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14. ABSTRACT

This research uses an infrastructure and cohort of over 100 previously deployed military personnel with symptoms of lung disease who are currently treated in the Center for Deployment Lung Disease, led by Dr. Cecile Rose at National Jewish Health. Our focus is on how exposure to airborne particles acting in concert with physical forces (e.g. blast injury) chemical toxicants, and allergens may damage the lung epithelial cells and may lead to lung disease.

Divided into four projects, we use existing clinical data and biological samples from previously deployed military personnel with symptoms of lung disease. These samples are used to determine what kind and how much lung injury results from deployment-related exposures. In parallel, using a combination of cell culture and animal models of lung injury, we characterize using cellular and molecular techniques, how these exposures contribute to lung damage. We are investigating an innovative approach to the treatment of this lung damage using experimental drugs that promote healing of injured lungs. Our goal is to be able to predict which military personnel are susceptible to the toxic effects of airborne particulate matter, use preventative measures to minimize any damage to the lung, and initiate treatment at the earliest signs of disease.

15. SUBJECT TERMS
Lung injury, particulate matter, burn pits, viral respiratory infections, small molecule inhibitors, respiratory health, cigarette smoke exposure, respiratory epithelium

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1. INTRODUCTION:

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

2. KEYWORDS:

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

3. ACCOMPLISHMENTS:

What were the major goals of the project?

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

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

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

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

What was accomplished under these goals?

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

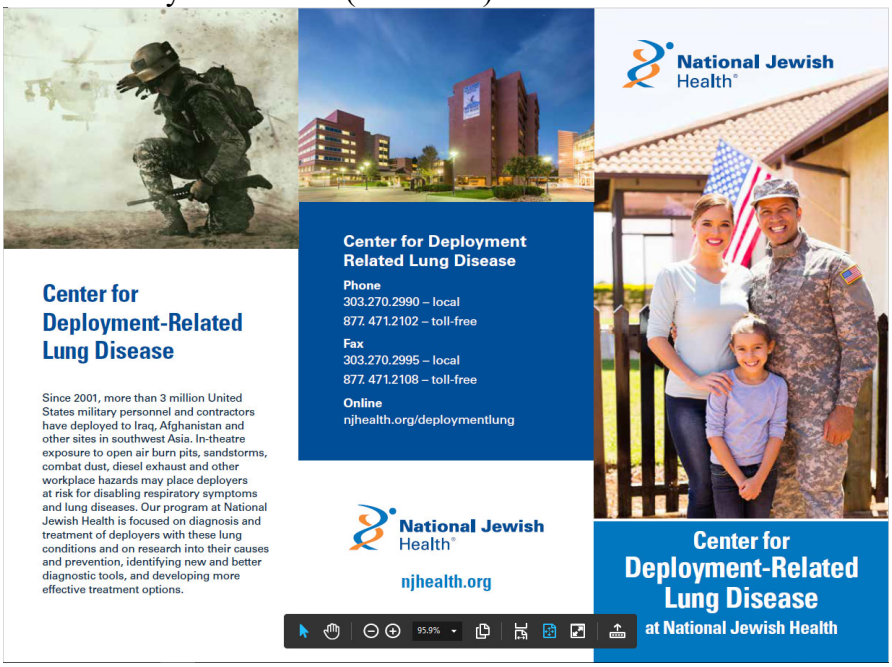
What were the major goals of the project?

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

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

Progress

1. NJH IRB approved the GLIDE Study in December 2016. GLIDE Study documents were submitted to HRPO in March 2017 and approved in August 2017. National Jewish Health switched to Biomedical Research Alliance of New York (BRANY) Institutional Review Board in June 2020. We recently received Continuing Review approval for the GLIDE Study from BRANY through 9/15/2022.
2. We continue to enter data from our Deployment Lung Disease Registry and Biorepository into a REDCap database.
3. We are using a printed Deployment-Related Lung Disease brochure for GLIDE Study recruitment (see below).



4. 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).
5. 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.

6. The GLIDE Study Outreach Committee continues to meet regularly to develop and implement plans for study subject recruitment.
7. 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.
8. The GLIDE Study coordinator facilitates scheduling deployer research subjects for GLIDE study participation, including for lung clearance index testing (LCI), chest CT image acquisition, research bronchoscopies and nasal epithelial cell brushings.
9. We continue to successfully consent and enroll deployers for LCI. We have met our target for LCI controls and are no longer actively recruiting control subjects.
10. Data for deployer study subject recruitment is indicated below. We defined **"screened"** as the total number of deployers who have signed consent and completed the deployment research questionnaire. We defined **"enrolled"** as deployers who signed informed consent and agreed to complete the research questionnaire and other Project 1 specific aims – that is, LCI, quantitative chest CT imaging, nasal epithelial brushing and/or fiberoptic bronchoscopy. We defined **"completed"** as those who completed testing after having been enrolled. There are a number of study subjects who have consented to GLIDE Study bronchoscopies and/or nasal brushings who have not completed these procedures because of the COVID-19 pandemic.
    - Number of subjects **screened**/target: **255/250**
    - Number of patients **enrolled**/target: **135/250**
    - Number of patients **completed**/target: **118/250**

Difficulties encountered

Due to the COVID-19 pandemic, National Jewish Health implemented an institutional pause in recruitment of research study subjects March 2020 to minimize risk of viral transmission for subjects and study personnel. Clinical research study recruitment resumed (with multiple COVID-19 protocols in place) in July 2020, with the exception of research bronchoscopies that resumed in August 2021.

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 and implemented the protocols and procedures.
2. We work with the Minimally Invasive Diagnostic Center (MIDC) staff where these procedures are performed. GLIDE Study bronchoscopists use the compatible electronic medical record documentation process (using a study-specific NJH Provation program) to record research bronchoscopies performed in the MIDC, ensuring accurate documentation.
3. We continue to meet as needed with MIDC personnel who are involved in GLIDE Study bronchoscopies to familiarize them with research protocols and study staff.

4. We worked closely with MIDC personnel to address technical issues and refine our protocols during the first round of research bronchoscopies.
5. We communicate regularly with laboratory technicians who process study samples to ensure that sample collection yield is maximized.
6. Study bronchoscopists adjusted brushing techniques to enhance the nasal and bronchial epithelial cell collection from deployer subjects based on feedback from laboratory-based co-investigators.
7. We have worked with Drs. Downey and Chu (Project 2 and 3) and Dr. William Janssen to collaborate on obtaining bronchoalveolar lavage specimens from normal human volunteers (collected under a separate grant and IRB protocol). The samples are de-identified and stored in our institutional biobank under our Honest Broker protocol. These samples can be used as controls to compare with deployer bronchoalveolar lavage specimens. Additional control sample acquisition is planed with resumption of research bronchoscopies under the, "Innate Immunity and Deployment-Related Lung Diseases"; Proposal Number PR201498, Award # W81XWH2110666, HRPO Log Number E02433
8. Protocols for collecting and analyzing nasal and bronchial brushing and BAL samples during COVID-19 have been established to assure that study subjects and staff are protected from viral exposure risk.
9. We have sent pilot deployer and control BAL samples to the University of Colorado Anschutz Medical Campus for preliminary flow cytometry (CyTOF) analysis to characterize dominant cell profiles.

Difficulties encountered

Due to the COVID-19 pandemic, National Jewish Health implemented an institutional pause in recruitment of research study subjects in March 2020 to minimize risk of viral transmission for study subjects and personnel. Research bronchoscopies resumed in July 2021 as part of an institutional pilot program with additional COVID-19 precautions including a negative COVID-19 test 96 hours prior to the bronchoscopy and a fully completed COVID-19 vaccination status. Performing bronchoscopists have been specially trained in use of controlled air-purifying respirators (CAPRs) and other COVID-19 prevention techniques to assure optimal study subject airway and aerosol controls.

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

Progress

1. GLIDE Study staff established protocols for the collection and distribution of BAL fluid, bronchial epithelial cell (BEC) brushings, and nasal epithelial cell (NEC) brushings and updated guidance based on the potential for COVID-19 exposure when these procedures are performed at National Jewish Health. This includes use of negative pressure rooms for NEC brushings and processing of samples in BSL2+ if COVID status of study participant is unknown (or positive).
2. To date, we have performed 33 research bronchoscopies with paired nasal brushings plus an additional 21 unpaired nasal brushings on deployer study subjects.

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. Protocols have been established including an IRB-approved consent form for collection of lung tissue blocks for GLIDE Study purposes from VATS biopsies performed for clinical diagnosis. Potential study participants have been contacted and consented.

Difficulties encountered

None.

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

Progress

1. Using our Deployment Lung Disease Registry and Biorepository, we continue to track which subjects have had a VATS biopsy and meet other requirements for participation in the GLIDE study.
2. When possible, we obtain informed consent for participation in the GLIDE Study from Registry participants who have had a VATS biopsy. During this reporting period, we continued to collect and analyze clinical VATS lung biopsies on symptomatic deployers seen in our clinical center of excellence.
3. Using a standardized REDCap-based scoring form, the study pathologist has completed scoring on 65 GLIDE Study lung tissue samples.
4. We have obtained and the study pathologist has scored non-deployer VATS lung tissue samples (31 positive and 11 negative controls) for comparison with deployer samples.
5. Statistical analysis and manuscript draft are in progress describing histologic findings in deployer lung tissue samples compared to controls.
6. Control lung tissue samples were sent to the USGS for pilot testing of analytical methods. After successful completion of the pilot testing, 81 total de-identified deployer and control lung tissue samples have been sent to the USGS for analysis.

Difficulties encountered

None.

Subtask 6: Establish LCI using Multiple Breath Washout technique.

Progress

1. We continue to use the Procedures Manual for LCI measurements developed for this study.
2. We continue to use a dedicated LCI testing space in the Clinical Translational Research Center at NJH where we will also perform GLIDE study subject consenting.

3. Our study staff continues to perform LCI testing on deployers, allowing each staff member to gain additional proficiency in LCI performance and quality.
4. We confer regularly with LCI experts in Toronto to assure that testing is meeting the highest quality standards.
5. We developed a revised safety protocol for LCI testing (an aerosol-generating procedure) targeted to the COVID-19 pandemic to protect study subjects and staff. This included ensuring adequate room filtration/negative pressure ventilation, disinfection, training, and personal protective equipment as well as screening of study participants.

Difficulties encountered

None.

Subtask 7: Complete LCI measurement from deployers and controls.

Progress

1. We used a SAS program that enabled us to match deployer subjects to controls and that aided in targeted study subject recruitment.
2. We met target GLIDE Study enrollment goals for both symptomatic military deployers and control study subjects.
3. Our manuscript was published in Respiratory Medicine, describing the utility of LCI as a noninvasive tool for diagnosis of large and small airways disease in symptomatic military personnel following deployment to austere environments.
4. An additional article was published in an open access journal, 'Data in Brief', to provide normative data on multiple breath washout (MBW)/LCI for 103 healthy U.S. adults, including histogram distribution of LCI scores based on sex and BMI.
5. We continue to perform LCI testing on deployer study subjects to support enhanced data acquisition and potential future analyses.
6. Recently, EcoMedics, the company that manufactures the machine we use for LCI testing, released major new software updates. We have been working directly with them to upgrade our database and understand impacts the changes in SIROWARE® 3.3 may have on our continued work.

Difficulties encountered

None.

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

Progress

1. Working with the NJH Imaging Department staff, we developed and implemented protocols for scheduling and consenting GLIDE Study participants for study chest CT imaging.
2. We established a protocol for transmitting study imaging acquisition data with de-identified study subject numbers and linkage to the GLIDE Study database for future analysis.

3. Study investigators and research staff created and implemented a process for matching deployers and controls and devised methods to blind radiologists during batched scoring of chest CT scans.
4. We designed, pilot-tested, revised and implemented a scoring system for use in REDCap by the study radiologists for qualitative chest CT image interpretation.
5. All three GLIDE Study radiologists have scored 96 chest CT images (blinded to deployer vs control status).
6. We added another 35 chest CT images from consenting symptomatic deployers (all obtained using the GLIDE Study protocol and all with informed consent) to the total number for which independent scores will be obtained by the three study radiologists in order to enhance statistical power for data analysis.
7. A manuscript comparing CT findings between controls and deployers with deployment-related lung disease has been submitted for publication and is currently under review.

Difficulties encountered

None.

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

Progress

1. As described in subtask 8 above, we have acquired and scored a total of 83 deployer study subject chest CT images for use in quantitative analysis, all using the GLIDE image acquisition and scoring protocols.
2. We have queried the control chest CT scan database used for the GLIDE Study to establish demographic linkages (including age, smoking status and gender) for matching with deployer study subject images.
3. We have received complete quantitative analysis data from Thirona Lung quantification software (Nijimegen, The Netherlands) for a total of 83 deployer CT images and 44 control images. We are preparing a manuscript comparing airway wall thickness (measured via Pi10) and emphysema between deployers and controls and linking those results with lung function measurements.

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

None.

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

Progress

Mineralogic analysis of VATS biopsies for deployer and control samples are in progress; see subtask 5 for more details.

Difficulties encountered

None.

Subtask 12: Prepare and submit manuscripts for publication.

Progress

1. Our manuscript titled "Military Occupational Specialty Codes: Utility in predicting Inhalation Exposures in Post-9/11 Military Personnel Following Southwest Asia Deployment" on the use of a respiratory hazard exposure matrix and Military Occupational Specialty (MOS) Codes to identify risk factors for respiratory symptoms and diseases in military deployers was published in the Journal of Occupational and Environmental Medicine in December 2019. DOI: 10.1097/JOM.0000000000001731
2. A large case series manuscript on clinical findings in symptomatic deployers titled "Respiratory Diseases in Post-9/11 Military Personnel Following Southwest Asia Deployment" was published in the May 2020 issue of Journal of Environmental and Occupational Medicine (JOEM) DOI: 10.1097/JOM.0000000000001817
3. We submitted two abstracts to the 2020 American Thoracic Society (ATS) International Conference using data from CT imaging of deployers. The first described preliminary findings from quantitative analysis of airway wall thickening in deployers compared to controls; the second described severity, morphology and risk factors for expiratory central airways collapse (ECAC) in our population of symptomatic deployers. Both were accepted for presentation at a scientific symposium at the ATS meeting.
4. Our manuscript titled "Multiple Breath Washout: A Noninvasive Tool for Identifying Lung Disease in Symptomatic Military Deployers" demonstrating the utility of LCI as a noninvasive tool to detect both small and large airways disease in deployers was published in Respiratory Medicine in January 2021.DOI: 10.1016/j.rmed.2020.106281.
5. The accompanying healthy control data manuscript titled "Multiple breath washout test data for healthy controls" was published in Data in Brief in February 2021. DOI: 10.1016/j.dib.2020.106641.
6. Our manuscript titled Semiquantitative Chest Computed Tomography Assessment Identifies Expiratory Central Airway Collapse in Symptomatic

Military Personnel Deployed to Iraq and Afghanistan" on aggregate ECAC findings in deployers was published in the Journal of Thoracic Imaging in June 2021. DOI: 10.1097/RTI.0000000000000596.

7. We submitted an abstract that was presented at the 2021 ATS International Conference describing unanticipated findings of skeletal muscle mitochondrial myopathy in a subgroup of symptomatic deployers based on findings using cardiopulmonary exercise testing. This abstract, titled 'Decreased Exercise Tolerance and Unexplained Dyspnea May Be Linked to Mitochondrial Myopathy Following Military Deployment to Iraq and Afghanistan', was accepted for poster presentation at the MHSRS (Military Health Systems Research) meeting in August 2021.
8. We submitted a manuscript titled "Sensitivity of High Resolution Computed Tomography for Detecting Deployment Related Lung Disease" which is now undergoing peer review.
9. We have begun data analysis and manuscript preparation on two additional GLIDE Study Project 1 focus areas: (1) quantitative analysis of Pi10 on CT imaging in deployers vs controls; and (2) the spectrum of lung histologic abnormalities in symptomatic deployers compared to positive and negative controls.

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

The studies using the murine cell line, MLE-12 in models of PM-induced cytotoxicity and effects of scratch wound closure were completed in previous years. During the fifth year, we have developed a model using a more robust cell line, rat lung epithelial (RLE) cells, obtained from ATCC. The RLE cells have many features of human alveolar type II cells including formation of tight junctions with readily measurable transepithelial resistance and we have optimized their cell culture conditions. We have used RLE cells to study the effects of blast overpressure injury on cell toxicity, recovery, and reformation of intercellular junctions, specifically tight and adherens junctions.

During this year, we have continued to develop an *in vitro* system of blast overpressure injury to cultured lung epithelial cells using our advanced blast simulator (ORA Inc, Fredericksburg, VA). In last year's report we described a system in which cultured cells grown in a sterile tissue culture dish are then placed into a mesh holder that is secured in the blast chamber. We have studied

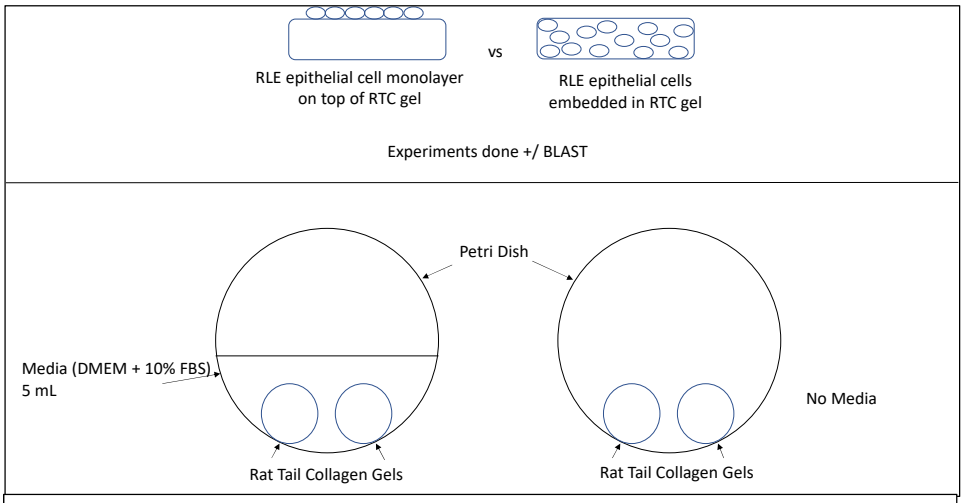


Figure 1. Culture conditions for lung epithelial cells exposed to blast overpressure injury. Left panel illustrates epithelial cells grown in submerged culture as a monolayer on top of a collagen gel. Right panel illustrates epithelial cells grown embedded within a collagen gel. We have used rat lung epithelial cells because they are a robust cell line to use to optimize the culture and blast conditions.

cells grown submerged in tissue culture medium, grown in an air liquid interface, grown as monolayers on the surface of a collagen gel, or cultured and embedded within a collagen gel matrix (**Figure 1**). Our most promising system consists of

rat lung epithelial (RLE) cells embedded within a collagen gel matrix in a Petri dish. A thin layer of agarose is then overlaid on top of the cells and allowed to solidify. The gels are then transferred to sterile sealable plastic bags and placed in a mesh holder in the advanced blast simulator (ABS). Cells were then subject to blast overpressure injury using Mylar membranes (these membranes generate approximately 14-15 PSI peak pressure). Under these conditions, we observed some fragmentation of the collagen gels but no fracture of the Petri dishes (**Figure 2**).

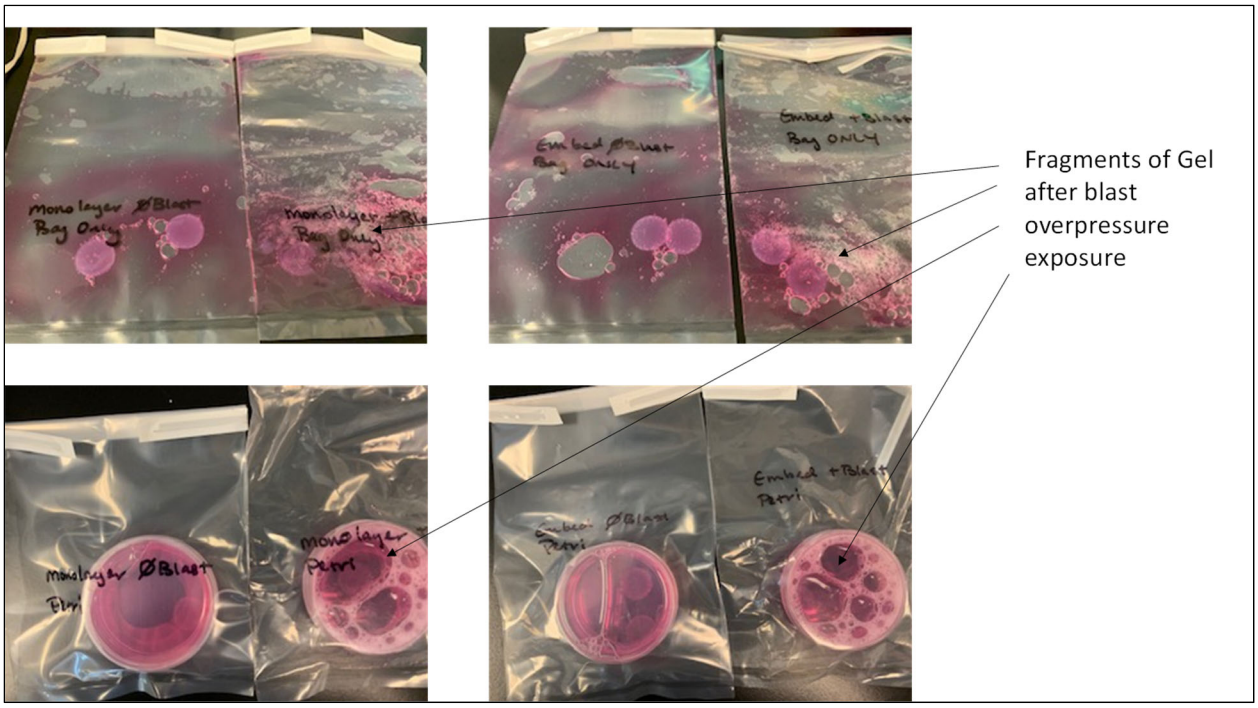


Figure 2. Cultured lung epithelial cells exposed to blast overpressure injury. **Top left panel:** epithelial cells grown in submerged culture as a monolayer on top of a collagen gel, removed from the petri dish and then exposed to blast overpressure in a sealed bag. **Top right panel**: epithelial cells grown embedded within a collagen gel, removed from the petri dish and then exposed to blast overpressure in a sterile sealed bag. **Bottom left panel**: epithelial cells grown in submerged culture as a monolayer on top of a collagen gel in a petri dish. The petri dish was placed in a plastic bag and then exposed to blast overpressure. **Bottom right panel**: epithelial cells grown embedded within a collagen gel in a petri dish. The petri dish was placed in a plastic bag and exposed to blast overpressure in the ABS.

After blast overpressure exposure, the bags were removed from the ABS and taken to the tissue culture facility where the cells were removed from the Matrigel by proteinase digestion, washed, and then transferred to tissue culture plastic flasks. Figure 3 illustrates the % of cells recovered (**Fig. 3A**) as well as their viability (**Fig. 3B**). The studies demonstrate that cultured RLE epithelial cells can be exposed to blast overpressure injury under controlled circumstances and that they can subsequently be cultured study the effects of blast overpressure injury.

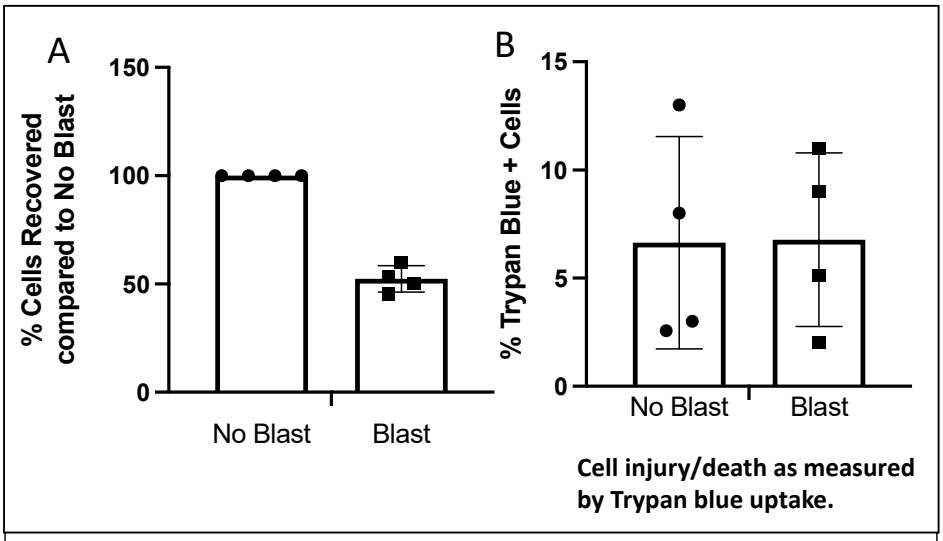


Figure 3. RLE Cell recovery and viability after blast overpressure injury. RLE cells were cultured on Matrigel and then overlain with a layer of agarose. The gels were removed from Petri dish and transferred into sterile sealable plastic bags. The bags containing the cells were transferred to a mesh holder within the advanced blast simulator (ABS) and subject to blast overpressure injury (Mylar membranes generating approximately 14-15 PSI peak pressure). The plastic bags containing the gels were removed from the ABS and taken to the tissue culture facility. The gels were digested, the cells recovered and then plated on tissue culture plastic.

We have now been successful both in recovering intact cells from the gel and in culturing the cells for up to 5 days after blast overpressure exposure (**Figures 4 and 5**).

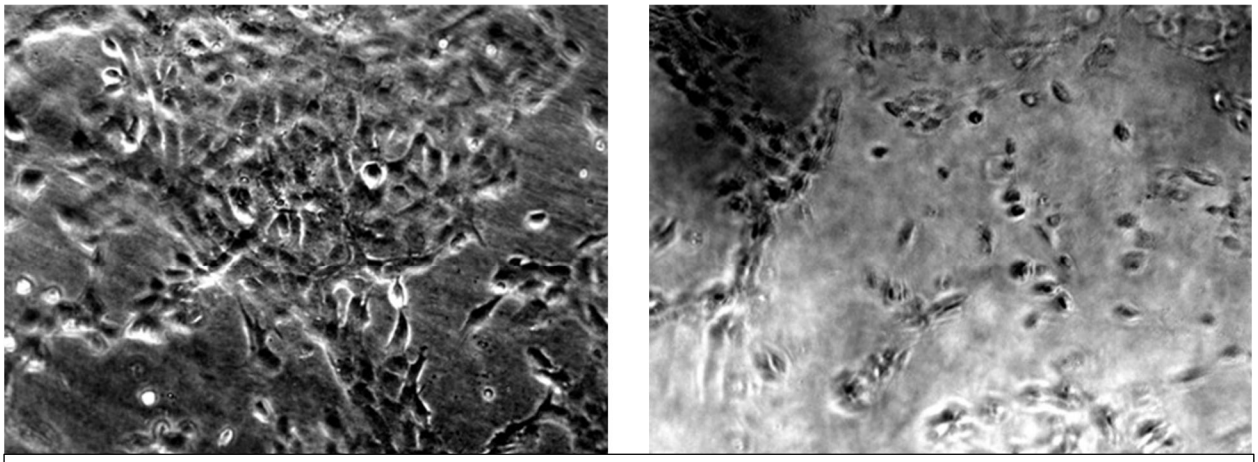


Figure 4. Rat lung epithelial cells grown on collagen gels as a monolayer and then exposed to blast overpressure ('blast') or sham ('no blast') in an advanced blast simulator. After blast overpressure exposure, cells were removed from the collagen gel using protease digestion and then cultured in tissue culture plates. **Left panel** illustrates epithelial cells not exposed to blast overpressure (sham). **Right panel** illustrates cells exposed to blast overpressure. Note sparseness of cells exposed to blast overpressure injury. Image taken 24 hours after plating the cells in tissue culture plates.

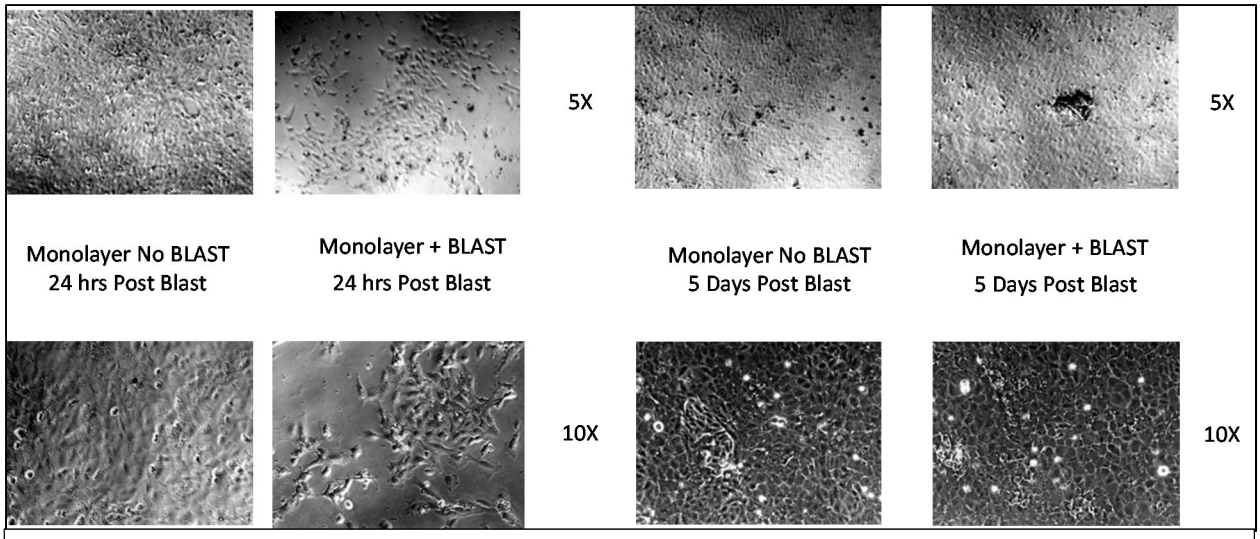


Figure 5. Rat lung epithelial cells grown as a monolayer on collagen gels and then exposed to blast overpressure ('blast') or sham ('no blast') in an advanced blast simulator. After blast overpressure exposure, cells were removed from the collagen gel using protease digestion and then cultured in tissue culture plates for 24 hrs or 5 days. **Left 4 panels** illustrate epithelial cells exposed to blast overpressure ('blast') or to sham blast (No blast) and then cultured for 24 hrs. **Right 4 panels** illustrate epithelial cells exposed to blast overpressure ('blast') or to sham blast ('no blast') and cultured for 5 days.

For the last year, we have encountered supply chain problems with the manufacturers of these membranes during the COVID-19 pandemic. We have worked with Stumptown Research, and have tried several different lots of membranes from different manufacturers. We have tested both Mylar and Valmex PVDF-type membranes and have found both Mylar and

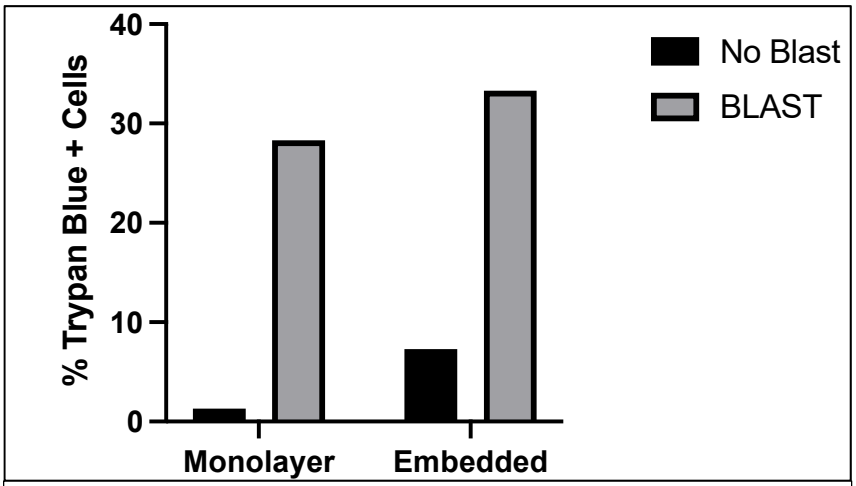


Figure 6. Viability of rat lung epithelial cells grown as a monolayer on top of or embedded within collagen gels and then exposed to blast overpressure injury in an advanced blast simulator. After blast overpressure exposure, the cells were removed from the collagen gel using protease digestion, dissociated into a single cell suspension, incubated with trypan blue and then counted in a hemocytometer. Cells grown in monolayer culture had a low basal rate of cell death and 28% cell death in response to blast overpressure injury. Cells grown embedded within the gel had a slightly higher basal rate of cell death and a 32% rate of cell death in response to blast overpressure injury.

Valmex PVDF membranes that give us reproducible peak blast pressures of 14-16 PSI (Mylar) and 15-18 PSI (Valmex PVDF) with a good blast overpressure wave profile. We have acquired the membranes in bulk material and Stumptown Research has cut them to fit our blast simulator so as to minimize variation. Using the Mylar membranes to provide blast overpressure exposure to the cultured lung epithelial cells grown either on (monolayer) or in (embedded) collagen gels, we observe approximately 30% cell death as determined by trypan blue staining (**Figure 6**). Cells grown in monolayer culture had a low basal rate of cell death and in response to blast overpressure injury, there was approximately 28% cell death. Cells grown embedded within the gel had a slightly higher basal rate of cell death (likely related to the processes involved in digesting the cells out of the collagen gel) and in response to blast overpressure exposure, there was 32% rate of cell death.

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

Transcriptional Effects of PM Exposure on Cultured Lung Epithelial Cells.
During the last year, we have continued our studies with Drs. Chu, Day, Seibold, Janssen, and Fingerlin characterizing the changes in global mRNA expression (transcriptional profile) of primary human airway and alveolar epithelial cells exposed to PM from Afghanistan, Iraq, and California using RNA-Seq. We chose the 4 hr and 24 hr time points after PM exposure to capture both early and more delayed alterations in gene expression based on preliminary experiments. We completed exposure studies on primary cells from five different non-smoking donors including NextGen RNA-sequencing. The advanced bioinformatics analysis of these data sets is currently ongoing. We have used DAVID, Hallmark, KEGG, and Ingenuity Pathway Analysis (IPA: QIAGEN) to characterize the signaling pathways that control the cellular responses to particulate matter exposure.

We identified several novel and physiologically important pathways that were activated in response to exposure to PM from Southwest Asia. Some of these pathways have been mentioned in previous reports and additional analysis has confirmed their importance, and that they are strongly altered by exposure to PM. We examined differences in transcriptional responses to PM exposure between large airway, small airway, and alveolar epithelial cells and observed distinct patterns. As examples, in large airway epithelial cells PM exposure induced alterations in expression of Tumor Necrosis Factor (TNF), C5, IL-1β, IL36, IL25, IFNG, IL17, IL33, IL4, and IL22. In small airway epithelial cells, the highest scores in Master Regulators include Tumor Necrosis Factor (TNF), TLR6, TLR1, ICOSLG/L, lipopolysaccharide, IL-1β, Aldose Reductase, NFκB, Wnt5B, TEAD, CLOCK, and NOTCH. In alveolar type II cells, the Master Regulators include lipopolysaccharide, TNF, IL1β, NFκB, IL1α, TRADD, JUN, MYD88, MTOR, IL17. Thus, while there are clearly common Master Regulator pathways activated in response to PM exposure by the three types of lung epithelial cells (large airway, small airway, and alveolar type II epithelial cells) such as TNF, IL-1β,

LPS, MYD88, there are differences in the magnitude of the transcriptional response comparing each cell type as well as distinct responses in each cell type (e.g. large airway epithelial cells express IL36, small airway epithelial cells express ICOSLG/L, TEAD, CLOCK, and NOTCH and alveolar type II epithelial cells express TRADD, MTOR, and JUN). We are in the process of linking these transcriptional responses in the epithelial cells with the phenotypic changes we see in deployers and in the preclinical murine models of respiratory PM exposure where we observe inflammation followed by fibrosis.

We next confirmed and validated the results of the RNA-seq analysis using quantitative (q)PCR with TaqMan probes. In general, all of the genes that were identified using RNA-seq analysis were confirmed by (q)PCR with TaqMan probes. We observed that for some genes, there was a difference in the magnitude of the increase or decrease in mRNA abundance of certain genes as assessed by RNA-seq compared to (q)PCR. To determine if some of this apparent discrepancy was due to alternate splicing of genes, we designed TaqMan probes to different parts of the mature mRNA based on known alternatively spliced transcripts to see if there were differences in these transcripts. We observed that for most of the genes we studied, alternate splicing was not responsible for the observed differences in magnitude of mRNA levels between RNA-seq and qPCR. Another potential source of discrepancy between RNA-seq and qPCR is the use of "housekeeping genes" to normalize the target mRNA. Specifically, if the level of expression of a gene used as a housekeeping gene in fact changed under the conditions of the experiment, this would result in an inaccurate assessment of the level of expression of the gene of interest. Indeed, we did observe differences in the level of expression of several housekeeping genes under the experimental conditions. This led us to assess the stability of expression of a panel of 9 housekeeping genes. Based on this assessment, we selected 4 genes that had stable levels of expression and we now incorporate all 4 in our qPCR analysis. A manuscript that describes the effects of PM exposure on these epithelial responses has been completed and will be submitted for publication.

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

Using the *in vitro* blast overpressure injury model system described above in **Subtask 1**, we sought to determine whether ICG-001 can protect the epithelial cells from blast overpressure injury. Initial experiments demonstrated that the cells tolerate incubation with the ICG-001 without any apparent toxicity and are able to grow to confluence in the continued presence of ICG-001 after blast overpressure exposure (**Figure 7**). We also observed that cells incubated with ICG-001 obtained confluence slightly earlier than the cells incubated with DMSO (vehicle) although we will need to do quantitative kinetic experiments with time lapse video microscopy to be certain of this. Finally, we observed that cells pretreated with ICG-001 prior to blast overpressure injury proliferated more rapidly and attained confluence more quickly than cells treated with vehicle control and then subject to blast overpressure injury (**Figure 7**).

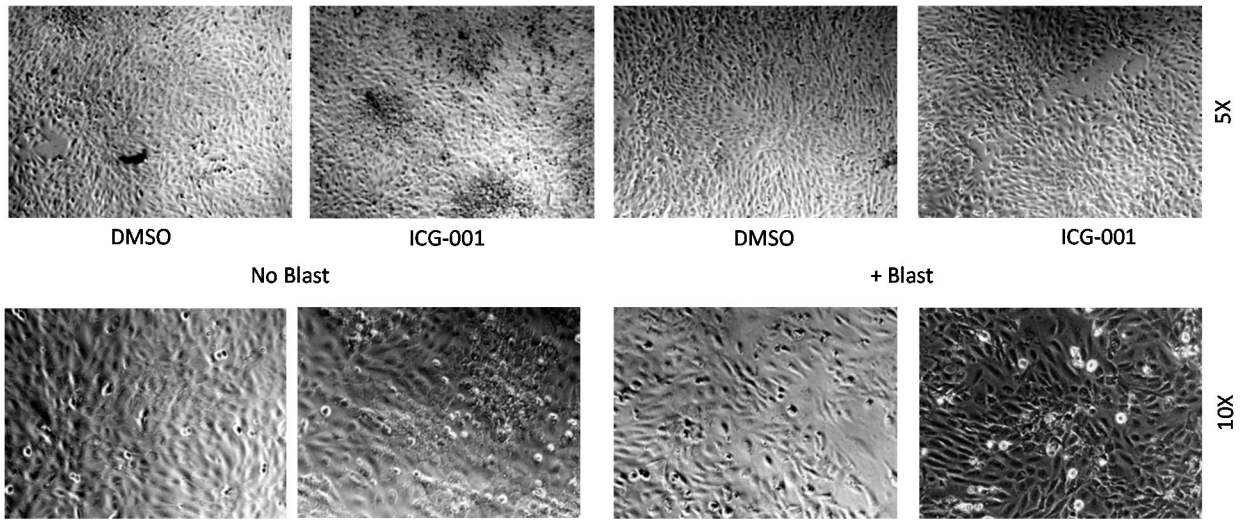


Figure 7. Rat lung epithelial cells grown as a monolayer on collagen gels and then exposed to blast overpressure ('blast') or sham ('no blast') in an advanced blast simulator in the presence or absence of ICG-001 or vehicle (DMSO) control. After blast overpressure exposure, cells were removed from the collagen gel using protease digestion and then cultured in tissue culture plates for 24 hr. **Left 4 panels** illustrate epithelial cells not exposed to blast overpressure (sham) and cultured for 24 hr. **Right 4 panels** illustrate epithelial cells exposed to blast overpressure ('blast') and cultured for 24 hr in the continued presence of ICG-001 or DMSO (vehicle control).

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 have continued to refine our model of blast over-pressure acute lung injury using an advanced blast simulator (ABS) purchased the blast simulator from ORA Inc. The initial blast exposures were done using membranes cut from a single roll of polyvinylidene fluoride (PVDF)-lacquer (Valmex or equivalent). These membranes generated reproducible peak blast overpressure waves of 14-17 PSI and resulted in a consistent degree of acute lung injury in mice. We used up our entire supply of these membranes for our initial studies looking at the effects of PM exposure prior to blast overpressure injury (summarized in previous progress reports). We tried to obtain additional rolls of this Valmex material but the company who supplied the membranes (Mehler Technologies) ran out of stock and this material has not been available for the last year, in part due to supply chain interruptions from the COVID-19 pandemic. We sourced similar material from other manufacturers and tested a variety of PVDF and polypropylene-reinforced vinyl membranes in our blast simulator but none were comparable to the original Valmex (16 oz/yard) membranes in terms of generating reproducible blast overpressure waves with peak pressures in the 14-17 psi range. We tried a lot of Mylar of higher tensile strength but it did not rupture in our simulated blast chamber (**Figure 8**).

Figure 8. Failure of rupture of high tensile strength Mylar membrane in our blast simulator (Ora Inc). Note that the membrane was deformed (stretched) but did not rupture in our system that uses compressed gas to pressurize the compression chamber.

We recently obtained PVDF membrane from an alternate supplier and used this to expose mice to blast overpressure injury. This test membrane resulted in a peak blast pressure of 14-17 PSI. As illustrated in **Figures 9-11**, mice developed a significant degree of acute inflammation and lung injury 24 hr after the blast

injury as indicated by an increase in IgM levels and an increase in total cells and neutrophils in bronchoalveolar lavage fluid.

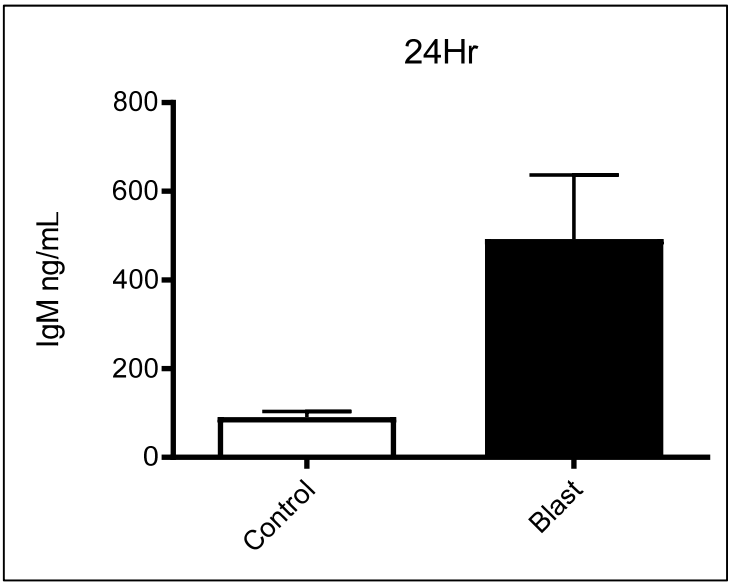


Figure 9. Acute lung injury at 24 hr in mice induced by blast over pressure injury using new PVDF membranes in our blast simulator. Increased lung permeability is measured by leak of IgM from the blood into the alveolar space as assessed by IgM concentrations in bronchoalveolar lavage fluid.

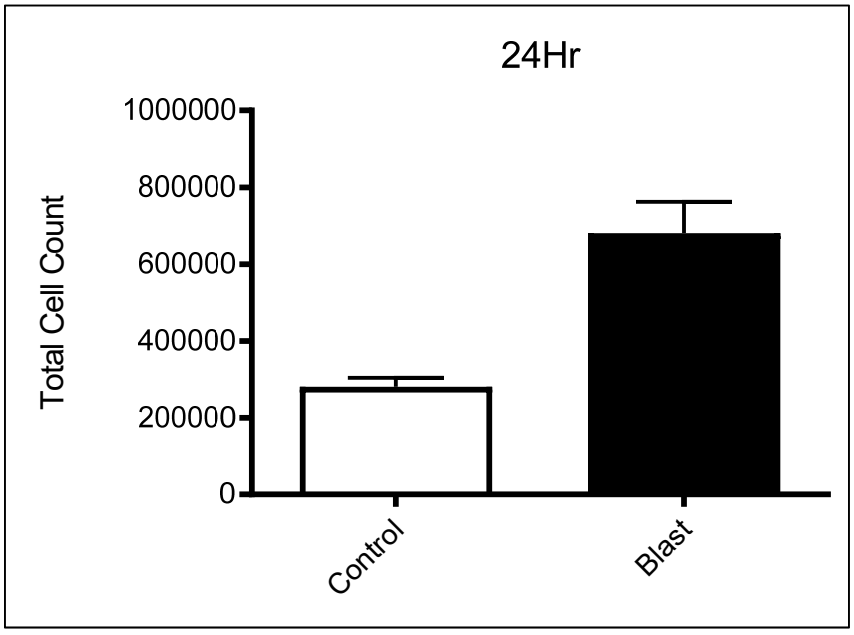


Figure 10. Acute lung injury at 24 hr in mice induced by blast over pressure injury using new PVDF membranes in our blast simulator. Lung inflammation is measured by an increase in total cells recovered by bronchoalveolar lavage.

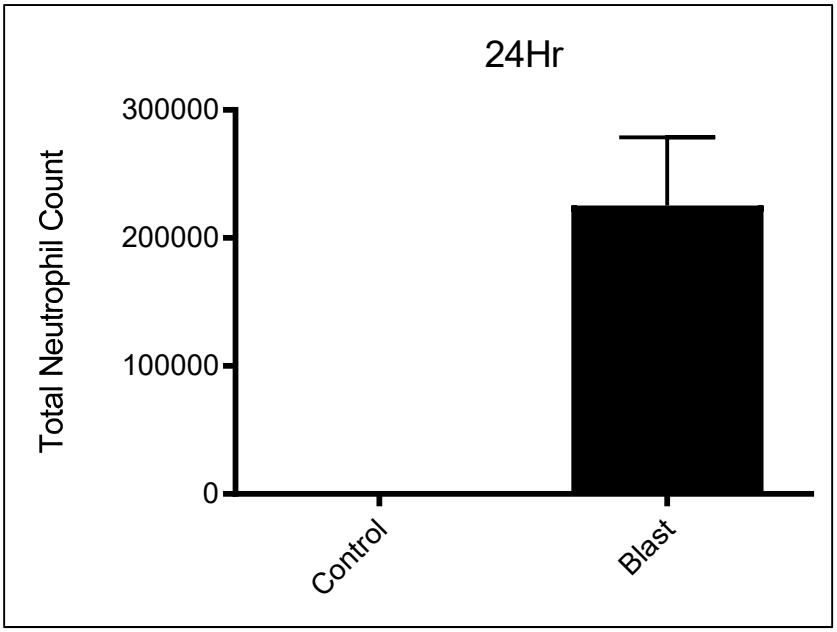


Figure 11. Acute lung injury at 24 hr in mice induced by blast over pressure injury using new PVDF membranes in our blast simulator. Lung inflammation is measured by an increase in neutrophils recovered by bronchoalveolar lavage.

Despite these substantial improvements, we continue to observe some variability in the degree of lung injury in mice – specifically that approximately 20% of mice exposed to blast overpressure do not develop evidence of injury. We observe this variability even in mice that are not otherwise treated and even between 2 mice that are exposed to the same overpressure blast wave in our dual 'hammock' restraint system.

To address this problem, we have been working closely with blast scientists at Stumptown Research Incorporated (they have taken over all the projects from ORA Inc) and Dave Ritzel to optimize the system. Our initial approach was to use high-speed video image capture to determine the movement of the specimen (mice) during the blast wave. The concern raised by Dave Ritzel was that the specimens could be moving with the blast wave thus attenuating the effects of the incident wave. In addition, the original specimen holder that we obtained with the advanced blast simulator was designed to hold 2 mice (one above the other). The studies using the high-speed video demonstrated that the mice moved considerably in the blast wave and that they sometimes moved in opposite directions. In order to prevent movement of the mice in the and the specimen holder, blast scientists at Stumptown Research designed a new specimen holder with a high porosity expanded metal panel held between two aluminum struts (**Figure 12**). The mice will be held in a high tensile strength material fastened to the struts. The metal panel will prevent motion of the mice with the incident blast wave. We have tested this apparatus and a pilot experiment and have observed that mice held in this new specimen holder developed lung injury as assessed by leak of IgM into the bronchoalveolar lavage fluid. Histological sections of the lungs of these mice also demonstrate evidence of acute lung injury 24 hr after the blast (**Figure 13**). This was confirmed by micro CT scan demonstrating a large

area of fluid density in lungs of mice exposed to the blast overpressure (**Figure 14**).

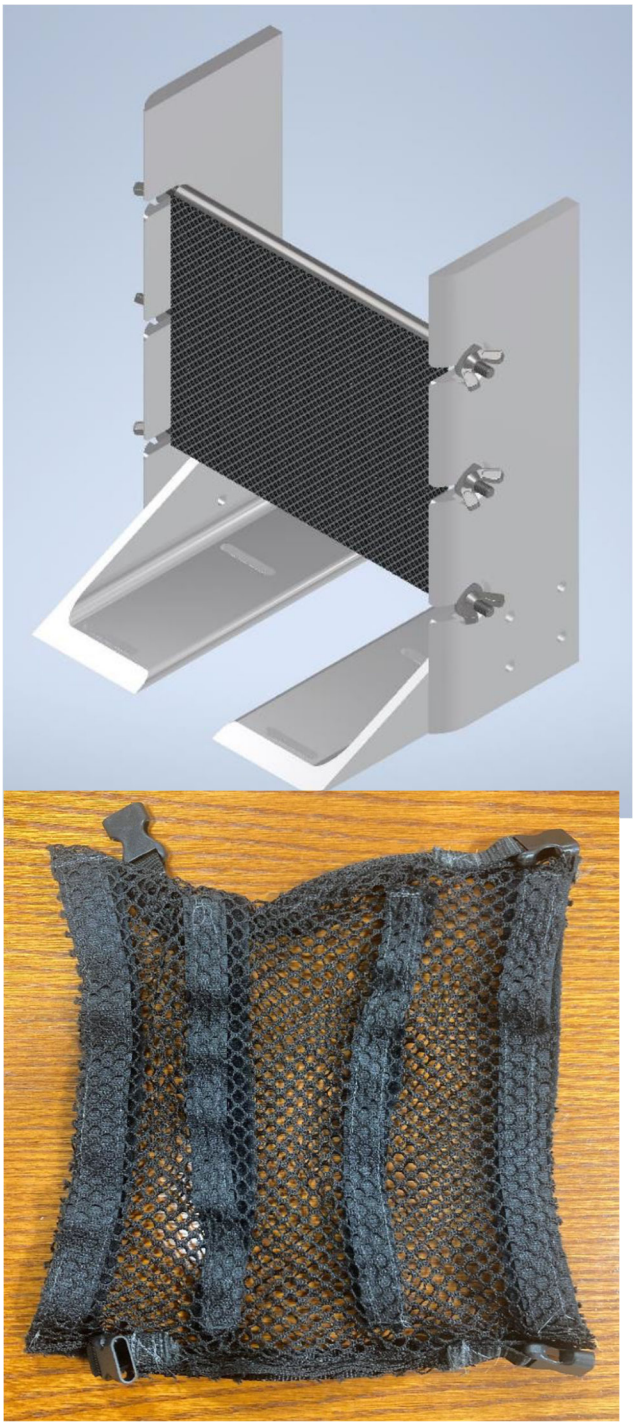


Figure 12. Redesign of the specimen (mouse) holder to be fastened into the advanced blast simulator. **Top:** aluminum struts with a high porosity expanded metal panel welded to the aluminum struts. **Bottom:** sling into which anesthetized mice will be placed and then the sling will be fastened to the aluminum struts using high tensile strength straps.

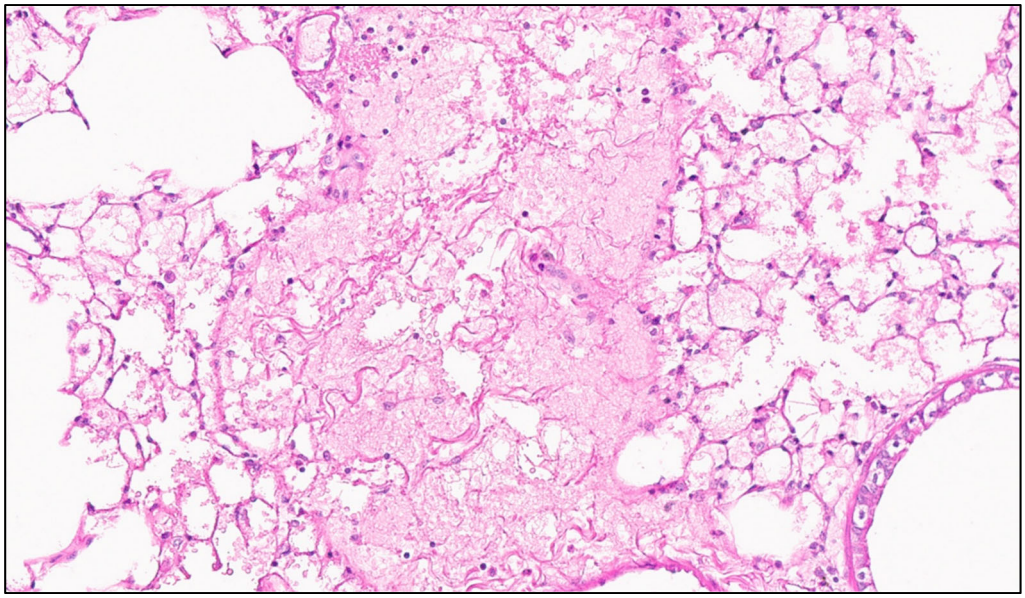


Figure 13. Acute lung injury at 24 hr in mice induced by blast over pressure injury using new PVDF membranes in our blast simulator. H&E staining illustrates proteinacious material in the alveolar spaces. 20X.

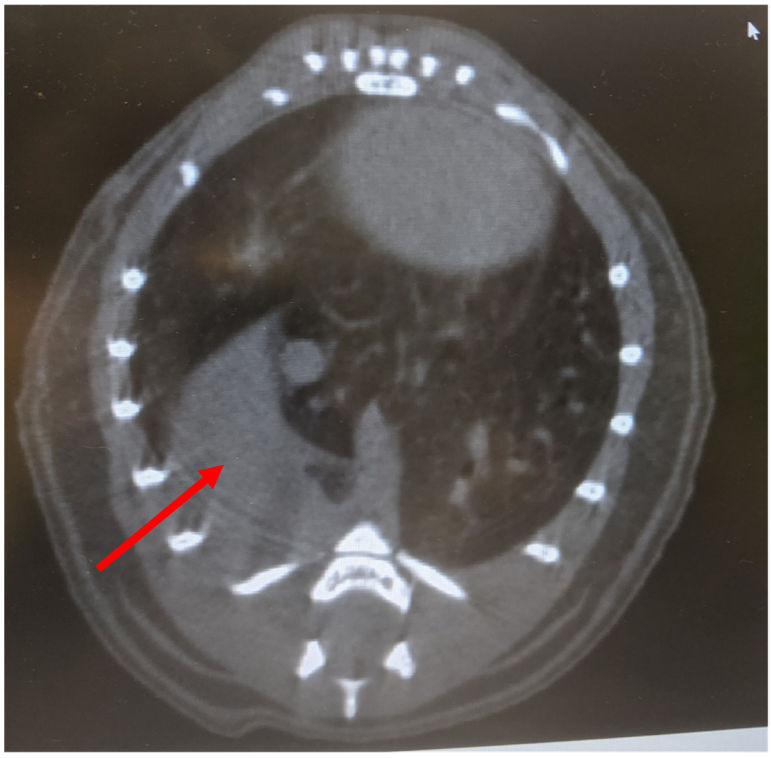


Figure 14. Micro-CT scan of the thorax of a mouse exposed to blast overpressure injury 24 hr after the blast. Note area of water density in the right lung (red arrow) indicating lung injury with pulmonary edema and/or hemorrhage.

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

Effect of ICG-001 on blast overpressure lung injury.

We completed a set of experiments using PVDF membranes from an alternate supplier to test whether ICG-001 attenuated blast overpressure lung injury. Test membranes from this material resulted in a peak blast pressure of 15-17 PSI. Mice were treated with ICG-001 50 mg/kg or vehicle control 60 min after the blast and the extent of lung injury 24 hr after the blast was assessed using bronchoalveolar lavage total cell and neutrophil counts, IgM levels (used as an indicator of alveolar-capillary unit permeability), and Receptor for Advanced Glycation Endproducts (RAGE) levels (indicator or alveolar epithelial injury). The results are illustrated in **Figures 15-17** below.

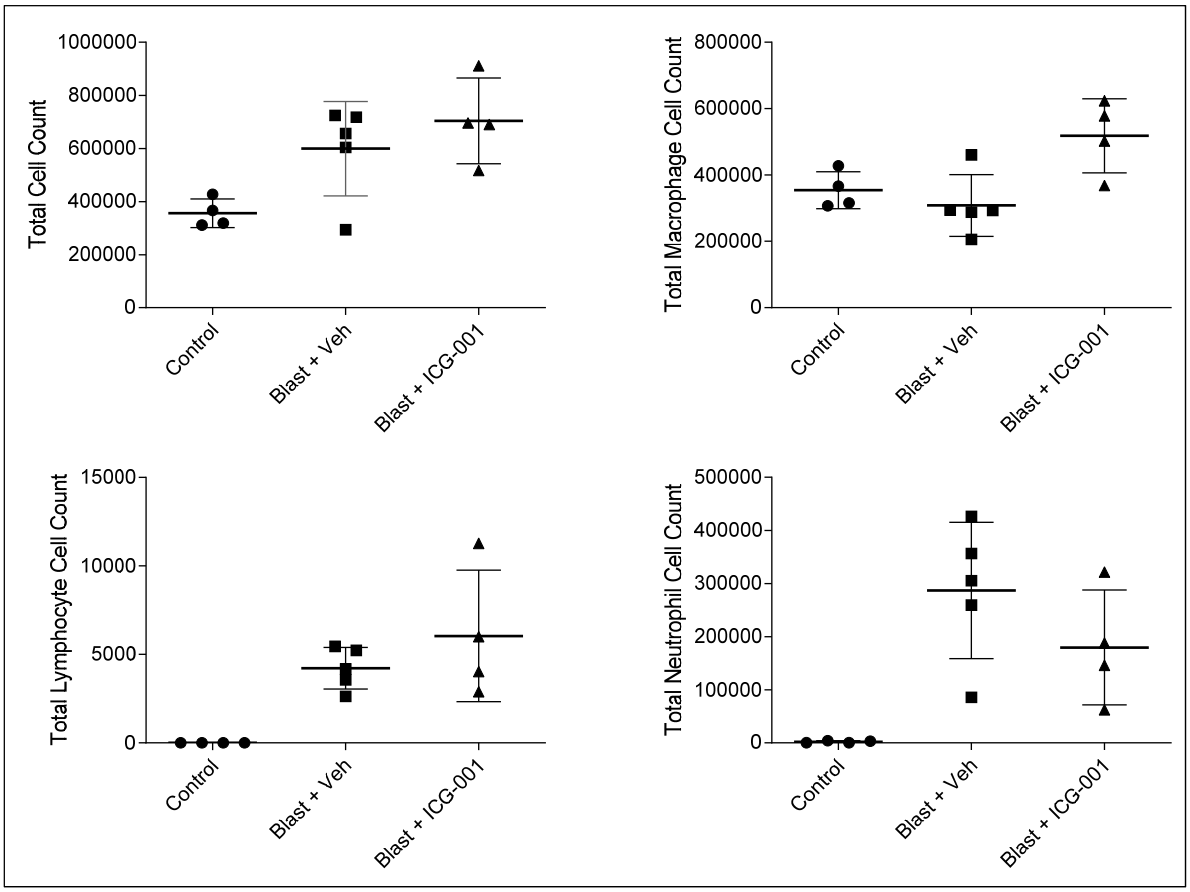
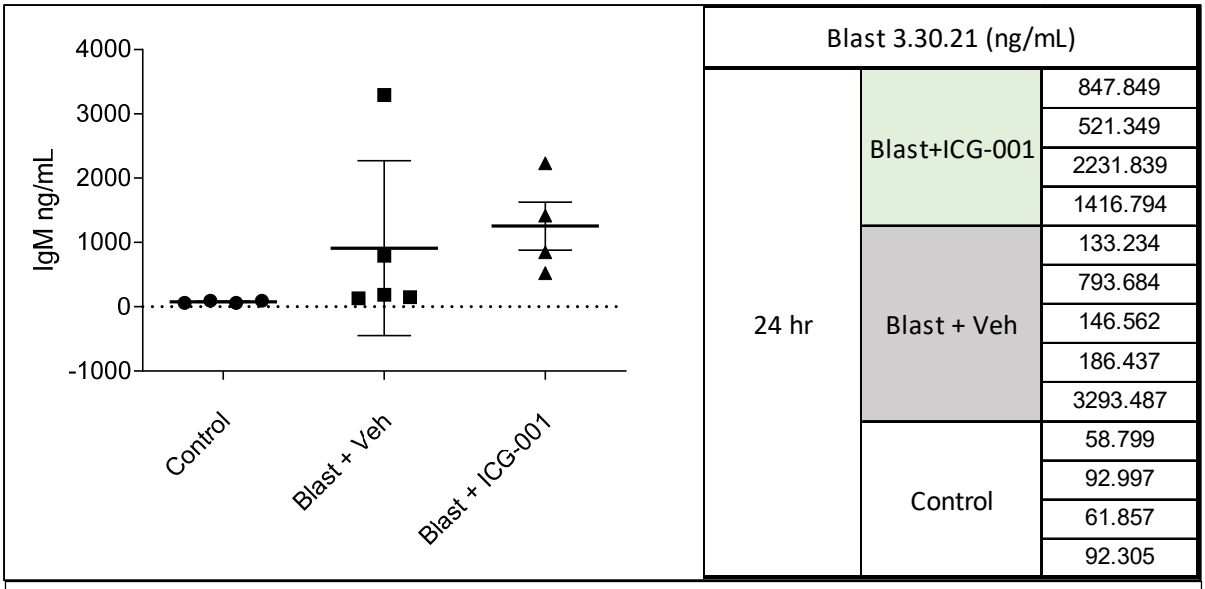


Figure 15. Effects of ICG-001 on blast overpressure lung injury at 24 hr in C57Bl/6 mice. Mice were exposed to the blast overpressure injury and then treated with ICG-001 50 mg/kg or vehicle control 60 min after the blast. Mice were euthanized 24 hr after blast exposure (n = 4-5 per group). Bronchoalveolar lavage was conducted and cell count was measured using a Coulter Counter and cell differential was measured using Wright Giemsa staining of cytospin slides.



Blast 3.30.21 (ng/mL)		
24 hr	Blast+ICG-001	847.849
		521.349
		2231.839
		1416.794
	Blast + Veh	133.234
		793.684
		146.562
		186.437
		3293.487
	Control	58.799
		92.997
		61.857
		92.305

Figure 16. Effects of ICG-001 on blast overpressure lung injury at 24 hr in C57Bl/6 mice. Mice were exposed to the blast overpressure injury and then treated with ICG-001 50 mg/kg or vehicle control 60 min after the blast. Mice were euthanized 24 hr after blast exposure (n = 4-5 per group). Bronchoalveolar lavage (BAL) was conducted and IgM levels in BAL fluid were measured by ELISA.

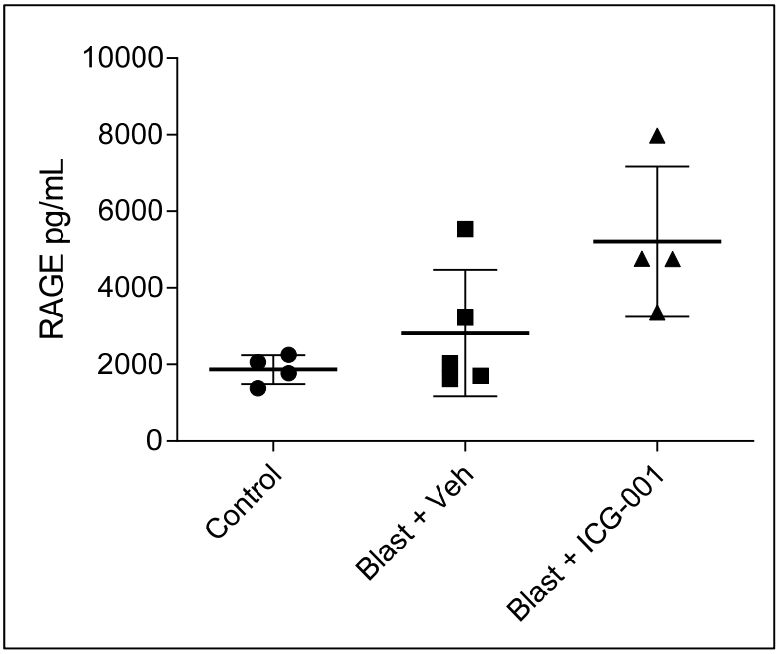


Figure 17. Effects of ICG-001 on blast overpressure lung injury at 24 hr in C57Bl/6 mice. Mice were exposed to the blast overpressure injury and then treated with ICG-001 50 mg/kg or vehicle control 60 min after the blast. Mice were euthanized 24 hr after blast exposure (n = 4-5 per group). Bronchoalveolar lavage (BAL) was conducted and RAGE levels in BAL fluid were measured by ELISA.

To summarize these results, the experiments show that blast overpressure induces lung inflammation (neutrophils in BAL fluid) and enhanced permeability (IgM). Treatment of animals 1 hr after blast overpressure with ICG-001 resulted in

diminished inflammation (neutrophil counts) and no change or increase in BAL IgM (permeability) and BAL RAGE (epithelial injury) levels. As in previous experiments, there was considerable inter-animal variability, even in the group of animals exposed to blast alone (vehicle treated) - one animal exhibited high levels of BAL IgM (increased lung permeability) and the remaining animals displaying much lower levels of BAL IgM. Similar variability was observed in BAL neutrophils and BAL RAGE levels.

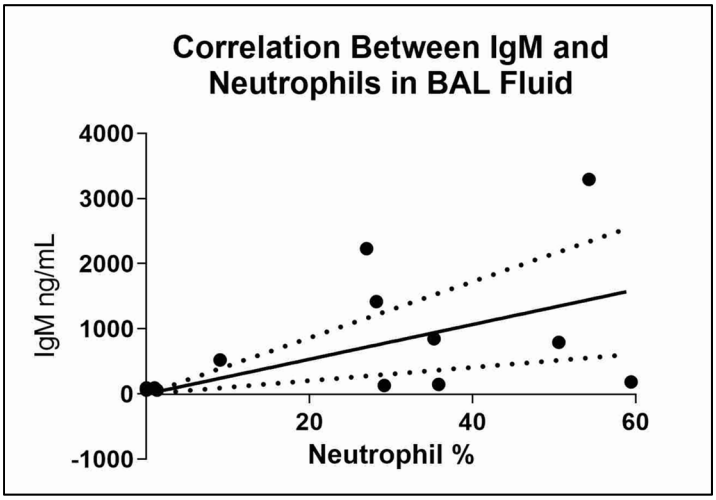


Figure 18. Correlation between BAL fluid IgM levels (indicator of lung permeability) and BAL neutrophil count (indicator of lung inflammation. The dotted lines show the 95% confidence intervals. While there is a strong correlation between BAL IgM levels and BAL neutrophil counts ($P< 0.001$, $R^2=1$), there is considerable variability.

Thinking about the potential reasons for the variability, we considered several possibilities including (i) variation in the magnitude or shape of the pressure wave, (ii) variation in the orientation or position of the animals relative to the blast wave, (iii) variation in the animal responses to blast overpressure, and (iv) technical problems with the BAL cell count or the ELISA assays for IgM and RAGE. To begin to sort through the reasons for the variability, we plotted BAL IgM levels (lung permeability) against BAL neutrophil counts (lung inflammation) as illustrated in **Figure 18**. This analysis reveals that while there is good correlation between BAL IgM (permeability) and BAL neutrophils (inflammation) $p < 0.0001$ and R^2 of 1, there are definitely outliers. We interpret this to mean that there is variation both in individual animal responses to the blast overpressure and that there are also technical problems with the assay(s) of BAL cell counts, IgM and RAGE. For the former, we have fabricated a new animal holder (described above) that ensures constant orientation of the animals relative to the blast wave. For the latter, we think that part of the problem is the extensive hemorrhage into the lung and presence of large numbers of red blood cells in BAL fluid. This may interfere with cell counts (artifact in the Coulter Counter) and possibly in the ELISAs. We will use several methods to lyse red cells (Zapoglobin, hypotonic lysis, and ammonium chloride) as well as use cell counting instruments (ThermoFisher Countess) that are less subject to artifact from red cell fragments.

We will continue to work with blast scientists at Stumptown Research Incorporated to source and test other types of PVDF membranes of varying tensile strength and to optimize the animal restraint system to minimize movement in the blast overpressure wave.

Difficulties encountered

We have continued to have some variability in the reproducibility of blast overpressure injury as outlined above. Our recent improvements to the advance blast simulator will hopefully allow us to achieve reproducibility and then we can complete the experiments with the combined PM exposure and 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

The USGS staff has completed the analysis of the particulate matter from Afghanistan and China Lake (summarized in previous reports) and developed techniques to assess the metal and mineral content of lungs from mice treated with PM from Afghanistan and Southwest Asia. They are currently using these techniques to quantify the metal and mineral content of VATS lung biopsies from previously deployed military personnel and from controls.

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

The team from the USGS are analyzing human lung tissue from the VATS lung biopsies from previously deployed military personnel and from controls using LA-ICP-MS, and FE-SEM.

Subtask 8. Prepare and submit manuscripts for publication.

Progress

Three manuscripts have been published related to the preclinical studies and another manuscript is in revision. We submitted an abstract to the MHSRS Symposium in August 2021. This was accepted for a poster presentation in the session on Acute Lung Injury in Trauma and Critical Illness. (MHSRS-21-02327. Prior Exposure to Airborne Particulate Matter from Afghanistan Increases the Susceptibility to Blast Overpressure Lung Injury in Mice). Unfortunately, the conference was cancelled due to COVID concerns.

Milestones Achieved:

1. The ('2-hit') *in vitro* epithelial cell culture model protocols have been completed characterizing the combined effects of PM and bleomycin, blast overpressure, and influenza infection on lung epithelial cell toxicity.
2. We have completed the molecular and bioinformatics analysis of the effects of PM exposure on primary human macrophages using RNA-Seq.
3. We have completed the NexGen sequencing for the RNA-Seq of lung epithelial cells (large airway, small airway, and alveolar type II cells) exposed to PM. We have completed the bioinformatics analysis for these samples.

4. The experiments with the murine model protocols exposed to PM from Iraq and Afghanistan using oropharyngeal aspiration and i.t. administration have been completed. These studies revealed evidence of small airways and parenchymal inflammation and fibrosis by PM from Iraq and Afghanistan when delivered by direct i.t. instillation or by oropharyngeal aspiration.
5. We conducted three separate inhalational exposure experiments in which mice were exposed to aerosolized PM from Afghanistan and from California in the NAMRU-Dayton facility. The third experiment has been completed and we observed only minor lung inflammation and no significant evidence of fibrosis (this has been detailed in previous reports).
6. Three manuscripts have been published and another submitted for publication. We submitted an abstract to the MHSRS Symposium to take place in August 2021. This was accepted for a poster presentation in the session on Acute Lung Injury in Trauma and Critical Illness. (MHSRS-21-02327. Prior Exposure to Airborne Particulate Matter from Afghanistan Increases the Susceptibility to Blast Overpressure Lung Injury in Mice). The conference was cancelled due to COVID concerns.

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.

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

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

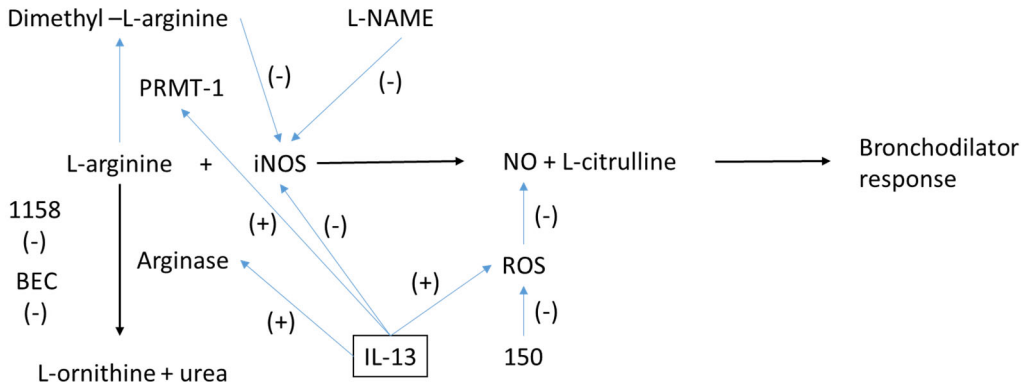
Progress

Completed.

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

Progress

1. Lack of additional effects of APM and cigarette smoke made us change our focus to study interactions between APM and allergic stimulus.
2. Afghanistan PM (APM) induces nitric oxide synthetase (iNOS) in macrophages along with increased levels of nitric oxide (NO) that is inhibited by IL-13 exposure. The mechanism behind the IL-13-mediated inhibition of nitric oxide, a known endogenous bronchodilator, is unknown.



Schematic 1 of several possible mechanisms by which IL-13 may mediate the inhibition of nitric oxide (NO) formation and pharmacological approaches to test possible mechanisms.

- Verification that APM increase in nitrite and nitrate (NOx) was produced by increased iNOS activity through inhibition with iNOS inhibitor, LNAME. We also verified that APM increased apoprotein levels of iNOS in the J744 cells and that IL-13 had little effect on this increased apoprotein expression.
- We moved from cultured macrophage cells to mouse precision cut lung slices (PCLS) and showed in this more complex and physiological lung organoid system that APM increases nitric oxide (NO) production and this

was further elevated with IL-13 co-treatment. It was interesting that responses were different depending on which exposure came first.

NO responses in mouse PCLS

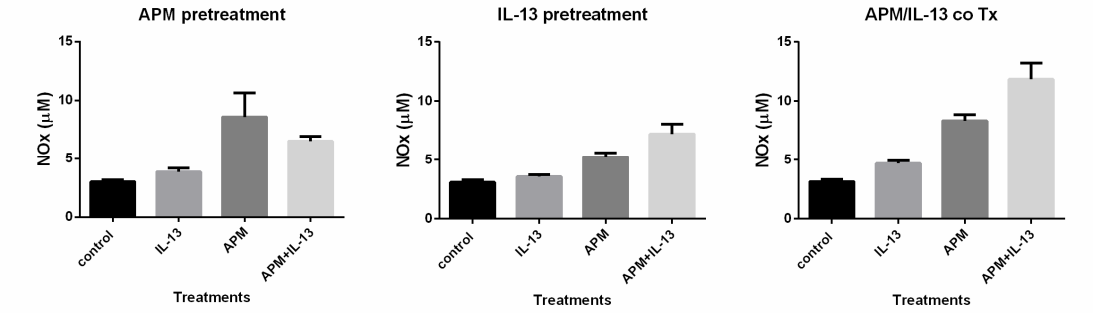
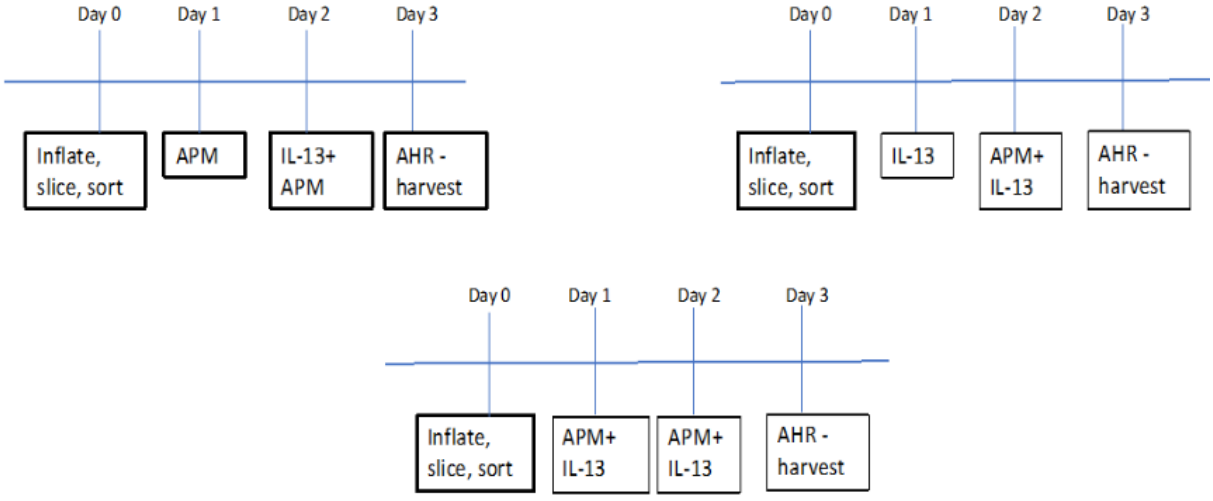


Figure 1. Nitric oxide (NO) production in mouse PCLS medium after APM and IL-13 treatments. Left panel PCLS were pre-treated with APM before IL-13 treatment. Middle panel PCLS were pre-treated with IL-13 before APM treatment. Right panel PCLS were treated at same time with APM and IL-13. Both IL-13 pre-treatment and co-treatments groups had elevated NO production.

- We used eotaxin and LIX s to IL-13 treatment to validate the PCLS system. Eotaxin-2 and LIX (chemokines) are well-established biomarkers for IL-13 response. We used this biomarker to assess different timings of APM and IL-13 treatments and durations to optimize the system.

Study Design



Eotaxin 2 response in mouse PCLS system

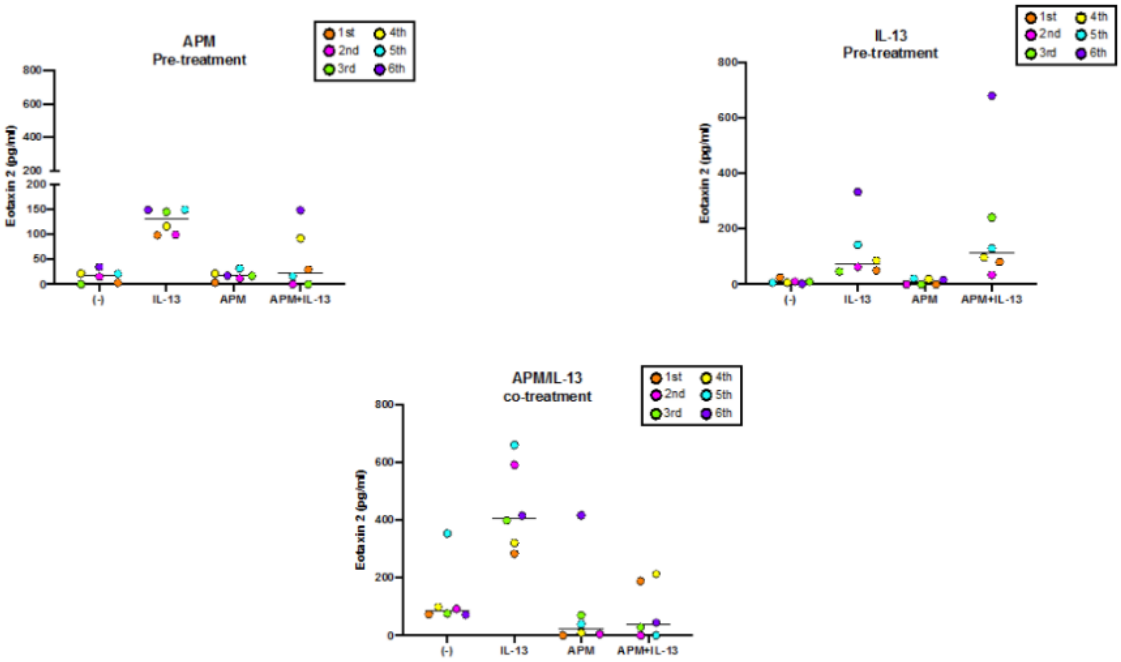


Figure 2. Mouse PCLS were cut and placed in culture wells. Assessment of slice sequence is denoted by color to ascertain any variability of slice order. IL-13 treatment resulted in the expected increase in eotaxin 2 release into the slice media under all three treatment scenarios. APM alone had little effect on eotaxin 2 release. The combination of IL-13 and APM had variable effects depending on treatment scheme. Eotaxin 2 was measured using an ELISA assay.

LIX responses in mouse PCLS system

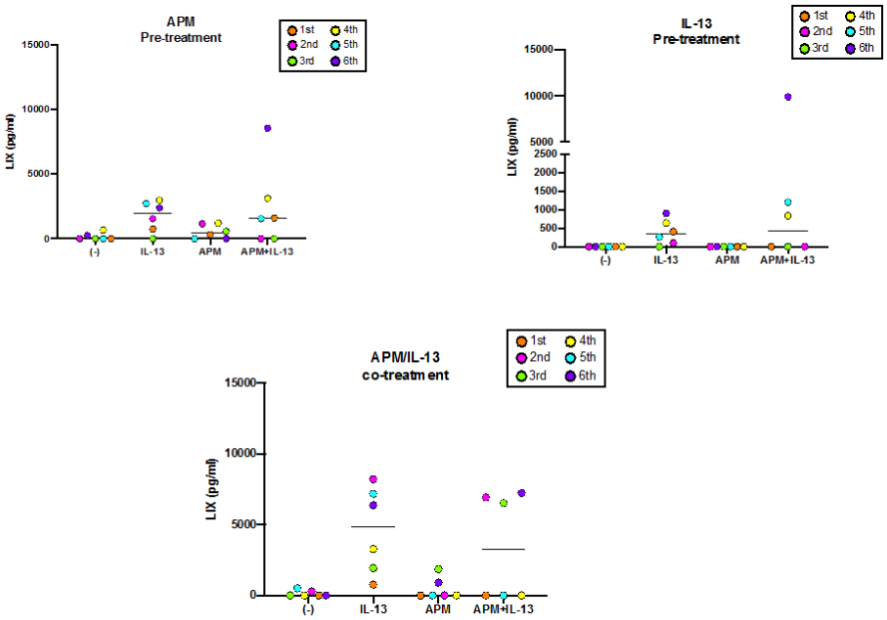
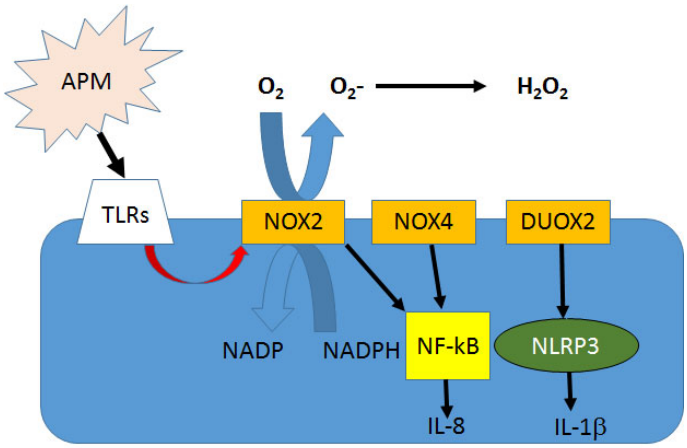


Figure 3. Mouse PCLS were cut and placed in culture wells. Assessment of slice sequence is denoted by color to ascertain any variability of slice order. IL-13 treatment resulted in the expected increase in LIX release into the slice media under all three treatment scenarios. APM alone had little effect on LIX release. The combination of IL-13 and APM had variable effects depending on treatment scheme. LIX was measured using an ELISA assay.

3. We have started studies looking at how IL-13 and APM can modulate ROS formation through activation and expression of NADPH oxidases (NOXs) and duel oxidases (DUOXs). Our previously published research showed a strong signaling of APM through the TLR pathway and we will use this to understand whether this pathway also drives ROS formation in airway epithelium and macrophages.



Schematic 2. Several possible mechanisms by which APM may mediate the activation and expression of NOXs and DUOXs to increase the formation of reactive oxygen species such as superoxide and hydrogen peroxide that are known activators of NF-kB and NLRP3 that drive airway inflammation.

4. Preliminary data supporting the ability of APM to increase the formation of ROS in macrophages exposed to APM, IL-13 and the combination. A macrophage cell line (J744) was grown to confluence in 24-well plates and treated with PBS, APM, IL-13 or the combination and cellular oxidative stress was measured using a redox fluorescent probe (CellROX green, Invitrogen) 24 hours after treatments. APM treated cells showed increased number and intensity of green fluorescence. The intensity of fluorescent cells was further increased in the APM +IL-13 treatment groups.

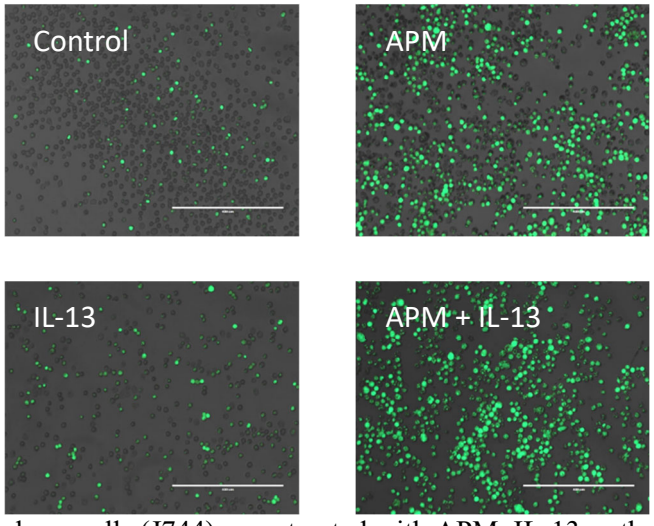


Figure 4. Macrophage cells (J744) were treated with APM, IL-13 or the combination and cellular oxidative stress was assessed using a redox fluorescent probe (CellROX green) 24

hours after treatment and green fluorescent intensity assessed by photo microscopy (magnification bars, 400 um).

5. We have detected Afghan PM (APM)-mediated hydrogen peroxide production in the mouse macrophage cell line (J744) and in mouse primary lung macrophages (**Figure 5**). The two most prominent sources of cellular ROS are NADPH oxidases (NOXs) and mitochondrial production. We looked at whether the hydrogen peroxide production could be inhibited by diphenylene iodonoum (DPI). DPI is a wide spectrum inhibitor of NOXs. APM increased hydrogen peroxide production over basal levels in both macrophage cultures, where the signal was much more pronounced in the primary lung macrophage cultures. IL-13 treatment had no effect on hydrogen peroxide production and in combination with APM had a small increasing effect in the J744 cells and no effect in the primary lung macrophage cultures. Interestingly, DPI had no effect on basal hydrogen peroxide production and slightly increased signals in the APM treatment group and the APM/IL-13 treatment groups in the J744 cells. This effect was not seen in the primary lung macrophage cultures where DPI produced a slight decrease in APM-mediated hydrogen peroxide production. Our data suggest that APM-mediated oxidative stress could be differentially regulated by IL-13 depending on the sources of macrophages, which may be independent of NOXs.

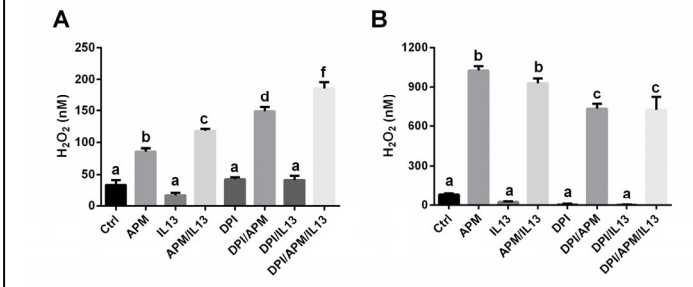


Figure 5. Effect of Afghan PM (APM) on production of hydrogen peroxide in J744 cell line (A) and primary lung macrophages (B). Cells were treated with APM (50ug/cm^2), IL-13 (50ng/ml), diphenylene iodonium (NOX inhibitor, 10uM), or their combinations for 24 hours. Hydrogen peroxide was measured using the Amplex red assay in imaging buffer over 3 hours. Different letter indicates a significant difference between treatment groups, $p<0.05$.

6. The lack of effect of a broad spectrum NOX inhibitor on APM-mediated hydrogen peroxide production suggested that the APM may be stimulating mitochondrial hydrogen peroxide production. We tested this idea using an inhibitor of mitochondrial ROS formation that blocks electron flow through the electron transport chain, (FCCP). We cultured J744 cells in a 48 well plate and treated with APM and/or IL-13 in the presence or absence of FCCP (**Figure 6**). APM treatment increased hydrogen peroxide formation over basal levels. IL-13 treatment had little effect by itself, but increased hydrogen peroxide production slightly in the APM/IL-13 treatment group. FCCP treatment decreased basal hydrogen peroxide formation, but had little effect on the APM-mediated hydrogen peroxide formation. Our data suggest that in a macrophage cell line, mitochondrial is unlikely a major source of hydrogen peroxide following APM stimulation.

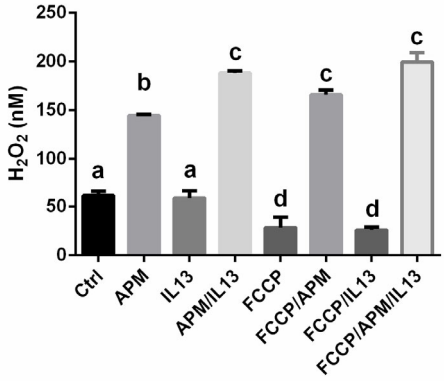


Figure 6. Effect of Afghan PM (APM) on production of hydrogen peroxide in J744 cell line. Cells were treated with APM (50ug/cm^2), IL-13 (50ng/ml), FCCP (10uM), or their combinations for 24 hours. Hydrogen peroxide was measured using the Amplex red assay in imaging buffer over 1 hours. Different letter indicates a significant difference between treatment groups, $p<0.05$.

7. The lack of effect of a mitochondrial hydrogen peroxide inhibitor suggested some other source. Another possible source of cellular hydrogen peroxide is the conversion of xanthine dehydrogenase to xanthine oxidase. We tested this idea using a xanthine oxidase inhibitor, allopurinol that is used in humans to treat gout. We cultured J744 cells in a 48 well plate and treated with APM and/or IL-13 in the presence or absence of allopurinol (**Figure 7**). APM treatment increased hydrogen peroxide formation over basal levels. IL-13 treatment had little effect by itself or in the APM/IL-13 treatment group. Allopurinol treatment no effect on basal hydrogen peroxide formation. Allopurinol in combination with APM greatly increased both APM-mediated hydrogen peroxide formation and further increased hydrogen peroxide formation

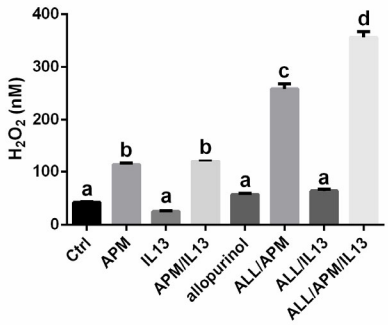


Figure 7. Effect of Afghan PM (APM) on production of hydrogen peroxide in J744 cell line. Cells were treated with APM (50ug/cm^2), IL-13 (50ng/ml), allopurinol (25uM), or their combinations for 24 hours. Hydrogen peroxide was measured using the Amplex red assay in imaging buffer over 1 hours. Different letter indicates a significant difference between treatment groups, $p<0.05$.

combination with APM/IL-13. It is unclear the mechanism behind these findings.

8. We have not seen any of these inhibitors affect the APM-mediated hydrogen peroxide signal. There was some concern on whether artifacts could be contributing to some of our findings related to the Amplex Red assay. We tested whether the Amplex red signal could be quenched by catalase which is specific for hydrogen peroxide and whether the hydrogen peroxide signal required cells. We tested this in a 48 well plate where two rows did not contain cells with and without APM under our study conditions (**Figure 8**). APM treatment increased hydrogen peroxide formation over basal levels and catalase treatment inhibited the basal Amplex red signal completely. Catalase also completely inhibited the APM Amplex red signal indicating both of these signals were due to hydrogen peroxide production. It was very interesting that in the wells with no cells the APM produced more Amplex red signal that was completely inhibited by catalase. These data suggest that the APM itself is the predominant source of hydrogen peroxide and that the cells were actually consuming some of this hydrogen peroxide production.

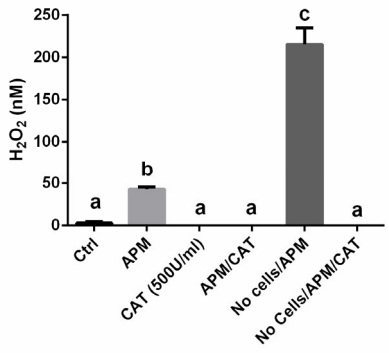


Figure 8. Effect of Afghan PM (APM) on production of hydrogen peroxide in J744 cell line or cell free system. Cells or cell free system were treated with APM (50ug/cm^2), catalase (CAT, 500U/ml), or their combinations for 24 hours. Hydrogen peroxide was measured using the Amplex red

9. We have recently turned our attention to the APM itself and found that the APM could produce hydrogen peroxide in a cell free system. We found that the cell free system generated much more hydrogen peroxide than the cell system and that all the signal could be quenched by catalase. We next compared the hydrogen peroxide production in cell and cell free systems of different PM sources (**Figure 9**). We compared the APM to Iraqi PM (IPM) and California PM (CPM) in J744 cells and in cell free systems. In the J744 cell system CPM had the highest hydrogen peroxide signal and both APM and IPM were similar. In the cell free system APM had the highest signal followed by the California and then the Iraqi PM. All signals could be quenched 100% by catalase.

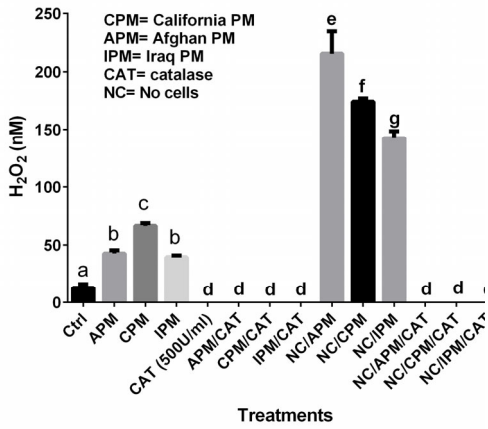


Figure 9. Effect of three different PMs on production of hydrogen peroxide in J744 cell line or cell free system. Cells or cell free system were treated with APM (50ug/cm^2), CPM (50ug/cm^2), IPM (50ug/cm^2), catalase (500U/ml), or their combinations for 24 hours. Hydrogen peroxide was measured using the Amplex red assay in imaging buffer over 1 hours. Different letters indicate a significant difference between treatment groups, $p<0.05$.

10. The literature has reported the ability of some PM to produce hydrogen peroxide and suggested the sources are from transition metals and polyaromatic hydrocarbons components of the PM (see Guan et al. *Int J Environ Res Public Health* 13:483, 2016; Rodriqnez-Cotto et al. *Environ Toxicol Pharmacol* 39:845, 2015). To test whether this is also a possibility with our APM, we used a metal chelator that is clinical approved for use in humans, desferrioxamine (Desferal, DFO) (**Figure 10**). DFO attenuated 70% of basal hydrogen peroxide signal and 100% of the APM signal in the J744 cell system. DFO treatment attenuated 75% of the APM-mediated hydrogen peroxide production in the cell free system. These data suggest that the type and amount of transition metals in the PM can contribute to the ROS signaling involved in the activation of the IL33/ST2 pathway. We are currently exploring these new exciting findings in our precision cut lung slice (PCLS) system and its effect on airway hyperreactivity.

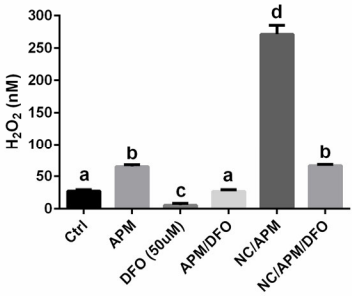


Figure 10. Effect metal chelation on APM-mediated production of hydrogen peroxide in J744 cell line or cell free system. Cells or cell free system were treated with APM (50ug/cm^2), desferal (50uM), or their combinations for 24 hours. Hydrogen peroxide was measured using the Amplex red assay in imaging buffer over 1 hours. Different letters indicate a significant difference between treatment groups, $p<0.05$.

11. We tested whether the pathways for APM-mediated stimulation of cytokine release and hydrogen peroxide generation signaled through the TLR2 receptor. We used a pharmacological approach that blocks TLR2 receptor from downstream partner signaling using C29 compound. We first performed a dose response with C29 (10, 25, 50 uM) for 24 hours and looked at hydrogen peroxide production in imaging buffer as previously described. C29 treatment had no effect on APM-mediated hydrogen peroxide in J774 cells (**Figure 11A**). To examine the role of APM stimulating cytokine (KC, IL-8 human analog) we treated J744 cells with APM (50ug/cm2) and C29 (50uM) for 24 hours and then looked at cytokine production 24 hours later. C29 treatment produced a robust inhibition of APM-mediated increases in KC levels (**Figure 11B**). This data suggest that the APM-mediated production of hydrogen peroxide are not through TLR2 signaling, but KC production is partly TLR2 dependent.

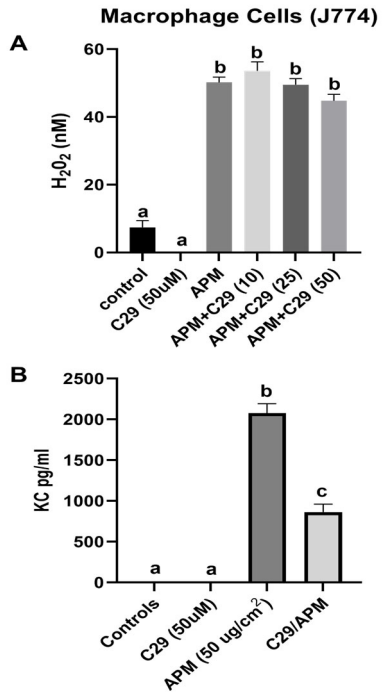


Figure 11. Effect of TLR2 inhibition on hydrogen peroxide (**A**) and (**B**) cytokine (KC) production mediated by APM. J774 macrophage cell line treated with APM or C29 for 24 hours and hydrogen peroxide measured with Amplex red assay and KC by ELISA.

12. We are currently moving studies to precision cut lung slice (PCLS) model that is a more complete testing system containing all of the lung cell types and structures. This system will also allow us to test in human PCLS to translate mouse findings to humans. Airway hyperresponsiveness (AHR) is a one of the most important research outcomes to study how particulate matter affects airway function such as airway flow limitation in deployers with asthma-like disease. Recently, we extensively optimized the experimental conditions to induce small airway contraction in human distal lung tissue using the PLCS approach. We obtained lungs from two human donors, one from a non-smoking subject and another one from a smoking subject. We cut lung slices at different thickness, such as 300, 350 and 450 µm and treated them

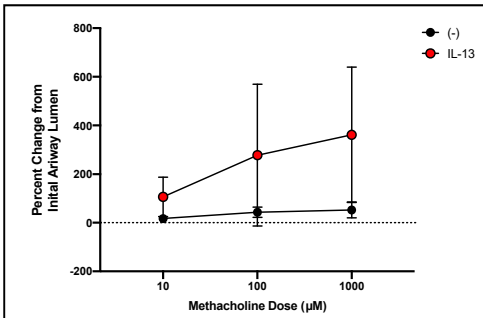


Figure 12. IL-13 increases airway hyperresponsiveness (AHR) in human PCLS after 7 days of IL-13 (25 ng/ml). PCLS was stimulated with increasing concentrations of methacholine.

with methacholine from 10 to 1000 μM. We were able to identify small airways in about 80% of the lung slices, and observed methacholine dose-dependent small airway contraction. With our optimized AHR measurement, we will test if Afghanistan particulate matter (APM) in combination with type 2 cytokines and viral infection (e.g., influenza A virus) enhances airway contraction, leading to more severe airway obstruction seen in deployers with asthma-like disease. IL-13 increased airway hyperresponsiveness in human PCLS (**Figure 12**).

Difficulties encountered:

None.

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

Progress

1. Most of the goals of this subtask have been completed and published: Berman et al IL-33/ST2 signaling modulates Afghanistan particulate matter-induced airway reactivity in mice. Toxicol Appl Pharmacol 404:115186, 2020.
   - This paper details the investigation the IL-33/ST2 pathway in IL-13 and APM induced inflammation using an IL-33 decoy receptor soluble ST2 (sST2).
   - This paper also tested the effects of using sST2 as a possible treatment for APM-mediated airway hyperresponsiveness.
   - This paper also describes a role for RGS2 in the downstream pathway associated with APM-mediated airway hyperresponsiveness.
2. New developments related to this subtask is development of mouse (**Figure 13**) and human (**Figure 14**) PCLS ex vivo systems to explore interactions between APM and IL-13 and mechanisms on airway hyperresponsiveness.
   - Mouse PCLS model system

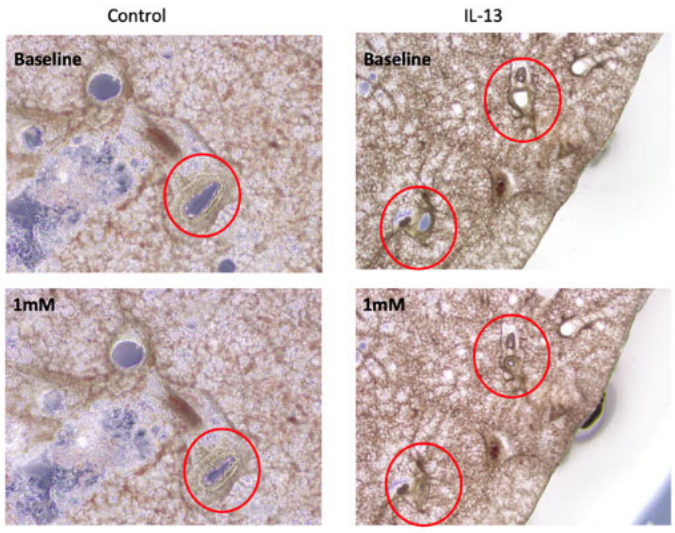


Figure 13. Mouse PCLS response to methacholine challenge. Left panels are mouse PCLS control (left top) and treated with 1mM methacholine (bottom left). Right panels are mouse

PCLS pre-treated with IL-13 (right top) and treated with 1 mM methacholine (bottom right). Circles indicates the airway contraction response to methacholine. A 3 day IL-13 pretreated PCLS revealed complete constriction of small airways compared with only a partial constriction in the control PCLS.

- Human PCLS model system

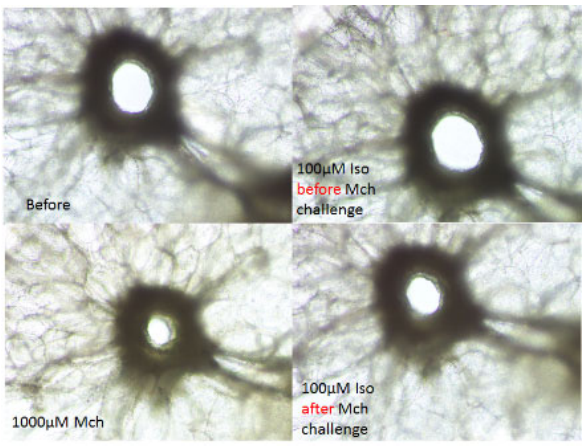


Figure 14. Micrographs of human PCLS constricted with methacholine and relaxed with isoproterenol (Iso).

Difficulties encountered:

None.

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

Progress

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

Difficulties encountered:

None.

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

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

Methodology is done. See **Project 2** for update

Subtask 7.

Progress

Published manuscript: Berman et al. IL-33 /ST2 signaling modulates Afghanistan particulate matter-induced airway reactivity in mice. *Toxicol. Appl Pharmacol*, 2020;404:115186.

Milestones achieved:

1. Mouse and human PCLS model systems developed with capability to measure ex vivo airway hyperresponsiveness.
2. Molecular mechanisms of APM-mediated hydrogen peroxide and nitric oxide production explored with evidence of direct APM formation of hydrogen peroxide and a TLR2 receptor dependent signaling pathway for cytokine production.
3. Role for metals in APM contributing to hydrogen peroxide formation.
4. Possible intervention of cytokine and hydrogen peroxide production.

Major activities:

1. Setting up more integrated model system using precision cut lung slices that have all the lung cell types in proper anatomical locations.
2. Using the precision cut lung slices to validate earlier findings between APM and allergic asthma.
3. Using precision cut lung slices from mouse and humans to validate findings in our rodent models to humans.
4. Using human and mouse PCLS to explore molecular mechanisms of hydrogen peroxide and cytokine production by APM.

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 reporting year, this study has resumed recruitment and enrollment of patients who fulfill the requirements for deployed subjects following the halt due to the COVID-19 pandemic. Beginning in quarter 2, enhanced safety protocols were implemented in order to allow for the re-initiation of nasal and bronchial brush collection. These precautions include pre-testing subjects to show SARS-CoV-2 negative status prior to sample collection, or processing under BSL2+ conditions if no test can be performed prior to sample collection. Due to increased precautions, and limitations in space and timing, recruitment has continued, though at a limited rate. 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. Within the last reporting year, we have successfully collected samples from all of the recruited subjects as listed below:

Table 1: Samples Collected from Deployed Subjects (as of 9/29/2021)

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

2. In the past reporting year, this study has resumed recruitment and enrollment of healthy subjects and non-deployed asthmatic patients who fulfill the requirements following the halt due to the COVID-19 pandemic. Beginning in quarter 2, enhanced safety protocols were implemented, in order to allow for the re-initiation of nasal and bronchial brush collection, including pre-testing subjects to show SARS-CoV-2 negative status prior to sample collection. Due to increased precautions, and limitations in space and timing, recruitment has continued, though at a limited rate. From these subjects, we continue to collect and isolate RNA and DNA samples from nasal and bronchial brushings collected from non-deployed subjects to fulfill Subtask 1. Within the last reporting year, we have successfully collected samples from all of the recruited subjects as listed below:

Table 2: Samples Collected from Non-Deployed Subjects (as of 9/2021)

	Total # Nasal Samples	Total # Bronchial Samples	Number of Paired Nasal/Bronchial Samples
Controls	10	3	6
Asthmatics	3 (Target # achieved)	3 (Target # achieved)	3 (Target # achieved)

3. Recruitment of deployers and non-deployers was halted until mid-February 2021 as handling of samples from patients with unknown SARS-CoV-2 status must be handled under BSL2+ safety conditions until qPCR can be completed. Upon testing, negative SARS-CoV-2 status confirmation has allowed samples to be handled under standard BSL2 conditions. In order to proceed with recruitment, we implemented the follow safety protocols and facilities:
   a. Developed appropriate PPE requirements and working guidelines have been established for a BSL2+ facility in order to process samples that are low risk but untested for SARS-CoV-2
   b. Allocation/purchasing of instrumentation required for this lab space has been completed and we have furnished and implemented a functional BSL2+ workspace
   c. Institutional approval was received to handle such BSL2+ samples before qPCR testing has been obtained and approved
   d. (If available) Pre-testing patients was added, before brush collection, for SARS-CoV-2 by either qPCR or rapid antigen testing
   e. (If pre-testing not available) Development of qPCR assays for SARS-CoV-2 virus was completed and is now being implemented to test samples either A: after collection and freezing of specimens, to proceed with culture of frozen specimens in BSL2 conditions, or B: immediately after collection, to allow for fresh samples processed under BSL2 conditions (if test is negative)

4. We have completed collection of the number of targeted samples for both paired nasal and bronchial (n=10), and nasal (n=50) samples from our non-deployed asthmatic group. We have also collected 70% of our target number for our non-deployed healthy controls. In this last year, we have compiled all samples currently available and have constructed RNA sequencing libraries, with each set including samples from all groups in the study to avoid batch effects.
   a. We have successfully completed the library preparation for 206 samples for this study from both nasal and bronchial brushes collected from deployed and non-deployed subjects.
   b. We have successfully extracted and QCd the ~50 additional samples currently available from recruited subjects, which are in our lab queue for library preparation and sequencing.

5. We have successfully sequenced all 206 of these samples. High quality RNA-seq data was obtained for all samples, with an average genome mapping rate

of 87.5% and an in-gene mapping rate of 80.5%. We are now analyzing the gene expression data, performing differential expression and network analyses, with the goal to identify gene expression changes associated with obese asthma.

Difficulties Encountered

1. Due to increased safety measures put into place due to the COVID-19 pandemic, recruitment and sample collection slowed momentarily, but has since begun again. Safety guidelines including pre-testing patients for SARS-CoV-2, or internal qPCR testing if pre-testing is not available have been initiated. Additionally, a BSL2+ working environment have been put into place to facilitate resuming recruitment and sample processing if pre-testing is not available.
2. Re-query of non-deployed controls resulted in removal of nearly half our consented control subjects due to confounding diagnoses that resulted from their medical visit. However, we have been able to recruit and collect healthy subjects from other similar airway studies, and have been able to increase the rate of recruitment of healthy control subjects, and we are confident this will aid in reaching our recruitment goals soon.

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

Progress

1. We continue to work on "grade of membership models" analysis of our datasets which we believe will help us to deconvolute mechanisms operating across different cell types in the airway brushings collected from the deployers. We are testing a large number of parameters across four datasets to determine the ideal parameters to analyze the DOD data set once it is available.

2. Additionally, our lab has recently published a manuscript describing the single cell transcriptome profile of 8 distinct cell populations, and their developmental intermediates, found within the *in vivo* human tracheal airway epithelium (Goldfarbmuren and Jackson, Nat Comm 2020). Using these datasets, we are implementing the use of these gene sets and cell type-specific markers to further investigate, understand, and deconvolute bulk RNAseq data sets. These analyses will give us a much clearer understanding of the responses we see in the nasal and bronchial brushes collected in Subtask 1.

3. We have been optimizing implementation of topic modeling as an analytic method for in vivo patient brushing RNA-seq data. This method (i) identifies latent programs of biological function driving expression profiles and (ii) enables subtyping of donors based on the relative expression of these coherent programs. Early results demonstrate the capacity to identify pathobiological mechanisms (endotypes) in asthma, and we anticipate a shared capacity to investigate mechanisms driving DLD and DDLD.

4. We have begun preliminary analysis of the nasal brushing *in vivo* data generated thus far to identify DEGs associated with both deployed lung diseases. To accomplish this, we used DESeq2 in the comparison of expression levels for all variant genes between available non-deployed healthy control (n=20) and DDLD+DRA (n=12) samples or DDLD only (n=23) samples, identifying 4,729 and 4,860 DEGs for these groups, respectively. Enrichment analyses on these DEGs revealed strong upregulation of the aryl-hydrocarbon receptor (AHR) pathway, which is involved in response to PM and other xenobiotics, as well as activation of interferon inflammation pathways. We also found that *IL33*, a master cytokine driving type 2 inflammation in asthma, was strongly upregulated.

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. Plans for genotyping of samples for patients recruited into this study were defined to using the Infinium Multi-Ethnic Genotyping Array (MEGA) chip. A project manager responsible for samples and analysis was assigned for our project in collaboration with the Research Genetics Core at the University of Colorado. These chips include all the necessary negative and positive controls for appropriate data analysis.
   a. Genotyping kits were ordered, and following backorder delays due to high demand for Agilent Technologies reagents and shipping backlogs, sample kits and chips have arrived and are now in house and ready to use

2. For all deployer subjects, collected blood samples have been processed for DNA isolation. DNA samples have been quantified, normalized, and arrayed into submission plates for analysis. Submission is currently pending the collection of final samples from 2021 to complete the 96well plate.

3. For all non-deployed subjects, we have completed extraction of all brush samples for DNA isolations. 35% of nasal/bronchial brush samples do not have adequate mass for submission and analysis. For these subjects, expansion of basal cells has begun to be used for DNA isolations for analysis of those donors.
   a. In this reporting period, we have finished ~1/2 of the isolations and QC for the subjects that required this extra step for DNA for genotyping.
   b. Submission of these samples, as with those from the deployers, are awaiting additional subjects from 2021 to fill out the submission plate, and are anticipated to be submitted in the next month.

Difficulties Encountered

The majority of the deployer samples (60% of deployed subjects) did not yield high enough DNA concentrations from the nasal or bronchial brushes. However, for all GLIDE subjects in the study, PBMCs from blood samples were collected and DNA isolated and stored. DNA yields from these samples are quite high and more than enough for genotyping analysis. We obtained these banked DNA

samples for genotyping of the deployers and submission of samples, which will be submitted for analysis in the next month.

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 are currently working to combine both batches of ICP-MS data, ensuring no batch effects are present, and that the data was analyzed in a similar manner before combining for final analysis. We will be working with the USGS to confirm combined data and interpretations.

Difficulties Encountered

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

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

Cell Expansion

1. Due to the COVID-19 pandemic, no recruitment of deployers was done in the first quarter of this past reporting year. In Q2, our BSL2+ labs were established, which allowed for the collection and processing of samples from subjects without a negative SARS-CoV-2 test to be processed following collection. Furthermore, implementation of pre-testing patients prior to collection of brushes (if available), has allowed for fresh processing of samples under BSL2 laboratory safety conditions. In the past reporting year, we have collected and successfully expanded cells from nasal brushes as listed below:

Table 3: Nasal Samples Cultured from Deployed Subjects: 9/2020 – 9/2021

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

2. Due to the COVID-19 pandemic, no recruitment of patients for any cohort was done in the first quarter of this past reporting year. In Q2, our BSL2+

labs were established, which allowed for the collection and processing of samples from subjects without a negative SARS-CoV-2 test to be processed following collection. Furthermore, implementation of pre-testing patients prior to collection of brushes (if available), has allowed for fresh processing of samples under BSL2 laboratory safety conditions. In the past reporting year, we have collected and successfully expanded cells from nasal brushes as listed below from non-deployed control and asthmatic subject:

Table 4: Nasal Samples Cultured from Non-Deployed Subjects: 9/2020 – 9/2021

	# Nasal Samples	Successfully Expanded Nasal Samples
Controls	10	10
Asthmatics	3	2

3. In concordance with Subtask 1, we have achieved our targeted number of expanded nasal brushes for non-deployed asthmatics (n=50), and are at the halfway point (n=25) for culture expansion of non-deployed controls.
4. Working toward our ultimate target of 50 non-deployed controls – we have successfully expanded cells from the majority of our collected subjects, and are 65% of the way toward our target goal for in vitro cell culture exposure models and analyses.
5. Following discussion with all project leads, for *in vitro* stimulations we decided to combine deployers in groups diagnosed with asthma, and those diagnosed with both asthma and DDLD, into a single category to study deployers with asthma and their response to Iraq sourced PM for Subtask 5.
6. Recruitment of deployers and non-deployers, in part, was halted through February 2021 as handling of samples from patients with unknown SARS-CoV-2 status must be handled under BSL2+ safety conditions until qPCR can be completed. Upon testing, negative SARS-CoV-2 status confirmation has allowed samples to be handled under standard BSL2 conditions. Implementation of these new protocols allowed us to handle, process, and culture nasal and bronchial cell samples under BSL2 safety conditions following a negative SARS-CoV-2 qPCR test. As such, we have completed the assembly of a functional BSL2+ room including:
   a. A BSL2+ facility was established including appropriate instrumentation required for this lab space
   b. Institutional approval to handle such BSL2+ samples before qPCR testing was obtained and approved.
   c. Implementation of CDC qPCR assays for SARS-CoV-2 virus has been completed and is now being used to test samples either A: after collection, to proceed with culture of frozen specimens in BSL2 conditions, or B: after collection, to allow for fresh samples processed under BSL2+ conditions to be moved into BSL2 lab space (if test is negative).

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

1. Using our acute model of airway epithelial exposure to Iraq PM, we have continued to expand on the biological responses of the epithelium after exposure. We found a significant downregulation in gene ontology terms important in cell-cell junctional structure, including *Cadherin Binding* (p-adj = 6.16e-20) and *Focal adhesion* (p-adj = 8.0e-13). Analysis of specific components involved in these junctional structures found the following:
    a. Downregulation of genes involved in adherens junctions, including the main junctional molecule cadherin (*CDH1*), and factors important in the stabilization of the cadherin complexes (*SCRIB* and *LLGL1*) (Figure 1).
    b. Disruption of genes coding for important desmosome and gap junction proteins involved in cell-cell and cell-basement membrane contact and structure. Downregulated signals include desmoplakin (*DSP*), periplakin (*PKP1, PKP2*), and plakoglobin (*JUP)* (Figure 1).

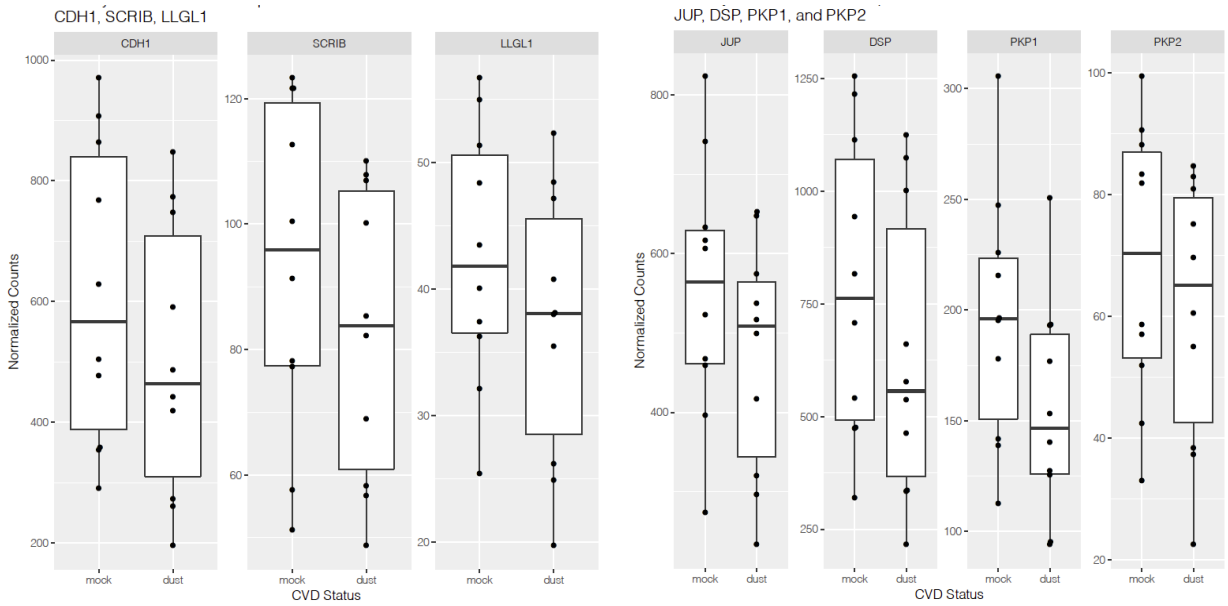


Figure 1: Gene expression changes in adherens, gap, and desmosome junctional components following acute PM-exposure of the airway epithelium

2. Further investigation into basal cell dysfunction induced by PM exposure was performed following evidence that both acute and chronic PM exposure negatively affects basal cells by decreasing genes important in maintaining basal cell health, and by increasing inflammatory signals.
    a. RNA fluorescent in situ hybridization (RNAscope) analysis of basal cells exposed to Iraq PM for 24hrs confirmed that IL-1 family cytokines, including *IL1A*, *IL1B* and *IL36G* are upregulated following acute PM exposure (Figure 2; left panel)
    b. Scratch-initiated wound healing assays were also performed on basal cells following exposure to Iraq PM for 24hrs. Following scratch initiation, we found that PM-exposure decreased the % wound healing by 38% at 12hrs post-scratch initiation ($p<0.05$; Figure 2; right panel)

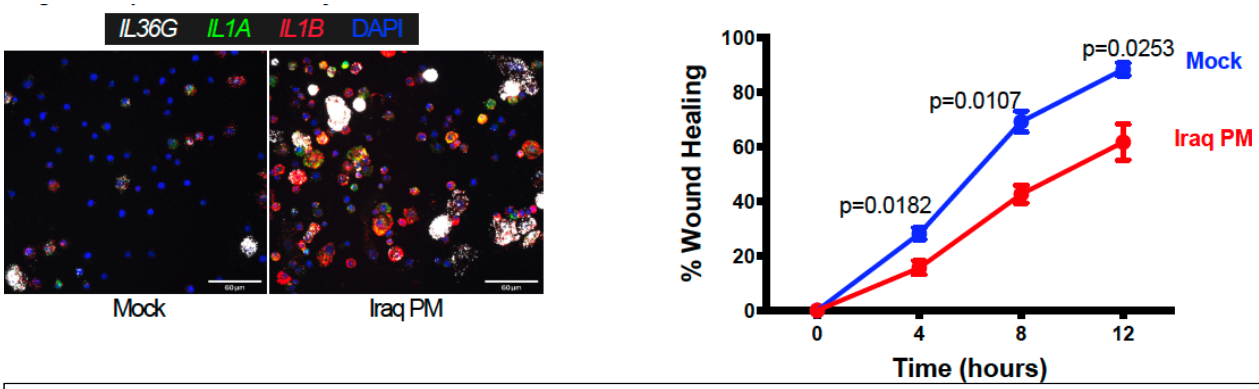


Figure 2: (Left Panel) RNA fluorescent in situ hybridization (RNAscope) labeling of *IL1B, IL36G,* and *IL1A* in basal cells exposed to control media or Iraq PM for 24hrs. (Right Panel) Wound healing assay measurement of % healing of nasal basal cells following 24hr exposure to mock vs Iraq PM treatment.

3. We previously performed a pilot experiment using a 96hr stimulation model. We first performed either mock or Iraq V3 PM stimulations for 72hours. Following initial 72hr stims, we continued mock or Iraq PM stimulation for 24hours, and added in IL-13 exposure for the final 24hours. This model aimed to measure, 1: the remodeling response of Iraq PM following chronic exposure, and 2: if this remodeling effect by chronic Iraq PM exposure alters the airway epithelial response to the potent asthma-associated cytokine IL-13 (Figure 3.)

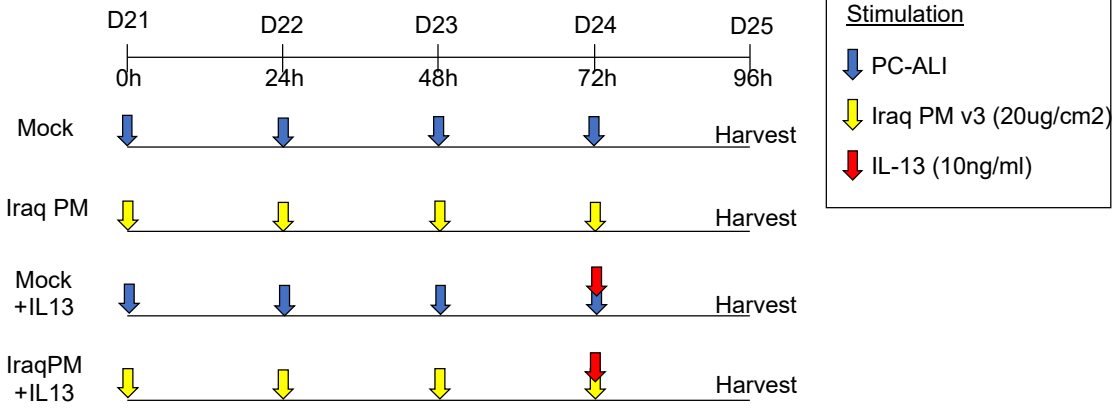


Figure 3: Timeline of chronic PM exposure followed by IL-13 stimulation to investigate 'two-hit' hypothesis for Project 4 Subtask

4. RNA-sequencing of samples from this chronic PM exposure pilot was completed on stimulations from nasal cells differentiated from 4 non-deployed subjects. Our analysis found that chronic PM exposure resulted in 4,003 significant differentially expressed genes compared to mock treated control samples.
   a. Significant remodeling was observed, as chronic PM increased several secretory-associated mucin genes including *MUC5AC, MUC4, MUC1*,

MUC16, and *MUC5B*. The average of these mucin expression values are plotted in Figure 4, and are significantly higher in PM-treated cultures ($p<0.05$).

b. Using gene signatures identified by single cell sequencing of cell types found within the tracheal airway epithelium, we found a significant enrichment of downregulated genes belonging to several basal cell populations including proliferating, proteasomal, and differentiating basal cell populations (Figure 5), suggesting further remodeling of the epithelium compared to control stimulated cultures.

c. Further analysis is currently being conducted to understand this remodeling response, including follow-up analyses looking at histological staining for cell populations, structural/organizational changes, and protein responses to chronic PM.

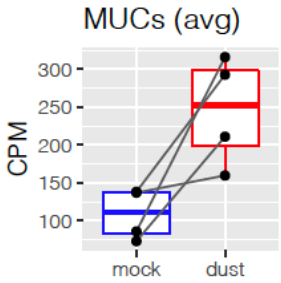


Figure 4: Average (composite) expression of 8 mucin genes with increased expression following chronic PM exposure of the mucociliary epithelium (n=4 donors); $p<0.05$

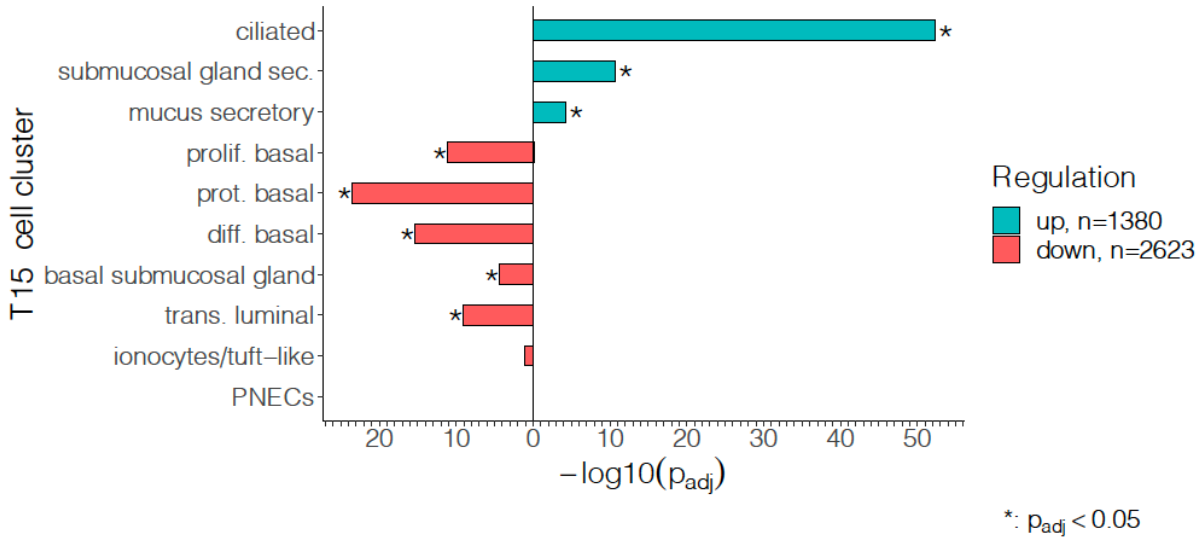


Figure 5: Cell type enrichment analysis using single cell data sets from tracheal epithelium demonstrates a significant enrichment in downregulated genes specific to proliferating (prolif), proteasomal (prot), and differentiating (diff) basal cell populations in response to chronic PM exposure (FDR <7.12E-12)

5. Several different types of analyses are in the process of being completed to determine how the repeated exposure to PM, alters the IL13 response of mucociliary epithelium.

a. Preliminary data identified that several genes induced by PM or by IL13 alone, have an additive effect following PM+IL13 stimulation, including several mucin genes (*MUC2, MUC5AC, FCGBP*) and inflammatory mediators.
b. Alternatively, several IL-13 induced genes are dampened in samples pre-exposed to PM compared to controls, suggesting pre-exposure to PM may have a complex effect different aspects of the IL13 response in the airway epithelium.

6. Analyses are being conducted using topic modeling, as is being done with in vitro RNA-seq analysis. This approach enables subtyping of epithelial cultures with unique responses, as well as identification of the primary biological programs of the cultures, with emphasis on treatment effects. This allows us to investigate transcriptomic changes in biological programs among PM and PM+IL13 treatments stratified by deployment status, asthma status, and DDLD status.

7. Project 4 personnel are currently working on completing the manuscript describing these acute and chronic effects for PM-exposure on the airway epithelium and its impact of a feed forward mechanism by which the basal stem cells are negatively affected following PM-exposure.

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 the past year, we completed our large-scale stimulations on the entire nasal cohort we had available for culture (n=127 donors). Each set of stimulations contained a randomized set of donors across all sample groups to prevent batch effects by disease status or by deployment status.
   a. Stimulations were completed in a 96 well ALI format which allowed for high throughput stimulations to be conducted, while being the most efficient with the limited PM we have available, as to conserve it for future mechanistic follow-up experiments. We completed stimulations on the following number of donors in our cohort:
      i. 8 subjects failed to produce usable differentiated cultures at ALI (94% success rate), and were removed from the study and will not be used for further RNA-seq analysis in this aim.

2. We have finalized the development, optimization, and testing of an automated RNA extraction protocol on our liquid handler robots (Beckman i7 instrument), which produces RNA samples with good quantity from a low input volume and good quality (RIN score) which will be ideal for downstream RNA-seq library preparation and analysis.

3. We have implemented a data tracking system for these automated extractions utilizing the Beckman DART software system, and have designed a semi-automated pipeline for tracking these samples from tube scan of the lysates through final extracted RNA samples. Additionally, this pipeline tracks and

merges QC data from these RNA plates allowing for enhanced planning on downstream RNA-seq library preparation.
   a. Appropriate barcodes of both sample lysate tubes from these *in vitro* derived samples, as well as for the processing labware, and the final elution plates have been developed, tested, and implemented for these extractions and sample management.

4. RNA extractions from these in vitro derived samples has been initiated and will be completed in the next couple of months.
   a. Moreover, library preparation for these samples has been planned out, reagent kits ordered, and optimization of the automated instrumentation required has been completed for these samples.

5. 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.

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

Subtask 7. Prepare and submit manuscripts for publication.

Progress

1. Project 4 presented a virtual abstract, at the international American Thoracic Society (ATS) conference held in May 2021 entitled, “Southwest Asia desert particulate matter exposure induces basal cell dysfunction and inflammatory remodeling of the mucociliary airway epithelium”.

2. Dr. Jamie Everman (project 4 personnel) was invited to give a presentation as part of the scientific symposium session at ATS 2021 titled “Military Deployment Respiratory Health: Insights into Potential Exposures, Clinical Findings, and Mechanisms”, to present on the biological mechanisms responsible for airway epithelium dysfunction and lung disease following Southwest Asia PM exposure. There were ~80 attendees of the virtual seminar series and project 4 received helpful feedback, interest, and future

direction ideas stemming from this seminar series and discussions that have been held since.

3. Project 4 submitted an abstract for the Military Health System Research Symposium (MHSRS) Conference entitled "Southwest Asia desert particulate matter exposure induces basal cell dysfunction and inflammatory remodeling of the mucociliary airway epithelium".
   a. MHSRS conference was cancelled entirely (no virtual option) due to the COVID-19 pandemic

4. The first manuscript for Project 4 is nearly complete. The goal is to submit this manuscript laying out the groundwork for further composition of the Iraq PM, and how the differentiated airway epithelium and the individual cell types that compose it, respond to Iraq dust particulate matter; we are currently integrating the chronic, repeated exposure data into the manuscript for a more complete analysis of the effects of PM on the airway epithelium.

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, and nasal only brushes from non-deployed asthmatics has been achieved. 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 available nasal and bronchial brush samples available in the cohort have been made into RNA-seq libraries and have undergone quality control (QC) to move forward with RNA-seq.
8. All available nasal and bronchial brush libraries have been successfully sequenced and are undergoing analysis.
9. All individual analytical methods in the differential gene expression workflow are established.
10. All analytic methods for eQTL and RNAseq variant calling methods development have been established.

11. All samples available (airway brush-derived and blood-derived) have had DNA extracted and prepared for genoptying analysis
12. 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 and induce significant gene expression responses.
13. Chronic PM and PM+IL13 stimulations of currently available nasal cells in our cohort have been completed (n=127 donors).

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

Project 1

The Project 1 team has participated in a number of professional development and training activities over the past grant year despite continued constraints from the COVID-19 pandemic. In June 2021, Dr. Rose was the 30th Annual Kass Lecturer at the University of Nebraska Medical Center where she presented Internal Medicine Grand Rounds entitled "Emerging Occupational Lung Diseases: Coal, Countertops, and Combat." She was an invited speaker and panelist at the Big Sky [Montana] Pulmonary Conference in March 2021 on "The Spectrum of Post-9/11 Respiratory Diseases." In January 2021, Dr. Rose was the speaker at Denver Allergy/Immunology Grand Rounds on "Surging and Emerging Occupational Lung Diseases: stone fabrication, coal mining and post-9/11 military deployment." Dr. Krefft presented at the Denver-wide Pulmonary Research in Progress Conference in October 2020 on "Deployment LEAP Study: A Longitudinal Cohort of Veterans with Post-9/11 Southwest Asia Deployment Lung Disease." Dr. Claudia Onofrei presented research findings at the February 2021 National Jewish Health Academic Affairs Research Retreat on "Decreased Exercise Tolerance and Unexplained Dyspnea May be Linked to Mitochondrial Myopathy Following Military Deployment to Iraq and Afghanistan." Dr. Silpa Krefft and Lauren Zell-Baran presented on "Multiple Breath Washout: A Noninvasive Tool for Identifying Lung Disease in Symptomatic Military Deployers" to the Veterans Affairs Airborne Hazards and Burn Pits Center of Excellence. Dr. Silpa Krefft organized a symposium on Military Deployment Related Health: Insight into Potential Exposures, Clinical Findings, and Mechanisms at the 2021 American Thoracic Society International Conference, and was joined by Dr. Jamie Everman (Project 4 scientist) who presented our shared GLIDE-related work.

The Project 1 team continues to hold bi-monthly Deployment Lung Disease clinical case conferences attended by 15 – 20 participants. Cases who have undergone evaluation for deployment-related lung disease are presented for discussion and management (including review of chest imaging with a pulmonary radiologist and lung histology with a pulmonary pathologist), in partnership with clinicians and researchers at the Eastern Colorado Veterans Administration Health Care System (ECVAHCS).

Dr. Rose continues to provide primary research mentorship for Dr. Krefft who is the recipient of a Career Development Award at the Rocky Mountain Regional VAMC. She also provides clinical research mentorship for Dr. Onofrei, whose work focuses on cardiopulmonary exercise testing in symptomatic military deployers. Dr. Rose was an invited member of the National Academies of Sciences, Engineering and Medicine Committee on the Respiratory Health Effects of Airborne Hazards Exposures in the Southwest Asia Theater of Military Operations, with a final report published in September 2020.

Project 2

Dr. Downey attended the 2021 American Thoracic Society annual international conference virtually and participated in the symposium on Military Deployment Related Health: Insight into Potential Exposures, Clinical Findings, and Mechanisms. Dr. Downey submitted an abstract to the 2021 Military Health System Research Symposium (MHSRS) in Orlando, FL. The abstract was accepted for a poster presentation but the meeting was cancelled due to COVID-19 concerns.

Project 3
Drs. Day and Chu trained and supervised Reena Berman who was a graduate student from 2016 to 2020. Dr. Berman graduated in June 2020 with publication of 3 first-authored manuscripts. She was trained in the human airway epithelial cell culture model of particulate matter exposure and IL-13 treatment, mouse models of allergen particulate matter exposures, molecular biology and single cell RNA-sequencing. In addition, she was mentored in writing a review article about the role of Southeast Asian particulate matter exposures in deployment-related lung disease. In addition, she submitted abstracts to the Military Health System Research Symposium (MHSRS) and the Society of Toxicology, and presented the posters at these meetings (2019 in Orlando, FL, and 2019 in Baltimore. MD).

Project 4
Dr. Everman has received training in the analysis of transcriptomic data in response to PM exposures and in other novel cell biology techniques as part of the conduct of this study. Furthermore, Dr. Everman submitted a poster abstract to the canceled 2021 Military Health System Research Symposium (MHSRS) in Orlando, FL on the transcriptomic responses of the airway epithelium to PM.

How were the results disseminated to communities of interest?

Project 1
An Outreach Committee meets regularly and has developed several tools for enhancing public understanding of deployment-related lung disease as well as for recruiting potential study subjects for participation in the GLIDE Study. As detailed above under "Accomplishments," these tools include a website and brochure as well as abstracts of peer-reviewed publications. Additionally, this year we reformatted our Center for Deployment-Related Lung Disease website (https://www.nationaljewish.org/center-for-deployment-related-lung-disease/overview) to include findings from our GLIDE Study research.

Project 2
An abstract reporting the studies with PM exposure and blast overpressure lung injury in mice was submitted to and accepted as a poster presentation for the Military Health System Research Symposium (MHSRS) in Orlando, FL in August 2021. The symposium was cancelled due to COVID-19 concerns.

Project 3
Our results were disseminated to communities of interest mainly through publications and presentations at local and national scientific meetings. We have three peer-reviewed original publications to inform the community about the impact of Afghanistan particulate matter exposures on the development of asthma-like disease and underlying mechanisms as well as the potential therapeutic interventions to alleviate asthma-like symptoms related to particular matter exposures. Recently, we wrote a review article on "Role of particulate matter from Afghanistan and Iraq in Deployment Related Lung Disease", which has been revised and resubmitted in October 2021 to the journal *Chemical Research in Toxicology* for consideration of publication.

Project 4

We submitted an abstract reporting changes to human airway epithelial biology in response to PM exposure to the Military Health System Research Symposium (MHSRS) in Orlando, FL in August 2021. The symposium was cancelled due to COVID-19 concerns.

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

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

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

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

Subtask 1. Consent patients from our NJH Deployment Clinic.

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

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

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

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

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

Subtask 7. Complete LCI measurements from deployers and controls.

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

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

> **Plans for the next year.** We plan to finalize our analysis and submit these findings for publication during the next project year.

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 analyze study data on quantitative CT imaging and lung pathology findings and submit manuscripts for peer review and publication in the next project 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 will complete the analysis of the experiments with *in vitro* cell culture models of exposure of lung epithelial cells to airborne PM and combined effects of physical (blast), 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 complete the studies of viral infection with influenza virus *in vitro* with cultured human 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 have spent much of the last year optimizing the blast overpressure advanced blast simulator system to be able to expose cultured human and rat lung epithelial cells to a reproducible blast overpressure wave as described above. In the next year we will repeat the experiment to determine if ICG-001, a small molecule modulator of the WNT pathway, can prevent the blast overpressure injury to lung epithelial cells.

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 have spent much of the last year optimizing the blast overpressure advanced blast simulator system to be able to expose intact mice to a

reproducible blast overpressure wave as described above. 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 and whether ICG-001, a small molecule modulator of the WNT pathway, can prevent the 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 complete the studies measuring the particulate matter and mineral content of the lungs 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.

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

Plans for the next year.
As we found that particulate matter did not distribute well at the apical surface of human airway epithelial cells cultured at the air-liquid interface, we decided to change the cell culture model to human lung tissue culture model where particulate matter distributes well and thus allows us to accurately study the role of particulate matter exposures in deployment-related lung disease. Specifically, we will use human precision-cut lung slice (PCLS) culture model that reflects the nature of human distal lung exposures to particulate matter. We plan to perform cultures of human PCLS from 5 to 8 donors. Human PCLS will be exposed to IL-13, particulate matter, influenza virus and combination. We will measure airway contraction in response to methacholine challenge as an indication of airway hyperresponsiveness (AHR) as well as cytokines and reactive oxygen species (ROS) to determine if excessive ROS generation serves as a mechanism of AHR and inflammation.

Subtask 1. Examine mechanism of APM's ability to enhance house dust mite airway hyperreactivity *in vivo*.

Plans for the next year.
We have published mouse model work to demonstrate the effect of particulate matter exposures on allergen-mediated airway inflammation and AHR. To further our mechanistic studies, we plan to use mouse PLCS model to see if it mimics some of the key findings from our initial in vivo mouse model work, and also determine if mouse PLCS model

reflects major findings from the human PCLS work as described above. Moreover, we will add inhibitors of TLR2 and antioxidants in mouse PLCS exposed to particulate matter. We will use PLCS from wild-type C57 mice (n = 10) to test if airway inflammation and AHR induced by particulate matter, IL-13 and influenza virus depend on TLR2 signaling and reactive oxygen species.

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.

Plans for the next year. Complete the collection of nasal and bronchial brushes from deployers led by Project 1, and of non-deployed subjects through the NJH biobank. Finish quality assays of biomolecules isolated 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.

Plans for the next year. The final set of *in vivo* samples will be prepped and analyzed for gene expression studies. Final analyses of biomarker and disease mechanisms will be conducted with the full dataset.

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.

Plans for the next year. Genetics data will be generated in the first part of the year to complement the trace metal/PM data.

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.

Plans for the next year. The generated genetics and trace metal/PM data will be associated with molecular changes to the airway in subjects with DLDs.

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.

Plans for the next year. All methods for the establishment of *in vitro* culture are developed/standardized and being applied in Subtask 6.

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.

Plans for the next year. All *in vitro* stimulations will be completed. Final analysis of PM/IL-13 transcriptomic responses will be completed.

Subtask 7. Prepare and submit manuscripts for publication.

Plans for the next year. We expect to complete and submit for publication at least two manuscripts, one covering *in vitro* responses to PM/IL-13, and one covering airway biomarkers and mechanisms underlying DLDs.

4. IMPACT:

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

- We have a busy Deployment Lung Disease Clinical Center and have evaluated over 250 patients, many of whom are participants in the GLIDE Study.
- We have described the spectrum of respiratory diseases that can affect military service members who have deployed to Southwest Asia and Afghanistan, including asthma, bronchiolitis (inflammation of the small airways), rhinosinusitis, vocal cord dysfunction, and expiratory central airways collapse.
- We have implemented a noninvasive assessment of small airways injury disease using lung clearance index (LCI) testing (a simple breathing test that takes around 30 minutes to perform), and have successfully applied this technique to screen over 70 previously deployed military personnel and over 100 healthy controls.
- We have identified quantitative methods to analyze chest CT scans from previously deployed military personnel with persistent respiratory symptoms and are using this approach to assess lung injury in patients who agree to participate in the study.
- We have developed a systematic scoring system for VATS lung biopsies and are analyzing these data in comparison to positive controls and normal subjects in order to identify the primary histologic features of deployment-related lung disease.
- 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?

PROJECT 1.
Nothing to Report.

PROJECT 2.
Nothing to Report.

PROJECT 3.
Nothing to Report.

PROJECT 4.
Nothing to Report.

What was the impact on technology transfer?

PROJECT 1.
Nothing to Report.

PROJECT 2.
Nothing to Report.

PROJECT 3.
Nothing to Report.

PROJECT 4.
Nothing to Report.

What was the impact on society beyond science and technology?

PROJECT 1.
Nothing to Report.

PROJECT 2.
Nothing to Report.

PROJECT 3.
Nothing to Report.

PROJECT 4.
Nothing to Report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

PROJECT 1.
Nothing to Report.

PROJECT 2.
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

PROJECT 1.
Nothing to Report.

PROJECT 2.
Nothing to Report.

PROJECT 3.
Nothing to Report.

PROJECT 4.
Adjustments to recruiting and processing of the deployer samples have been made in response to pandemic restrictions. Therefore, we anticipate receiving the remaining deployer samples in this coming year, which will be analyzed to accomplish our stated goals.

Changes that had a significant impact on expenditures

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

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals.

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications.

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

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

3. **Zell-Baran LM**, **Meehan R**, **Wolff J**, **Strand M, Krefft SD, Gottschall EB**, Macedonia TV, Gross JE, Sanders OL, Pepper GC, **Rose CS**. Military Occupational Specialty Codes. Journal of Occupational and Environmental Medicine 2019; 61: 1036-1040. DOI: 10.1097/JOM.0000000000001731

4. **Krefft SD**, **Wolff J**, **Zell-Baran L**, **Strand M**, **Gottschall EB**, **Meehan R**, **Rose CS**. Respiratory Diseases in Post-9/11 Military Personnel Following Southwest Asia Deployment. J Occup Environ Med 2020. DOI: 10.1097/JOM.0000000000001817

5. **Zell-Baran L, Krefft SD**, Moore CM, **Wolff J, Meehan R, Rose CS**. Multiple Breath Washout: A Noninvasive Tool for Identifying Lung Disease in Symptomatic Military Deployers. Respiratory Medicine. January 2021, 176:106281. DOI: https://doi.org/10.1016/j.rmed.2020.106281

6. **Zell-Baran L, Krefft SD**, Moore CM, **Wolff J, Meehan R, Rose CS.** Multiple breath washout test data for healthy controls. Data in Brief, February 2021, 34:106641. DOI: https://doi.org/10.1016/j.dib.2020.106641

7. **Krefft SD**, Oh A, **Zell-Baran L, Wolff J**, Moore CM, Macedonia TV, **Rose CS**. Semiquantitative Chest Computed Tomography Assessment Identifies Expiratory Central Airway Collapse in Symptomatic Military Personnel Deployed to Iraq and

Afghanistan. Journal of Thoracic Imaging, June 2021. DOI: 10.1097/RTI.0000000000000596

8. **Berman R, Kopf KW, Min E, Huang J, Downey GP, Alam R, Chu HW, and Day BJ**. IL-33/ST2 signaling modulates Afghanistan particulate matter-induced airway reactivity in mice. *Toxicol. Appl. Pharmacol*. 404:115186, 2020. Federal support acknowledged.

9. **Berman R, Min E, Huang J,** Kopf K**, Downey GP,** Riemondy K, Smith HA**, Rose CS, Siebold MA, Chu HW, and Day BJ**. Single-cell RNA sequencing reveals a unique monocyte population in bronchoalveolar lavage cells of mice challenged with Afghanistan particulate matter and allergen. Toxicol. Sci. 182:297-309, 2021. Federal support acknowledged.

10. **Berman R, Rose CS, Downey GP, Day BJ, and Chu HW**. Role of particulate matter from Afghanistan and Iraq in deployment related lung disease. Under review at *Chemical Research in Toxicology, 2021.*

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

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

2. Everman JL, NJ Jackson, B Saef, K Li, C Rios, MT Montgomery, CS Rose, BJ Day, H Chu, GP Downey, MA Seibold (2019) Desert dust exposure induces epidermal differentiation of both human airway epithelial stem cells and the mucociliary airway epithelium. MHSRS 2019 Poster Abstract

3. Onofrei C, Gottschall EB, Kraus R, Zell-Baran L, Pang K, Krefft SD, Rose CS. Decreased Exercise Tolerance and Unexplained Dyspnea May Be Linked to Mitochondrial Myopathy Following Military Deployment to Iraq and Afghanistan. American Thoracic Society Conference 2021. Abstract.

- **Other publications, conference papers, and presentations**.

  Nothing to Report.

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

  Nothing to Report.

- **Technologies or techniques**

Nothing to Report.

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

  Nothing to Report.

- **Other Products**

  Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

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

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

Name:	James Crooks, PhD
Project Role:	Biostatistician, Project 1
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1 (annually)

Contribution to Project: No change

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

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

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

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

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

Name: Kathy Pang
Project Role: Research Staff, Project 1
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 4 (annually)
Contribution to Project: No change No change

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

Name: Keriann Beke
Project Role: Lab Researcher, Project 2
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 11 (annually)
Contribution to Project: No change

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

Name: Bradley Richards
Project Role: Lab Researcher, Project 2
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1 (annually)
Contribution to Project: No change

Name: Natalie Briones
Project Role: Lab Researcher, Project 2
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1 (annually)
Contribution to Project: No change

Name: Kelly Correll
Project Role: Senior Researcher, Project 2
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 6 (annually)
Contribution to Project: No change

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

Name: Yudong Teng
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: 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: Nicole Pavelka
Project Role: Research Associate, Project 3
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: <1 (annually)
Contribution to Project: No Change

Name: Christina Lisk
Project Role: Research Fellow, Project 3
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 5 (annually)
Contribution to Project: No change

Name: Bruce Berg
Project Role: Research Associate, Project 3
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 4 (annually)
Contribution to Project: No change

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

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

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

Name: Jamie Everman, PhD
Project Role: Faculty Scientist, Project 4
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 2 (annually)
Contribution to Project: No change

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

Name: Katherine Goldfarbmuren, PhD
Project Role: Senior Laboratory Researcher, Project 4
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1 (annually)
Contribution to Project: No Change

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

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

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

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

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

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

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

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

Dr. Downey's participation in project U01HL131755 entitled, "Multicenter Interventional Lymphangioleiomyomatosis Early Disease Trial (MILED)" has concluded. Dr. Downey's research project, R01HL132950 entitled, "Checkpoint Function of PTPalpha in Pathological Fibrogenesis in the Lung" has concluded.

Dr. Brian Day was awarded project, R21ES032951 entitled, "Repurposing thiosulfate for chlorine gas exposure". His effort on the Cystic Fibrosis Foundation award Day18GO entitled, "Manganese porphyrin selenocyanate salts as a novel antimicrobial therapy against CF pathogens" has concluded.

Dr. Hong Wei Chu was awarded project R01AI161296 entitled, "Novel Biased Beta2-AR Ligands as Asthma Therapeutics" and project U19AI125357 entitled, "Innate Immunity and Viral infection in Asthma: Tollip inhibits IL-33 signaling during airway influenza virus infection". Dr. Chu's work on project U19AI125357 entitled, "Dysfunction of Innate Immunity in Asthma: Role of Tollip in dysfunction of asthma airway innate immunity" and project R01HL122321 entitled, "Novel function of MUC18: amplification of inflammation in allergic lungs" has concluded.

Dr. Max Seibold began work on project U01AI160033 entitled, "Critical Windows in the Development of Asthma Endotypes and Phenotypes in High Risk Toddlers".

What other organizations were involved as partners?

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

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: 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: