Award Number: W81XWH-20-1-0158

TITLE: Lung Macrophage Populations and Functions in COPD-Susceptible Smokers

PRINCIPAL INVESTIGATOR: Dr. Mehrdad Arjomandi, MD

CONTRACTING ORGANIZATION: University of California, San Francisco

REPORT DATE: APRIL 2023

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
APRIL 2023	Annual	15MAR2022 - 14MAR2023

4. TITLE AND SUBTITLE
Lung Macrophage Populations and Functions in COPD-Susceptible Smokers

5a. CONTRACT NUMBER W81XWH-20-1-0158
5b. GRANT NUMBER
5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dr. Mehrdad Arjomandi, MD

E-Mail: Mehrdad.arjomandi@ucsf.edu

5d. PROJECT NUMBER
5e. TASK NUMBER
5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Regents of the University of California, San Francisco

AND ADDRESS(ES)
490 Illinois Street, 4th Floor
San Francisco, CA 94143-0000

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Chronic obstructive pulmonary disease (COPD) is a heterogeneous disease that affects only a subset of those who smoke tobacco. Recent studies have documented that the "susceptible" smokers are more likely to show evidence of air trapping before any other pathologies of COPD, such as spirometric airflow obstruction, manifest themselves. However, the underlying biology that predisposes the non-obstructed smokers with air trapping to develop COPD has not been elucidated. The main objective of this proposal is to develop methodology and preliminary data that would lay the foundation for a longitudinal cohort study to examine the underlying biological processes that may render the non-obstructed smokers with air trapping their susceptibility to develop COPD. This pilot project focuses on development of mass cytometry (Cytometry by Time of Flight or CyTOF) methodologies for phenotypical and functional characterization of lung myeloid cells and generating preliminary data on how lung myeloid cells may contribute to disease progression in susceptible smokers.

The project had three major aims including development of CyTOF methodologies (Aim 1), followed by deployment of those methodologies for molecular and functional phenotyping of airway myeloid cells (luminal cells obtained by bronchoalveolar lavage or BAL) (Aim 2) and lung tissue cells (tissue resident cells obtained from surgically resected lung samples) (Aim 3). In the previous years, we performed BAL in volunteer human research subjects with history of smoking but without COPD (non-obstructed smokers) with and without air trapping, optimized our CyTOF panel for molecular phenotyping of airway (BAL) myeloid cells, performed CyTOF analysis of BAL myeloid cells, and generated a publication from those data.

Over the course of the past year, we continued to collect lung tissue samples from donors who were undergoing surgical lung resection and managed to achieve the target number of subjects planned. We also optimized our mass cytometry methodology for analysis of myeloid cells from lung tissue samples, and then used those methods to perform CyTOF analysis of lung tissue myeloid cells. Monocytes from matching peripheral blood samples collected from lung tissue donors and volunteers undergoing BAL were also collected and analyzed using CyTOF over the past year. Currently, we are in process of analyzing the data generated from the lung tissue CyTOF experiments described above and preparing a manuscript for publication. In addition, we are working on performing single cell gene expression analysis of the lung tissue samples.

15. SUBJECT TERMS NONE LISTED

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** USAMRDC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	17	

INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) is a heterogeneous disease that affects only a fraction of those who smoke tobacco. The origin of this variability in susceptibility to develop COPD is unclear, but understanding its underlying biology has important implications for our ability to design suitable preventative and therapeutic strategies for its management. This DOD discovery research proposed to develop methodologies and generate preliminary data needed to lay the foundation for a large study that would investigate the underlying biological susceptibility of those who smoke tobacco to develop COPD.

KEYWORDS

COPD; smoking; innate immunity; lung macrophages; bronchoalveolar lavage; spirometry; air trapping.

ACCOMPLISHMENTS

What were the major goals of the project?

The main objective of this proposal was to develop methodology and preliminary data that would lay the foundation for a longitudinal cohort study to examine the underlying biological processes, in particular lung myeloid cells characteristics, that may render the smokers with air trapping their susceptibility to develop COPD. This pilot project would then position us to examine our focused hypothesis that the susceptible smokers (those with air trapping) have an altered airway innate immune system with distinct lung macrophage phenotype and function, which predisposes them to develop COPD. We proposed to examine the above hypothesis through the following specific aims:

Specific Aim 1- Develop and validate additional methodology for molecular and functional phenotyping of lung myeloid cells in patients with history of heavy smoking. We previously developed a 44-marker time of flight mass cytometry (CyTOF) panel and flow cytometry-based phagocytosis and efferocytosis assays for characterization of lung macrophages from smokers. In this application, we proposed to develop an additional CyTOF panel to allow for more comprehensive single-cell proteomics examination of the diverse subpopulations of lung myeloid cells. Furthermore, we planned to develop CyTOF-based phagocytosis and efferocytosis assays to allow for functional evaluation of macrophage subpopulations identified by CyTOF. We planned to optimize these assays for use in both luminal (alveolar) and non-luminal (tissue) lung myeloid cells.

Specific Aim 2- Generate pilot molecular and functional phenotyping data of luminal (alveolar) macrophages from a cohort of non-obstructed smokers with and without air trapping. In this application, we planned to recruit and perform bronchoalveolar lavage (BAL) in a pilot cohort of smokers without COPD and perform molecular and functional phenotyping of their BAL alveolar macrophages (AM) using CyTOF (single-cell proteomics), transcriptomics (single-cell RNA sequencing), protease activity measures, and phagocytosis and efferocytosis assays. We also planned to perform detailed clinical and physiologic characterization of subjects and then examine whether distinct AM signatures exist that show trend association with air trapping phenotype, or other clinical characteristics, in non-obstructed smokers.

Specific Aim 3- Generate pilot molecular and functional phenotyping data of non-luminal (tissue) lung resident myeloid cells, including macrophages and dendritic cells, from a cohort of non-obstructed smokers with and without air trapping. Through an associated active lung cancer screening and treatment program, we have developed protocols to obtain resident myeloid cells from non-cancerous (“healthy”) portions of lung tissue resected from patients as part of their definitive non-small cell lung cancer (NSCLC) treatment. Nearly half of these patients have been current or former smokers without spirometric COPD. We have been developing a biorepository of live myeloid cell from these lung tissue samples. In this application, we planned to continue with active recruitment of these patients, obtain lung tissue myeloid cells from their resected lungs, and perform molecular and functional phenotyping of non-luminal lung myeloid cells. We planned to then examine whether air trapping, or other clinical characteristics, could identify distinct signatures of lung myeloid cells in non-obstructed smokers.

What was accomplished under these goals?

SPECIFIC AIM 1: Development of CyTOF methodology under the following **major tasks**:

1. **Development of CyTOF Panel II**: Proposed Milestone- Optimization and operationalizing of CyTOF Panel II: Two CyTOF panels were developed: one for characterization of airway luminal myeloid cells obtained via BAL, and one for characterization of tissue myeloid cells obtained from surgically resected lung tissue samples.

A. The methodology for CyTOF on BAL myeloid cells is now published in September 2020 edition of the American Journal of Respiratory Cell and Molecular Biology (*Vasudevan S, Vasquez J, Chen W, Aguilar-Rodriguez B, Niemi E, Zeng S, Tamaki W, Nakamura M,* ***Arjomandi M****. Lower PDL1/2 and AXL expression on lung myeloid cells suggests inflammatory bias in smoking and COPD. Am J Respir Cell Mol Biol. 2020 Sep 11. doi: 10.1165/rcmb.2020-0085OC. Epub ahead of print. PMID: 32915645. PMCID: PMC7790144*). Figure 1 and Table 1 below provide a summary of the methodology developed.

CyTOF Methodologies for BAL Myeloid Cells Analysis

Figure 1. Experimental design and data deconvolution. BAL was performed on subjects without chronic obstructive pulmonary disease (COPD) (control; *n* = 12) and with COPD (*n* = 20), and BAL immune cells were isolated and made into single-cell suspensions. The cells were stained with metal isotope–labeled antibodies and analyzed on a time of flight mass cytometry 2 mass cytometer. Data generated were normalized and debarcoded for data deconvolution and quality control. Cells were identified using DNA intercalator, normalization beads were gated out, live cells were identified by gating out cisplatin (live–dead)-positive cells, and immune cells were gated in using CD45 positivity. The $CD45^+$ cells were used for downstream high-dimensional analysis using Rphenograph (*Published in Vasudevan S, et al. Am J Respir Cell Mol Biol. 2020 Sep 11).*

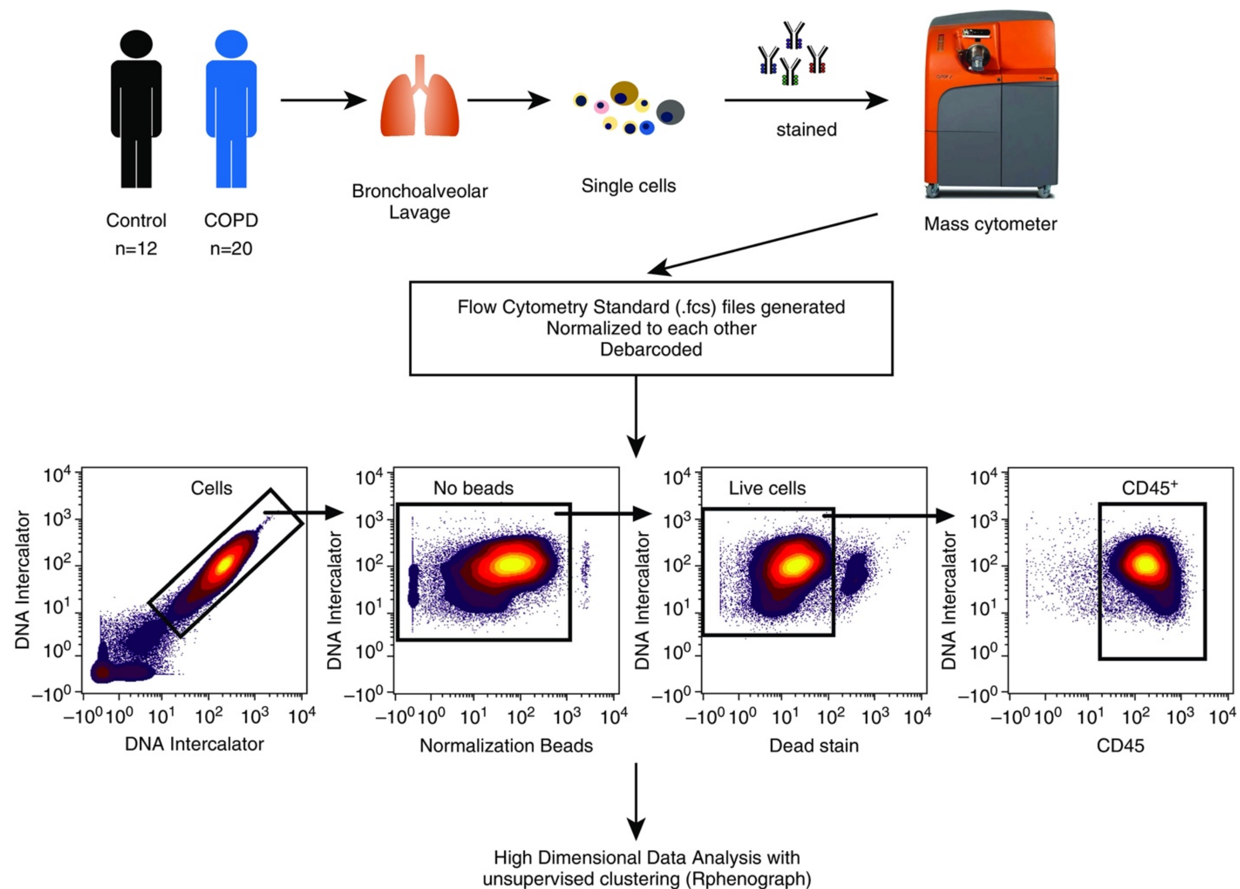


Table 1 List of Mass Cytometry Antibodies and Channels

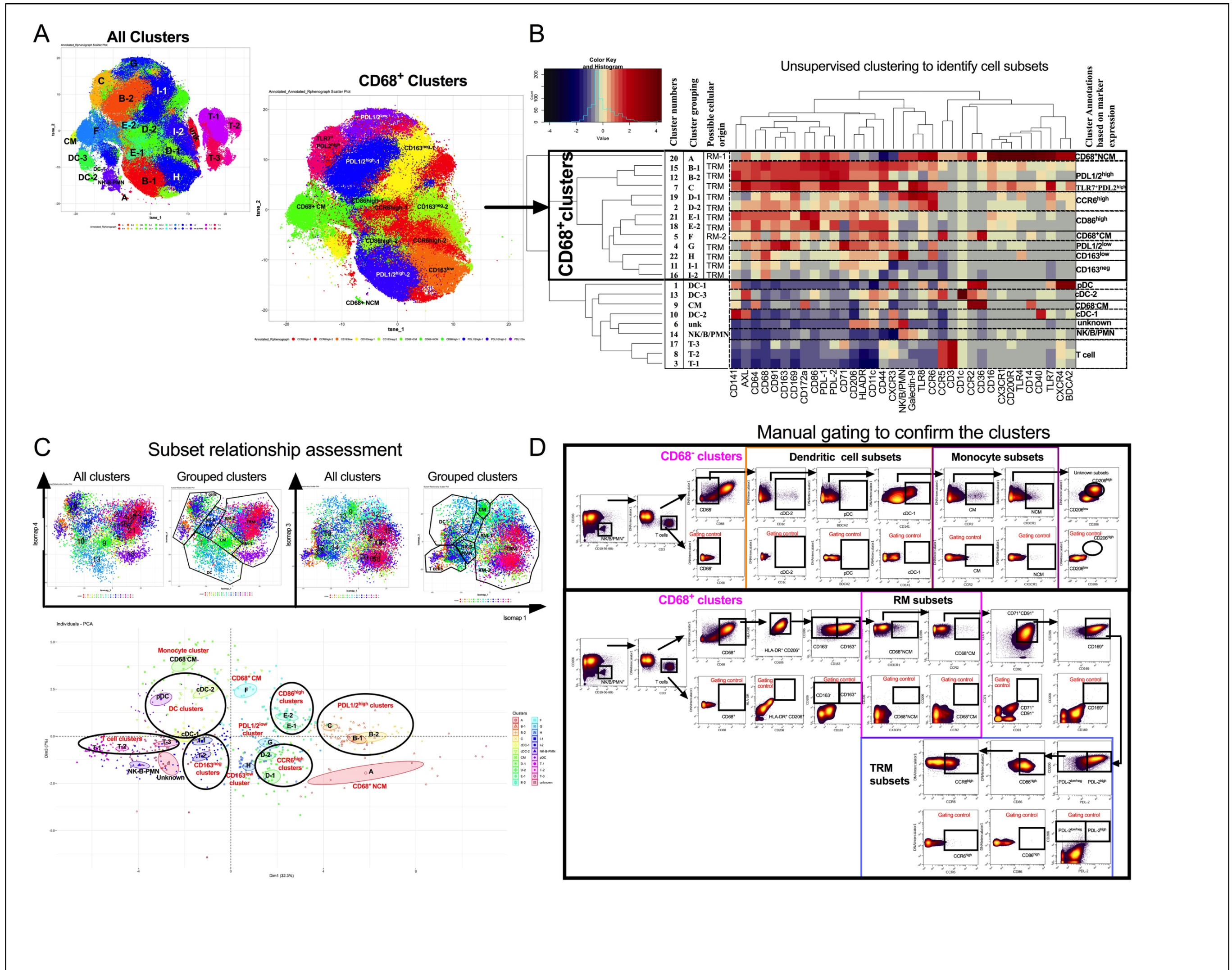
Channel	Target	Clone Designation	Manufacturer
^{89}Y	CD45	H130	Fluidigm
^{140}Ce	CD8α	RPA-T8	BioLegend
^{142}Nd	CD19	HIB19	Fluidigm
^{142}Nd	CD56	My31.13	BD Biosciences
^{142}Nd	CD66b	80H3	Bio-Rad
^{145}Nd	CD16	3G8	Fluidigm
^{149}Sm	CD141	M80	BioLegend
^{157}Gd	BDCA2	201A	BioLegend
^{160}Gd	CD14	M5E2	Fluidigm
^{168}Er	CD206 (MMR)	15-2	Fluidigm
^{170}Er	CD3	UCHT1	Fluidigm
^{176}Yb	CD1c	L161	BioLegend
^{143}Nd	CD91	A2MR-A2	BD Biosciences
^{147}Sm	TLR8	935166	R&D Systems
^{148}Nd	CD274 (PD-L1)	29E.2A3	Fluidigm
^{154}Sm	CD163	GHI/61	Fluidigm
^{158}Gd	CD284 (TLR4)	HTA125	Fluidigm
^{162}Dy	CD172a (SIRPa)	602411	R&D Systems
^{163}Dy	Galectin-9	9M1-3	Fluidigm
^{164}Dy	TLR7	533707	R&D Systems
^{166}Er	CD200R	Polyclonal	R&D Systems
^{167}Er	AXL	Polyclonal	R&D Systems
^{172}Yb	CD273 (PD-L2)	24F.10C12	Fluidigm
^{174}Yb	HLA-DR	L243	Fluidigm
^{113}In	CD68	Y1/82A	BD Biosciences
^{146}Nd	CD64	10.1	Fluidigm
^{152}Sm	CD36	5-271	Fluidigm
^{159}Tb	CD11c	Bu15	Fluidigm
^{169}Tm	CD169	7-239	BioLegend
^{171}Yb	CD44	IM7	Fluidigm
^{175}Lu	CD71	OKY-9	Fluidigm
^{141}Pr	CD196 (CCR6)	11A9	Fluidigm
^{144}Nd	CD195 (CCR5)	NP-6G4	Fluidigm
^{150}Nd	CD86	IT2.2	Fluidigm
^{153}Eu	CD192 (CCR2)	K036C2	Fluidigm
^{156}Gd	CXCR3	G025H7	Fluidigm
^{161}Dy	CX3CR1	2A9-1	BioLegend
^{165}Ho	CD40	5C3	Fluidigm
^{173}Yb	CD184 (CXCR4)	12G5	Fluidigm

Definition of abbreviations: BDCA2 = blood dendritic cell antigen 2; CX3CR1 = CX3C chemokine receptor 1; HLA-DR = human leukocyte antigen-DR isotype; MMR = macrophage mannose receptor; TLR4 = Toll-like receptor 4.

Published in Vasudevan S, et al. Am J Respir Cell Mol Biol. 2020 Sep 11.

Figure 2: Identification of BAL immune cell subtypes and confirmation using manual gating. $CD45^{+}$ immune cells from all subjects (n=32 samples) were analyzed using tsne dimension reduction algorithm and were found to be composed of 22 different clusters and 13 of them were positive for CD68 marker (A). Heatmap (B) of hierarchical clustering of the median intensities of markers (n=35 markers) and Rphenograph generated clusters (n=22 clusters) were grouped and annotated based on dendrograms and marker expression. The clusters which are $CD68^{+}$ (macrophages) are highlighted within the black box. The inter-cluster relationship assessed by ISOMAP and PCA are shown in C. 2-dimensional scatter graphs (D) from a representative sample are shown for manual gating and confirmation of identified cell subpopulations of $CD68^{-}$ and $CD68^{+}$ clusters. The gating controls are

based on the same population in T cells and are shown below the identified populations. RM subsets denote the possible recruited macrophages while TRM subsets denote tissue resident macrophages (*Published in Vasudevan S, et al. Am J Respir Cell Mol Biol. 2020 Sep 11).*



B. The methodology for CyTOF on lung tissue myeloid cells was very recently completed but is not published yet. Preliminary methodology is as follow:

<u>Preliminary CyTOF Methodologies for Lung Tissue Sample Myeloid Cell Analysis</u>

Mass Cytometry

Sample preparation and staining

Cryopreserved patient lung tissue samples were thawed and assessed for viability. Samples with less than 50% viability were excluded from further analysis. To evaluate viability, cells were stained with live-dead Cell-ID Cisplatin (201064; Fluidigm) at a dilution of 1:10,000 for 5 minutes. After staining, cells were quenched and washed with CSM containing 0.5% BSA, centrifuged, and fixed with 1.6% paraformaldehyde in PBS for 10 minutes.

To enable multiplexing, cells were barcoded using the 20-plex Palladium barcoding kit (UCSF core facility) and pooled together. Before barcoding, a separate aliquot of the sample was set aside as an unstained control for quality control purposes. The remaining cells were incubated with a serum cocktail containing human, rat, and mouse serums to block non-specific binding.

After blocking, the cells were labeled with surface antibodies for 30 minutes. Subsequently, the cells were permeabilized by adding methanol while vortexing the samples and incubating at 4˚C for exactly 5 min. Samples were quenched and centrifuged. Intracellular antibody labeling was then performed for

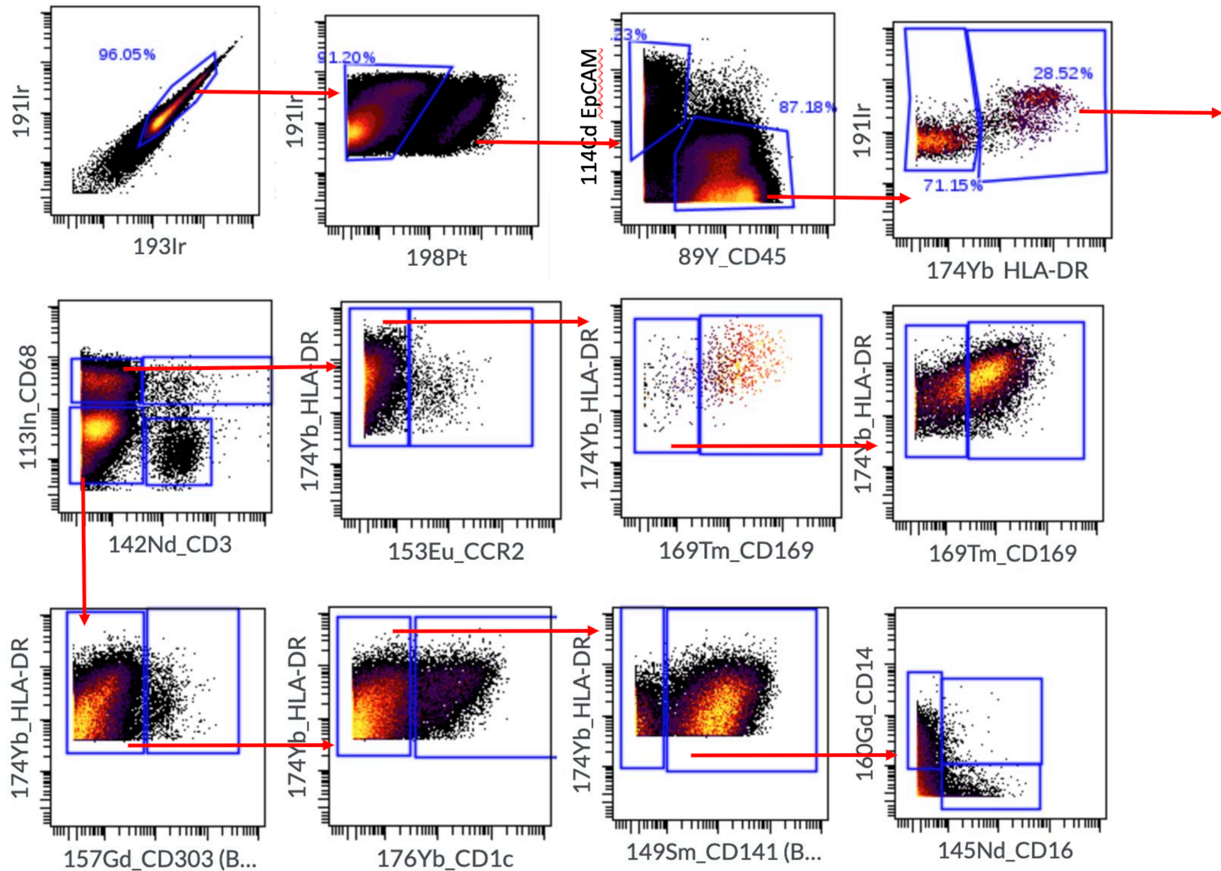
45 minutes. To enable nuclear identification, both stained and unstained cells were labeled with Cell-ID Intercalator-Ir (201192A; Fluidigm) overnight.

Cell analysis was conducted using a CyTOF2 mass cytometer (UCSF single-cell analysis core). EQ four-element calibration beads (1:10, 201078; Fluidigm) were added prior to data collection to ensure accurate calibration. The acquired data were saved as flow cytometry standard (FCS) files for subsequent analysis.

Data deconvolution and analysis

Acquired data were normalized and debarcoded using the Matlab-based open access package (https://github.com/ParkerICI/premessa). High-dimensional data analysis was performed on $CD45^+$ cells from all samples after the exclusion of dead cells and debris. Sample files underwent manual gating to investigate the immune cell population.

Figure 3- Data deconvolution for lung tissue sample CyTOF.



2. **Development of CyTOF-based phagocytosis and efferocytosis functional assays.** The development of this assay encountered multiple logistical obstacles. While the experimental methodologies for performing phagocytosis and efferocytosis using BAL and lung tissue myeloid cells were established, the further steps incorporating CyTOF into the assay for measurement of outcomes was hampered by initial unavailability of CyTOF machine during the first year of the pandemic and then departure of the staff with appropriate scientific expertise. While we are continuing to pursue this line of investigation, we are unsure whether the task would be completed before the end of the current second-year no-cost-extension funding period. A summary of the methodologies so far developed are detailed below.

Preliminary Methodology Development for Myeloid Cell Phagocytosis and Efferocytosis Assay

Bronchoscopy and BAL sample preparation

Our laboratory's procedures of bronchoscopy and bronchoalveolar lavage (BAL) have been previously discussed in detail (Ref). Briefly, intravenous access was established, supplemental O_2 was delivered, and the upper airways were anesthetized with topical lidocaine. Sedation with intravenous midazolam and fentanyl was used as needed for subject comfort. The bronchoscope was introduced through the mouth

and vocal cords into the airways. The bronchoscope was then directed into the right middle lobe (RML) where lavage was performed with two 60-ml aliquots (total of 120 mL) of pre-warmed 0.9% saline in each of medial and lateral segments of RML (total of 240 mL). The BAL was collected in a polyethylene tube and placed on ice transiently during transport to laboratory. After bronchoscopy, each subject was observed for an approximate 2-hour recovery period. A small aliquot (1 ml) of the BAL was separated for performance of cell count, and the remainder was immediately fractionated into cells and fluid using centrifugation at 180 g for 15 minutes at 4° C. The supernatant (BAL fluid) was separated and frozen at -80° C. The cell pellets were washed and counted using a hemocytometer. The cells were re-suspended in freezing medium (10% DMSO in FBS) and either shipped to Five Prime Therapeutic Inc., South San Francisco, CA for further studies or frozen at -80° C.

Bacteria labeling and preparation: Non-typeable Hemphilus influenza (NTHi; ATCC 53600) was cultured in Brain Heart Infusion broth to obtain a 0.6 OD600. The culture

media was removed, bacteria were re-suspended in PBS, and heat killed for 2 hours at 60°C. After heat killing, the bacteria were pelleted by centrifugation and lyophilized overnight. The dry pellet was weighed and re-suspended to 20mg/ml in sodium bicarbonate buffer. Labeling of the bacteria was performed using pHrodo Red SE labeling kit (P36600) from Invitrogen per the manufacturer's instructions.

Phagocytosis assay and Automated Imaging: Within 4 hours of bronchoscopy, alveolar macrophages (AM) were plated at a density of 3000 cells/well in a 384-well optically clear plate (Aurora IQ-EB, #1032-11300) with RPMI and 10% heat-inactivated FBS. Cells were cultured overnight at 37°C, 5% CO2. Media was changed and after 18 hours of treatment, the cells were washed and stained with Cell Tracker Green (# C7025, Life Technologies) and Hoechst (# H3570, Life Technologies) per the manufacturer's instruction. pHrodo-labeled heat-killed NTHi was added to the designated wells and incubated for 2hr at 37°C. Cells were fixed prior to imaging using the IN Cell Analyzer 2000. Images, containing ~75 cells/field, were analyzed with a multi-parameter protocol using IN Cell developer software. The key read outs that were evaluated include percent responding cells, phagocytosis index and pHrodo density. Illustration of the analysis algorithm and examples of segmentation are shown in supplementary figure Sx. Data analysis was determined using z-score-like statistics based on median, strictly standardized mean difference (SSMD) and reproducibility in samples from multiple COPD donors.

SPECIFIC AIM 2: Molecular and Functional phenotyping of BAL macrophage under the following **major tasks**:

1. **Development of IRB protocol and application**. The appropriate IRB protocol was developed and was approved by the University of California San Francisco Institutional Review Board (IRB) and the San Francisco Veterans Health Care System (SFVAHCS) Administration Research and Development Committee. The IRB protocol has been shared and approved by Department of Defense Human Research Protection Office (HPRO).
2. **Subject Recruitment and characterization**. A total of 17 subjects out of the planned 20 subjects were recruited and completed participation in the BAL arm of the study (Aim 2). The recruitment for this study was hampered by multiple and nationwide and institutional repeated holds that were place on academic and Department of Veterans Affairs clinical research activities during the first year of COVID-19 pandemic as well as difficulties with recruiting additional research subjects in the setting of potential participants' concerns about the possibility of contracting infection by coming into the medical centers and undergoing research bronchoscopy. No further BAL sampling will be done at this point but the CyTOF analysis will be performed on the samples that have been collected.
3. **Performance of bronchoscopies**. Bronchoscopy with bronchoalveolar lavage (BAL) was performed in 17 subjects and BAL samples were live-frozen and archived as planned for later batch experimentation by CyTOF.
4. **Molecular phenotyping of BAL macrophages**. Molecular phenotyping of BAL myeloid cells along with matching samples of peripheral blood mononuclear cells (PBMC) were performed using the CyTOF panel developed. We are currently analyzing the data to test our hypothesis. Preliminary unadjusted analysis has shown potentially significant cell cluster differences between the BAL myeloid cells from unobstructed smokers with or without clinical phenotype of air trapping, as defined by high versus low RV/TLC ratio, respectively. Regression analysis with adjustment for covariates is in progress and is pending.

5. **Functional phenotyping of BAL macrophages**. Functional phenotyping of the BAL myeloid cells will not be performed as the phagocytosis and efferocytosis procedures have not been developed- No data provided here.
6. **Data analysis and manuscript preparation**. We are in process of analyzing the data and preparing a manuscript.

Figure 4- Mass cytometry reveals differences in BAL myeloid cells. While similar myeloid cell clusters were present in BAL samples from unobstructed smokers with and without air trapping (as measured by high and low ratio of residual volume to total lung capacity (RV/TLC) measurement), different abundance of those clusters were identified. N=17; 7 with low RV/TLC and 10 with high RV/TLC; RV/TLC of 0.35 was used as a cut-off based on previous studies by our group (Arjomandi et al. BMJ Resp Res 2018).

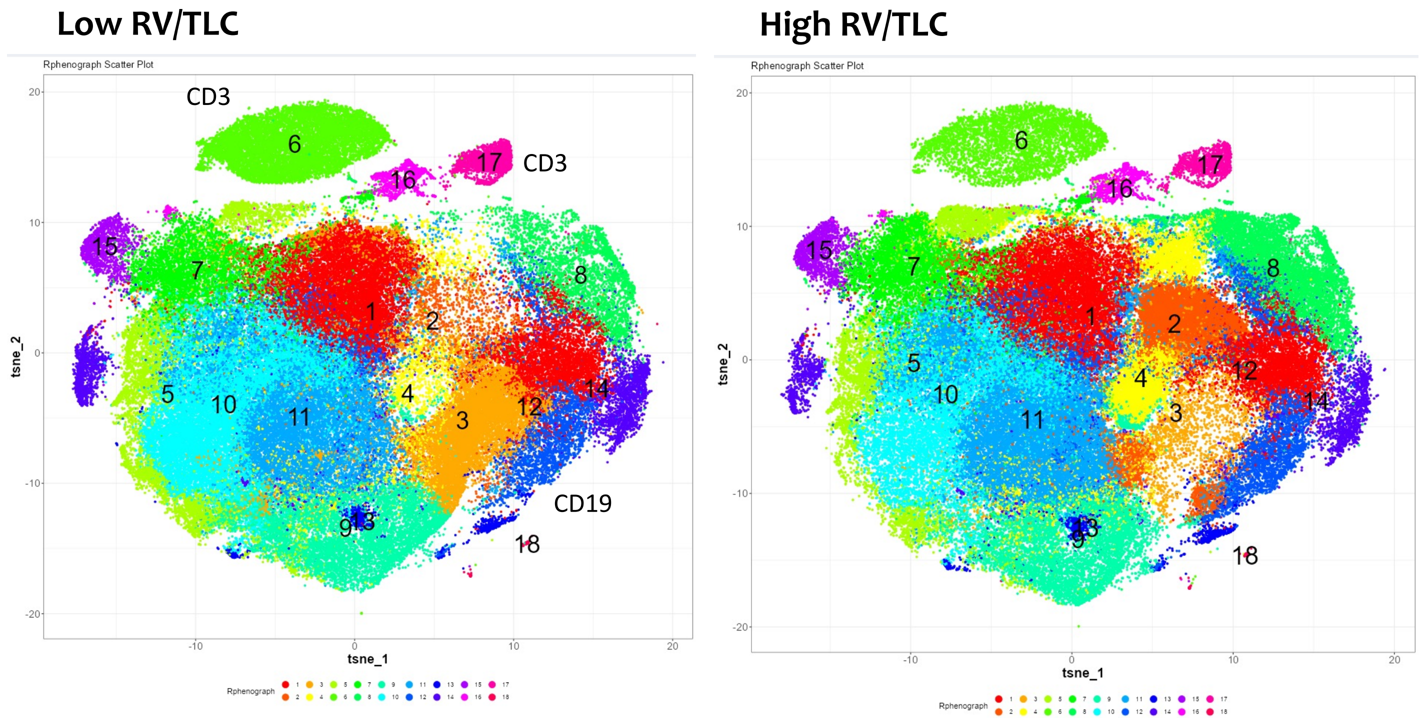


Figure 5- Mass cytometry reveals differences in BAL myeloid cells. BAL myeloid cell clusters 3, 4, and 12 had higher abundance in those with high RV/TLC, while cluster 2 had lower abundance. There was no significant difference in cluster 1 cells.

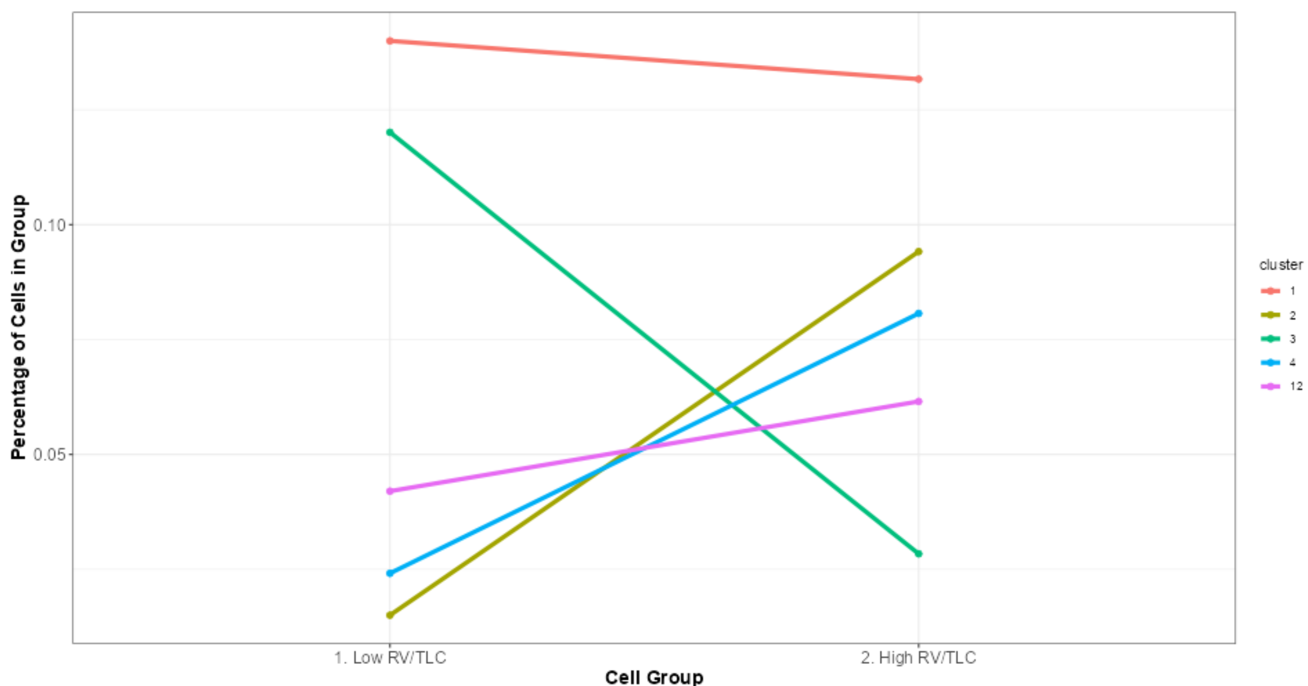


Table 2- Participants' characteristics. RV: residual volume in liters; TLC: total lung capacity in Liters; RV/TLC: the ratio of RV to TLC as a measure of air trapping. RV/TLC ratio of 0.35 was used as a cut-off for presence of air trapping based on previous studies by our group (Arjomandi et al. BMJ Resp Res 2018).

ID #	Age (years)	Gender	RV (L)	TLC (L)	RV/TLC
216.2	71	Male	2.04	6.90	30
80.2	59	Female	1.73	5.03	34
262	69	Male	2.30	6.84	34
251	74	Male	1.90	5.45	35
256	62	Male	2.23	6.38	35
257	67	Male	2.51	7.48	35
259	71	Male	2.47	7.01	35
261	76	Male	2.55	6.82	37
63R-2	63	Male	2.34	6.13	38
258	72	Male	2.10	5.56	38
108R-2	68	Male	2.42	6.44	38
83.2	60	Male	2.70	6.97	39
253	67	Male	2.76	6.93	40
255	70	Male	2.72	6.45	42
192.2	63	Male	2.54	5.96	43
254	67	Male	3.42	7.80	44
39.2	58	Male	2.64	5.71	46

SPECIFIC AIM 3: Molecular and Functional phenotyping of lung tissue myeloid cells under the following **major tasks**:

1. **Development of IRB protocol and application**. The appropriate IRB protocol was developed and was approved by the University of California San Francisco Institutional Review Board (IRB) and the San Francisco Veterans Health Care System (SFVAHCS) Administration Research and Development Committee. The IRB protocol has been shared and approved by Department of Defense Human Research Protection Office (HPRO).

2. **Subject Recruitment and characterization.** A total of 28 subjects with history of tobacco use, with no spirometric COPD, and with and without air trapping (as determined by high and low RV/TLC measurement) who were planned to undergo curative lung resection to remove a small malignant nodule were recruited into the study. The subjects completed full pulmonary function testing and standardized respiratory questionnaires as planned.

3. **Obtaining lung tissue sample and preparation of myeloid cells.** Lung tissue samples and peripheral blood mononuclear cells from the 28 unobstructed former and current smoking subjects with and without air trapping was collected. The single cell suspension of lung tissue myeloid cells and PBMC samples were live-frozen and archived as planned for later batch experimentation by CyTOF.

4. **Molecular phenotyping of lung tissue macrophages**. Molecular phenotyping of lung tissue myeloid cells along with matching samples of peripheral blood mononuclear cells (PBMC) were performed using the CyTOF panel that was developed as part of Aim 1. We are currently analyzing the data to test our hypothesis.

5. **Functional phenotyping of lung tissue macrophages**. Functional phenotyping of the lung tissue myeloid cells will not be performed as the phagocytosis and efferocytosis procedures have not been developed.

7. **Data analysis and manuscript preparation**. We are in process of analyzing the data and preparing a manuscript- No data provided here.

Table 3- Participants characteristics- Characteristics of the unobstructed former and current smoking subjects with and without air trapping (as determined by high and low RV/TLC, respectively) who donated lung tissue and blood samples. RV: residual volume in liters; TLC: total lung capacity in Liters; RV/TLC: the ratio of RV to TLC as a measure of air trapping. RV/TLC ratio of 0.35 was used as a cut-off for presence of air trapping based on previous studies by our group (Arjomandi et al. BMJ Resp Res 2018).

Study ID	Gender_Mal	Age	TLC	RV	RV/TLC
028	0	60	4.84	0.64	13
014	0	59	6.20	1.66	27
008	0	53	6.26	1.85	30
085	0	69	6.58	1.96	30
107	1	38	5.54	1.70	31
004	0	62	8.06	2.56	32
047	1	78	5.25	1.69	32
087	0	59	6.60	2.14	32
036	0	58	6.57	2.23	34
102	0	70	6.34	2.25	35
063	1	63	3.98	1.42	36
090	0	65	5.27	1.90	36
026	1	53	4.38	1.60	37
044	0	74	5.74	2.15	37
019	0	63	8.64	3.25	38
055	0	78	6.03	2.30	38
068	1	77	4.98	1.96	39
024	1	51	5.03	2.01	40
093	0	66	5.73	2.31	40
103	0	73	5.63	2.29	41
089	0	68	7.34	3.00	41
032	1	70	4.86	2.02	42
076	0	85	7.48	3.14	42
116	0	62	6.13	2.73	45
017	0	57	8.09	3.67	45
039	0	73	5.80	2.64	46
128	0	68	8.43	4.07	48
013	0	61	6.47	3.14	49

Other Accomplishments: In addition, and in a collaborative effort with another investigator at UCSF (Arun Prakash. MD, PhD), we demonstrated that the lung myeloid cells immune tone could be modulated by the metabolites produced by gut microbiomes, or so-called “gut-lung immune axis”, identifying an additional method via which smoking may affect the myeloid cells phenotype in lungs. The findings were recently accepted for publication in the American Journal of Physiology- Lung Cellular and Molecular Physiology (*Liu et al. Am J Physiol Lung Cell Mol Physiol. 2021 Jul 1;321(1):L65-L78; PMID: 33851870*).

Through another collaboration with Dr. Melanie Ott, MD, PhD, at the Gladstone Institute of Virology, we were able to use the lung tissue samples collected through this project to develop three-dimensional lung structure or organoids, which were then used by Dr. Ott’s lab as lung primary cell models for research involving viral infection including COVID-19. The findings were recently submitted for consideration for publication and are currently available in the pre-print format at bioRxiv (*bioRxiv. 2022 Aug 2:2022.08.02.502100. doi: 10.1101/2022.08.02.502100. Preprint. PMID: 35982664*).

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

This study expanded the field of COPD and myeloid immunobiology by establishing a framework to evaluate airway myeloid subpopulations for future studies involving disease progression and treatment response.

How were the results disseminated to communities of interest?

The findings were published in the American Journal of Respiratory Cell and Molecular Biology (Vasudevan et al. Am J Respir Cell Mol Biol. 2020 Dec;63(6):780-793). The findings from collaboration with Dr. Arun Prakash were recently accepted for publication in the American Journal of Physiology- Lung Cellular and Molecular Physiology (*Liu et al. Am J Physiol Lung Cell Mol Physiol. 2021 Jul 1;321(1):L65-L78*). The findings from collaboration with Dr. Melanie Ott were posted on a pre-print platform (*bioRxiv. 2022 Aug 2:2022.08.02.502100. doi: 10.1101/2022.08.02.502100. Preprint*) and are currently under consideration for publication at a scientific journal.

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

During the second year no-cost-extension period, we will be focusing on performance of Aim 3. The lung tissue and matching blood samples for experiments proposed in Aim 3 of the project have been collected and mass cytometry (CyTOF) experiments on these samples have been completed. We plan to proceed with a global analysis of the high dimensional data generated from this work. In addition, further co-variate adjusted analysis on data collected on BAL myeloid cells as part of Aim 2 will be performed.

Furthermore, we have performed single-cell gene expression on RNA samples from 7 subjects who donated lung tissue samples. This work is ongoing and analysis of the data is pending. We have established collaboration with a biostatistician with expertise in computational biology who will be assisting us with the analysis and interpretation of the genomic data.

IMPACT

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

Our findings to date generate reference baseline data that will be used as reference values for future evaluation of lung immune cells in smoking-related lung diseases including the long-term goal of this research, which is to investigate the underlying biological susceptibility to develop COPD in smokers.

What was the impact on other disciplines?

Our collaborative effort with Dr. Prakash has established additional evidence for the role of gut-lung immune axis in setting the diverse immune responses in lungs. In addition, our collaborative effort with Dr. Ott has generated lung organoids that maintain three-dimensional architecture and cellular diversity of the airway epithelium and could be used for understanding pathobiology of viral infections.

What was the impact on technology transfer?

Not applicable.

What was the impact on society beyond science and technology?

At this point, there is no direct impact on society; however, understanding the diversity of the lung myeloid cell population and how they may contribute to the lung response to inhalation of tobacco smoke has potential direct relevance to the lung response to inhalation of other pollutants such as secondhand tobacco smoke, general air pollution, and occupational inhalational exposure including military and combat-related exposures such as exposure to burn pit smoke, and thus the findings of this study will have direct public health as well as military personnel health relevance.

CHANGES AND PROBLEMS

Changes in approach and reasons for change

None.

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

The main obstacle for this research has been the safety and regulatory restrictions that have been applied to us through the UCSF and San Francisco VA Health Care System (SF VA), which is the primary location of our research laboratory, as described below:

A. To comply with governmental policies and public health guidelines concerning COVID-19 pandemic, beginning in March 2020, both UCSF and the SF VA placed holds on all research activities and restricted the presence of research staff in both medical centers. This affected not only the research activities themselves, but also the research administration and IRB and slowed down those processes due to the many restrictions that were placed in effect.

B. Later in June 2020, UCSF began research activities in a phased format. In July 2020, UCSF allowed the start of clinical research procedures with high-risk for the spread of COVID-19 infection (aerosol generating procedures [AGP] such as lung function testing, exercise testing, and bronchoscopy) with certain requirements including the strict use of PPE, COVID-19 symptom screening, and COVID-19 testing of subjects. However, based on a national level mandate, the SF VA continued to have a ban on all clinical research due to public health concerns about a shortage of PPE and COVID-19 testing. As a result, the current research, which includes AGP including exercise testing remained on hold. During the COVID-19 disease peaks of post-Thanksgiving and Winter holidays, UCSF research groups again ceased all AGP research procedures due to concerns for public health and safety.

C. Anticipating normalization of research activities as COVID-19 vaccines became available, we began generating and establishing procedures for the safe performance of the research plan. Because the SF VA's current policy is to not provided PPE and COVID-19 testing for clinical research, we modified our IRB research protocol and obtained permission from UCSF to obtain PPE and COVID-19 testing through UCSF resources. This process has been extremely bureaucratic and required tremendous amounts of effort, but we have now established IRB-approved and UCSF-sanctioned procedures to obtain PPE and COVID-19 testing for the purpose of this study.

D. In November 2020, the SF VA agreed to allow limited (12.5% capacity) clinical research activity for all the projects of our research group on the VA campus. However, in December, due to concerns about high risk AGP of this research, public health matters, and safety of our staff and subjects, and in concordance with the UCSF research community, the AGP research procedures including bronchoscopy were again restricted for the duration of the COVID-19 disease peaks of post-Thanksgiving and Winter holidays.

E. Over the course of the past year and half, we were able to proceed with recruiting a qualified technician, train her in performance of mass cytometry, and re-validate our antibody panels that were outdated due to the passage of time. Of note, productions of some essential mass cytometry antibodies were delayed due to COVID-19 pandemic and supply chain problems until late 2022. We are now in the process of pushing forward with the mass cytometry experiments that we had proposed, completing Aim 1 and nearly completing Aims 2 and 3 of the project.

F. We requested and obtained approval for a second year of no-cost-extension to be able to complete this project, which we firmly believe is important research with significant impact for Veterans' health as well as for the general population.

Changes that had a significant impact on expenditures

As the study progress was hindered due to the COVID-19 pandemic, a significant amount of the budget had remained unused. We requested a carryforward of funds. No additional funds are being requested to complete the work on this project.

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

As described above, we have had to halt all human research activities due to COVID-19 pandemic, and invent and incorporate major safety protocols to ensure subject and staff safety as we have slowly returned to in-person clinical research and subjects' recruitment.

To ensure that we can continue with the human research experiments in this study that includes bronchoscopy in the setting of COVID-19 pandemic, we spent major efforts to generate and implement safety protocols, as described below.

A. Prepare IRB application modifications of the COVID-19 safety guidelines and protocols and obtain UCSF IRB approval.

B. Obtain SF VA Research and Development Subcommittees and Committee approvals.

C. Replace the staff who left our group during the pandemic and retrain the new staff.

D. Obtain the required institutional (UCSF and SFVA) research logistics including appropriately sized lab space with adequate ventilation required for (1) safe return of staff and (2) safe conduct of aerosol generating research procedures (AGP).

E. Secure ability to perform and operationalize COVID-19 testing for asymptomatic research subjects within 96 hours prior to each research visit.

F. Secure personal protective equipment (PPE) and COVID-19 vaccination for research staff to be able to perform aerosol generating procedures (AGP) involved in this study.

Significant changes in use or care of human subjects

We now have to incorporate safety protocols to ensure subjects and staff safety in the setting of COVID-19 pandemic. These include symptoms screening and COVID-19 testing prior to each subject visit, extensive cleaning and sterilization of space and devices used for human subject experimentation, and protection of staff using appropriate PPE and vaccination.

Significant changes in use or care of vertebrate animals

Not applicable.

Significant changes in use of biohazards and/or select agents

Not applicable.

PRODUCTS

Publications, conference papers, and presentations

Please see below.

Journal publications

1. The findings were published in the American Journal of Respiratory Cell and Molecular Biology (Vasudevan et al. Am J Respir Cell Mol Biol. 2020 Dec;63(6):780-793).
2. The findings from collaboration with Dr. Arun Prakash were recently accepted for publication in the American Journal of Physiology- Lung Cellular and Molecular Physiology (Liu et al. Am J Physiol Lung Cell Mol Physiol. 2021 Jul 1;321(1):L65-L78).
3. The findings from collaboration with Dr. Melanie Ott were posted on a pre-print platform (bioRxiv. 2022 Aug 2:2022.08.02.502100. doi: 10.1101/2022.08.02.502100. Preprint) and are currently under consideration for publication at a scientific journal.

Books or other non-periodical, one-time publications

Not applicable.

Other publications, conference papers, and presentations

Not applicable.

Website(s) or other Internet site(s)

Not applicable.

Technologies or techniques

Not applicable.

Inventions, patent applications, and/or licenses

Not applicable.

Other Products

Not applicable.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Mehrdad Arjomandi, MD
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-0116-9217
Nearest person month worked:	3
Contribution to Project:	Provided consultation about the interpretation of the high dimensional analysis of generated data.
Funding Support:	Department of Veterans Affairs, BLRD

Name:	Christine Hsieh, PhD
Project Role:	Co-investigator
Researcher Identifier (e.g. ORCID ID):	0000-0003-3620-784X
Nearest person month worked:	1
Contribution to Project:	Provided consultation about the interpretation of the high dimensional analysis of generated data.
Funding Support:	Department of Veterans Affairs, BLRD

Name:	Arun Prakash, MD, PhD
Project Role:	Collaborator
Researcher Identifier (e.g. ORCID ID):	0000-0002-9720-8179
Nearest person month worked:	1
Contribution to Project:	Collaborated on examining the diversity of myeloid cell populations in lung contributed by the gut-lung immune axis.
Funding Support:	National Institute of Health, R01

Name:	Joshua Vasquez, MD
Project Role:	Collaborator

Researcher Identifier (e.g. ORCID ID):	0000-0002-7165-5739
Nearest person month worked:	2
Contribution to Project:	Provided consultation about the design and optimization of the CyTOF panel and interpretation of the high dimensional analysis of generated data, and with presentation and publication of findings.
Funding Support:	National Institute of Health, K08

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

No.

What other organizations were involved as partners?

None other than the original institutions of UCSF and San Francisco VA Medical Center.

SPECIAL REPORTING REQUIREMENTS

Award chart is provided in the Appendix.

APPENDICES

Award Chart is provided below.

Proposed budget/spending plan: The remaining funds in this project will be used to mainly support the effort of research staff and the costs associated with cytometry assays (please find our budget documents attached here).

Outline of the remaining tasks: In the following table, which outlines the original scope of work and its timeline, we have highlighted the work accomplished and pending.

Specific Aims (specified in proposal)	**Timeline**	**Accomplished or Achieved**	**Pending**	**Not Achieved**
Specific Aim 1: Development of additional methodology				
Major tasks:				
Development of CyTOF Panel II	FY 1	Done (100%)		
Development of CyTOF-based phagocytosis and efferocytosis assays	FY 2	Done (40%)		
Milestones achieved:				
Optimization and operationalizing of CyTOF Panel II	FY 1	Achieved		
Optimization and operationalizing of CyTOF-based phagocytosis and efferocytosis assays				Not Achieved
Manuscript preparation	2nd yr NCE		1 Pending	1 Pending
Specific Aim 2: Molecular and Functional phenotyping of BAL macrophage				

Major tasks:				
UCSF IRB and VA R&D Approval	FY 1	Done (100%)		
Subject Recruitment and characterization	FY 1 & 2; 1st yr NCE	Done (85%)		
Performance of bronchoscopies	FY 1 & 2; 1st yr NCE	Done (85%)		
Molecular phenotyping of macrophages	FY 1 & 2; 1st yr NCE	Done (85%)		
Functional phenotyping of macrophages		Not Done		
Data analysis and manuscript preparation	1st yr NCE	Being Done (50%)	Pending	
Milestones achieved:				
Single-cell proteomics analysis of BAL macrophage and their association with subjects' clinical phenotype	1st yr NCE	Achieved		
Single-cell genomics analysis of BAL macrophage and their association with subjects' clinical phenotype	2nd yr NCE		Pending	
Functional analysis of BAL macrophage and their association with subjects' clinical phenotype				Not Achieved
Manuscript preparation	2nd yr NCE	1 Achieved	1 Pending	
Specific Aim 3: Molecular and Functional phenotyping of lung tissue myeloid cells				
Major tasks:				
UCSF IRB and VA R&D Approval	FY 1	Done (100%)		
Subject Recruitment and characterization	FY 1 & 2; 1st yr NCE	Done (100%)		
Obtaining lung tissue sample and preparation of myeloid cells	FY 1 & 2; 1st yr NCE	Done (100%)		
Molecular phenotyping of macrophages	FY 1 & 2; 1st yr NCE	Done (100%)		
Functional phenotyping of macrophages		Not Done		
Data analysis and manuscript preparation	1st yr NCE		Pending	
Milestones achieved:				
Single-cell proteomics analysis of BAL macrophage and their association with subjects' clinical phenotype	1st yr NCE	Achieved		
Single-cell genomics analysis of BAL macrophage and their association with subjects' clinical phenotype	2nd yr NCE		Pending	
Functional analysis of BAL macrophage and their association with subjects' clinical phenotype				Not Achieved
Manuscript preparation	2nd yr NCE		1 Pending	