AWARD NUMBER: W81XWH-17-1-0442

TITLE: Herpesviruses and Immune Dysregulation in Pulmonary Fibrosis

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CONTRACTING ORGANIZATION: Vanderbilt University Medical Center
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REPORT DATE: October 2019

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command

Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE: Oct 2019	**2. REPORT TYPE: Annual**	**3. DATES COVERED** 09/30/2018 – 09/29/2019

4. TITLE AND SUBTITLE: Herpesviruses and Immune Dysregulation in Pulmonary Fibrosis

5a. CONTRACT NUMBER

5b. GRANT NUMBER W81XWH-17-1-0442

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S): Timothy S. Blackwell, MD

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

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

U.S. Army Medical Research and Materiel 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:

The objective of these studies is to determine the role of human herpesviruses and antiherpesvirus immunity in the development of idiopathic pulmonary fibrosis. The first aim of the study is to collect peripheral blood and broncholalveolar lavage fluid from patients with IPF or other ILDs undergoing clinical bronchoscopy, quantify viral load and perform immunophenotyping of circulating and BAL immune cells. To date, enrollment is continuing and we are proceeding with characterization of immune cell populations as planned. The second aim is to use a model of genetic risk for IPF to investigate the mechanisms of herpesvirus-driven experimental fibrosis. Our first objective was to determine the T-cell profiles during the time course of MHV68 infection. We found that there is expansion of the T-cell compartment along with upregulation of T-cell exhaustion markers that peaks 14-28 days after infection. We plan to continue studies as outlined in the SOW for the next funding period.

15. SUBJECT TERMS: Idiopathic pulmonary fibrosis, Interstitial lung disease, Bronchoalveolar lavage, Alveolar epithelial cells, Cytomegalovirus, Epstein-Barr virus, Endoplasmic reticulum stress, T lymphocytes, Programmed Death-1

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	15	

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1. INTRODUCTION:

It is widely recognized that recurrent injury to alveolar epithelial cells (AECs) initiates the process of progressive fibrotic remodeling in Idiopathic Pulmonary Fibrosis (IPF). Although the environmental factors that cause repetitive AEC injury in IPF are not well understood, both observational and experimental data support a role for herpesvirus reactivation in this process. Our preliminary data show that cytomegalovirus (CMV) and Epstein-Barr virus (EBV) antigens can be detected in AECs lining areas of fibrosis in the majority of IPF patients, whereas herpesvirus protein expression is not found in AECs in normal lungs. Herpesvirus antigens in AECs from IPF patients co-localize with markers of endoplasmic reticulum (ER) stress, which is a nearly universal finding in IPF lungs and contributes to AEC dysfunction and apoptosis. Our studies test the hypothesis that: 1) re-activation of latent herpesviruses, particularly CMV and EBV, in dysfunctional type II AECs contributes to a recurrent injury-repair cycle that drives progressive fibrosis in patients with IPF, 2) targeted anti-herpesvirus therapy or interventions to restore immunosurveillance will limit progression of lung fibrosis. Specific aims of this project are: 1) to determine viral load and immune response to herpesviruses in pulmonary fibrosis patients and 2) to investigate the mechanisms of herpesvirus-induced lung fibrosis in mice with AEC dysfunction.

2. KEYWORDS:

Idiopathic Pulmonary Fibrosis
Interstitial lung disease
Bronchoalveolar lavage
Alveolar epithelial cells
Cytomegalovirus
Epstein-Barr virus Endoplasmic reticulum stress T lymphocytes
Programmed Death-1

3. ACCOMPLISHMENTS:

What were the major goals of the project?

This project consists of 2 specific aims:

Aim 1. To determine viral load and immune response to herpesviruses in pulmonary fibrosis patients.

Major task 1: Enroll 100 subjects with pulmonary fibrosis in prospective cohort study.
Major task 2: Determine whether herpesvirus loads differ in IPF lungs compared to controls.
Major task 3: Define the herpesvirus-related T-cell phenotypes.

Aim 2. To investigate the mechanisms of herpesvirus-induced lung fibrosis in mice with AEC dysfunction.

Major task 1: Determine whether alveolar epithelial endoplasmic reticulum stress affects quantitative or functional T cell responses to herpesvirus infection.
Major task 2: Determine whether antiviral treatment can prevent or reverse T-cell exhaustion.
Major task 3: Test whether therapies designed to prevent or reverse T-cell exhaustion can enhance viral clearance and prevent lung fibrosis.

What was accomplished under these goals?

Aim 1:

Major task 1: Enroll 100 subjects with pulmonary fibrosis in prospective cohort study.

In the first year of this study, we receive regulatory approval (April 2018) and began subject enrollment and biospecimen collection. To date, we have enrolled 49 subjects with pulmonary fibrosis. We have collected clinical information as planned and entered these data into a study Redcap database. The demographics of the current study population is shown in **Table 1**. We plan to continue enrolling patients as they present for evaluation in our interstitial lung disease (ILD) clinic.

Table 1. Characteristics of Enrolled Subjects

	n=49
Age	66.12 (8.58)
Sex (female)	17 (35%)
Smoking History	
Current	2 (4%)
Former	27 (55%)
Never	20 (41%)
Pulmonary Function Tests	
FVC%	66.84 (17.57)
FEV1%	73.86 (19.12)
TLC%	67.16 (17.24)
DLCO%	44.77 (15.08)

Major task 2: Determine whether herpesvirus loads differ in IPF lungs compared to controls.
We have collected BAL samples and plan to batch samples for measuring viral load in the lungs. CMV and EBV serology testing has been done on samples collected to date. We have also optimized an in-situ hybridization-assay that we believe has improved sensitivity for detection of active CMV in tissue (**Figure 1**). Analysis of biopsy sections is ongoing.

Major task 3: Define the herpesvirus-related T-cell phenotypes.
Initially, we used standard flow cytometry to investigate for differences in T lymphocyte subsets in the peripheral blood samples from patients with IPF and non-diseased controls. As shown in **Figure 2**, we did not find substantial differences in major lymphocyte subsets between IPF and control samples. Therefore, we chose to perform more in-depth analysis using time of flight mass spectrometry (cyTOF) to more specifically determine subsets of immune/inflammatory cells. We optimized a 34 analyte panel (as shown in **Table 2**) and used this approach to evaluate T cells in blood from IPF patients and non-diseased control subjects. Using ViSNE and FlowSOM analysis, we evaluated peripheral blood mononuclear cells (PBMCs) and compared cellular subsets from IPF patients and control subjects. This approach allows us to define major categories of immune cells and then subcategorize these cell types based on additional markers from our panel (**Figure 3**). In initial analysis, we focused on T cell subsets and identified differences in IPF compared to controls in effector memory (T_{EM}) populations of CD4 and CD8 cells, which were increased in IPF (**Figure 4**). We then subdivided IPF patients based on serology status for CMV and found that a subset of CD4 T_{EM} cells ($PD1^{+}CD28^{-}$) was increased only in CMV+ IPF patients (**Figure 5**). In contrast, CMV- IPF patients showed a selective increase in CD8 T_{EM} cells ($PD1^{+}CD28^{-}$). Although substantially more work is needed, these findings suggest that we can identify a CMV immune response signature in IPF. Of note, all but 1 IPF patients with serology testing so far have been EBV positive, so it is not yet possible to examine for an EBV immune response signature in IPF.

Table 2: CyTOF panel analytes

Live cells/ Leukocytes	Innate Immune	Lymphocytes	Ligands/ receptors		Chemokine receptors	Transcription factor	Stem Cell
Cisplatin	CD11b	CD19	PDL2	PDL1	CCR4	FOXP3	CD34
Intercalator	CD11c	CD4	CD127	CD169	CCR7		
CD45	CD16	CD8a	CD86	CD103	CCR2		
	CD14	CD45RO	HLA-DR	CD28			
	CD24	CD3	CD36	CD206			
	CD68	CD56	CD163	CD25			
			PD-1	CD38			

In addition to identification of immune/inflammatory cell subsets in peripheral blood, we have examined global T cell phenotypes in IPF patients and controls (**Figure 6**). Using CD3+ T cells isolated from blood, we found no differences in T cell proliferation or cytokine production following stimulation with CD3/CD28 beads. Together, current studies suggest that, despite the lack of differences in major immune/inflammatory cell populations or global T cell functional capacity in IPF, herpesvirus-related differences in T cell subsets are present in IPF. These findings will continue to be explored in the upcoming year of funding.

Aim 1 figures

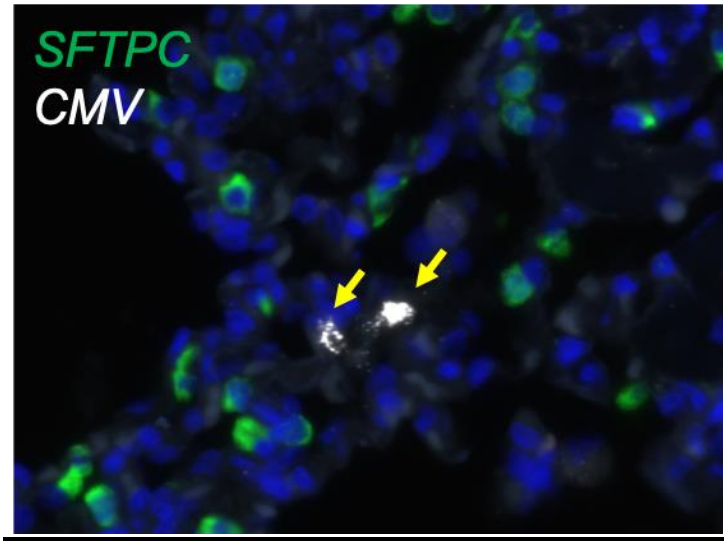

Figure 1. Multiplexed RNA in-situ hybridization localizing CMV- expressing cells in lung biopsy sections. Original magnification 400x. Yellow arrows indicate CMV+ cells.

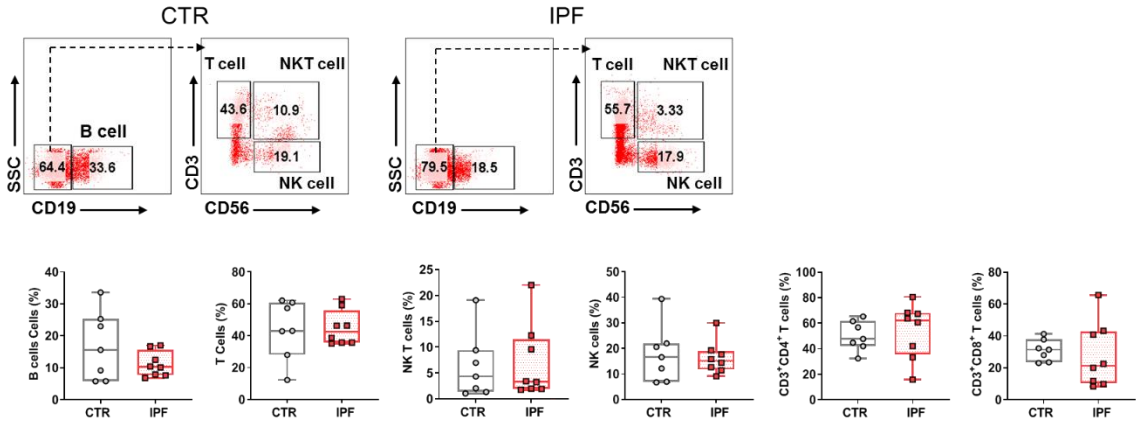

Figure 2: Characterization of lymphocyte subsets in peripheral blood from IPF and controls using flow-cytometry. To identify different subsets of B cells, T cells and NK cells in peripheral blood mononuclear cells (PBMC), total PBMCs were stained with viability dye, followed by fluorescent-conjugated antibodies and then analyzed by flow-cytomentry. **A)** Viable PBMCs were gated on B cells ($CD19^+$), T cells ($CD3^+CD56^-$), NK ($CD3^-CD56^+$) and NK T cells ($CD3^+CD56^+$) as indicated. **B**) Percentage of B cells, T cells, NK cells, and the subsets of CD4 and CD8 T cells were determined in control (CTR) and IPF patients. Box plots represent median, 25^{th}-75^{th}

percentile, and range. n=7 control and n=8 IPF.

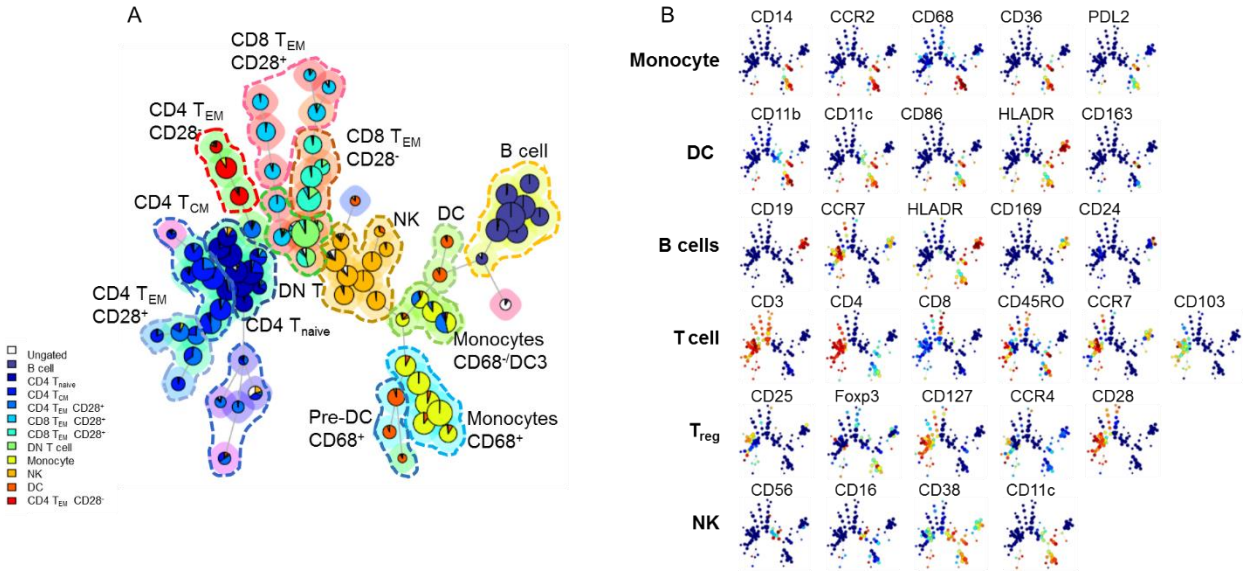

Figure 3: Characterization of leukocyte subsets in peripheral blood from IPF and controls using mass cytometry by time-of-flight (Cytof). To identify different subsets of leukocytes, total PBMCs were stained with viability dye, followed by incubation with metal-conjugated antibodies specific for different subpopulations of leukocytes, as indicated. **A)** Unsupervised self-organized map (minimum spanning tree) of viable PBMCs obtained by viSNE followed by FlowSOM analysis depicting cell subpopulations clustered according to mean markers values. **B**) Identification of single clusters using patterns of expression markers previously defined for different subpopulations of immune cells (monocytes, DCs, B lymphocytes, T cells and NK cells). n=23 control and n=25 IPF.

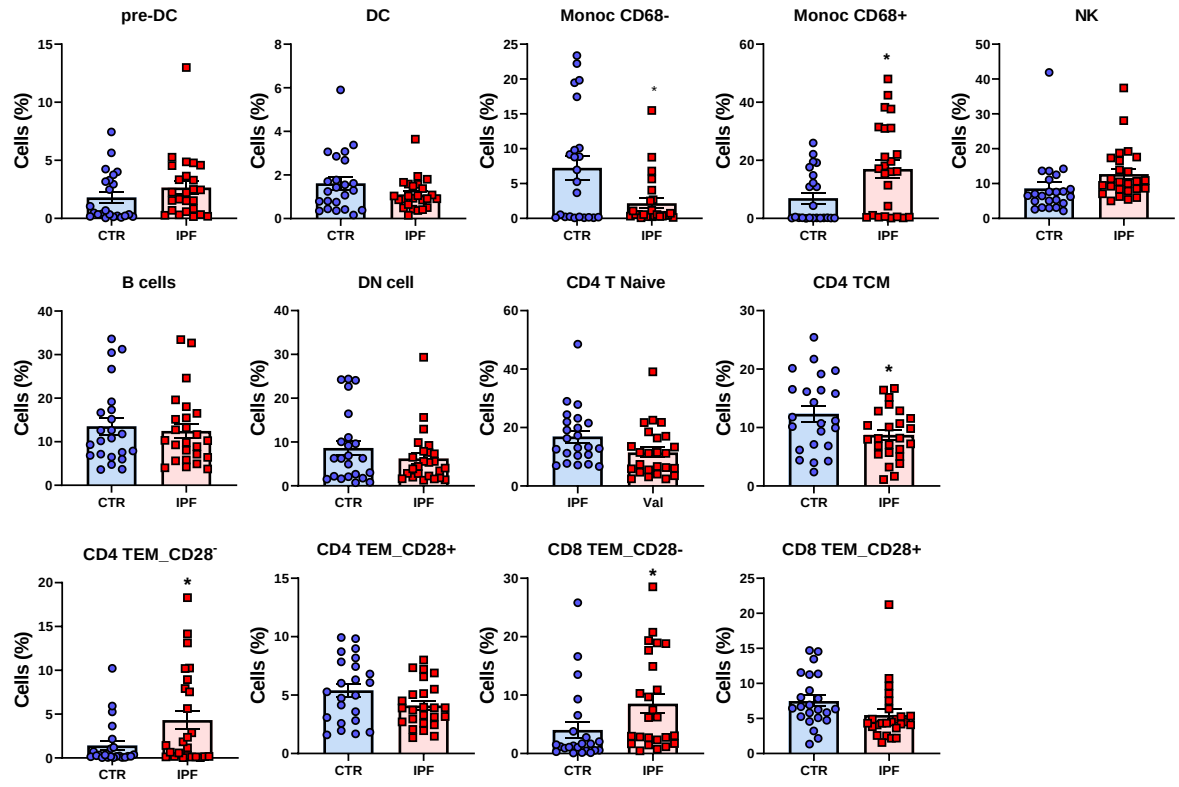

Figure 4: Characterization of leukocyte subsets in peripheral blood from IPF and controls using mass cytometry by time-of-flight (Cytof). Percentage of different subpopulations of leukocytes in PBMC of IPF and controls using unsupervised FlowSOM analysis. (TEM- effector memory T cell; TCM- central memory T cell; DN- $CD4^{-}CD8^{-}$ T cell). n=23 control and n=25 IPF. *=p<0.05.

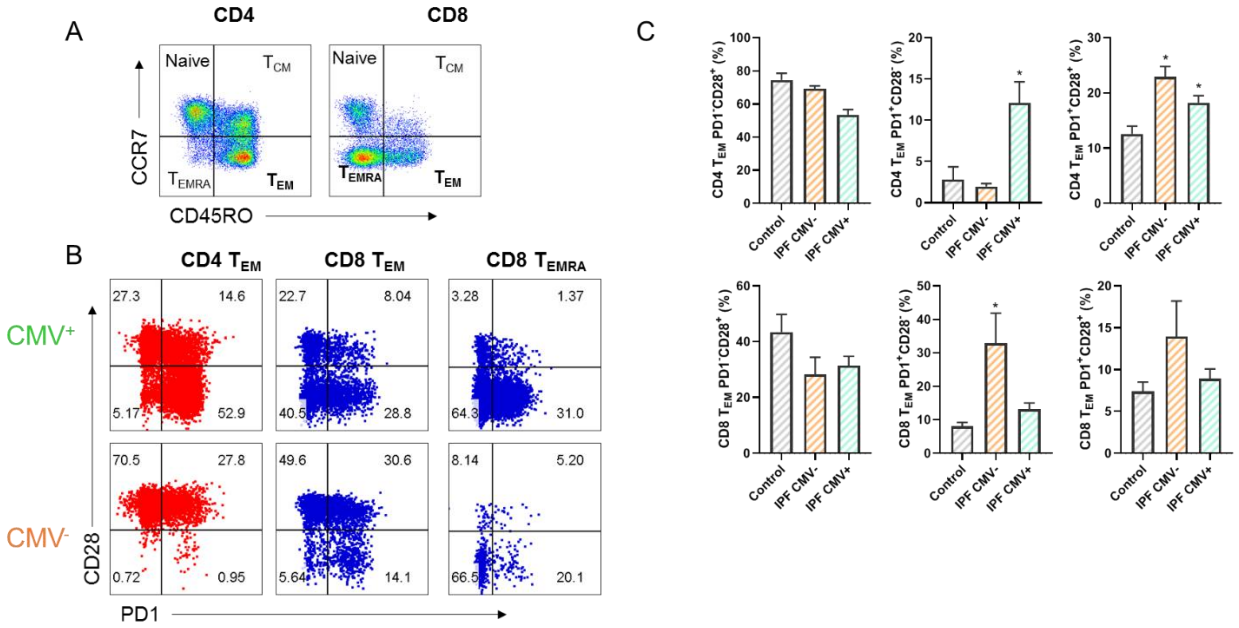

Figure 5: Herpesvirus serology status dictates the appearance of T_{EM} cells subsets lacking CD28 expression in peripheral blood in IPF. A) Gate representation of naïve, T_{CM}, T_{EM} and T_{EMRA} cells. **B**) Gate representation of CD28 and PD1 expression on CD4 T_{EM}, CD8 T_{EM}, and CD8 T_{EMRA} cells in CMV+ and CMV- patients with IPF. **C**) Percentage of CD4 T_{EM} and CD8 T_{EM} expressing CD28+/- and PD1+/-. Bars represent mean ±SEM. *p<0.05

compared with control (T_{EM}- effector memory T cell; T_{CM}- central memory T cell; T_{EMRA} effector memory T cell expressing RA). n=11 controls, IPF: n=21 CMV+, and n=8 CMV-.

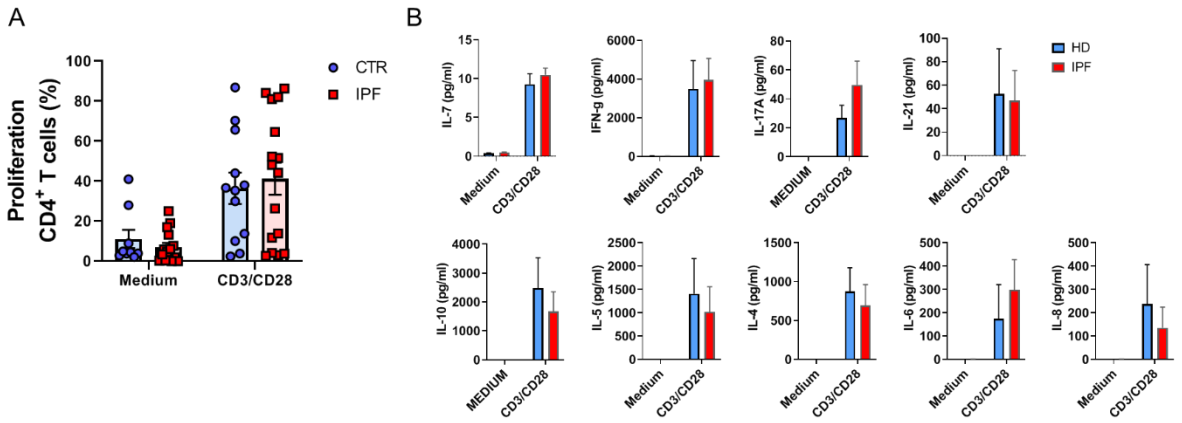

Figure 6: T cell proliferation is similar in IPF and control subjects. CD3 T cells were isolated from PBMC from controls and IPF patients, stained with cell trace violet and cultured in a density of $5x10^5$ cells with medium alone or with CD3/CD28 beads. **A**) After 5 days, the percentage of proliferating CD4 T cell was determined by flow-cytometry. **B**) Different cytokines were measured in the supernatant by Luminex after 48 hours of culture. Bars represent mean ±SEM. n=11 control, n= 16 IPF.

Aim 2:

Major task 1: Determine whether alveolar epithelial endoplasmic reticulum stress affects quantitative or functional T cell responses to herpesvirus infection. We have performed a series of experiments analyzing the time-course of immune activation following MHV68 infection using the L188Q SFTPC mice. We have found that peak CD4 and CD8 induction occurs 7-10 days after infection, and largely resolves by 28 days after infection. While activation and expression of exhaustion markers occurs 10-14 days after infection, we have not observed significant differences in specific T-cell subsets but rather a global increase in lymphocytic inflammation (**Figure 7**). This suggested to us that functional, rather than quantitative, immune response is the predominant factor regulated by AEC ER stress. To this end, 28 days after infection, we isolated whole-lung lymphocytes and performed intracellular cytokine staining after stimulation with MHV68 peptides and/or PMA/ionomycin. We observed a significant increase in interferon gamma production by CD4 cells from L188Q SFTPC mice, but no differences in IL4 or IL17 expression (**Figure 8).** This led us to examine the epithelial response to MHV68 more closely. We isolated CD45-/CD31-/CD326+ primary lung epithelial cells from WT and L188Q SFTPC mice, and infected them ex-vivo with MHV68. Compared to WT mice, L188Q SFTPC mice demonstrated an enhanced, autonomous interferon response characterized by induction of interferon-alpha receptor 1 and the interferon-gamma receptor, as well as interferon alpha expression (**Figure 9**).

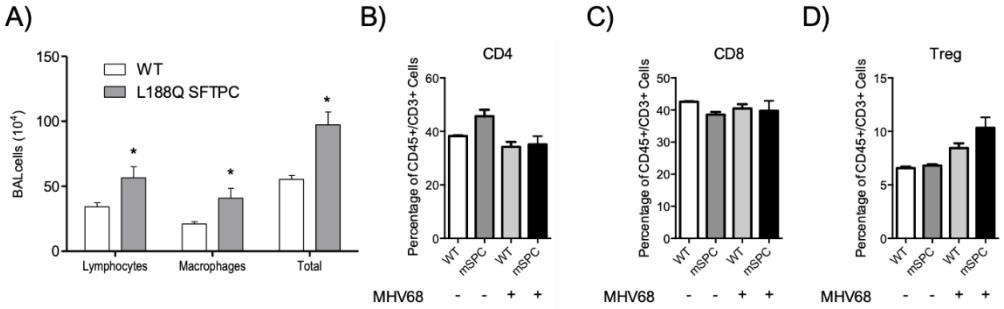

Figure 7. A) BAL cell count and differential from WT and L188Q SFTPC mice 14 days post infection (dpi). B-D) Quantification of T-cell subsets from WT and L188Q SFTPC mice 28 dpi demonstrated resolution of the most inflammation with a modest increase in regulatory T-cells.

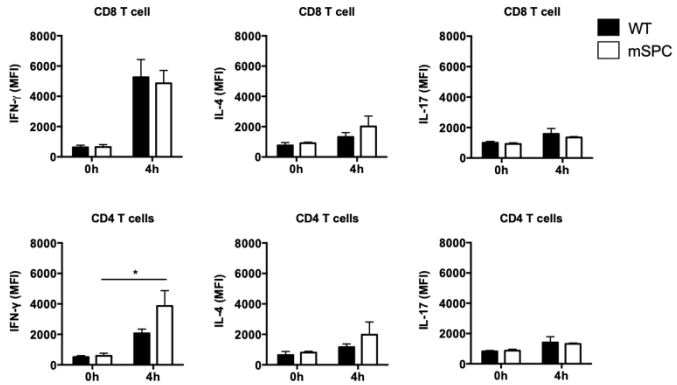

Figure 8). T-cells were isolated from L188Q SFTPC and WT mice 28 dpi, and stimulated ex-vivo with MHV68 peptides or PMA/ionomycin. Intracellular cytokine staining was performed and quantified by flow cytometry.

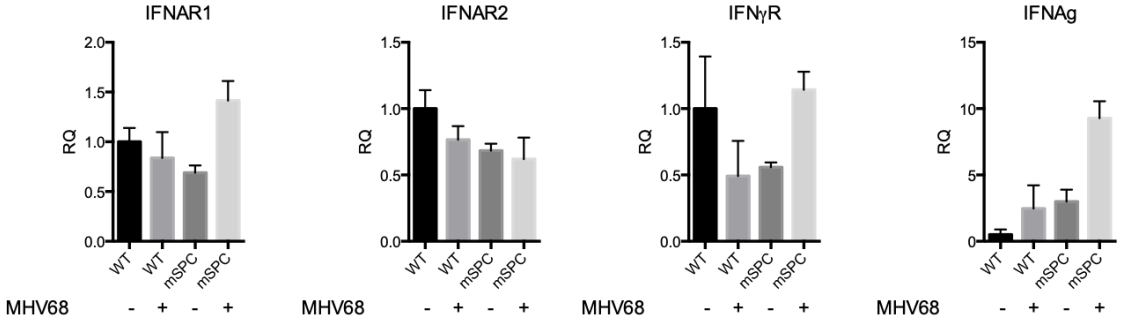

Figure 9). Primary alveolar epithelial cells were isolated from L188Q SFTPC and WT mice (CD45-/CD31-/CD326+, MACS Microbeads, Miltenyi, Inc), and 5 x 10^5 cells were placed in 50% Matrigel then cultured with BEGM media. Twenty-four hours after cultures were established, cells were infected with MHV68 (MOI 0.1), and 48 hours post-infection RNA-was isolated for gene expression analysis.

Major task 2: Determine whether antiviral treatment can prevent or reverse T-cell exhaustion. These studies are ongoing.

Major task 3: Test whether therapies designed to prevent or reverse T-cell exhaustion can enhance viral clearance and prevent lung fibrosis.
These studies are ongoing.

What opportunities for training and professional development has the project provided
Dr. Carla Copeland (Fellow, Pulmonary/Critical Care Medicine) has recently joined the project and taken on the primary role of leading multidisciplinary committee discussion for diagnosis adjudication. Dr. Serezani, an early-career investigator, has presented this work at a major international conference, and developed advanced computational skills analyzing mass-cytometry data.

How were the results disseminated to communities of interest?
The following abstract was presented at the American Thoracic Society meeting in May 2019:
Serezani A, Pascoalino B, Vowell K, Kropski J, Blackwell TS. T Cell Phenotype in Peripheral Blood of Patients with Idiopathic Pulmonary Fibrosis. Proc. Am. Thorac. Soc. 199:A2425, 2019.

What do you plan to do during the next reporting period to accomplish the goals?
We plan to continue studies as outlined in the SOW. We will continue to recruit ILD patients and perform immunophenotyping as planned and presented above. We anticipate submitting a manuscript (or possibly 2) regarding our results from immunophenotyping of IPF patients.

We will continue animal studies as planned and begin treatment studies as outlined in the SOW.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
Nothing to Report.

What was the impact on other disciplines?
Nothing to Report.

What was the impact on technology transfer?
Nothing to Report.

What was the impact on society beyond science and technology?
Nothing to Report.

5. CHANGES/PROBLEMS:
Nothing to Report.

6. PRODUCTS

Publications, conference papers, and presentations:

- **Journal publications.**

Nothing to Report.

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

Nothing to Report.

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

Serezani A, Pascoalino B, Vowell K, Kropski J, Blackwell TS. T Cell Phenotype in Peripheral Blood of Patients with Idiopathic Pulmonary Fibrosis. Proc. Am. Thorac. Soc. 199:A2425, 2019.

- **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: Timothy S. Blackwell, M.D.
Project Role: P.I.
Nearest person month worked: 2.69
Contribution to project: Dr. Blackwell manages the project. Dr. Blackwell oversees studies measuring the immune response to herpesviruses and he oversee all animal experiments. He participates in data interpretation, presentation, and publication.
Funding support: N/A

Name: Carla Calvi
Project Role: Sr. Research Specialist
Nearest person month worked: 3.0
Contribution to project: Serves as a research specialist for the project and is responsible for the performance of animal experiments including flow cytometry studies. She is also responsible for lab management and supplies related to this project.
Funding support: N/A

Name: Linda Gleaves
Project Role: Lab Manager
Nearest person month worked: 1.2
Contribution to project: Ms. Gleaves no longer works for VUMC. During her time on the project, Ms. Gleaves was responsible for histological processing of samples, as well as performing basic staining of study samples.
Funding support: N/A

Name: Arun Habermann
Project Role: Research Assistant I Nearest person month worked:4.53
Contribution to project: Mr. Habermann is a research assistant for the project. He assists with animal experiments and sample processing.
Funding support: N/A

Name: Raphael Hunt
Project Role: Research Assistant III Nearest person month worked: 2.53
Contribution to project: Mr. Hunt oversees the animal colony and is responsible for breeding and maintenance of the animal colony.
Funding support: N/A

Name: Susan Martin
Project Role: LPN Research
Researcher Identifier: N/A
Nearest person month worked: 6.0
Contribution to project: Ms. Martin serves as the study coordinator for this project. She is responsible for enrollment, organization of study visits, and data collection.
Funding support: N/A

Name: Bruno Pascoalino
Project Role: Research Fellow
Researcher Identifier: N/A
Nearest person month worked: 5.6
Contribution to project: Dr. Pascoalino no longer works on this grant. During his time he assisted Dr. Serezani with processing of human samples, performing flow cytometry, cytokine bead arrays, lymphocyte culture experiments.
Funding support: N/A

Name: Taylor Sherrill
Project Role: Lab Manager
Nearest person month worked: 3.15
Contribution to project: Mr. Sherrill is assisting with mouse model studies.
Funding support: N/A

Name: Ana Serezani
Project Role: Co-investigator
Nearest person month worked: 8.6
Contribution to project: Dr. Serezani is responsible for performing and interpreting the human and animal T-cell functional studies.
Funding support: N/A

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

Other Support Changes:

Timothy S. Blackwell, M.D.
New: None.
Ended: None.

Wonder Drake, M.D.
Dr. Drake has been unavailable to participate in this project.

Jonathan A. Kropski, M.D.
New: R01 HL 145372-01 (Banovich, Kropski), W81XWH-19-1-0415 (Banovich, Kropski)
Ended: None.

Fabien Maldonado, M.D.
New: 1 R41 HL140709-01 (Hendrick)
Ended: None.

Vasiliy V. Polosukhin, M.D., Ph.D., Sc.D.
New: - 1 U01 HL145561-01 (Shaykhiev)
Ended: None.

Ana Serezani, Ph.D.
New: None.
Ended: None.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:** N/A
- **QUAD CHARTS:** N/A

9. APPENDICES: N/A