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**14. ABSTRACT**
Purpose: To test the hypothesis that the replacement of tissue-resident alveolar macrophages with monocyte-derived macrophages in response to repeated injury over the lifespan explains the delayed onset of lung fibrosis until the later decades of life. We will use our experimental findings in mice to guide an unbiased assessment of macrophage heterogeneity in normal human lungs and lungs from patients with pulmonary fibrosis. The overarching goal of these studies is to identify clinically applicable biomarkers that can guide therapy and factors released from tissue-resident macrophages or bone marrow-derived macrophages that prevent or promote fibrosis, respectively so they can be targeted for prevention or therapy.

Aim 1: To determine whether replacement of tissue-resident alveolar macrophages by monocyte-derived alveolar macrophages during aging contributes to the enhanced susceptibility to lung fibrosis in aged mice. Mice are currently being aged. The work is continuing according to SOW, no major findings to report. However, we have generated improved, more economical and robust mouse model to distinguish tissue-resident and monocyte-derived alveolar macrophages.

Aim 2: To determine whether tissue-resident alveolar macrophages or monocyte-derived alveolar macrophages differentially respond to epithelial injury in a murine model of accelerated pulmonary fibrosis. The work on this aim is in progress, in accordance with SOW, no major findings to report at the moment (expected in the year 2 of award).

Aim 3: To identify novel biomarkers expressed by human tissue-resident and monocyte-derived alveolar macrophages based on single cell molecular classification in patients with pulmonary fibrosis. We have recruited a planned number of patients into the study and performed transcriptional profiling by means of RNA-seq on single cell and bulk sorted populations or alveolar macrophages. Major findings: In agreement with our hypothesis, using single cell RNA-seq, we found substantial heterogeneity of alveolar macrophages in the healthy donor lung, we now sequencing and analyzing data from patients with lung fibrosis. In addition, we found that gene expression signature of profibrotic monocyte-derived alveolar macrophages generated in mouse models of lung fibrosis was present in the alveolar macrophages of patients with various forms of lung fibrosis.

**15. SUBJECT TERMS**
alveolar macrophages, pulmonary fibrosis

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Introduction

Purpose: To test the hypothesis that the replacement of tissue-resident alveolar macrophages with monocyte-derived macrophages in response to repeated injury over the lifespan explains the delayed onset of lung fibrosis until the later decades of life. We will use our experimental findings in mice to guide an unbiased assessment of macrophage heterogeneity in normal human lungs and lungs from patients with pulmonary fibrosis. The overarching goal of these studies is to identify clinically applicable biomarkers that can guide therapy and factors released from tissue-resident macrophages or bone marrow-derived macrophages that prevent or promote fibrosis, respectively so they can be targeted for prevention or therapy. This hypothesis will be tested in the following aims: Aim 1: To determine whether replacement of tissue-resident alveolar macrophages by monocyte-derived alveolar macrophages during aging contributes to the enhanced susceptibility to lung fibrosis in aged mice. Aim 2: To determine whether tissue-resident alveolar macrophages or monocyte-derived alveolar macrophages differentially respond to epithelial injury in a murine model of accelerated pulmonary fibrosis. Aim 3: To identify novel biomarkers expressed by human tissue-resident and monocyte-derived alveolar macrophages based on single cell molecular classification in patients with pulmonary fibrosis.
Keywords

Alveolar macrophages, pulmonary fibrosis, idiopathic pulmonary fibrosis
Accomplishments

**Major Goal: Specific Aim 1:** To determine whether replacement of tissue-resident alveolar macrophages by monocyte-derived alveolar macrophages during aging contributes to the enhanced susceptibility to lung fibrosis in aged mice.

**Major Task 1:** Are tissue-resident alveolar macrophages replaced by monocyte-derived alveolar macrophages during normal aging?  
**Subtask 1:** Generate cohorts of shielded bone marrow chimeric mice. Dr. Misharin will perform the procedure, Drs. Chen and Soberanes will monitor animals, Dr. Budinger will oversee the project.  
**Accomplishments:** Generation of the shielded bone marrow chimeras is complete (100%). While we have finished harvesting tissues from most of the animals, some mice are currently being aged as planned. We have generated preliminary data from the initial cohort of animals demonstrating that monocyte-derived alveolar macrophages recruited during the lung injury exhibit activated profile for several months after the initial injury. The causal experiments involving adoptive transfer of these cells are currently under way.

**Other achievements:** As an indirect result of this project, we have developed a novel, more robust and economic, fate mapping system based on CD11c-ER-Cre and CX3CR1-ER-Cre mice crossed to fluorescent reporter (see Figure 1). This system should be able to replace shielded bone marrow chimeras in the future studies and increase throughput, accuracy and cost-effectiveness of our research. We are currently testing these reporter mice to validate our findings from shielded chimeras.

**Subtask 2:** Aging of shielded bone marrow chimeric mice. Monitor shielded bone marrow chimeric mice during aging. Track mortality (Drs. Misharin, Soberanes, Chen). Harvest lungs from shielded chimeric at designated time points (6 weeks, 6, 12, 18 and 24 months), FACSort alveolar macrophages, isolate RNA (Drs. Misharin, Soberanes and Chen). Prepare libraries for RNA-seq, perform sequencing and analyze the data (Drs. Budinger, Misharin, Soberanes, Bartom and Chen).

**Accomplishments:** Generation of the shielded bone marrow chimeras is complete (100%), and 6 weeks, 6 and 12 months time points have already

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**Figure 1.** Alternative approaches for tracking TR-AM and Mo-AM.  
A: CX3CR1-ER-Cre x zsGreen system. Administration of tamoxifen (Tx) permanently labels 100% of circulating monocytes and their progeny as GFP+. Thus, monocyte-derived alveolar macrophages will be GFP-positive, while tissue-resident (TR-AM) will be GFP-negative.  
B: CX3CR1-ER-Cre x zsGreen mice (N=3) were maintained on tamoxifen chow and peripheral blood was analyzed by flow cytometry for % of GFP+ cells.  
C: GFP+ Mo-AM persist in the lung after influenza A-induced lung injury. Representative contour plots are shown.  
D: CD11c-ER-Cre x zsGreen system. Administration of tamoxifen labels 100% of TR-AM, while recruited Mo-AM will be GFP-negative.  
E: Left panel: CD11c-ER-Cre x zsGreen mice were maintained on regular or tamoxifen chow, which results in labeling of 100% of TR-AM (CD64+), representative contour plots are shown. Right panel: GFP signal is bright enough to separate TR-AM from Mo-AM by FACS.
been harvested and FACSorted. Animals for 18 and 24 months are being aged. No technical difficulties were encountered.

**Major Task 2:** Do insults associated with normal aging accelerate the replacement of tissue-resident alveolar macrophages with monocyte-derived alveolar macrophages?

**Subtask 1:** Generate cohorts of shielded bone marrow chimeric mice. Dr. Misharin will perform the procedure, Drs. Soberanes and Chen will assist and monitor animals, Dr. Budinger will oversee the project.

**Accomplishments:** As reported previously, generation of the shielded bone marrow chimeras is complete (100%). While we have finished harvesting tissues from most of the animals, some mice are currently being aged as planned. Our preliminary data indicate that insults associated with normal aging indeed accelerate replacement of tissue-resident alveolar macrophages with monocyte-derived macrophages.

**Subtask 2:** Infect shielded chimeric mice with three strains of influenza A (A/WSN/33 and A/PR8/34 and A/Udorn/72) at 2, 4 and 6 months of age. Dr. Soberanes will perform the infections, Drs. Soberanes and Chen will assist and monitor animals. Dr. Budinger will oversee the project. Harvest lungs from shielded chimeric at designated time points (6 weeks, 6, 12, 18 and 24 months), FACSort alveolar macrophages, isolate RNA (Drs. Misharin, Soberanes and Chen). Prepare libraries for RNA-seq, perform sequencing and analyze the data (Drs. Budinger, Misharin, Bartom, Soberanes and Chen).

**Accomplishments:** Shielded bone marrow chimeras were infected with influenza A virus (WSN strain). Six weeks, 6 and 12 months time points have already been harvested and FACSorted. Animals for 18 and 24 months are being aged. We have observed similar results with both WSN and PR8 influenza A viruses.

**Major Task 3:** Does replacement of tissue-resident alveolar macrophages by monocyte-derived alveolar macrophages lead to exaggerated fibrosis in mice?

**Subtask 1:** Generate cohorts of shielded bone marrow chimeric mice on using CD11c-DTR mice as a host. Dr. Misharin will perform the procedure, Drs. Soberanes and Chen will assist and monitor animals, Dr. Budinger will oversee the project.

**Subtask 2:** Initiate bleomycin-induced lung fibrosis in shielded chimeric mice and harvest macrophage populations. Dr. Soberanes will instill mice with bleomycin, Drs. Chen and Soberanes will assist and monitor animals. Dr. Budinger will oversee the project. Harvest lungs, FACSort alveolar macrophages, isolate RNA (Drs. Misharin, Soberanes and Chen). Prepare libraries for RNA-seq, perform sequencing and analyze the data (Drs. Budinger, Misharin, Bartom, Soberanes and Chen).

**Accomplishments:** As reported during the last year, during the initial phase experiments, we found out that CD11c-DTR mice develop severe lung injury upon depletion of CD11c-DTR-positive alveolar macrophages. These findings were recently confirmed and reported by another group (Mann et al., Eur J Immunol, 2016). To overcome this issue, we have developed an alternative system for depletion of tissue-resident alveolar macrophages using intratracheal instillation of clodronate-loaded liposomes. We found that intratracheal instillation of 50 ul of clodronate-loaded liposomes efficiently depletes tissue-resident alveolar macrophages and leads to the repopulation of the niche by monocyte-derived alveolar macrophages. Thus, we were able to overcome the technical issues and use regular shielded bone marrow chimeras for this subtask. We have generated sufficient number of shielded bone marrow chimeras and treated them with...
clodronate-loaded liposomes to deplete tissue-resident alveolar macrophages and induce repopulation with monocyte-derived alveolar macrophages.

While final experiments are still under way, our preliminary data indicate that environment during the niche repopulation plays a crucial role in programming monocyte-derived alveolar macrophages. While monocyte-derived alveolar macrophages recruited into non-inflammatory environment after sterile depletion with clodronate-loaded liposomes did not exhibit pro-fibrotic activity and behaved similarly to tissue-resident alveolar macrophages, monocyte-derived alveolar macrophages recruited during bleomycin-induced or influenza A-induced lung injury exhibited marked profibrotic activity, resulting in marked transcriptional changes (as determined by RNA-seq) as well as changes in lung function (as determined by direct measurement of lung mechanics with Flexivent).

**Major Task 4:** Can adoptive transfer of tissue-resident or monocyte-derived alveolar macrophages rescue or exacerbate, respectively, the severity of experimental lung fibrosis?

**Subtask 1:** Sort alveolar macrophages, generate bone marrow derived macrophages, perform adoptive transfer and induce lung fibrosis. Dr. Misharin will perform sorting for alveolar macrophages and generate bone marrow derived macrophages in vitro. Dr. Soberanes will perform adoptive transfer of alveolar and bone marrow-derived macrophages and instill mice with bleomycin. Drs. Chen and Soberanes will assist and monitor animals. Harvest lungs, FACSort alveolar macrophages, isolate RNA (Drs. Misharin, Soberanes and Chen). Prepare libraries for RNA-seq, perform sequencing and analyze the data (Drs. Budinger, Perlman, Misharin, Bartom, Soberanes and Chen).

**Accomplishments:** We have established protocol for adoptive transfer of alveolar macrophages and performed initial experiments. Our data suggest that adoptive transfer of tissue-resident alveolar macrophages does not rescue experimental lung fibrosis. The experiments with adoptively-transferred monocyte-derived alveolar macrophages are currently ongoing. We expect to complete them in a timely manner during the third year of the project and include in our second publication.

We have recently published summary of our work for the Aim 1 of the current project in the Journal of Experimental Medicine (Misharin et al., JEM, 2017). We also presented our work at American Thoracic Society meeting in Washington, DC and 2017 MHSRS meeting in Florida. Importantly, our findings were independently confirmed by Dr. Janssen’s group (McCubbrey et al., AJRCMB, 2017), which speaks of high reproducibility of our data.

**Specific Aim 2:** To determine whether tissue-resident alveolar macrophages or monocyte-derived alveolar macrophages differentially respond to epithelial injury in a murine model of the accelerated pulmonary fibrosis.

**Major Task 1:** Do macrophages from young and aged mice differentially regulate chronic stress in the lung epithelium?

**Subtask 1:** Generate cohorts of shielded bone marrow chimeric mice with tissue-resident or monocyte-derived alveolar macrophages using young and aged CD11c-DTR mice. Dr. Misharin will perform the procedure, Drs. Chen and Soberanes will monitor animals, Dr. Budinger will oversee the project.

**Subtask 2:** Sort tissue-resident alveolar macrophages, perform adoptive transfer and induce lung fibrosis. Dr. Misharin will perform sorting for alveolar macrophages. Dr. Soberanes will perform
adoptive transfer of alveolar and instill mice with bleomycin. Dr. Soberanes will assist and monitor animals. Harvest lungs, FACSsort alveolar macrophages, isolate RNA (Drs. Misharin, Soberanes and Chen). Prepare libraries for RNA-seq, perform sequencing and analyze the data (Drs. Budinger, Misharin, Bartom, Soberanes and Chen).

**Subtask 3:** Bleomycin-induced lung fibrosis in bone marrow chimeric mice. Monitor shielded bone marrow chimeric mice during aging. Track mortality (Drs. Misharin, Soberanes, Chen). Harvest lungs from shielded chimeric at designated time points, FACSsort alveolar macrophages, isolate RNA (Drs. Misharin, Soberanes and Chen). Prepare libraries for RNA-seq, perform sequencing and analyze the data (Drs. Budinger, Perlman, Misharin, Soberanes and Chen).

**Accomplishments:** See above, Aim 1, Major task 4. During the initial phase experiments, we found out that CD11c-DTR mice develop severe lung injury upon depletion of CD11c-DTR-positive alveolar macrophages. These findings were recently confirmed and reported by another group ([Mann et al., Eur J Immunol, 2016](#)). To overcome this issue, we have developed an alternative system for depletion of tissue-resident alveolar macrophages using intratracheal instillation of clodronate-loaded liposomes. We found that intratracheal instillation of 50 ul of clodronate-loaded liposomes efficiently depletes tissue-resident alveolar macrophages and leads to the repopulation of the niche by monocyte-derived alveolar macrophages. Thus, we were able to overcome the technical issues and use regular shielded bone marrow chimeras for this subtask. We have generated sufficient number of shielded bone marrow chimeras and treated them with clodronate-loaded liposomes to deplete tissue-resident alveolar macrophages and induce repopulation with monocyte-derived alveolar macrophages.

**Major Task 2:** Does chronic stress in the epithelium accelerate the replacement of tissue-resident alveolar macrophages with monocyte-derived alveolar macrophages during aging?

**Subtask 1:** Generate cohorts of shielded bone marrow chimeric mice using HPS-/- mice. Dr. Misharin will perform the procedure, Drs. Chen and Soberanes will assist and monitor animals, Dr. Budinger will oversee the project.

**Accomplishments:** See above, Major Task 2. While HPS-/- mice provided a valuable insight during the preliminary work, their utility was undermined because HPS deficiency was global and affecting not only epithelial cells, but all other cell types as well (including alveolar macrophages). Fortunately, soon after the work on the grant was initiated, a new strain of mice specifically causing stress in the lung epithelium by targeting telomerase (Trf1) in alveolar type 2 epithelial cells using highly specific CreSftpc driver became available ([Povedano et al., 2015, Cell Rep](#)). These mice develop spontaneous age-dependent lung fibrosis. We have initiated collaboration with Dr. Mary Blasco and obtained Trf1-floxed mice for our colony. We have now finished crossing these mice to CreSftpc mice and initiated the proposed experiments. While this change of tools delayed the work on this subtask, it did not change the scope of our work. Moreover, this approach is much more specific and will allow us to reduce the number of required animals.

We have discussed our approach with Dr. Mary Armanios, one of the world’s experts in telomerase deficiency-associated pulmonary fibrosis. This consultation has confirmed our current direction.

**Subtask 2:** Harvest and analyze the lungs. Harvest lungs from shielded chimeric at designated time points (6 weeks, 4, 12 and 18 months), FACSsort alveolar macrophages, isolate RNA (Drs. Misharin, Soberanes and Chen). Prepare libraries for RNA-seq, perform sequencing and analyze the data (Drs. Budinger, Perlman, Misharin, Bartom, Soberanes and Chen).

**Accomplishments:** The work on this subtask was delayed because of the change in the mouse strain (see above). We have started our experiments using mice with Trf1-deficiency in alveolar
type 2 cells. Our initial experiments confirmed successful deletion of telomerase-1 gene in alveolar type 2 cells. The proposed experiments involving second hit (influenza A and bleomycin) are currently under way.

**Major Task 3:** Can the adoptive transfer of tissue-resident alveolar macrophages improve chronic stress in the lung epithelium?  
**Subtask 1:** Generate cohorts of shielded bone marrow chimeric mice using HPS-/- mice. Dr. Misharin will perform the procedure, Drs. Chen and Soberanes will assist and monitor animals, Dr. Budinger will oversee the project.  
**Accomplishments:** See above, Major Task 2. While HPS-/- mice provided a valuable insight during the preliminary work, their utility was undermined because HPS deficiency was global and affecting not only epithelial cells, but all other cell types as well (including alveolar macrophages). Fortunately, soon after the work on the grant was initiated, a new strain of mice specifically causing stress in the lung epithelium by targeting telomerase (Trf1) in alveolar type 2 epithelial cells using highly specific Cre<sup>Sftpc</sup> driver became available (Povedano et al., 2015, Cell Rep). These mice develop spontaneous age-dependent lung fibrosis. We have initiated collaboration with Dr. Mary Blasch and obtained Trf1-floxed mice for our colony. We have now finished crossing these mice to Cre<sup>Sftpc</sup> mice and initiated the proposed experiments. While this change of tools delayed the work on this subtask, it did not change the scope of our work. Moreover, this approach is much more specific and will allow us to reduce the number of required animals.  
**Subtask 2:** Sort alveolar macrophages, perform adoptive transfer and induce lung fibrosis. Dr. Misharin will perform sorting for alveolar macrophages. Dr. Soberanes will perform adoptive transfer of alveolar and instill mice with bleomycin. Ms. Saber and Dr. Soberanes will assist and monitor animals. Harvest lungs, FACSort alveolar macrophages, isolate RNA (Drs. Misharin, Soberanes and Chen). Prepare libraries for RNA-seq, perform sequencing and analyze the data (Drs. Budinger, Perlman, Misharin, Soberanes and Chen).  
**Accomplishments:** The work on this subtask was delayed because of the change in the mouse strain (see above). Once the experiments in Major Task 2 are complete, we will perform experiment in this Subtask.

**Specific Aim 3:** To identify novel biomarkers expressed by human tissue-resident and monocyte-derived alveolar macrophages based on single cell molecular classification in patients with IPF.  
**Major Task 1:** Collect samples, perform RNA-seq on populations of lung macrophages, perform single cell RNA-seq and analyze the data.  
**Subtask 1:** Submit documents for local IRB review. Organize workflow, establish standard operating procedures, meeting with all co-investigators.  
**Accomplishments:** Our study protocol was approved by the local IRB on 2016/06/05. This protocol was reviewed by the US Army Medical Research and Materiel Command (USAMRMC), Office of Research Protections (ORP), Human Research Protection Office (HRPO) and found to comply with applicable DOD, US Army, and USAMRMC human subjects protection requirements. We received an initial note of approval on 2015/12/01 and HRPO Approval Memorandum on 2016/1/9, HRPO Log Number A-18899.b. During the frist part of the project, we have completed all optimization studies and established standard operation procedures. We have published the results of these preliminary studies (Bharat et al., 2016, AJRCMB) and presented these data at the American Thoracic Society meeting (San Francisco, CA) in the the poster format. Our publication was accompanied by the editorial
and at the time of this report listed among highly accessed on AJRCMB website. Importantly, our protocol and findings were independently validated by two groups (Yu et al., 2016, AJRCMB; Desch et al., 2016, AJRCCM).

During 2015-2016 we have conducted monthly meetings between all collaborators (Drs. Mutlu, Bhorade, Budinger) to align the work on the project on both study sites and coordinate efforts. We have successfully obtained multiple samples from our collaborators at University of Chicago for both bulk sorting and RNA-seq as well as for single cell RNA-seq.

We have established a reliable and reproducible single cell RNA-seq protocol for analysis of alveolar macrophage heterogeneity using Chromium platform from 10x Genomics. We have also optimized sequencing procedures to decrease cost and increase throughput and developed computational pipelines for the multi-sample integration and analysis.

We have recently published preliminary data on bulk-sorted alveolar macrophages from patients with pulmonary fibrosis (related to our work for the Aim 3 of the current project) in the Journal of Experimental Medicine (Misharin et al., JEM, 2017), funding from DoD was fully acknowledged. We also presented our work at American Thoracic Society meeting in Washington, DC and 2017 MHSRS meeting in Florida. Importantly, our findings in human subjects were independently confirmed by Dr. Janssen’s group (McCubbrey et al., AJRCMB, 2017), which speaks of high reproducibility of our data. Second manuscript, containing data on the extended cohort of patients and including single cell RNA-seq data is currently in preparation.

**Subtask 2:** Begin study. Recruit first patients to the study (Drs. Mutlu, Bhorade, Budinger). Perform pilot RNA-seq on populations of pulmonary macrophages, establish optimal workflow and data analysis pipelines (Drs. Misharin, Budinger, Bartom, Berdnikovs, Perlman).

**Figure 2:** Integrative analysis of the whole human lung (left panel), FACSorted alveolar type 2 cells (middle panel) and FACSorted alveolar macrophages (right panel) from donors and patients with pulmonary fibrosis. Manuscript in preparation.
Accomplishments: We have achieved a significant progress on this subtask. Since approval of the protocol by IRB and HRPO over 70 lung samples were processed, including donor lungs and lungs from patients with various forms of lung fibrosis (SSc-ILD, IPF, ILD, MCTD, hypersensitivity pneumonitis, pneumoconiosis, CPFE). As proposed, we have FACSorted alveolar macrophages and alveolar type 2 cells from these samples and extracted RNA for gene expression profiling using RNA-seq. In addition, to emphasize the advantages of the focusing on specific cellular populations we analyzed gene expression profiles from the whole lung tissue (traditional approach implemented by other groups). All samples have yielded high-quality (RIN over 7) RNA. Libraries for RNA-seq were prepared using a high-throughput automated robotic platform (Agilent Bravo) to minimize a batch effect, all libraries have passed the QC. Libraries from were sequenced on Illumina NextSeq 500 instrument. Data was processed using an established bioinformatic pipeline on high-performance computational cluster QUEST (Dr. Bartom). Integrative analysis of the whole lung tissue, alveolar macrophages and alveolar type 2 cells demonstrated that alveolar macrophages and alveolar type 2 cells carry transcriptional signature of pulmonary fibrosis and can be used for the diagnostics (Figure 2). We are finishing preparation of the manuscript, describing these data, it will soon be submitted for review.


Accomplishments: During preliminary phase we have established collaboration with Dr. Ido Amit, Weizmann Institute of Science, Israel, one of the world’s leading experts in single cell RNA-seq. Using the materials provided by Amit lab, we have performed single-cell sorting on a subset of donor and fibrotic lungs and, subsequently, performed single-cell RNA-seq using MARS-seq protocol on two donor lungs. These data were presented during the previous year report. However, as the new technologies emerged and became commercially available, we have switched to single cell RNA-seq using GemCoder technology, commercially available through 10x Genomics. This allowed us do decrease the cost, increase throughput and improve turnaround time for sample processing. We have successfully prepared, sequenced and performed integrative analysis of 9 samples from healthy donor lungs and from patients with pulmonary fibrosis (Figure 3). Manuscript, describing these data, is currently in preparation and will soon be submitted for review.

Major Task 2: Design diagnostic flow cytometry panel.
Subtask 1: Select prospective markers based on RNA-seq data (Drs. Misharin, Budinger, Berdnikovs, Perlman). Screen antibodies for panel design (Dr. Misharin). Design and validate the panel (Drs. Misharin, Budinger).

Accomplishments: Since this task is interdependent on the results from Major Task 1, currently, we have nothing to report.

Opportunities for training and professional development:
On March 30th, 2016 Dr. Misharin participated as a panel member at the webinar “Flow Cytometric Analysis of Myeloid Cells in Human Blood, Bronchoalveolar Lavage, and Lung Tissues” organized by American Thoracic Society. He presented findings from our group (Aim 3, Task 2; Bharat et al., AJRCMB, 2016).
On May 19th, 2017 during the Postgraduate course on flow cytometry in the mouse and human lung at American Thoracic Society meeting in Washington, DC, Dr. Misharin gave lecture on FACSorting for isolation of material for genomic research. He presented data related to Aim 1, Aim 2 and Aim 3 of this proposal.

**Impact on society beyond science and technology:** Nothing to report.

**Figure 3:** Integrative analysis of the single cell RNA-seq samples from donors and patients with pulmonary fibrosis. Data demonstrates accurate identification of multiple cell types across patients and conditions as well as fibrosis-associated signature. Manuscript in preparation.
Impact

Impact on the development of the principal discipline(s) of the project: Nothing to report.

Impact on other disciplines: Nothing to report.

Impact on technology transfer: Nothing to report.

Impact on society beyond science and technology: Nothing to report.
Changes/Problems

Changes in approach:

**Aim 1, Major task 4.** During the initial phase experiments, we found out that CD11c-DTR mice develop severe lung injury upon depletion of CD11c-DTR-positive alveolar macrophages. These findings were recently confirmed and reported by another group (Mann et al., *Eur J Immunol*, 2016). To overcome this issue, we have developed an alternative system for depletion of tissue-resident alveolar macrophages using intratracheal instillation of clodronate-loaded liposomes. We found that intratracheal instillation of 50 ul of clodronate-loaded liposomes safely and efficiently depletes tissue-resident alveolar macrophages and leads to the repopulation of the niche by monocyte-derived alveolar macrophages. Thus, we were able to overcome the technical issues and use regular shielded bone marrow chimeras for this subtask. We have generated sufficient number of shielded bone marrow chimeras and treated them with clodronate-loaded liposomes to deplete tissue-resident alveolar macrophages and induce repopulation with monocyte-derived alveolar macrophages.

**Aim 2, Major Task 2.** Initially, we proposed to use HPS-/- mice and a series of adoptive transfer experiments to dissect relationship between stress in alveolar epithelial cells, alveolar macrophages and susceptibility to lung fibrosis. While HPS-/- mice provided a valuable insight during the preliminary work, their utility was undermined because HPS deficiency was global and affecting not only epithelial cells, but all other cell types as well (including alveolar macrophages). Fortunately, soon after the work on the grant was initiated, a new strain of mice specifically causing stress in the lung epithelium by targeting telomerase (*Trf1*) in alveolar type 2 epithelial cells using highly specific *Cre*<sub>Sftpc</sub> driver became available (Povedano et al., 2015, *Cell Rep*), these mice develop spontaneous age-dependent lung fibrosis. We have initiated collaboration with Dr. Mary Blasco and obtained *Trf1*-floxed mice for our colony. We have now finished crossing these mice to *Cre*<sub>Sftpc</sub> mice and initiated the proposed experiments. While this change of tools delayed the work on this task, it did not change the scope of our work. Moreover, this approach is much more specific and will allow us to reduce the number of required animals.

**Aim 3, all major tasks.** During the first year of the project, we have conducted pilot single cell RNA-seq experiments using MARS-seq technology. However, during the second year we have switched to GumeCode technology and Chromium 10x platform. This switch allowed us to decrease the operation cost, increase throughput and turnaround time for sample processing. All procedures are not performed in house and not being outsourced.

**Actual or anticipated problems or delays and actions or plans to resolve them:** Nothing to report.
**Changes that had a significant impact on expenditures:** Nothing to report.
**Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents:** Nothing to report.
Products

Journal publications:


Presentations:

1. MHSRS 2017: August 27–30, 2017, Kissimmee, Florida, USA
Poster: MHSRS-17-0822 Macrophage responses to epithelial dysfunction promote lung fibrosis in aging.
GR Scott Budinger, Alexander V Misharin
Participants and other collaborating organizations

Name: **GR Scott Budinger**  
Project Role: Principal Investigator  
Researcher Identifier: NA  
Nearest person month worked: 1.2  
Contribution to Project: Dr. Budinger has prepared IRB protocol and worked on its revisions. He supervised generation of the shielded bone marrow chimeras and development of the new fate-mapping mouse models. He performed acquisition of the human lung samples for FACSorting, interpretation of RNA-seq results and presented data at the conferences.

Name: **Raul Piseaux**  
Project Role: Postdoctoral Researches  
Researcher Identifier: NA  
Nearest person month worked: 3  
Contribution to Project: Dr. Piseaux monitored shielded bone marrow chimeras, managed mouse colony and worked on generating new fate-mapping mouse models.

Name: **Hiroaki Matsuda**  
Project Role: Postdoctoral Researches  
Researcher Identifier: NA  
Nearest person month worked: 1  
Contribution to Project: Dr. Matsuda has been working on developing bioinformatics pipelines for analysis of the population-based and single cell RNA-seq data. Dr. Matsuda coordinates monthly bioinformatics meetings for our group.

Name: **Sergejs Berdnikovs**  
Project Role: Co-Investigator  
Researcher Identifier: NA  
Nearest person month worked: 1.0  
Contribution to Project: Dr. Berdnikovs provided his expertise in transcriptomic analysis of the population-based (mouse and human) and single cell RNA-seq (human) data.

Name: **Elizabeth Bartom**  
Project Role: Co-Investigator  
Researcher Identifier: NA  
Nearest person month worked: 1.2  
Contribution to Project: Dr. Bartom developed and continuing to provide support to bioinformatics pipelines for analysis of the population-based and single cell RNA-seq data. She plays crucial role in integrating our sequencing pipeline into current computational infrastructure at Northwestern (Hundred Genomic Nodes/QUEST project).

Name: **Sangeeta Bhorade**  
Project Role: Co-Investigator  
Researcher Identifier: NA  
Nearest person month worked: 0.6
Contribution to Project: Dr. Bhorade coordinate recruitment of the human subjects into lung transplant program and provided us with invaluable human samples. She is one of the first co-authors our manuscript on flow cytometric analysis of macrophages in the human lung.

Name: **Runyan, Constance E**  
Project Role: Res Lab Mgr 1  
Researcher Identifier: NA  
Nearest person month worked: 3  
Contribution to Project: Ms. Rynyan processed human lung specimens for single cell RNA-seq and assisted with mouse experiments.

Name: **Gokhan Mutlu**  
Project Role: Co-Investigator  
Researcher Identifier: NA  
Nearest person month worked: 1.2  
Contribution to Project: Dr. Mutlu coordinated recruitment of the human subjects into lung transplant program (University of Chicago site) and provided us with invaluable human samples.

**Special reporting requirements**

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

**Dr. Budinger:**
- The project “Macrophage Modulation of Lung Fibrosis” (1R01HL134375-01A1) was funded to support 0.72 CM effort
- The project “Regulation of IL-6 and DNA and RNA methylation by PM-Induced Mitochondrias ROS” (R21ES025644) was ended by 4/30/2017. The effort was 0.6 CM.

**Gokhan Mutlu**
- The project “Regulation of IL-6 and DNA and RNA methylation by PM-Induced Mitochondrias ROS” (R21ES025644) was ended by 4/30/2017. The effort was 0.96 CM.

**Other organizations involved as partners:**
Organization Name: University of Chicago, Section of Pulmonary and Critical Care Medicine, Dr. Gokhan Mutlu  
Location of Organization: Chicago, IL  
Partner's contribution to the project: Dr. Mutlu provided us with human lung specimens for to fulfill the research as described in Aim 3.  
Financial support: not applicable

**Appendices**
Nothing to report.