuring changes in lung tissue elastase activity. Mice exposed to 15 days of cigarette smoke had increased signs of lung inflammation (**Figure 9A**) and lung injury (**Figure 9B**). These studies demonstrate that we can successfully expose mice to cigarette smoke and will be able to test the effects of PM's interactions with cigarette smoke in vivo.

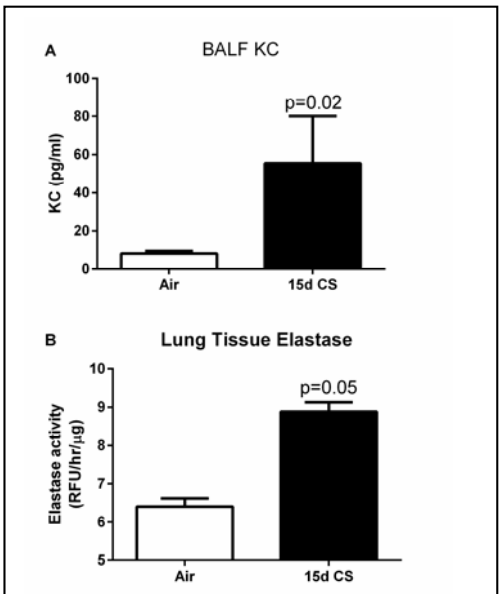


Figure 9. Mice exposed to 15 days of cigarette smoke (CS) had increased levels of lung inflammation marker KC (A) in their bronchoalveolar lavage fluid (BALF) and increased damage marker (elastase activity) in their lung tissue (B).

- Optimization of a mouse model of asthma induced by house dust mite (HDM) challenges. To optimize a mouse model of asthma or airway allergic inflammation, we performed a HDM dose response study, as well as a time course study. Two strains of mice (BALB/c and C57, male and female, 8 to 14 weeks old) were included in our optimization experiments. Mice, after isoflurane anesthesia, were intranasally sensitized with endotoxin-low HDM at 10, 20 or 100 µg/mouse in PBS or PBS (control) at day 1 and day 7, and then intranasally challenged with HDM at 10, 20 or 100 µg/mouse in 50 µl PBS or PBS (control). After 1, 3, 5 and 7 days of the last HDM challenge, we performed bronchoalveolar lavage (BAL) and lung histopathology to evaluate allergic inflammation including eosinophils and mucus production.

  We found that BAL eosinophil level is higher in C57 mice than BALB/c mice. There is a HDM dose response regarding BAL eosinophil levels. For example, in C57 mice, the mean % of BAL eosinophils at day 1 post 10 µg/mouse HDM challenge is about 20%, but it reaches about 60% post 100 µg/mouse HDM challenge. Notably, eosinophils decrease from day 1 to day 7 after the last HDM challenge. Our data suggest that we have successfully developed a mouse model of allergen challenges, which will be very useful for our upcoming experiments to test the in vivo effects of particulate matter (PM) from

Afghanistan on airway allergic inflammation, a key pathological feature in human asthma patients. Also, we have successfully measured airways hyperresponsiveness (AHR) to methacholine challenge in HDM-challenged mice by using the flexiVent system. As AHR is another key feature in human asthma, our expertise in AHR measurement will allow us to determine the detrimental effect of PM exposure on pulmonary function.

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

Progress

- Pending NAMRU's IACUC and ACURO approvals.

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

- We are working with the US Geological Survey to set this system up. Pending HRPO approval

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

Progress

- This will be done after Subtask 5 is completed. Pending HRPO approval

Subtask 7.

Progress

- No manuscripts have been submitted to date.

Milestones achieved:

1) Approval of local IACUC protocols for all Task 3 animal procedures (AS2831-06-19)
2) In vitro epithelial cell culture model protocol development.
3) In vivo animal exposure protocol development for cigarette smoke exposures and house dust mite allergen sensitization.

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

- In this first year developed laboratory methods to automate on a robot the production of RNA-seq libraries from the airway epithelial brushings to be used in this project. Specifically, we developed a robotic method to produce 96 PolyA-selected whole transcriptome RNA-seq libraries in a two-day protocol on the Beckman Coulter Biomek Fp instrument. This method produces libraries in a robust fashion, without biases and cross-contamination, and with a minimum of sample handling. This method was tested using primary airway epithelial, A549, and virus infected airway epithelial cells. Success of library preparation was judged by concentration of libraries generated, Agilent bioanalyzer traces, sequencing reads generated and transcriptome of the detected libraries. Additionally, we performed metagenomic analysis of the samples screened and confirmed that only virus infected samples contained virus reads and not the uninfected samples. This confirmed that virus RNA was not being transferred from the infected to uninfected samples during library preparation on the robot. In another project, this method was used to generate over 300 RNA-seq libraries with only 2 failures. Therefore the method is fully tested and implemented in our laboratory. As soon as the deployers are recruited this method will be apply to the RNA collected from nasal and bronchial epithelial cells.

- Additionally, we have been working with Drs. Rose, Chu, and Downey to set up the standard operating procedures for collection and processing of the airway epithelial brushings to be used in the biomarker and disease mechanism analyses. This includes designation of sampling area and technique, set up of sample barcoding system, biomolecule extraction protocol and distribution to investigators, and initial expansion of the airway epithelial cells.

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

Progress

- In this first year we have worked on generation of the analytical programs and computing code needed to run the biomarker and disease mechanism analyses of the RNA-seq data. Specifically, we have established code to run RNAseqQC on all fastq sequence files to be generated in this project. We have also established a pipeline to map the raw fastq data with GSNAP. Finally we have established code to extract the RNAseqQC results and GSNAP mapping results into a summary file. We have also established code to run HTSEQ on mapped RNA-seq files to create gene expression matrices that contain expression levels for all genes in all subjects. We have established code to run normalization of the gene expression matrices with DESEQ and to perform differential expression analysis. To measure alternative transcript gene expression levels we have optimized and put in place code to run Kalisto software. We are currently generating a consolidated pipeline to run all the code we have generated in this first year, for accomplishing the first level RNA-seq analysis of: (1) demultiplexing, (2) RNAseQC, (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

In this first year 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.

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

Progress

- We have been working with US Geological survey personnel to prepare airway samples for development of protocols to measure metal/PM in airway brushing specimens by LA-ICP-MS. Determination of the genetic factors will be undertaken once Subtask 3 is completed.

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.

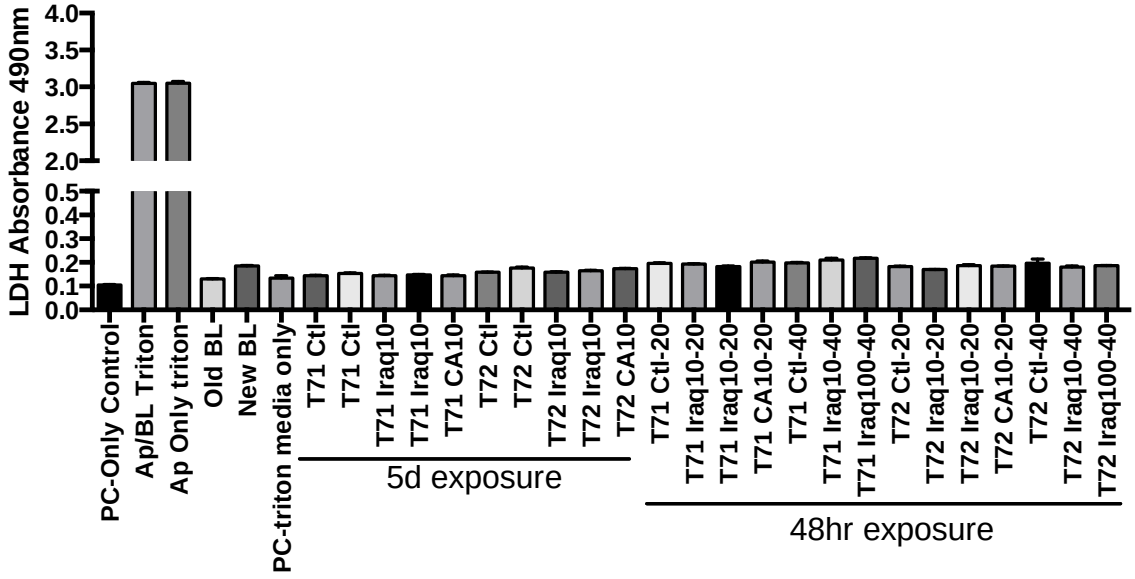
Progress

- In this first year, we have formalized efficient methods for the recovery and expansion of airway epithelial cells from airway brushings using modified Schlegel methods. These methods can be used in our project. However, a new protocol has been devised by STEMCELL technologies that promises even better expansion and recovery of airway stem cells from our airway epithelial brushings. We are currently testing this protocol and will determine in the coming months whether its performance exceeds our currently developed protocol.
- In the first year we have tested several different culture methods for nasal and bronchial epithelial cells at air-liquid interface. This testing has resulted in our adoption of the Pneumocult culture media and method for this project.
- We have settled on a protocol that allows both isolation of total RNA (sufficient for WTS) and generation of airway epithelial cultures. The extraction is performed with special processing and isolation of total RNA and DNA from an All-prep column available through Qiagen.
- We are working on a robotic protocol to automate RNA-seq library preparation from the in vitro airway epithelial cultures. This method is based on the Ampliseq whole transcriptome RNA-seq library prep and will be performed on the Beckman Coulter

Biomek Fp instrument which was used to develop the polyA RNA-seq library method detailed in subtask 1.

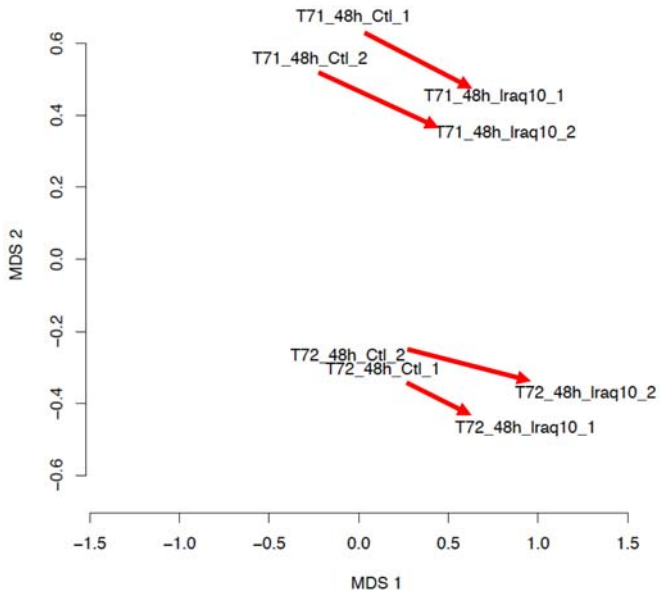
In the project first year we have performed airway epithelial exposure trials of PM matter from Iraq and California military bases. Preliminary experiments aimed to establish parameters for PM stimulation and delivery, of which the following variables were tested: dust delivery volume, concentration of dust used for stimulations, duration of dust stimulation, and source of dust. To measure the cytotoxic effect of the PM matter on the ALI cultures, a lactate dehydrogenase (LDH) assay was utilized to measure the presence of LDH in culture media in the event of cell lysis and death. Using PM concentrations ranging from 1ug/ml to 100ug/ml during stimulation, over the course of 48hrs and 5 days, none of the cultures exhibited LDH activity in the basolateral chamber of the cultures greater than that of unstimulated controls during each respective time course of treatment (Figure 1).

Figure 1. Toxicity of PM treatments of ALI airway epithelial cell cultures.



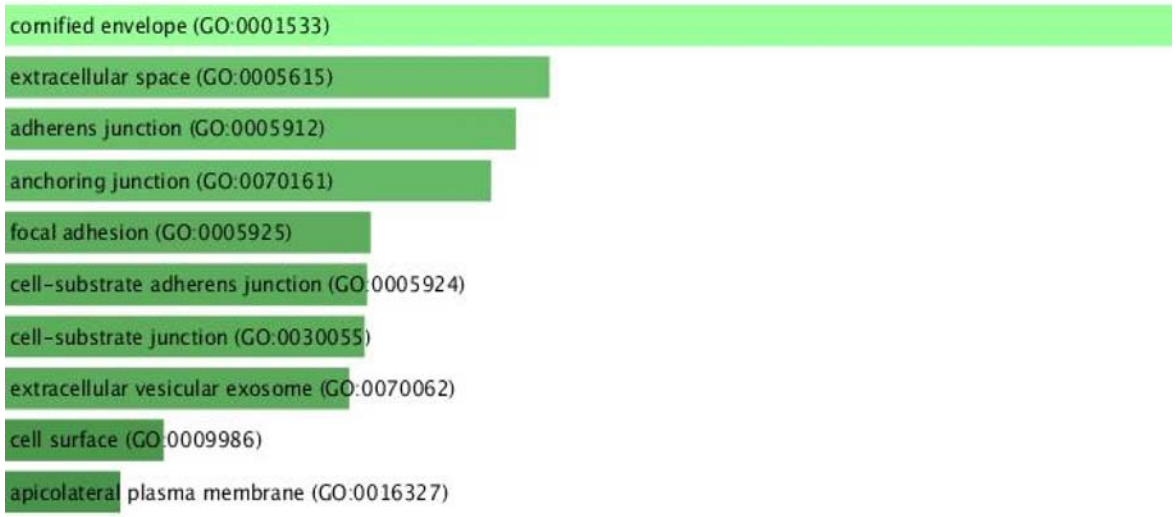
RNA-seq analysis was conducted on the 48-hour control and Iraq PM dust (100ug/ml) stimulated cultures to analyze the effects of delivery volume of PM, as well as to analyze the transcriptome changes that occur during stimulation. Comparing the apical delivery volumes of 20ul and 40ul for PM stimulation, differential expression analysis found no differentially expressed genes between the 2 treatment delivery volumes. When the 48 hour PM dust stimulated cultures were compared to 48 hour mock treated cultures, 2,543 genes were found to be differentially expressed, with 1,442 genes upregulated, and 1,101 gene down-regulated in dust treated airway epithelial cultures (FDR <0.05) (Figure 2).

Figure 2. MDS plot of samples using the most variant genes in the dataset.



Gene ontology analysis identified cellular components involved in cornified envelope development, secretion of extracellular components, and cell-substrate adherens junctions to be the most highly enriched gene ontology categories after Iraq APM dust stimulation (Figure 3).

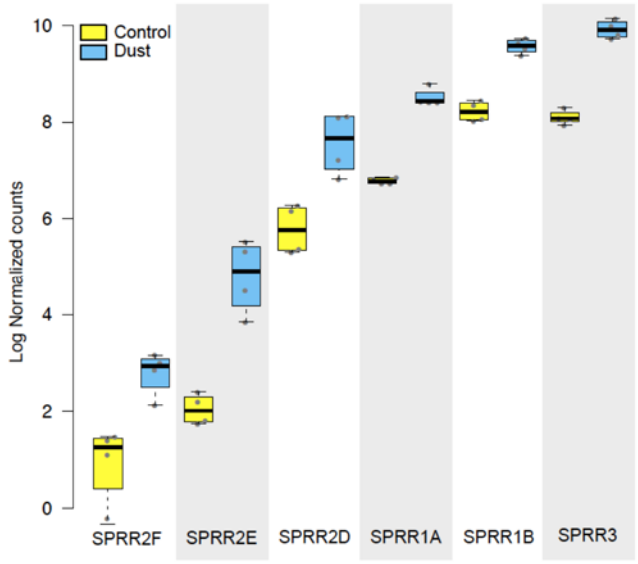
Figure 3. Significant Gene Ontology categories for PM stimulation upregulated genes.



Cellular cornification, otherwise known as squamous cell metaplasia or terminal cornification, is driven by a large complex of genes known as the Epidermal Differentiation Complex (EDC). This complex is activated by a variety of transcription factors, many of which are upregulated in response to Iraq PM stimulation (KLF3 log2FC=0.98, GRHL3 log2FC=1.78, ARNT log2FC=0.6). In a broader context, the EDC contains many families of genes involved in cornification, of which a large number of genes are upregulated upon Iraq PM dust stimulations. These families include the S100 protein family (7 upregulated genes), SFTP protein family (2

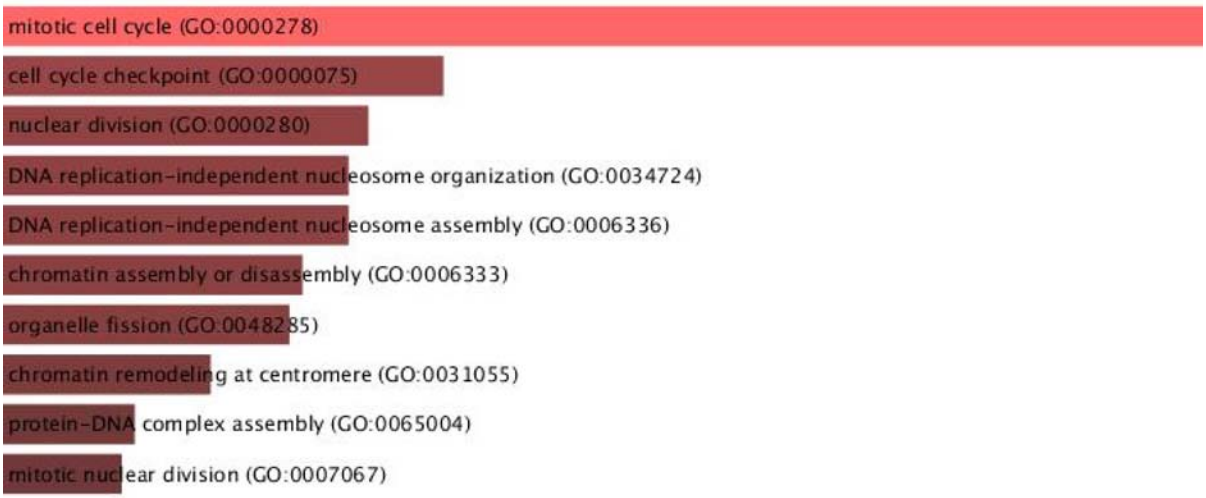
upregulated genes), and the small proline rich-repeat protein family (SPRR) (6 upregulated genes) (Figure 4).

Figure 4. SPRR genes upregulated in airway epithelial cells with PM treatment.



Additional upregulated genes involved in cornification but not found within the EDC include TGM1, cornifelin (CNFN), and involucrin (IVN) which encode markers, proteins, and enzymes involved in building the cornified cell envelope. Iraq PM dust stimulation also upregulates a variety of genes involved in the secretion of inflammatory mediators including IL36a, IL36g, and IL-8, which have all been implicated in epithelial injury and skin disease pathogenesis, and TGF-B, which can encourage the progression of squamous cell metaplasia. Alternatively, Iraq PM stimulation also resulted in the downregulation of a wide variety of genes. Most notably, the vast majority of downregulated genes are members of cell functions including the mitotic cell cycle, nuclear division, and DNA replication (Figure 5).

Figure 5. Significant Gene Ontology categories for PM stimulation downregulated genes.



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

- We have nearly completed testing of a robotic protocol to automate RNA-seq library preparation from the in vitro airway epithelial cultures. This method is based on the Ampliseq whole transcriptome RNA-seq library prep. We anticipate running stimulated airway epithelial RNA samples using this method in the next report period.

- This actual analysis in this task will be undertaken after Subtask 5 is completed.

Subtask 7. Prepare and submit manuscripts for publication.

Progress

- No manuscripts have been completed to date.

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.
We hired two Clinical Research Coordinators to support the GLIDE Study. Both have been or are being trained to perform high quality, NIOSH-certified spirometry; Lung Clearance Index testing; REDCap database management and data entry; questionnaire interviewing techniques; sample acquisition for deployers; IRB reporting; study subject consenting; and all other tasks relevant to supporting the GLIDE Study. They are supervised and guided by the Project 1 Lead (Dr. Rose), with support from other Project 1 faculty (including Dr. Meehan for Outreach; Dr. Krefft for LCI testing; Drs. Richards, Humphries and Lynch for CT imaging and textural analysis; Dr. Gottschall for cardiopulmonary exercise test data acquisition; and Drs. Strand and Crooks for biostatistical support and data management).

The Project 1 team has participated in a number of Professional Development activities. Drs. Rose and Krefft attended the 2017 American Thoracic Society annual international conference and presented data on clinical findings in patients with deployment-related lung disease. They attended and presented relevant data at a July 2017 Veterans Affairs Field Meeting titled "Developing an Airways Disease VA research Network (ADVARN)." Dr. Krefft attended the 2017 Aspen Lung Conference and presented preliminary data on LCI testing in symptomatic

deployers. The Project 1 team presents cases with deployment-related lung disease for discussion and management at a monthly Deployment Lung Disease clinical case conference at NJH.

PROJECT 2.
We hired two professional research associates and trained them to conduct the *in vitro* and *in vivo* laboratory experiments proposed in the grant application. Dr. Downey attended the VA/DoD-sponsored Airborne Hazards Symposium in Washington DC in May 2017 and led a discussion of progress on the GLIDE study. Dr. Downey also attended the 2017 Aspen Lung Conference on environmental lung disease at which deployment lung disease was discussed .

PROJECT 3.
We have recruited a Toxicology PhD graduate student who is mainly working on in vitro experiments involving cultures and treatment of airway epithelial cells from healthy human subjects, non-deployed and deployed asthma subjects. Drs. Chu and Day mentor the student. We have biweekly meetings with the student to discuss research ideas and progress. The student will also learn about the PM exposure animal models and measure airway hyperresponsiveness. This project will provide a great opportunity for the student to develop a research career that will benefit patients with deployment-related asthma and other lung diseases.

Dr. Day attended the 2017 annual meeting sponsored by the Society of Toxicology in Baltimore, MD, and exchanged research ideas about our Project.

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.

A substantial goal of Project 1 during the first year was to develop tools and processes for GLIDE Study outreach. An Outreach Committee has been assembled, has met multiple times, and has developed a number of 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. We are also looking into social medial outreach options, particularly since most deployers are younger and likely to access social media for information. These tools will be supplemented with presentations and meetings with interested physicians, military personnel and civilians. For example, Dr. Krefft recently gave a presentation about GLIDE Study efforts at Buckley Air Force Base at their Warrior Symposium.

Drs. Rose and Downey participated in the development of a workshop to be chaired by Dr. Eric Garshick entitled 'Respiratory Health and Service in Southwest Asia and Afghanistan' that has been submitted for consideration for the 2018 annual meeting of the American Thoracic Society

in San Diego, CA in May 2018. The decision of the executive committee will be announced in October 2017.

Drs. Rose and Downey also participated in the development of a noon symposium to be chaired by Dr. Eric Garshick entitled 'Veterans Health Studies and Programs in Pulmonary Deployment Health: Opportunities for Research'.

Dr. Downey attended the VA/DoD-sponsored Airborne Hazards Symposium on May 8-9, 2017 in Washington DC in May 2017 and led a discussion on the GLIDE study and on animal models of airborne PM exposure.

Drs. Downey, Chu, and Day visited NAMRU Dayton in July 2017 for discussions with Drs. Mumy and Wong on animal models of airborne PM exposure using aerosol exposure chambers.

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

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

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

As outlined in the Scope of Work, the following subtasks under Major Task 1 will be undertaken during the next reporting period to accomplish our goals. The major rate-limiting step to accomplishing these goals is obtaining HRPO approval for study subject recruitment and sample accession.

Subtask 1. Begin recruitment and consent patients from our NJH Deployment Clinic.

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

Subtask 7. Complete LCI measurements from deployers and controls.

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

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

Subtask 12. Prepare and submit manuscripts for publication.

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 will continue to refine our methods to quantify epithelial toxicity including cell death and cellular apoptosis. We plan to complete the studies examining the combined effects of PM and bleomycin and PM and physical force using the scratch wound. We will implement the shock tube to generate a simulated blast wave as form of physical injury. We will continue to develop the technique for influenza viral infection of lung epithelial cells and then study the combined effects of PM and influenza infection on epithelial injury.

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 use primary human alveolar epithelial cells to confirm key observations made in cell lines.

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. Once we have established the magnitude of epithelial injury induced by particulate matter, physical forces, chemical (bleomycin), and infectious agents (influenza virus) we will test whether small molecule modulators of the WNT pathway can prevent the injury. These studies will likely begin next year.

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

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.

Plans for the next year. These studies will be undertaken after we complete the studies examining the effects of single and combined injurious agents on 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. We plan to begin these studies as soon as we obtain HRPO approval to proceed.

Subtask 8. Prepare and submit manuscripts for publication.

Plans for the next year. We hope to have sufficient data to prepare a manuscript by the end of this next year.

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.

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

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

Plans for the next year. We have accomplished this. No further work needs to be planned.

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

Plans for the next year. The goal is to test if PM exposure enhances human primary bronchial epithelial responses (injurious/reparative, pro-inflammatory, oxidative) to allergic stimuli (e.g. IL-13) and cigarette smoke exposure. We will expand our initial air-liquid interface (ALI) culture experiments by including cells from more normal control subjects and asthmatics (both non-deployed and deployed). We plan to finish ALI and submerged cell culture experiments using brushed bronchial epithelial cells from five normals, five non-deployed and five deployed asthmatics. Results will allow us to perform statistical analyses to conclude if PM from Afghanistan versus from California will increase the severity of airway epithelial injury and remodeling such as production of pro-fibrotic cytokine TGF-beta.

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).

Plans for the next year. Once we obtain NAMRU's IACUC and ACURO approvals, we will develop and refine methods to expose mice to PM from Afghanistan as well as from California (control).

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

Plans for the next year. We have optimized the mouse exposure to cigarette smoke and allergen, as well as measured airway hyperresponsiveness (AHR) in allergen (e.g., house dust mite) challenged mice. Once we obtain mice from NAMRU that are exposed to PM from Afghanistan as well as from California (control), we will carry out our first mouse exposure experiment by exposing PM pre-exposed mice with cigarette smoke and house dust mite, and determine AHR, airway inflammation and remodeling (e.g., mucus production and airway wall fibrosis).

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.

Plans for the next year. We will continue to work with the US Geological Survey to set this system up.

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

Plans for the next year. This will start after Subtask 5 is completed.

Subtask 7. Prepare and submit manuscripts for publication.

Plans for the next year. We plan to summarize the data from experiments as described under Subtask 2, and write a manuscript for submission and publication.

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.

In the next reporting period we plan to continue of optimization of PM exposure methods, continue to develop our laboratory and analytical methods, and begin collecting and processing study samples as detailed in the project 4 subtasks.

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).

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 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.
Nothing to Report.

PROJECT 2.
Nothing to Report.

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.
The major rate-limiting factor for the delay in Project 1 deployer study subject recruitment has been lack of approval for the GLIDE Study by HRPO. The PI has been in regular communication with DOD/HRPO staff and provided an item that was identified as missing in the HRPO submission. We anticipate that HRPO approval will be forthcoming in the very near future. We are poised to begin study subject recruitment as soon as approval is received, and we have been keeping a list of potential GLIDE deployer study subjects who would like to be contacted to participate.

PROJECT 3
We have had a minor delay in getting dust-exposed animals from NAMRU due to delays related to NAMRU IACUC approval.

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.

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

Because of the delay in obtaining HRPO approval for GLIDE study subject recruitment, we have not begun using those parts of the grant budget that will cover costs for study subject reimbursements, deployer bronchoscopies with BALs and nasal brushings, and chest CT scans We anticipate that HRPO approval will be forthcoming in the very near future. We are poised to begin study subject recruitment as soon as approval is received, and we have been keeping a list of potential GLIDE deployer study subjects who would like to be contacted to participate. We will mobilize bronchoscopists and other staff once study subject recruitment and consent have been obtained.

PROJECT 4:
A delay in sample procurement means Project 4 spending was less than anticipated in this first year.

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

Not applicable.

Significant changes in use or care of vertebrate animals.

Not applicable.

Significant changes in use of biohazards and/or select agents

Not applicable.

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).*

  Nothing to Report

  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).*

  Nothing to Report

  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):
Nearest person month worked: 2 (annually)
Contribution to Project: No change

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

Name: Jennifer Smith
Project Role: Physician Assistant, Project 1
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 2 (annually)
Contribution to Project: No change

Name: Jenna Marcotte
Project Role: Clinical Research Coordinator, Project 1

Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 12 (annually)
Contribution to Project: Clinical Research Coordinator

Name: Meghan Nelson
Project Role: Lab Researcher, Project 2
Nearest person month worked: 6 (annually)
Contribution to Project: Perform analysis of gene expression in epithelial cells by qPCR

Name: Helen Roybal
Project Role: Lab Researcher, Project 2
Nearest person month worked: 4 (annually)
Contribution to Project: Perform and analyze experiments with epithelial cell culture including cytotoxicity and scratch wound assays.

Name: Maria Wong
Project Role: Lab Researcher, Project 2
Nearest person month worked: 3 (annually)
Contribution to Project: Perform and analyze experiments in mice exposed to PM

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

Name: Jie Huang
Project Role: Lab Researcher, Project 3
Nearest person month worked: 6 (annually)
Contribution to Project: No change

Name: Di Jiang
Project Role: Lab Researcher, Project 3
Nearest person month worked: 3 (annually)
Contribution to Project: No change

Name: Connor Stevenson
Project Role: Lab Researcher, Project 3
Nearest person month worked: 4 (annually)
Contribution to Project: No change

Name: Max Seibold, PhD
Project Role: Project Leader Project 4
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1 (annually)
Contribution to Project: No change

Name: Noah Zaitlen, PhD
Project Role: Co-investigator, University of San Francisco California, Project 4
Researcher Identifier (e.g. ORCID ID):
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):
Nearest person month worked: 1 (annually)
Contribution to Project: Dr. Cromie will lead the airway epithelial basal cell isolations from all brushings and the culture of these cells, which will be given to Dr. Everman for the ALI experiments and exposures.

Name: Andrew Dahl
Project Role: Postdoctoral Scholar
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 2 (annually)
Contribution to Project: Andrew has performed work for the analysis of RNA-sequencing data.

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: Karen Mumy
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: Less than 1
Contribution to Project: Dr. Mumy is currently planning the inhalation exposures and setting up the inhalational apparatus at NAMRU-Dayton.

Name: Brian Wong
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID): N/A

Nearest person month worked: Less than 1
Contribution to Project: Dr. Wong is assisting in the planning of inhalation exposures to particulate and setting up the inhalational apparatus at NAMRU-Dayton.

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.

Gregory Downey, MD: Dr. Downey's R21 (1R21 ES023932-02) ended on 4/30/2017. **Awarded:** R01 HL132950, PTPα as a Checkpoint in Fibrogenic Responses in the Lung; PR150015 Role of Matrix Metalloproteinase-3 in Deployment-Related Pulmonary Fibrosis.

Hong Wei Chu, MD: **Completed:** CIA – 130041Cigarette Smoke Impairs α1-antitrypsin Function ended on 6/30/2017; ADAM-17-NRG-1 Signaling in Acute Lung Injury (Finigan) ended on 6/30/2017. **Awarded:** U19AI125357 Role of Tollip in dysfunction of asthma airway innate immunity (Kraft)

Brian Day, PhD: Completed: U54 ES015678, Catalytic Antioxidants in Sulfur Mustard Toxicity ended on 8/31/2016; 20473901, Drug Discovery and Biomarker Assessments ended on 12/31/2016. Awarded: DAY16GO, Anti-inflammatory effects of thiocyanate in treating emerging CF pathogens.

Cecile Rose, MD: Awarded: U3ARH27243 University of Illinois at Chicago Black Lung Center of Excellence; AFC316-53 Linkage of Active Miner Surveillance, Former Miner Disability Evaluations, and Mortality Data Sets to Evaluate and Prevent Lifetime Risk of Cardiopulmonary Disease in US Miners; PR150115 Role of Matrix Metalloproteinase-3 in Deployment-Related Pulmonary Fibrosis.

Max Seibold, PhD: **Awarded:** R01 HL135156 Transcriptomic and Pharmacogenetic Asthma Endotypes in Minority Children (PI: Seibold); R01 DK111733 MerTK Mediated T Cell Suppression in the Pancreatic Islets during Type 1 Diabetes (PI: Friedman); R01 MD010443 Genes, air pollution, and asthma severity in minority children (MPI Seibold, Burchard); R01 RAR041256F The Role of Bacterial Toxins in Human Skin Disease (PI: Goleva Leung).

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.*

Nothing to Report

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.

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.