AWARD NUMBER: W81XWH-19-1-0375

TITLE: Neutrophil Elastase Reprograms Macrophage Function in Chronic Obstructive Pulmonary Disease

PRINCIPAL INVESTIGATOR: Judith Voynow

CONTRACTING ORGANIZATION:

Virginia Commonwealth University
Children's Hospital of Richmond at VCU

REPORT DATE: October 2020

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

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| October 2020 | Annual | 1SEPT2019 - 31AUG2020 |

**4. TITLE AND SUBTITLE**
Neutrophil Elastase Reprograms Macrophage Function in Chronic Obstructive

Pulmonary Disease

**5a. CONTRACT NUMBER**
W81XWH-19-1-0375

**5b. GRANT NUMBER**

**5c. PROGRAM ELEMENT NUMBER**

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**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

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**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**

**The central hypothesis of this proposal is that extracellular NE is taken up by macrophages and accumulates in both cytoplasmic organelles and the nucleus. NE activity degrades histone deacetylase 2 (HDAC2) and possibly other HDACS and Sirtuins resulting in increased acetylation of several targets including histone H3, High Mobility Group Box 1 (HMGB1) and nuclear factor kappa B (NFkB) p65, resulting in increased cytokine transcription and release of HMGB1 (AIM 1). Nuclear NE cleaves histone H3 and increases H3 citrulline resulting in chromatin decondensation and release of vital nuclear METs (AIM 2).**

**15. SUBJECT TERMS**

NONE LISTED

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRMC |
| Unclassified | Unclassified | Unclassified | Unclassified | 10 | 19b. TELEPHONE NUMBER *(include area code)* |

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1. **INTRODUCTION**:

The central hypothesis of this proposal is that extracellular NE is taken up by macrophages and accumulates in both cytoplasmic organelles and the nucleus. NE activity degrades histone deacetylase 2 (HDAC2) and possibly other HDACS and Sirtuins resulting in increased acetylation of several targets including histone H3, High Mobility Group Box 1 (HMGB1) and nuclear factor kappa B (NFkB) p65, resulting in increased cytokine transcription and release of HMGB1 (**AIM 1**). Nuclear NE cleaves histone H3 and increases H3 citrulline resulting in chromatin decondensation and release of vital nuclear METs (**AIM 2**).

2. **KEYWORDS**: Chronic obstructive pulmonary disease, macrophages, human blood monocyte derived macrophages (hBMDM), extracellular traps, histone deacetylase, sirtuin, High Mobility Group Box 1, Nuclear factor kappa B, Neutrophil elastase (NE)

3. **ACCOMPLISHMENTS**:

- **Major Goals of the project**:

**Major Task 1** : Neutrophil elastase localization and protease activity; Cytokine mRNA and protein expression
**Major Task 2:** HDAC and Sirtuin expression and activity; H3, p65, HMGB1 lysine acetylation; siRNA silencing of HDAC and/ or Sirt top candidates to test impact on cytokine and HMGB1 release; Alveolar macrophage isolation and characterization measurement of NE uptake, protease activity and HDAC or H3 modifications
**Major Task 3**: Quantitate DNA released into culture media; Determine nuclear H3 degradation, H3 citrulline, and PAD1-4 expression; Identify cationic protein candidates in conditioned media that are associated with METs by LC-MS

- **Accomplishments:**

Major Task 1 : Neutrophil elastase localization and protease activity; Cytokine mRNA and protein expression

We received approval from the HRPO on December 12, 2019 to initiate clinical recruitment. During the months of February and March 2020, we recruited 13 COPD patients to participate and collected clinical information and whole blood to isolate plasma and blood monocytes for culture to differentiate into human blood monocyte derived macrophages (hBMDM). The patient demographics are shown in **Table 1**.

We used these samples to perform the initial experiments for Aim 1: testing the intracellular NE activity following administration of NE to hBMDM (**Figure 1**) and after administration of fluorescent-labeled NE, we determined the intracellular localization by confocal microscopy (**Figure 2**). We found a dose-dependent increase in intracellular NE protease activity and that NE localized to both nuclei and cytoplasmic domains. Results are similar so far between different degrees of COPD severity, race or sex.

Patient recruitment summary:

We collected 13 COPD blood samples from VCU between 2-3-2020 and 3-12-2020, before the research lab was shut down and all elective out- patient appointments were halted due to COVID-19. The patients included both Caucasian and African-American, both male and female, and have GOLD scores ranging from A-D. The following table (**Table 1**) summarizes the participants.

**Table 1. Demographics of participants with COPD**

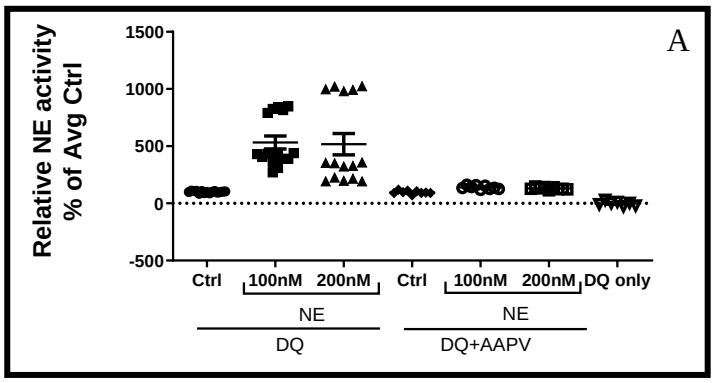
| COPD | GOLD | Smoking | Race | sex | date |
|---|---|---|---|---|---|
| COPD 1001 | D | Past | Caucasian | Male | 2/3/2020 |
| COPD 1002 | B | Current | African American | Female | 2/10/2020 |
| COPD 1003 | A | Current | Caucasian | Male | 2/13/2020 |
| COPD 1004 | B | Past | Caucasian | Male | 2/13/2020 |
| COPD 1005 | D | Past | African American | Female | 2/20/2020 |
| COPD 1006 | B | Past | African American | Male | 2/24/2020 |
| COPD 1007 | D | Current | African American | Female | 2/24/2020 |
| COPD 1008 | D | Current | Caucasian | Female | 2/27/2020 |

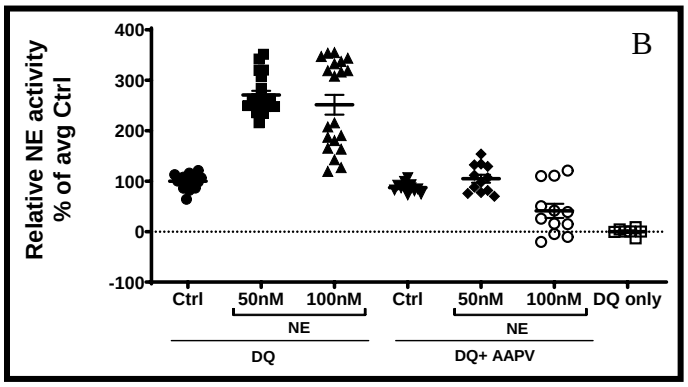
| | | | | | |
|---|---|---|---|---|---|
| COPD 1009 | B | Past | African American | Female | 2/27/2020 |
| COPD 1010 | A | Current | Caucasian | Male | 3/5/2020 |
| COPD 1011 | A | Current | African American | Female | 3/5/2020 |
| COPD 1012 | D | Current | African American | Male | 3/12/2020 |
| COPD 1013 | D | Past | Caucasian | Female | 3/12/2020 |

NE localization and activity determined by DQ-Elastin assay.

To determine whether NE taken up by macrophages was proteolytically active in the macrophage intracellular domains, we exposed cells to DQ-elastin, a substrate which fluoresces after NE cleavage to indicate NE proteolytic activity in hBMDM. To account for any inherent MMP12 elastase activity in macrophages, we exposed NE treated cells to a NE-specific inhibitor, Ala-Ala-Pro-Val-chloromethylketone (AAPV- CMK) as a control. COPD hBMDM were treated with NE (50nM, 100nM or 200 nM) or control vehicle for 2h, fixed and permeabilized (all in suspension), and then placed in a microtiter plate for DQ-elastin exposure and measurement of released DQ fluorescence (**Figure 1**). Control conditions included no treatment (Ctrl) or NE + the NE inhibitor (NE+ AAPV). Our results demonstrate that NE is active in the intracellular environment of COPD hBMDM and increased relative to control treated cells. NE uptake and localization in COPD hBMDM was determined by FITC-NE treatment followed by confocal microscopy. Human BMDM from subjects with COPD, were treated with FITC-labeled NE (100, 200 or 500 nM, 2 h) then followed by confocal microscopy detection of NE (**Figure 2**). FITC-NE was localized to both nuclei and cytoplasm in COPD hBMDM.

**Figure 1. NE activity was present intracellularly in COPD hBMDM**. Human BMDM were treated with NE (100nM and 200 nM, 2 h) (COPD 1001, 1003, 1004, Gold score D, A, B) (**A**) or NE (50nM and 100nM) (COPD 1006, 1007,1012, 1013, Gold score B, D, D, D) (**B**) or control vehicle (Ctrl). After fixation and permeabilization, cells were incubated with DQ-elastin 10µg/ml of 1x reaction buffer, (EnzChek, ThermoFisher) for 20 h, and fluorescence was determined using a TECAN fluorescence plate reader. Control conditions included treatment with NE followed by treatment with Ala-Ala-Pro-Val chloromethylketone (NE+AAPV), a specific NE inhibitor. Relative NE activities were calculated by normalizing to average Control after background reduction. Data are summarized; mean ± SEM; 3-4 experiments.



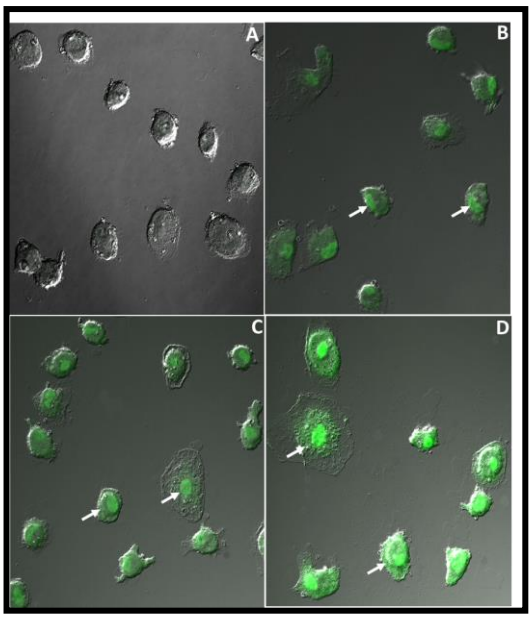




**Figure 2. NE taken up by COPD hBMDM was localized to the cytoplasm and nucleus.** Human blood monocyte derived macrophages (BMDM), isolated from COPD subjects were treated with FITC-labeled NE dose (100, 200, 500nM for 2h, Elastin Products) or vehicle control. After treatments, cells were washed with PBS, fixed and stained with DAPI for nuclear detection, analyzed NE uptake and localization by confocal microscopy. White arrows show positive FITC-NE localization in the nucleus. Confocal micrographs of FITC-DIC images (40x magnification) treated with control vehicle (A) or FITC-NE 100 nM (B), 200 nM (C) or 500nM (D) for 2h is shown and is representative of confocal images from 3 COPD donors. FITC-NE was localized in both cytoplasm and nucleus by 2 hours.

Major Task 3: Quantitate DNA released into culture media; Determine nuclear H3 degradation, H3 citrulline, and PAD1-4 expression; Identify cationic protein candidates in conditioned media that are associated with METs by LC-MS

To determine whether COPD hBMDM viable cells would release DNA after exposure to NE, we treated hBMDM with a dose curve of NE and measured extracellular DNA in the conditioned media by PicoGreen assay (**Figure 3**). We demonstrate in one donor, that NE triggered the release of DNA in a dose dependent manner consistent with release of macrophage extracellular traps. We then tested whether NE caused histone H3 or H4 clipping and whether NE stimulated H3 citrullination. We demonstrate that in healthy, non-COPD hBMDM, NE caused H3 clipping, but not H4 clipping (**Figure 4**). We also show that NE increased H3 citrullination (**Figure 5**). Both histone clipping and histone citrullination are precursors for macrophage extracellular traps. Finally, we show the surprising finding that the enzymes required for citrullination, peptidylarginine deiminase (PAD) 2 and 4 are degraded by NE (**Figure 6**). Therefore we will be evaluating for other mechanisms of transition from arginine to citrulline.

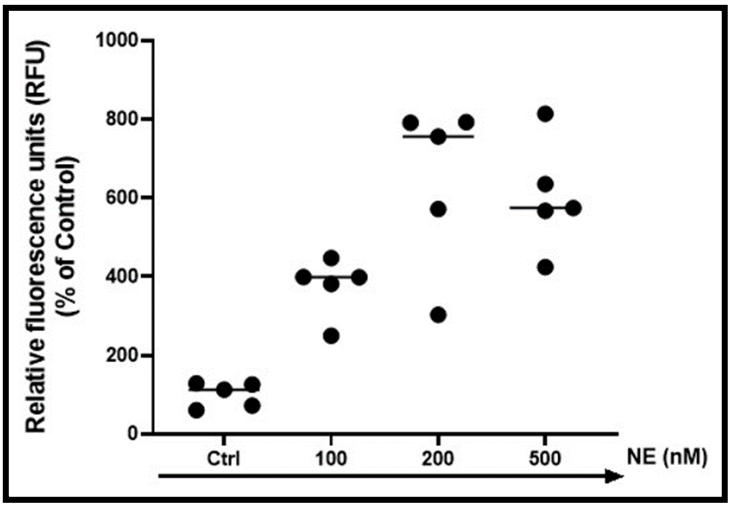



**Figure 3**. **NE-induced MET release in hBMDM from COPD was measured by PicoGreen staining of DNA .** hBMDM grown on 24 well plates were treated with NE 100, 200 or 500nM or control vehicle for 2h. Following NE treatment, cells were incubated with micrococcal nuclease (4U, 15 min, NEB). At the end of nuclease treatment, a 1:200 dilution of PicoGreen reagent (Quant-iT™ PicoGreen™ dsDNA Assay Kit, Invitrogen) was added to an equal volume of the nuclease treated culture supernatant. Fluorescence was quantified at the excitation wavelength of 480 nm and an emission wavelength of 520 nm using an automated plate reader (TECAN). Data summarized as mean ± SEM. Data is representative of 1 donor, n=5 replicates.

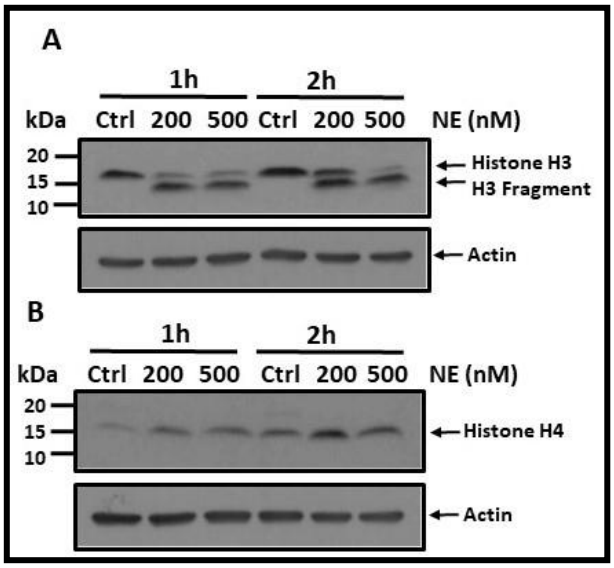



**Figure 4. NE induced the cleavage of histone H3, but not Histone H4, in Non-COPD hBMDM cells**. Human BMDM from non-COPD were treated with control vehicle (Ctrl) or NE (200 or 500nM) for 1 or 2 h and total cell proteins were collected. Total cell lysate protein (30 µg) was separated on a 4-20% SDS-PAGE and tested for histone H3 or H4 expression using Western analysis. Blots were probed with primary rabbit monoclonal for anti-H3 or anti-H4 (1:1000 dilution); secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (1:5000 dilution), and development by chemiluminescence (Lightning Ultra ECL (Perkin Elmer)). Histone H3 (A) and Histone H4 (B) in non-COPD hBMDM by western analyses. Western for β-actin (1:5000; #A5441, Sigma-Aldrich) were a control for equal loading of total protein on the gel. Western blots shown were representative of n= 2 non-COPD donors.

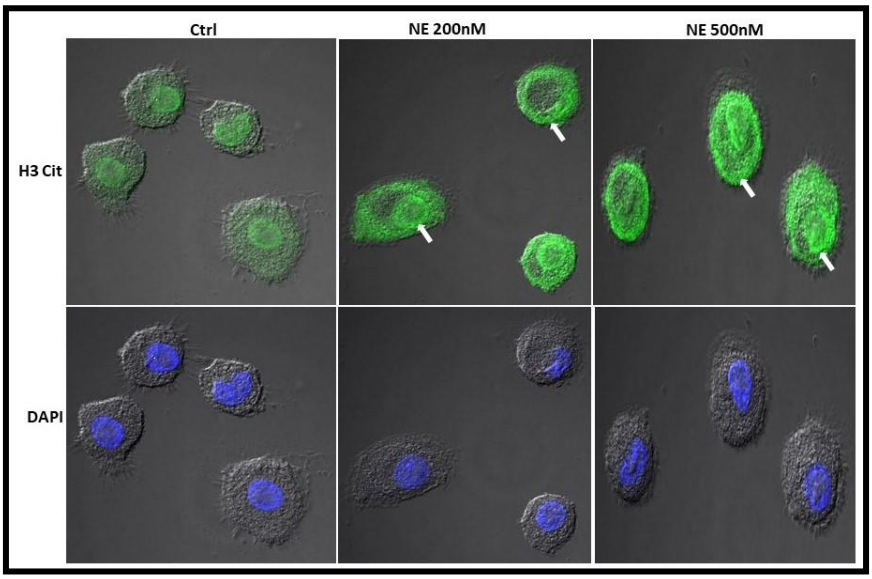



**Figure 5. NE increased H3Citrulline (citH3) in non-COPD hBMDM**. Human BMDM obtained from non-COPD were treated with NE (200 and 500nM, 2h) or control vehicle (Ctrl) on cover slips. Following fixation, cells were incubated with anti-Histone H3 citrulline R2+R8+R17 (1:100 dilution, overnight, Abcam), followed by Alexa Fluor 488-conjugated anti-rabbit IgG antibody (4µg/ml, Invitrogen) for 1h, RT, counterstained with DAPI, and evaluated by confocal microscopy (Zeiss LSM 700). Representative confocal micrographs of fluorescein-DIC mode (upper panel) and the corresponding DAPI nuclear fluorescence-DIC (Lower Panel) are shown for non-COPD hBMDM (63x magnification) . Images shown were representative of 2 non-COPD donors.

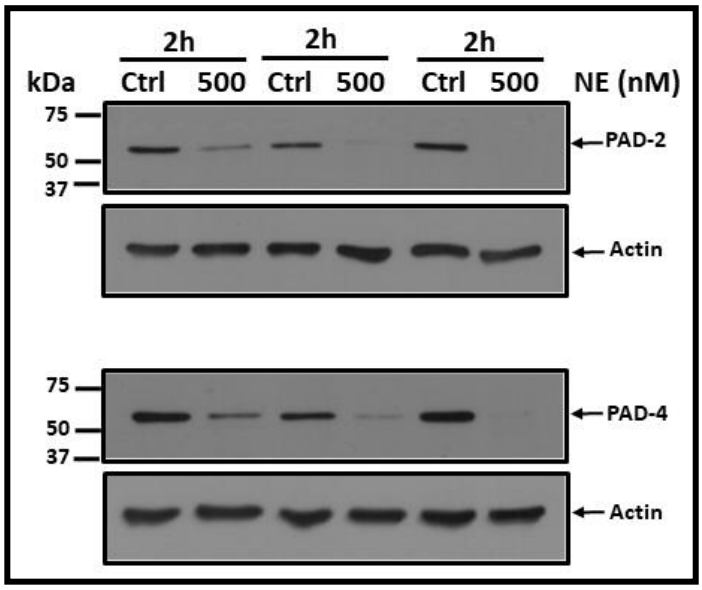



**Figure 6. NE decreased the expression of PAD2 and PAD4 in Non-COPD hBMDM cells.** Human BMDM from non-COPD were treated with control vehicle (Ctrl) or NE (500 nM) for 2h and total cell proteins were collected. Total cell lysate protein (30 µg) was separated on a 4-20% SDS-PAGE and tested for PAD2 or PAD4 expression using Western analysis. Blots were probed with primary monoclonal for anti-PAD2 (1:3000) or anti-PAD4 (1:500 dilution); secondary horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG antibody (1:5000 dilution), and development by chemiluminescence (Lightning Ultra ECL (Perkin Elmer)). PAD2 and PAD4 was analyzed in non-COPD hBMDM by western analyses. Western for β-actin (1:5000; #A5441, Sigma-Aldrich) were a control for equal loading of total protein on the gel. Western blots shown were representative of 3 non-COPD donors.

- **Opportunities for Training**: Nothing to Report
- **Results Disseminated to Communities of Interest**: A manuscript entitled, "Neutrophil Elastase Triggers the Release of Macrophage Extracellular Traps in Cystic Fibrosis" was submitted to the American Journal of Respiratory Cell and Molecular Biology for review. [manuscript attached]
- **Plans for next reporting period**: We plan to complete the scope of work for Major tasks 1 and 3 and initiate Major task 2 to determine the HDACs and Sirtuins regulated by NE or having altered expression at baseline in COPD and whether COPD severity, or participant race or sex will affect the expression of these epigenetic regulators.

4. **IMPACT**:
   - **Impact on development of the principle discipline of the project**: Nothing to Report
   - **Impact on other disciplines**: The concept that high NE concentrations in the airway may induce macrophage extracellular traps is relevant for other chronic mucoobstructive diseases like cystic fibrosis. Please see attached manuscript.
   - **Impact on technology transfer**: Nothing to Report
   - **Impact on society beyond science and technology**: Nothing to Report

**5. CHANGES/PROBLEMS**

- **Changes in approach and reasons for change:** Nothing to Report
- **Delays and actions to resolve them**: Due to COVID19, all outpatient facilities at VCU and the research laboratories were shut down between March 15 and August 30. We have reopened the laboratory and are starting to perform experiments with existing biospecimens. However, although the hospital is open for all outpatient activities, there is a delay in getting patients back into the clinic, and most COPD patients are being cared for by telehealth. Currently approximately 30% of patients are seen in person (personal communication, Dr. Aamer Syed, Co-investigator). To respond to this change in healthcare delivery, we received IRB approval for remote consent in outpatients and we will request that those patients who consent for participation in the study, provide a blood sample at one of the VCU clinical laboratories within a week of their telehealth visit. We are also working closely with Dr. Syed to approach all patients who arrive at VCU for in person visits to obtain in-person consent and a blood sample on the same day. Finally, due to COVID19, we were unable to complete the IRB consent process at the McGuire VA. I am meeting with Dr. Leonard Moses, Chief of Pulmonary and Critical Care Medicine at the McGuire VA, on October 9, to complete the IRB process so that we can recruit patients from the VA.
- **Changes that had a significant impact on expenditures**: Nothing to Report
- **Significant changes in use or care of human subjects**: We received permission from the VCU IRB to obtain consent remotely by telephone for participation in the study. The IRB letter of approval, and the updated IRB proposal and consent forms are attached.
- **Significant changes in use or care of vertebrate animals**: Nothing to Report
- **Significant changes in use of biohazards and/or select agents**: Nothing to Report

6. **PRODUCTS**:
- **Publications, conference papers, and presentations**: Nothing to Report
- **Websites or internet sites**: 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**

| **Name** | Judith Voynow, MD | Shuo Zheng PhD | Apparao Kummarapurugu PhD | Margaret Lessard | Aamer Syed, MD |
|---|---|---|---|---|---|
| **Project Role** | PI | Co-I | Co-I | Research Coordinator | Co-I |
| **Research Identifier (ORCID ID)** | 0000-0002-2437-2859 | | 0000-0002-6175-5293 | | |
| **Nearest person month worked:** | 7 | 4 | 4 | 4 | 4 |
| **Contribution to the project** | Evaluation of recruitment; Oversight of quality assurance for clinical data; review of experimental data | Isolation of monocytes and hBMDM culture; NE activity | FITC-NE cellular localization in COPD hBMDM; NE induced release of DNA from COPD hBMDM; NE | Recruit patients from clinic, obtain consent, obtain whole blood samples from clinical lab | Identify potential subjects from Pulmonary Clinic; inform faculty and fellows about the |

| | | | | | |
|---|---|---|---|---|---|
| | Weekly communication with research coordinators and laboratory staff | assays | cleavage of histone H3 in vivo and in vitro; H3 citrullination | | project to encourage recruitment |
| **Funding Support** | CF Foundation | CF Foundation | CF Foundation | CF Foundation | |

- **Change in the active other support of the PI:**

- The PI has been awarded a CF Foundation Research Grant, Voynow19G0, "Neutrophil Elastase and Macrophage Extracellular traps in CF". The period of performance is 11/1/2019- 10/31/2021. The Specific Aims follow: Aim 1. To evaluate whether NE taken up by CF macrophages is proteolytically active, cleaves histone H3, and upregulates PAD2 or 4 expression to catalyze H3 citrullination. Aim 2. To evaluate whether NE-generated reactive oxygen species (ROS) are associated with formation of CF Macrophage extracellular traps (METs), and whether inhibition of ROS blocks release of CF METs. There is no overlap with the project that is the subject of the project report.

- The PI has been awarded a R01 HL146811-01A1 from NHLBI, NIH, "The Trojan Horse Hypothesis: Neutrophil Elastase Reprograms Macrophage Function." The period of performance is 9/1/2020- 8/31/2024. The Specific Aims follow: Aim 1. To determine whether NE degrades HDACs in CF macrophages, resulting in acetylation of downstream targets, leading to transcriptional upregulation of TNFα and release of HMGB1 from CF macrophages. Aim 2. To evaluate whether NE protease activity and/or NE-generated reactive oxygen species (ROS) increase release of mitochondrial and nuclear METs from CF hBMDM. There is no overlap with the project that is the subject of the project report, nor the CFF-funded project.

- These awards do not impact the effort of personnel on the project that is the subject of the project report.

- **What other organizations were involved as partners**: Nothing to Report

8. **SPECIAL REPORTING REQUIREMENTS**: Nothing to Report

**9. APPENDICES:**

**Manuscript:** Neutrophil Elastase Triggers the Release of Macrophage Extracellular Traps in Cystic Fibrosis, submitted to American Journal of Respiratory Cell and Molecular Biology

**IRB revision**: HM20015308 Neutrophil Elastase Reprograms Macrophage Function in Chronic Obstructive Pulmonary Disease