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

PRINCIPAL INVESTIGATOR: Judith A. Voynow

CONTRACTING ORGANIZATION: Virginia Commonwealth University
Children's Hospital of Richmond at VCU

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Neutrophil Elastase Reprograms Macrophage Function in Chronic Obstructive Pulmonary Disease

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Judith A. Voynow, MD

E-Mail: Judith.voynow@vcuhealth.org

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

Virginia Commonwealth University
Children's Hospital of Richmond
at VCU
1000 East Broad Street
PO Box 980315 Richmond, VA 23298

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13. SUPPLEMENTARY NOTES

14. ABSTRACT: Background: The relentless progression of lung disease in COPD is due in part to the failure of lung macrophage innate immune function. The failure of the macrophage to clear infections and terminate inflammation is attributed to the airway milieu in COPD but the mechanisms are not completely understood. Neutrophil elastase (NE),present in the COPD airway, may subvert macrophage function from protective to pro-inflammatory. Rationale: We demonstrate that NE is taken up by macrophages and targets the cytoplasm and nucleus, and NE exerts intracellular protease activity. NE degrades or modifies epigenetic regulators resulting in release of potent pro-inflammatory proteins: cytokines and High Mobility Group Box 1 (HMGB1), and release of macrophage extracellular traps (METs). Extracellular traps are DNA strands decorated with chromatin binding proteins,myeloperoxidase and NE; they trap and kill bacteria but they are also highly pro-inflammatory. Airway extracellular traps are associated with worse COPD lung disease. Although macrophages are the major leukocyte in the airways, the presence of macrophage extracellular traps in the COPD airway has not previously been reported and the etiology of METs in COPD is not known.

15. SUBJECT TERMS NONE LISTED					
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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

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:** *Provide a brief list of keywords (limit to 20 words).*

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:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

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

Major Task 2: HDAC and Sirtuin expression and activity will be determined in hBMDM. Impact of loss of HDAC will be evaluated by identification of lysine acetylation of targets Histone H3, NFkB p65, or High Mobility Group Box 1 (HMGB1). To confirm impact of loss of HDACs/ Sirtuins, siRNA silencing of HDAC and/ or Sirtuin top candidates will be performed to test the impact on cytokine and HMGB1 release. Alveolar macrophages will be isolated and characterized to measure 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.

Please see copy of Scope of Work and Milestones listed below and indication of completion of

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Major Task 1. Neutrophil elastase (NE) localization and protease activity; Cytokine mRNA and protein expression.

Neutrophil elastase uptake and localization in COPD blood monocyte derived macrophages (bMDM). Using fluorescein-labeled NE, we demonstrated that NE was taken up by bMDM cytosol and nuclei in a dose dependent manner (**Figure 1**). Furthermore, we confirmed that the localization was specific by exposing cells to a dose curve of unlabeled NE and then performed immunofluorescence using a primary anti-human NE antibody and secondary rhodamine-labeled antibody to demonstrate that NE localized to both cytosol and the nuclei in a dose dependent manner (**Figure 2**). Thus, using two different methods, we demonstrated that NE is taken up by human blood monocyte derived macrophages.

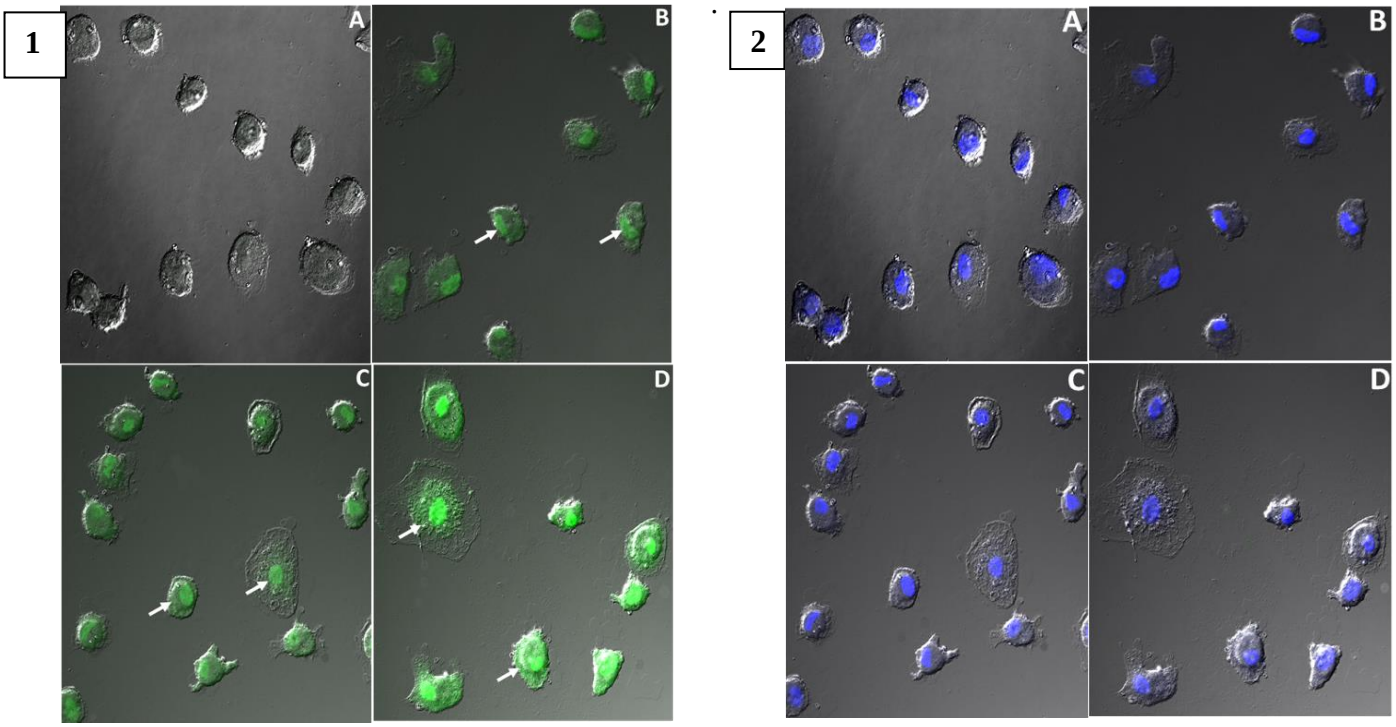
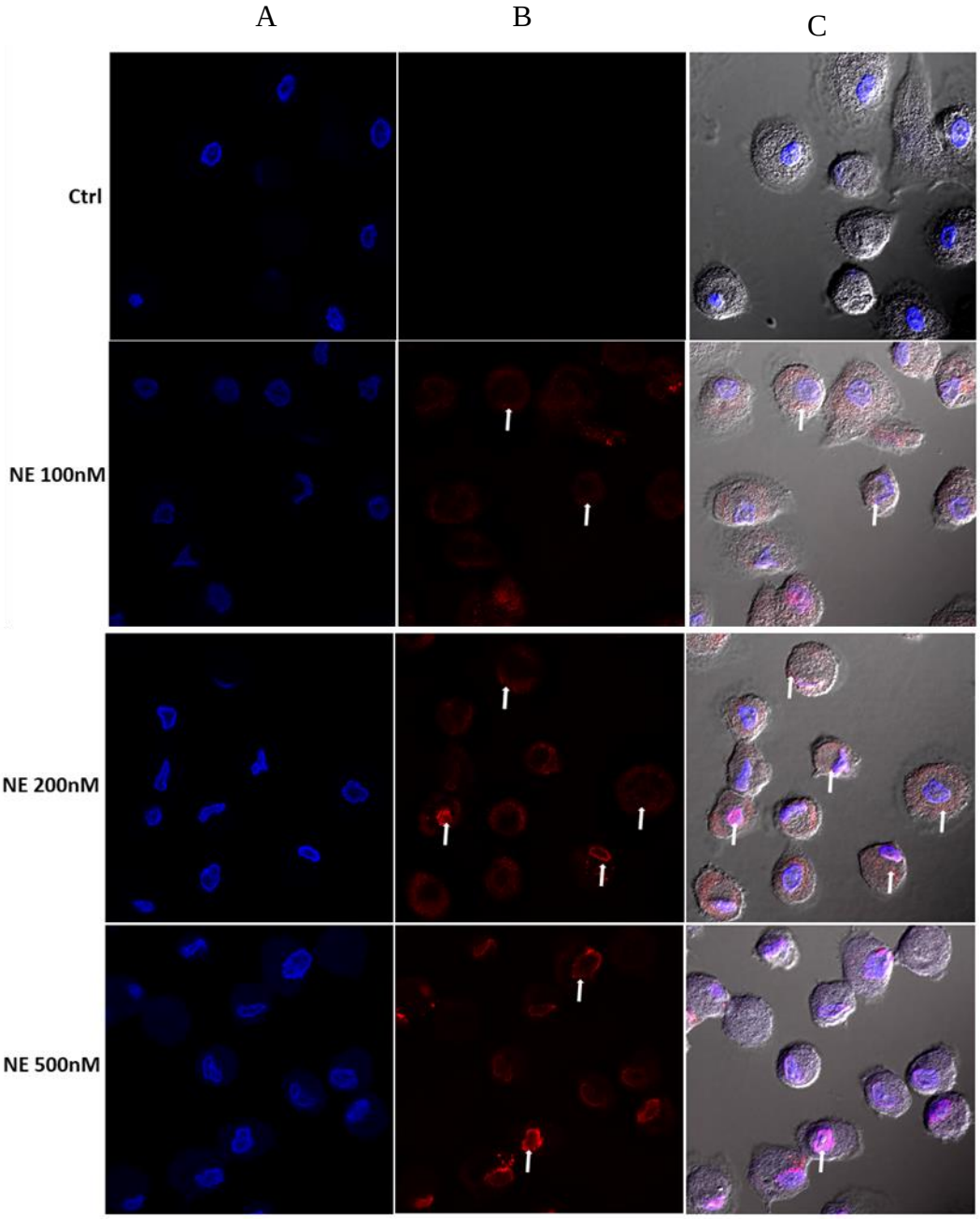


Figure 1. FITC-NE taken up by hBMDM was localized to the cytoplasm and nucleus. Human blood monocyte derived macrophages (BMDM), isolated from COPD subjects were treated with FITC-labeled NE dose (100, 200, 500nM for 2h, Elastin Products) or vehicle control. After treatments, cells were washed with PBS, fixed and stained with DAPI for nuclear detection, and NE uptake and localization were analyzed by confocal microscopy. White arrows show positive FITC-NE localization in the cell nuclei. Confocal micrographs of FITC-DIC (Differential Interference Contrast) images (40x magnification) (**1**) and the corresponding DAPI stained-DIC images (**2**) treated with control vehicle (**A**) or FITC-NE 100 nM (**B**), 200 nM (**C**) or 500nM (**D**) for 2h are shown and are representative of confocal images from 3 COPD donors. As shown below, FITC-NE was localized in both cytoplasm and predominantly in nucleus by 2 hours



<u>Figure 2</u>. NE is taken up by BMDM as detected by immunofluorescence confocal analysis. Human BMDM from a subject with COPD were adhered to poly-L-lysine coated coverslips and treated with control vehicle or NE (100, 200 or 500 nM) for 2 h. Following NE exposure cells were fixed, washed, and incubated with mouse anti-human neutrophil elastase primary antibody (1:100, DAKO, Clone NP57) and then incubated for 1h at RT with secondary goat anti-mouse Alexa Fluor 594 conjugated antibody (5µg/ml, Invitrogen). Following washes, cells were stained with DAPI to visualize the nuclei DNA staining. Fluorescent images (40x) showing NE localization (red) using confocal microcopy. **A**) DAPI stain (blue); **B**) NE staining (red) localized in cytoplasm and nucleus (arrows), **C**) Merged image showing nuclei (blue), NE localization (red) and differential interference contrast (DIC).

NE taken up by COPD BMDM had sustained intracellular protease activity**.** To determine the relative abundance of intracellular protease activity following NE exposure, BMDM were cultured in suspension, and exposed to a dose curve of NE (100 or 200 nM) or control vehicle for 2 h. Cells were then placed in a tissue culture 96 well plate for adherence and levels of elastase activity were quantified by an assay with DQ elastin, a substrate that when cleaved releases DQ which emits a fluorescent signal. Some replicates were treated with a NE-specific inhibitor, Ala-Ala-Pro-Val Chloromethylketone (AAPV) as a negative control to inhibit NE activity. We compared seven COPD subjects to test whether there were any differences in intracellular NE activity. The demographics of the 7 individuals are shown in **Table 1**. We summarized the DQ relative activity normalized to control-treated cells (**Figure 3**).

Table 1. Demographics of COPD subjects for intracellular NE protease activity assays

COPD	GOLD	Smoking	Race	sex
COPD 1001	D	Past	Caucasian	Male
COPD 1003	A	Current	Caucasian	Male
COPD 1004	B	Past	Caucasian	Male
COPD 1006	B	Past	African American	Male
COPD 1007	D	Current	African American	Female
COPD 1012	D	Current	African American	Male
COPD 1013	D	Past	Caucasian	Female

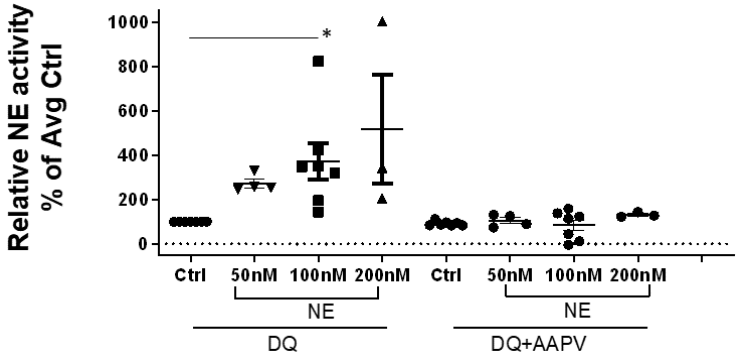


Figure 3. NE activity is sustained intracellularly in human COPD bMDM. Human BMDM from COPD 1001, 1003, 1004, 1006, 1007, 1012, 1013, grown in suspension was treated with Ctrl and NE 50 nM (n=4), 100 nM (n=7) or 200nM (n=3) for 2h. At the end of 2hr, cells were fixed in 2% PFA, permeabilized with 0.1% NP40 for 10min and resuspended in PBS and cell numbers determined. Equal numbers of cells from each treatment group were diluted in PBS and mixed with equal volume of DQ-elastin (20µg/ml in reaction buffer, EnzChek® Elastase Assay Kit, E-12056). The Cells/DQ-elastin mixtures (100µl/well) were incubated in a 96 well black plate. AAPV-CMK was added in some wells as a negative control. The plate was protected from light and incubated at room temperature overnight. Relative Fluorescence Intensity (RFU) was measured by TECAN fluorescence microplate reader (excitation/emission 505/515 nm). Relative NE activity was determined by RFU normalized to corresponding average control. Data are summarized as mean ± SD. Significant differences between control and NE treatments were determined by one-way, nonparametric ANOVA test and post hoc comparisons by the Wilcoxon rank sum test, and significant differences are indicated by *, $p<0.005$.

Major Task 2: HDAC and Sirtuin expression and activity will be determined in hBMDM. Impact of loss of HDAC will be evaluated by identification of lysine acetylation of targets Histone H3, NFkB p65, or High Mobility Group Box 1 (HMGB1). To confirm impact of loss of HDACs/ Sirtuins, siRNA silencing of HDAC and/ or Sirtuin top candidates will be performed to test the impact on cytokine and HMGB1 release. Alveolar macrophages will be isolated and characterized to measure NE uptake, protease activity and HDAC or H3 modifications

Patient Recruitment Summary: We have recruited a total of 41 participants with COPD. Nineteen of the subjects are African American and 22 are Caucasian. Nineteen are male and 22 are female. Severity of COPD includes 8 GOLD Score A, 19 GOLD Score B, and 14 GOLD Score D; patients with GOLD A or GOLD B have mild disease and patients with GOLD D have severe disease. Ages range from 43 to 79 years and 18/41 subjects are less than 60 years of age. Patient blood samples have been used to culture blood monocyte derived macrophages for experiments to determine MET release, mechanisms of MET release, and cell fractionation experiments to harvest protein lysates and conditioned media to complete experiments proposed for both Major Tasks 2 and 3.

Histone Deacetylases (HDAC) and Sirtuin expression in healthy blood monocyte derived macrophages. To analyze the impact of neutrophil elastase treatment on epigenetic enzymes from healthy blood monocyte derived macrophages, we obtained buffy coats from healthy deidentified individuals from the American Red Cross. We used 9 Buffy Coats from 5 females/ 4 males and 7 Caucasians/ 2 mixed race with ages that range from 20 to 82. Buffy coat Monocytes were isolated by double gradient method, and cultured in RPMI growth medium containing GM-CSF (20ng/ml) to differentiate into macrophages for treatment with NE (200 or 500 nM) or control vehicle for 2 h. We isolated nuclear protein or total cell lysates (TCL) to perform western analyses to quantitate HDAC (Class 1- nuclear HDACs) (**Figure 4**), HDAC Class II- cytosolic HDACs) (**Figure 5**), and Sirtuins 1 and 3 (**Figure 6**). The nuclear and TCL preparations were analyzed for protein concentration so that equal amounts of protein were used for analysis. To determine the relative changes in HDAC and Sirtuin levels, nuclear proteins were used to analyze Class 1 HDACs (HDAC 1, 2, 3, and 9). Because NE also cleaved other nuclear proteins used for normalization, we instead used Ponceau S, a protein stain, and quantified total protein per sample by ImageJ. For cytosolic or mitochondrial HDAC and Sirtuins, we used beta-actin as a control protein because actin was not cleaved by NE. Our results reveal that all the HDACs and Sirtuins evaluated thus far are cleaved by NE. HDAC9 is a small size than expected (160 kD) and additional experiments are required to determine whether we are detecting a smaller fragment of HDAC9. Our results summarize at least 2 separate individuals with assays performed in duplicate. We demonstrate that NE cleaves and/ or degrades all 11 HDAC enzymes and Sirtuin 1 and 3 under the conditions used for NE incubation.

Future experiments include 1) testing total HDAC activity and total histone acetyltransferase (HAT) activity after NE treatment compared to control vehicle treatment to evaluate whether cleavage depletes HDAC activity or increases HAT activity; 2) testing whether Sirtuin activity is depleted after NE treatment compared to control vehicle treatment; 3) evaluating whether HDACs resident in COPD blood monocyte derived macrophages (hBMDM) have the same sensitivity to NE exposure by measuring protein abundance as shown in Figures 3 and 4, and measuring HDAC and Sirtuin activities. Other experiments for Task 2 include testing whether NE increases lysine acetylation of Histone H3, p65 and High Mobility Group Box 1. If this is confirmed, then we will develop IP techniques to pull down these proteins for mass spectroscopy to evaluate the specific lysine domains acetylated after NE treatment and then we will test whether knockdown of specific HDAC or Sirtuin enzymes are sufficient to induce acetylation of these targets.

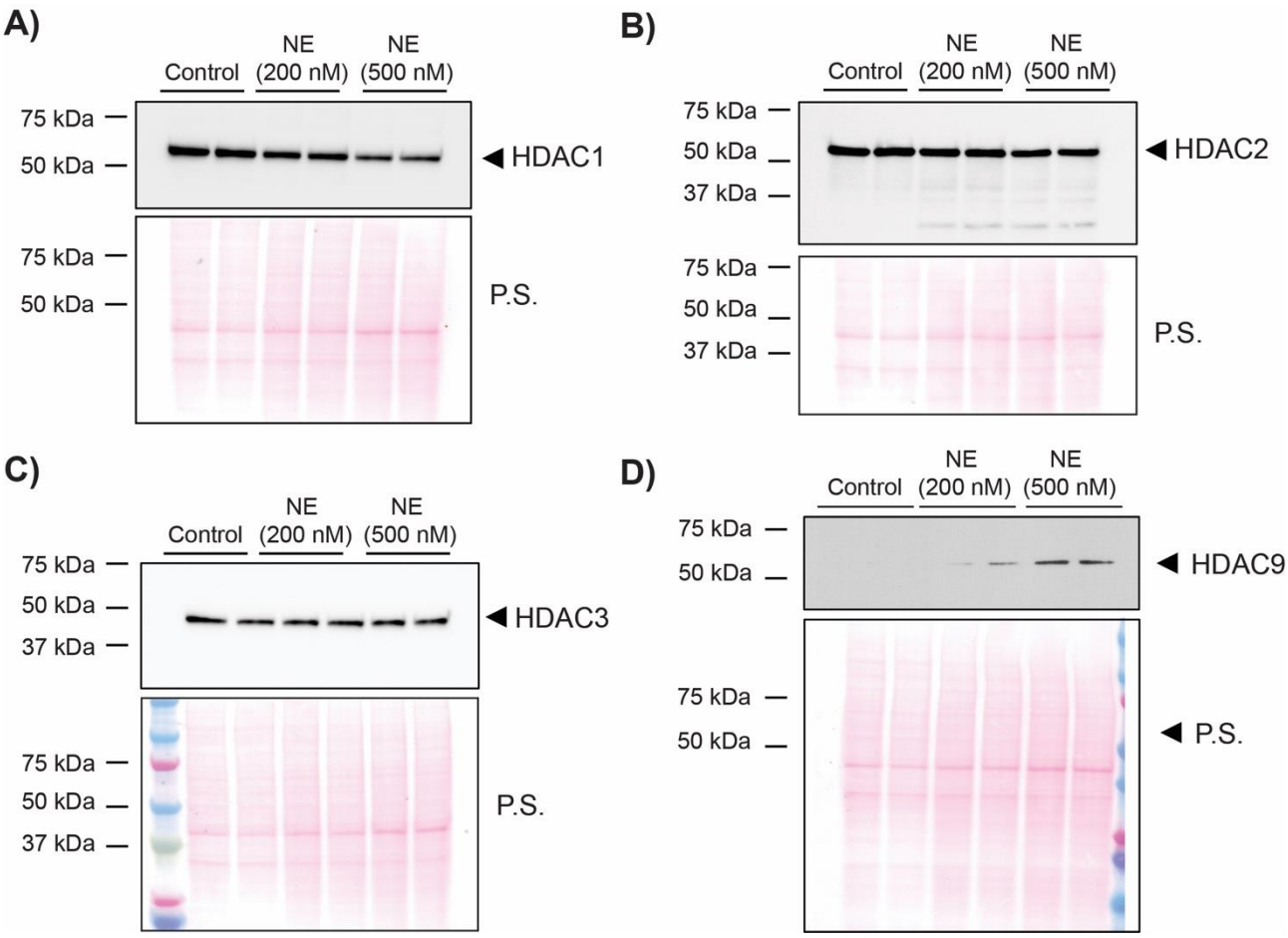


Figure 4. **Nuclear HDAC degradation profiles upon Neutrophil Elastase (NE) treatment in human blood monocyte derived macrophages (hBMDM).** Nuclear extract (25 ug) from hBMDM treated with control or NE as indicated, were resolved by SDS-PAGE and blotted using A) HDAC1 antibody (Santa-Cruz, 1:500 in 5% milk), B) HDAC2 antibody (Cell Signaling Technology, 1:1000 in 5% milk), C) HDAC3 antibody (Cell Signaling Technology, 1:1000 in 5% milk), D) HDAC9 antibody (Santa-Cruz, 1:500 in 5% milk), followed by anti-mouse HRP (Cell Signaling Technology, 1:4000 in 5% milk), and visualization using Pico-ECL reagent. Ponceau S-stained images from the same blots are included as a loading control.

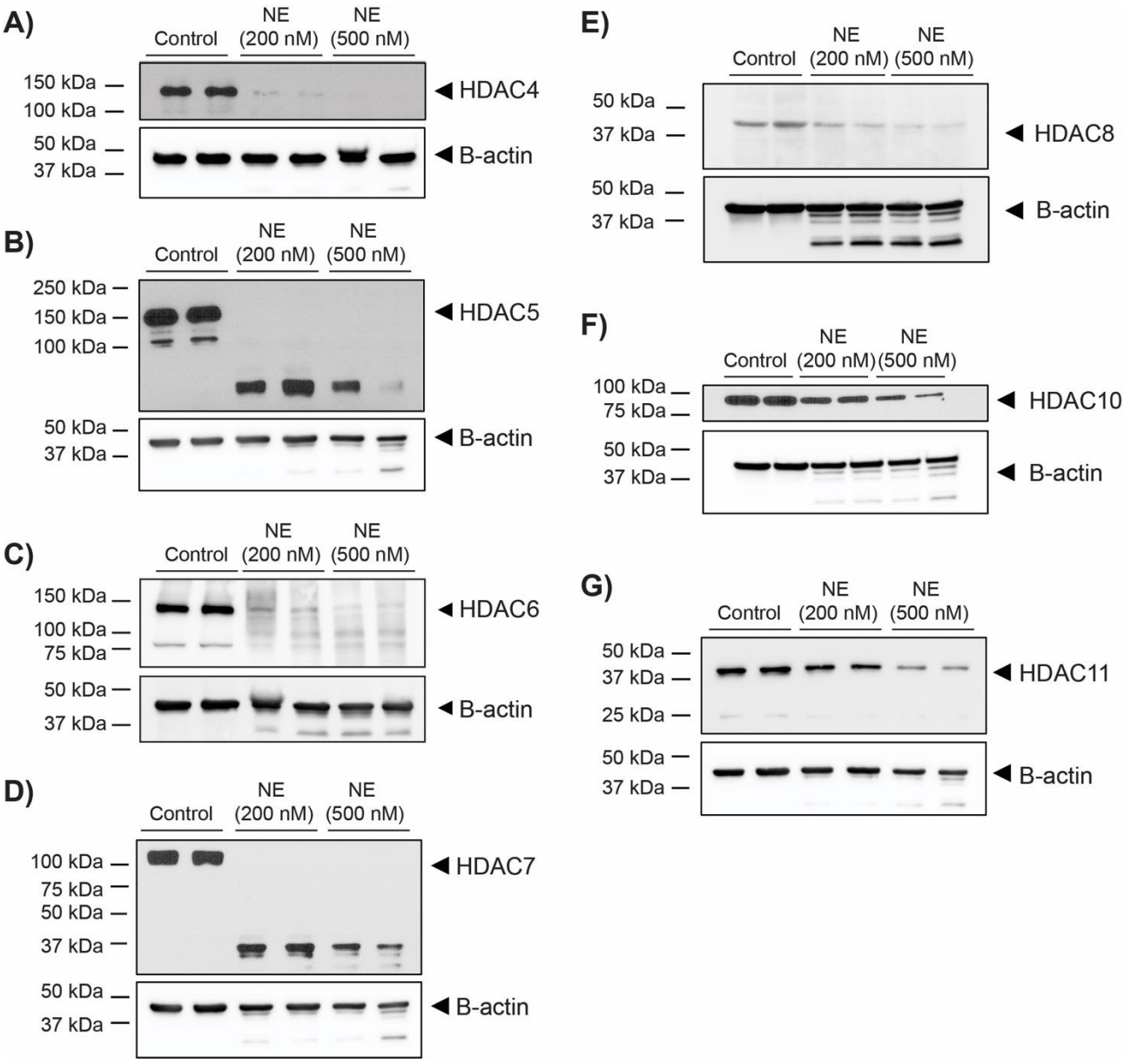


Figure 5. Total cell lysate HDAC degradation profiles upon Neutrophil Elastase (NE) treatment in human monocyte derived macrophages (hBMDM). Total cell lysates (25 ug) from hBMDM treated with control or NE as indicated were resolved by SDS-PAGE and blotted using A) HDAC4 antibody (Santa-Cruz 1:500 in 5% milk), B) HDAC5 antibody (Santa-Cruz, 1:500 in 5% milk), C) HDAC6 antibody (Cell Signaling Technology, 1:2000 in 5% milk), D) HDAC7 antibody (Santa-Cruz, 1:500 in 5% milk), E) HDAC8 antibody (50ug TCL, Santa-Cruz, 1:500 in 5% milk), F) HDAC10 antibody (Santa-Cruz, 1:500 in 5% milk), G) HDAC11 antibody (Santa-Cruz, 1:500 in 5% milk), followed by anti-mouse HRP (CST, 1:4000 in 5% milk, except for HDAC8 1:2000) were visualized using Pico-ECL reagent, except for HDAC4 and HDAC8 were visualized using Lightning ECL. Stripped blots were probed for B-actin (Sigma 1:8000) from the same blots are included as a loading control.

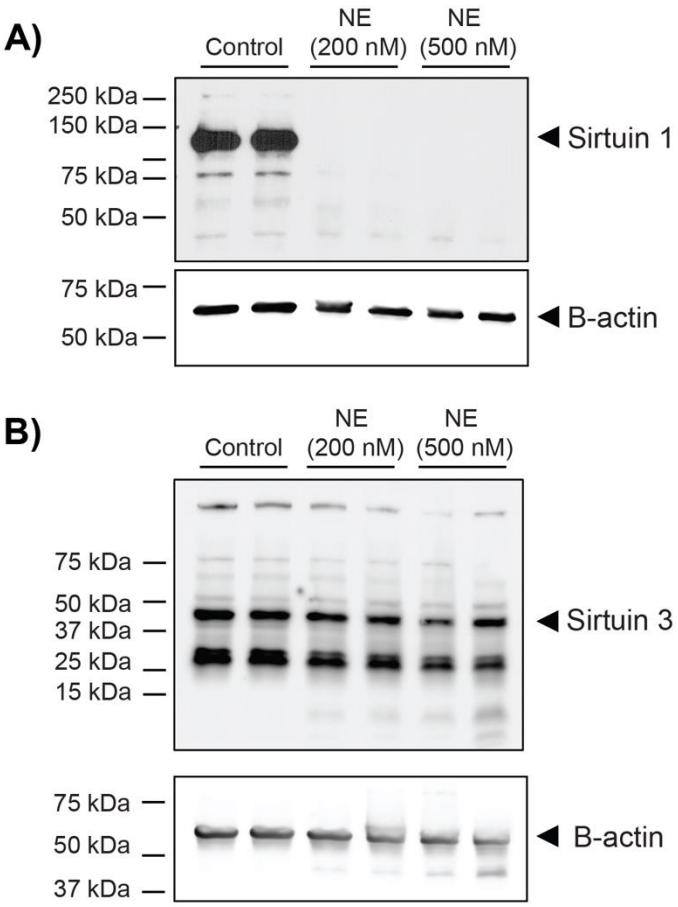


***Figure 6*. Total cell lysate Sirtuin degradation profiles upon Neutrophil Elastase (NE) treatment in human monocyte derived macrophages (hBMDM). A**) Total cell lysates (50 ug) from hBMDM treated with control or NE as indicated were resolved by SDS-PAGE and blotted using Sirtuin1 antibody (CST 1:1000 in 5% milk), **B**) Total cell lysates (25 ug) from hBMDM treated with control or NE as indicated were resolved by SDS-PAGE and blotted using Sirtuin 3 antibody (CST 1:1000 in 5% BSA), followed by anti-mouse HRP (CST, 1:4000 in 5% milk for Sirtuin 1, 1:8000 in 5% milk for Sirtuin 3, were visualized using Pico-ECL reagent. Stripped blots were probed for B-actin (Sigma 1:8000) and included as a loading control.

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

Macrophage extracellular trap (MET) release is increased by NE in a dose-dependent manner in COPD BMDM as detected by Picogreen quantitation of extracellular DNA. COPD BMDM from 8 individuals were exposed to a dose curve of NE (50, 100, 200, or 500 nM) or control vehicle for 2 h. Following exposures, the conditioned media was removed and the extracellular DNA still attached to cells was released with a treatment with micrococcal nuclease for microplate analysis of extracellular DNA by Picogreen fluorescent green detection. Five replicate experiments were prepared from cells derived for each individual. The demographic characteristics of the subjects with COPD are included in **Table 3**. The quantitation of MET release by Picogreen is summarized in **Figure 6**. Differences between treatment conditions were analyzed for significance using one-way nonparametric ANOVA and post-hoc comparisons by Wilcoxon rank sum test.

Table 3. Demographics of Subjects with COPD in the MET release Assay

COPD	GOLD	Smoking	Race	sex
1011	A	**Current**	**AA**	**female**
1016	B	past	AA	female
1019	D	past	C	female
1020	D	past	C	male

1021	B	Current	C	male
1024	D	past	C	male
1037	B	past	C	male
1038	B	Current	AA	male

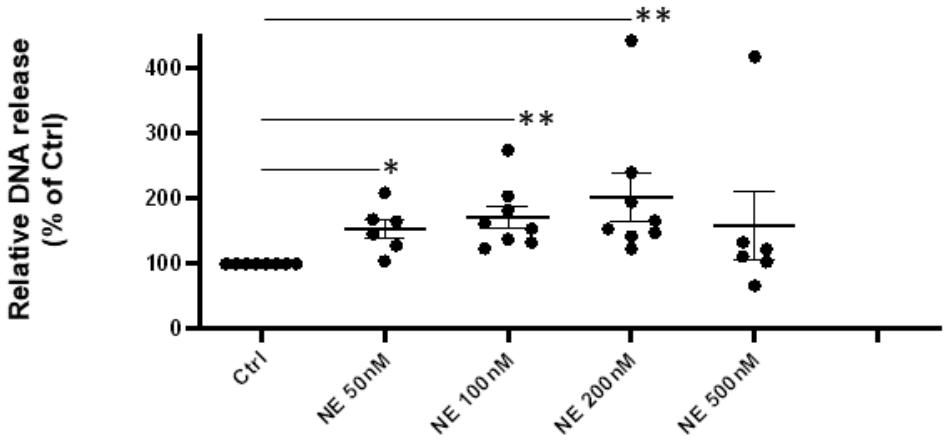


Figure 6. NE induced MET release in a dose-dependent manner from COPD hBMDM. Human BMDM from subjects with COPD, were cultured in suspension with GM-CSF for 10 days and then seeded in 48 well plates. Cells were treated with control vehicle or NE (50, 100, 200, or 500 nM) for 2 h. At the end of incubation, the conditioned media was removed and cells were treated with micrococcal nuclease (4U/well) to release adherent METs. The micrococcal nuclease treated conditioned media was collected and placed in a 96 well black plate and equal volume of Picogreen reagents were added. Picogreen detects extracellular ds DNA by generating green fluorescence. Relative Fluorescence Intensity (RFU) was measured by SPARK fluorescence microplate reader (excitation/emission 485/528 nm). Resulting RFU is linearly associated with extracellular DNA. The RFU were normalized to the control treated cell fluorescence and summarized for n=8, mean ± SD. Significant differences between control and NE treatments are indicated: *, $p<0.01$; **, $p<0.001$.

<u>NE clips Histone H3 but does not upregulate Peptidyl Arginine Deiminase (PAD) enzymes.</u> The mechanisms underlying NE-induced MET release may be due to NE-induced histone clipping or cleavage of other chromatin binding proteins. Extracellular trap release has also been attributed to increased histone citrullination related to upregulation of PAD 2 or 4 activity, which catalyzes the oxidation of arginine to citrulline in proteins. We examined these pathways in hBMDM from COPD subjects and discovered a NE-dose-dependent clipping of Histone 3 (**Figure 7**) but not Histone 4, and no change in the two major PAD enzymes 2 and 4 post-NE treatment (**Figure 7**). Therefore, we propose that NE enters the nucleus and clips Histone 3 which triggers the expulsion of nuclear DNA from the nucleus into the cytosol and eventually release from the cell as a MET.

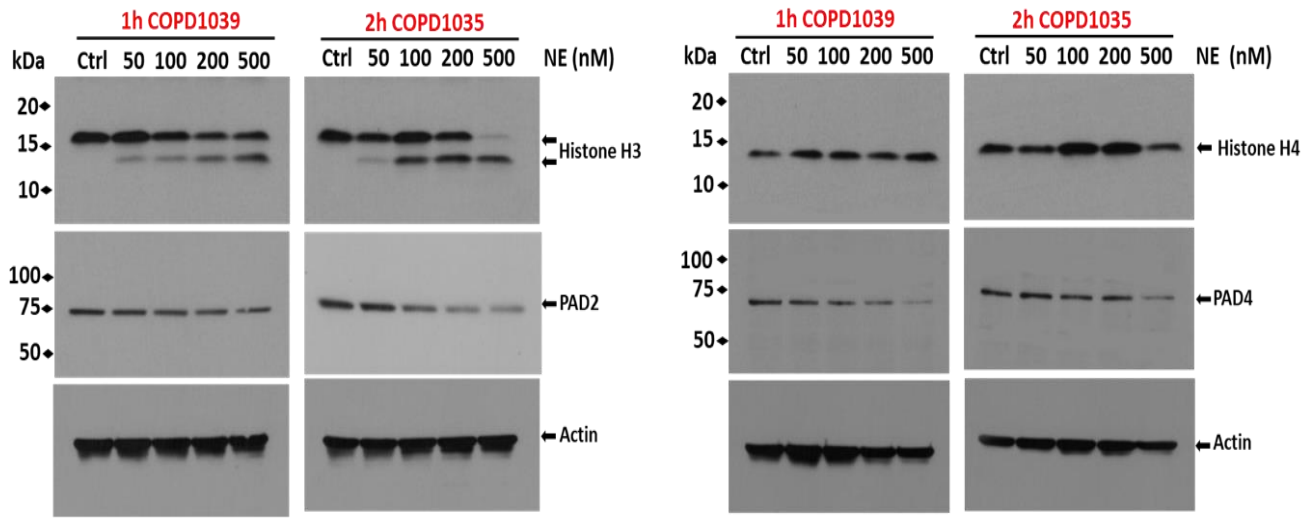


Figure 7. NE activates Histone H3 clipping but not Histone H4 cleavage, and does not upregulate PAD 2 or 4 protein expression. Human BMDM (n= 2 donors) were treated with control vehicle or NE (50, 100, 200, 500 nM) for 1 or 2 h. Total cell lysates (40µg) were separated on a 4-20% SDS-PAGE and tested for Histone H3, Histone H4, PAD2 or PAD4 expression using Western analysis. Blots were probed with primary rabbit monoclonal antibodies for Histone H3(1:1000, CST) and Histone H4 (1:1000, CST) and PAD2 (1:1000, CST) and mouse monoclonal antibody for PAD4 (1:500 dilution, Biolegend); secondary horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG antibody (1:5000 dilution, Amersham) or HRP-conjugated goat anti-mouse IgG antibody (1:5000, CST). Following washes, immunoreactive complexes were developed by chemiluminescence (Supersignal West Pico PLUS Chemiluminescent Substrate, Cat# 34580, Thermo Scientific). Western for β-actin (1:5000; #A5441, Sigma-Aldrich) were a control for equal loading of total protein on the gel.

STATEMENT OF WORK – Accomplishments on September 9, 2021
PROPOSED START DATE Sep 01, 2019
REVISED Aug 01, 2021
Tasks completed are marked in yellow and tasks in progress are marked in green

Site 1: **Virginia Commonwealth University**
1217 E. Marshall St.
KMSB Rm 621-623
PO Box 980315
Richmond, VA 23298
PI: Dr Judith Voynow

Specific Aim 1: To determine whether NE generates a pro-inflammatory macrophage phenotype by degrading histone deacetylases, resulting in transcriptional upregulation and release of cytokines and HMGB1.	**Timeline**	**Site 1**
Major Task 1: Neutrophil elastase localization and protease activity; Cytokine mRNA and protein expression	**Months**	

Subtask 1: [Kummarapurugu] Purchase Buffy coats and culture, characterize, and store aliquots of BMDM (3 male and 3 female) and cultured media for Task 1 and 2 experiments; Recruit first 20 subjects from each GOLD COPD group and first 20 healthy smokers (80 subjects): Culture and characterize BMDM; Use cells for Major Tasks 1 and 2.	**1-6**	**Dr. Voynow (Total 80 Subjects)**
Subtask 2: [Kummarapurug and Zheng] Determine optimal dose and time point for FITC-NE uptake , NE protease activity,	**1-6**	**Dr. Voynow**
Subtask 3: Zheng] cytokine qPCR and Multiplex assays; HMGB1 ELISA	**1-30**	**Dr. Voynow**
Milestone(s) Achieved 2: Comparison of healthy non-smoker, Healthy Smoker, and 3 COPD groups to determine if NE localization, activities or cytokine expression are modified by sex, race, COPD severity or smoking/ environmental exposures	**30**	**Dr. Voynow**
Local IRB/IACUC Approval	**1**	**Dr. Voynow**
Milestone Achieved 1: HRPO/ACURO Approval	**1**	**Dr. Voynow**
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		
Subtask 1: [Kummarapurugu] Purchase buffy coats and culture hBMDM (2 male and 2 female), and hBMDM cultured from HS, COPD (3 grades of severity; total 80 subjects); save cell nuclear and total cell lysates; save conditioned media for experiments for HNS; [Zheng] determine HDAC and Sirtuin expression by western and activity assays	**1-24**	**Dr. Voynow (total 80 subjects)**
Subtask 2: [Hawkridge] LC-MS analysis of lysine acetylation for H3, p65 and HMGB1 in cell lysates	**6-30**	**Dr. Voynow**
Subtask 3: [Zheng] siRNA knockdown of specific targets identified as degraded by NE exposure and subsequent analysis of lysine acetylation of H3, p65, and HMGB1	**24-36**	**Dr. Voynow**

Subtask 4: [Zheng] Isolate alveolar macrophages from BAL and evaluate for NE uptake, protease activity, BAL supernatant cytokines and HMGB1, HDAC2 degradation (15 male and 15 female) **Complete BAL Macrophage analysis (15 male and 15 female)**	**1-18** **18-36**	**Dr. Voynow (60 subjects undergoing bronchoscopy)**
Milestone(s) Achieved 3: Determine whether the measurements below are altered by NE treatment or modified by sex, race, COPD severity, smoking / airborne pollutants • **HDAC and Sirtuin analysis** • **LC-MS lysine acetylation** • **siRNA knockdown of HDAC or Sirtuin and subsequent lysine acetylation identification**	**24** **24** **36**	**Dr. Voynow**
Specific Aim 2: To evaluate whether NE protease activity alters histone structure or histone post-translational modifications leading to chromatin decondensation and increased release of nuclear METs.		
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		
Subtask 1: [Kummarapurugu] Culture BMDM from 30 subjects in each COPD group and HS **[Zheng]Quantitate DNA released into media and confirm change in nuclear gene expression**	**6-30**	**Dr. Voynow (Total 120 Subjects)**
Subtask 2: [Zheng and Kummarapurugu] Analyze nuclear lysates for H3 degradation, H3 citrullination, and PAD1-4 expression	**1-24**	**Dr Voynow**
Subtask 3:[Hawkridge] Analyze conditioned media proteome to identify potential released positively charged chromatin or granule proteins that may be associated with METs	**24-36**	**Dr. Voynow**
Milestone(s) Achieved 4: Determine whether the measurements below are altered by NE treatment or modified by sex, race, COPD severity, smoking / airborne pollutants • **DNA quantitation in conditioned media** • **H3 degradation; H3 citrullination** • **PAD1-4 upregulation**	**30** **30** **30**	**Dr. Voynow**

• **LC-MS of conditioned media** • **Publications to be completed and submitted**	**36** **36**	

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to Report

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

During the next reporting period, we plan the following experiments to complete our goals:

The Scope of Work for Major Task 1 is almost completed. The remaining experiments to be performed include cytokine and HMGB1 analyses in conditioned media with comparisons between demographic categories of subjects with COPD.

The Scope of Work for Major Task 2 is partially completed. We will continue to work on evaluating BMDM from COPD subjects to determine the impact of NE on HDAC and Sirtuin protein levels. We will use western analyses and LC-MS to identify lysine acetylation of targets- H3, p65 and HMGB1, and we will test the impact of siRNA suppression of specific HDAC enzymes to determine whether the loss of specific enzymes are associated with target lysine acetylation.

The Scope of Work for Major Task 3 is almost completed. We will obtain MET biospecimens from subjects with COPD to perform LC-MS and identify the protein components associated with METs.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Nothing to report

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

The persistence of the COVID19 pandemic worsened by the delta SARS-CoV-2 variant has hampered recruitment of subjects because the majority of patients with COPD have continued to seek routine care by telehealth. Therefore, potential subjects were not present in the clinic to provide a blood sample for study. With increased COVID19 vaccination over this past year, patients are now coming in person and are therefore willing to consent to the study and provide a blood sample.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

We are improving our recruitment success and hired a new research Associate April 1, 2021, to assist with carrying out blood monocyte cell culture and experiments. Our research productivity has markedly improved over the past 6 months so we believe we have resolved problems for subject recruitment and completion of experimental goals.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

Not applicable

Significant changes in use of biohazards and/or select agents

Not applicable

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

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

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1. Kummarapurugu AB, Zheng S, Ma J, Ghosh S, Hawkridge AM, **Voynow JA.** (2021) Neutrophil Elastase Triggers the Release of Macrophage Extracellular Traps: Relevance to CF. Am J Respir Cell Mol Biol. *In press*
2. Ma J, Kummarapurugu AB, Hawkridge A, Ghosh S, Zheng S, **Voynow JA.** (2021) Neutrophil elastase-regulated macrophage sheddome/secretome and phagocytic failure. Am J Physiol Lung Cell Mol Physiol. 1;321(3):L555-L565. doi: 10.1152/ajplung.00499.2019. PMID: 34261337
3. Voynow JA, Shinbashi M. (2021) Neutrophil Elastase and Chronic Lung Disease. Biomolecules. 2021 Jul 21;11(8):1065. doi: 10.3390/biom11081065. PMID: 34439732
4. Voynow, JA, Zheng, S, Kummarapurugu, AB (2020) Glycosaminoglycans as Multifunctional Anti-Elastase and Anti-Inflammatory Drugs in Cystic Fibrosis Lung Disease, Invited Review, Frontiers in Pharmacology, in press, doi: 10.3389/fphar.2020.01011.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to report

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report

- **Other Products**

  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name:	Shuo Zheng, PhD
Project Role:	Co-investigator
No change	
Name:	Apparao Kummarapurugu, PhD
Project Role:	Co-investigator
No change	
Name:	Gamze Bulut, PhD
Project Role:	Research Associate
Nearest person month worked:	6
Contribution to Project:	Dr. Bulut has completed the screen of HDAC and Sirtuin enzymes in control subject hBMDM
Funding Support:	Children's Hospital Foundation
Name:	Le Kang, PhD
Project Role:	Co-investigator
Nearest person month worked:	1
Contribution to Project:	Statistical analysis of MET release intracellular protease activity
Name:	Adam Hawkridge, PhD
Project Role:	Co-investigator
Nearest person month worked:	1
Contribution to Project:	HPLC-MS/MS analysis of MET constituents
Name:	Shobha Ghosh, PhD
Project Role:	Co-investigator
Nearest person month worked:	1
Contribution to Project:	Assistance with macrophage characterization

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Judith A. Voynow, MD	No Change in other support since the last reporting period
Shuo Zheng, PhD	No Change in other support since the last reporting period
Apparao Kummarapurugu, PhD	No Change in other support since the last reporting period
Le Kang, PhD	No Change in other support since the last reporting period
Adam Hawkridge, PhD	**P01 NIH NHLBI** (Hoffmeister, Desai, Lau) 1/1/21-12/31/2025 (Hawkridge, Co-investigator/ Core Director, (3.6 month effort) Title: Role of Glycosaminoglycans in Hematopoiesis The overall goal in this program grant applicatin is to study the role of heparan sulfate in stem cell differentiation in the bone marrow niche.
Shobha Ghosh, PhD	**AHA Transformational Project Award** 1/1/21-12/31/23 American Heart Association (Ghosh, PI) Title: Inhibition of Sterol Carrier Protein-2 (SCP2): A novel anti-dyslipidemic strategy The major goal of this project is to validate SCP2 inhibition as a novel strategy to prevent diet-induced dyslipidemia.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ebrap.org/eBRAP/public/index.htm*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil/Pages/Resources.aspx) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Journal articles accepted or in press:

1. Kummarapurugu AB, Zheng S, Ma J, Ghosh S, Hawkridge AM, **Voynow JA.** (2021) Neutrophil Elastase Triggers the Release of Macrophage Extracellular Traps: Relevance to CF. Am J Respir Cell Mol Biol. *In press*

2. Ma J, Kummarapurugu AB, Hawkridge A, Ghosh S, Zheng S, **Voynow JA.** (2021) Neutrophil elastase-regulated macrophage sheddome/secretome and phagocytic failure. Am J Physiol Lung Cell Mol Physiol. 1;321(3):L555-L565. doi: 10.1152/ajplung.00499.2019. PMID: 34261337

3. Voynow JA, Shinbashi M. (2021) Neutrophil Elastase and Chronic Lung Disease. Biomolecules. 2021 Jul 21;11(8):1065. doi: 10.3390/biom11081065. PMID: 34439732

4. Voynow, JA, Zheng, S, Kummarapurugu, AB (2020) Glycosaminoglycans as Multifunctional Anti-Elastase and Anti-Inflammatory Drugs in Cystic Fibrosis Lung Disease, Invited Review, Frontiers in Pharmacology, in press, doi: 10.3389/fphar.2020.01011.

American Journal of Respiratory Cell and Molecular Biology/AJRCMB

Neutrophil Elastase Triggers the Release of Macrophage Extracellular Traps: Relevance to CF

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Subject Category:	9.17 Cystic Fibrosis: Translational & Clinical Studies < LUNG DISEASES
Keywords:	neutrophil elastase, macrophage, extracellular traps, histone H3, cystic fibrosis

Neutrophil Elastase Triggers the Release of Macrophage Extracellular Traps: Relevance to CF

Apparao B. Kummarapurugu[1], Shuo Zheng[1], Jonathan Ma[1], Shobha Ghosh[2], Adam Hawkridge[3], Judith A. Voynow[1]

[1]Department of Pediatric Pulmonary Medicine, Children's Hospital of Richmond at Virginia Commonwealth University, Richmond, VA, [2]Department of Internal Medicine, Virginia Commonwealth University School of Medicine, Richmond, VA, [3]School of Pharmacy, Virginia Commonwealth University, Richmond, VA

Corresponding Author: Apparao B. Kummarapurugu PhD, Department of Pediatric Pulmonary Medicine, PO Box 980315, 1217 East Marshall St., Kontos Medical Science Building, Rm 623, Children's Hospital of Richmond at VCU, Richmond, VA 23298; email: apparao.kummarapurugu@vcuhealth.org; phone: 804-628-6983

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Contributions of Authors: Conception and Design: ABK and JAV, Analysis and Interpretation: ABK, SZ, JAV, Drafting the Manuscript for Important Intellectual Content: ABK, SZ, JM, SG, AH, JAV

Running Title: NE and Macrophage Extracellular Traps

Descriptor: 9.17 Cystic Fibrosis: Translational & Clinical Studies

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org

Abstract

Rationale: Neutrophil extracellular traps increase cystic fibrosis (CF) airway inflammation. We hypothesized that macrophage exposure to neutrophil elastase (NE) would trigger the release of macrophage extracellular traps (METs), a novel mechanism to augment NE-induced airway inflammation in CF.

Objectives: To test whether human blood monocyte derived macrophages (hBMDM) from CF and non-CF subjects take up proteolytically active NE resulting in clipping of chromatin binding proteins and the release of METs.

Methods and Measurements: Human BMDM from CF and non-CF subjects were treated with FITC-NE to determine NE localization. Intracellular NE activity was determined by DQ-elastin assay. MET DNA release was detected by Pico-green for hBMDM, and visualized by confocal microscopy for hBMDM, and for alveolar macrophages harvested from intratracheal NE-exposed *Cftr*-null and wild-type littermate mice. Immunofluorescence assays for histone citrullination and western analyses for histone clipping were performed.

Main Results: FITC-NE was localized to cytoplasmic and nuclear domains, and NE retained proteolytic activity in hBMDM. NE (100 to 500 nM) significantly increased extracellular DNA release from hBMDM. NE activated MET release by confocal microscopy in hBMDM, and in alveolar macrophages from *Cftr*-null and *Cftr* wild-type mice. NE-triggered MET release was associated with H3 citrullination and partial cleavage of Histone H3 but not H4.

Conclusions: Exposure to NE caused release of METs from both CF and non-CF hBMDM *in vitro* and murine alveolar macrophages *in vivo*. MET release was associated with NE-activated H3 clipping, a mechanism associated with chromatin decondensation, a prerequisite for METs.

Abstract Total Words: 243

Key Words: Neutrophil Elastase, Macrophage, Extracellular Traps, Histone H3, CF

Introduction

Innate immune dysfunction is a central component of the pathophysiology of lung disease in cystic fibrosis (CF) (1, 2). The macrophage and blood-recruited monocyte derived macrophage participate in multiple roles as the sentinel innate immune leukocytes in the airway. They detect and remove pathogens and noxious substances, they mediate subsequent immune responses through cytokines, and they resolve inflammation (3). Previous studies have shown that macrophages from patients with CF have deficient pathogen clearance via phagocytosis and defective efferocytosis, which leads to unchecked infection and inflammation (3). Furthermore, macrophages from patients with CF also promote a pro-inflammatory state through increased transcription of cytokines IL-6, TNF-α, IL-1β, and sCD14 (4). Macrophage dysfunction may be due in part to loss of CFTR function (5-8), however exposure to airway sputum supernatant promotes overexuberant inflammation (9). Neutrophil elastase (NE), a major proteinase in the CF airway surface liquid, is a likely culprit in CF sputum to impair macrophage innate immune function.

Several decades ago, two labs (10, 11) reported that NE is rapidly endocytosed by human alveolar macrophages and retains proteinase activity intracellularly. Since these early reports, neither the mechanisms required for NE trafficking, nor the sequelae of NE- intracellular proteinase activity have been identified. NE and myeloperoxidase activate the release of neutrophil extracellular traps (NETs) (12). Thus, we hypothesized that NE uptake by macrophages activates the release of macrophage extracellular traps (METs), a mechanism that may play an important role in CF lung disease progression (13).

Extracellular traps are a released complex of DNA strands decorated with chromatin binding proteins and granule proteins. They were originally described as a form of cell death in neutrophils with the capacity to bind and kill microorganisms (14). However, since the first reports, extracellular traps have been reported to be released from other leukocytes including macrophages, monocytes, eosinophils, mast cells and basophils (15), and can be released by

viable cells (16). METs are released following exposure to microbes, increased intracellular calcium, TNFα, interferon-γ, extracellular DNA and oxidative stress (15, 17, 18). However, it is not known whether NE proteinase activity triggers the release of METs *in vitro* or *in vivo*. Using primary human blood monocyte derived macrophages (hBMDM) from patients with CF and from subjects without CF (CF and non-CF hBMDM) and using bronchoalveolar macrophages from *Cftr*-null and *Cftr*-wild-type littermate mice, we tested whether NE was sufficient to release METs and investigated potential mechanisms for NE-induced MET release. Some of the results of these studies have been previously reported in the form of an abstract (19).

Materials and Methods. Please see complete methods in Supplemental Data Section.

Human BMDM cultures

Blood samples were obtained from CF and non-CF donors following VCU IRB-approved informed written consent. Subject demographics and clinical information are presented in **Table 1**. Mononuclear cells were isolated and cultured into monocyte derived macrophages (hBMDM) (20). All experiments with hBMDM were performed in serum free RPMI-1640 medium with no proteinase inhibitors unless otherwise specified.

Localization of FITC-NE in hBMDM by Confocal Microscopy

Human BMDM ($1x10^5$ cells/ glass coverslip) were treated with FITC-NE (200 nM, #FS563, Elastin Products) or control vehicle, 2 or 4h, 37°C, then AAPV-CMK (NE inhibitor, 10 μM, #M0398, Sigma), then fixed, counterstained with DAPI (1μg/ml, #9542, Sigma) and evaluated by confocal fluorescence microscopy (Zeiss LSM 700).

Detection of intracellular NE activity in hBMDM. Human BMDM were treated in suspension with control vehicle, or NE (200nM, #SE563, Elastin Products, 2hr, 37°C), then fixed, permeabilized and incubated with DQ-elastin (20μg/ml, EnzChek® Elastase Assay Kit, E-12056), RT, overnight. Relative Fluorescence Intensity was measured (excitation/emission 505/515 nm).

Quantification of NE- induced METs release by PicoGreen

Human hBMDM (1x 10^5 cells/ well) were treated with control vehicle or NE (100, 200 or 500 nM, 2 h, 37°C), micrococcal nuclease (16 U/ml, #M10247S, New England BioLabs, 37°C, 20 min), and then analyzed for extracellular dsDNA using the Quant-iT™ PicoGreen® dsDNA Assay kit (#P7589, Thermo Fisher Scientific).

Visualization of NE induced METs by Confocal Microscopy

Human BMDM (1x10^5 cells/ coverslip) were treated with NE (200 nM) or control vehicle for 2h, 37°C, fixed, counterstained with DAPI, and evaluated by confocal microscopy.

In a VCU IACUC-approved protocol, *Cftr*-null, FABP human CFTR Tg1/Jaw/J mice (JAX, Stock # 002364) and *Cftr*-wild-type (WT) littermates (8-12 weeks old, both male and female) were administered human NE (50 µg [43.75 U]/ 40 µl saline) or normal saline (NS) by oropharyngeal aspiration on Days 1, 4, and 7 (21, 22). On day 8 (24 h following the last dose of NE), bronchoalveolar lavage (BAL) macrophages were isolated, stained with DAPI and evaluated for MET structures by confocal microscopy.

Detection of Histone H3 Citrullination by Confocal Microscopy

Human BMDM (1x10^5 cells/ coverslip) were treated with NE (200 nM) or control vehicle, 2h, 37°C, fixed, permeabilized, and incubated with Histone H3 citrulline (CitH3) rabbit polyclonal antibody (1:100, #ab5103, Abcam), and secondary Alexa Fluor 488-conjugated goat anti-rabbit antibody (Invitrogen) for confocal microscopy.

Detection of Histone cleavage by Western Blot analyses

Human BMDM were treated with NE (200 or 500nM) or control vehicle, 1 or 2h, 37°C. Cell lysates (30µg) were analyzed by western analysis for Histone H3 (Primary, rabbit IgG,1:1000, #4499, CST) or Histone H4 (1:1000, #13919, CST), secondary HRP-conjugated goat anti-rabbit IgG (1:4000, #7074, CST), and SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific). Westerns for β-actin (1:5000; #A5441, Sigma-Aldrich) were a loading control.

In-vitro Histone H3 Cleavage assay

Purified H3.1 or H3.3 (500ng) was treated with NE (25 or 50nM) for 15 or 30 min, 37°C. Reaction products were separated by 12% SDS-PAGE, and detected on nitrocellulose membrane by colloidal gold staining (Bio-Rad).

Statistical Analysis

Data, mean ± SEM, were analyzed using a one-way, nonparametric ANOVA (Kruskal-Wallis) test, followed by post hoc comparisons using the Wilcoxon rank sum test (Statistix 8.0). $P < 0.05$ was considered statistically significant.

Results

Cellular localization of neutrophil elastase in macrophages

We first sought to determine the intracellular fate of NE taken up by hBMDM from both CF and non-CF subjects. To assess the localization of NE, macrophages were exposed to FITC- labeled NE (200 nM) or control vehicle for 2 or 4 h, then fixed and stained with DAPI to localize nuclei (**Figure 1A**), and the relative FITC intensity and cellular distribution by confocal microscopy (**Figure 1B**). At 2 hours, FITC-NE was localized in the cytosol as well as the nucleus with clearly enhanced FITC signal. By 4 hours, FITC-NE signal was stronger and preferentially located in the nucleus (as shown by DAPI immunofluorescence localized to the same nuclei) (**Figure 1B**). FITC-NE uptake in macrophages from non-CF donors was similar to that observed in macrophages from patients with CF (**Figure 1C**). These data demonstrate that NE was taken up by all the macrophages examined, and distributed into intracellular compartments, including the nucleus. As a control for specificity of localization, macrophages incubated with FITC- *E.coli* bioparticles had fluorescent particles only in cytosolic organelles but not the nucleus **(Figure E1**) while cells incubated with FITC alone did not emit a fluorescent signal (data not shown).

NE exhibited Intracellular proteinase activity

Following evidence of NE uptake by hBMDM, we sought to determine whether NE maintained intracellular proteinase activity by measuring the degradation of a modified substrate, DQ-elastin, which emits a fluorescent signal on cleavage. The cells exposed to NE (200nM, 2h), exhibited significantly increased fluorescence within 16h after adding DQ elastin to the cells (**Figure 1D**). Co-incubation of NE with Ala-Ala-Pro-Val-Chloromethylketone, a NE specific inhibitor, significantly quenched cell fluorescence to background levels indicating that cleavage of DQ-elastin in macrophages was NE-specific. These results confirm that NE retained detectable proteinase activity after uptake by hBMDM from both CF and non-CF subjects.

NE induced the release of extracellular DNA in macrophages

Although NE is a major airway inflammatory mediator in CF and is sufficient to trigger NETs (12), a role for NE in the formation of METs had not previously been described. To determine whether NE exposure induced the release of METs, we quantified extracellular DNA using Picogreen, a fluorescent probe that binds dsDNA. CF and non-CF hBMDM were incubated with NE (100, 200, or 500nM, 2.5h) or control vehicle and DNA content was quantitated in the culture supernatants by Picogreen relative fluorescence units. NE exposure significantly increased the amount of extracellular DNA in culture supernatants, at all NE concentrations compared to control treatment (**Figure 2**). However, non-CF MDMs released significantly higher amount of extracellular DNA in culture supernatants compared to CF MDMs at 100nM NE concentration. Cell viability was not affected by NE under the experimental conditions used as assessed by LDH release (**Figure E2**).

Visualization of NE induced METs by confocal microscopy

Confocal microscopy was used to confirm that increased extracellular DNA following NE treatment corresponded to MET structures. CF and non-CF hBMDM, seeded onto poly-L-lysine coated glass coverslips, were exposed to NE (200nM for 2h), stained with DAPI, and analyzed by confocal microscopy. No DAPI-stained extracellular DNA structures were found in control

vehicle-treated cells at 2h incubation, and nuclei had normal morphology. In contrast, we observed DAPI-stained web-like extracellular DNA structures (white arrows) in response to NE exposure in some macrophages consistent with MET release (**Figure 3**). To discern whether the macrophages that released ETs were alive or dead, cells were stained with live/dead Zombie Red fixable dye which revealed that MET-releasing macrophages were viable (**Figure E3**). Together, the Picogreen and confocal microscopy results show for the first time that NE mediated the release of METs from CF and non-CF hBMDM. Furthermore, we determined that the hBMDM exposed to NE increased release of TNFα, a cytokine typical of M1 polarized macrophages, and not CCL18, a cytokine typical of M2 polarized macrophages (23). Control- vehicle treated hBMDM did not express either TNFα or CCL18 (**Figure E4**).

Intratracheal NE induced the release of METs from murine BAL macrophages

To determine whether NE induced the release of METs *in vivo*, *Cftr*-null, gut-corrected mice, and *Cftr*- wild-type (WT) littermates were treated with intratracheal human NE (50μg) or normal saline on days 1, 4, and 7, and BAL was harvested on Day 8 (24). BAL cells were collected and alveolar macrophages were enriched by adherence to poly-L-lysine coated coverslips, fixed with 4% PFA, and stained with DAPI (1 μg/ml). No MET structures were observed in macrophages from normal saline (NS) exposed *Cftr*-null and *Cftr*-WT mice. However, *Cftr*-null and *Cftr*-WT murine alveolar macrophages exposed to NE *in vivo*, exhibited DAPI-stained web-like extracellular DNA structures (white arrows) confirming that NE mediated the release of extracellular traps *in vivo* (**Figure 4**). Thus, NE induced the release of extracellular traps in alveolar macrophages *in vivo*, consistent with the *in vitro* results that NE triggered MET release in both CF and non-CF hBMDM. NE-induced MET release did not occur in all cells. To estimate the frequency of NE-induced METs, we performed an *ex vivo* dose curve of NE treatment (0-500 nM) of alveolar macrophages from *Cftr*-null and *Cftr*-WT mice and determined that METs occurred at all doses of NE treatment (100-500 nM) at a range from 3-11% of NE-treated cells (**Table E1**).

NE increased Histone H3 Citrullination

NE exposure increased another signature marker of extracellular trap formation, namely histone citrullination. CitH3 was present at baseline levels in hBMDM. However, following NE treatment, there was increased CitH3 in both CF and non-CF hBMDM (**Figure 5**).

NE induced the cleavage of Histone H3 but not H4 in macrophages

The mechanisms for the release of METs remain elusive. NE generates NETs via histone H4 degradation (12) which is associated with de-condensation of chromatin. In addition, in neutrophils, NADPH oxidase activates the cleavage of Histone H4 (25, 26), which is associated with the generation of NETs (27). Based on these insights, we tested whether NE cleaves histones as a potential mechanism for chromatin de-condensation and release of extracellular traps in macrophages. Non-CF (**Figure 6A**) or CF (**Figure 6C**) hBMDM were exposed to NE (200 or 500nM for 1h or 2h) or control vehicle and total protein lysates were evaluated by Western analysis for histone H3 cleavage. The results demonstrated that NE treatment resulted in a cleaved fragment for H3, with a 15kDa band representing full length H3 and a smaller ~13kDa band representing a clipped H3 fragment. The pattern was consistent for all NE treatment conditions. In contrast, no H3 cleavage was observed in cells exposed to control vehicle treatment. We next investigated the effect of NE (200 or 500nM, for 1h or 2h) on histone H4 cleavage. NE did not cause H4 cleavage in either non-CF hBMDM **(Figure 6B)** or CF hBMDM **(Figure 6D**). These results support the association of histone H3 cleavage but not histone H4 cleavage with chromatin decondensation and MET release.

Histone H3 cleavage is directly dependent on proteolytic activity of NE

To test whether NE proteolytic activity is sufficient for H3 clipping, recombinant proteins of Histone H3 isoforms, H3.1 and H3.3, were incubated with NE (25 or 50nM) for 15 or 30min, and cleaved H3 proteins were resolved on SDS-PAGE, transferred to nitrocellulose membrane and bands

detected by colloidal gold staining. NE exposure at 15 or 30 min, resulted in two bands consisting of 15kDa band representing full length H3 and a smaller ~13kDa H3 fragment (**Figure 7**). The size of the bands obtained match those obtained by western analyses from NE –treated hBMDM (**Figure 6**). In contrast, no H3 cleavage was observed in cells exposed to control vehicle. A band at 29 kDa, consistent with the expected size for human NE on SDS PAGE, was present only in lysates treated with NE. These results suggest that NE proteinase activity directly cleaves H3 histone as part of the mechanism of MET generation.

Discussion

NE induces airway inflammation by several mechanisms (28). NE cleaves cell surface opsonins and opsonin receptors, and degrades innate immune proteins, resulting in failure of microbial killing. NE also activates the release of pro-inflammatory cytokines and High mobility group box 1 (HMGB1) (29, 30), resulting in increased airway inflammation. Herein, we report a novel mechanism by which extracellular NE amplifies inflammation in the lung: exogenous NE activates the release of METs. METs cause airway inflammation by exposing the airway and lung parenchyma to pro-inflammatory molecules including extracellular DNA, histones, proteinases, myeloperoxidase (17) and HMGB1 (31). It is important to note that macrophages are the major sentinel leukocyte in the lung and are increased in the lungs of patients with CF (32) and COPD (33). Therefore, METs may constitute a major contribution to the extracellular traps in the lungs of patients with CF and with COPD.

MET release varies depending on the source of macrophages and the clinical environment/ and polarization of the macrophage (17). METs are attributed to M1 polarized macrophages (17) and we demonstrate that NE-treated hBMDM released TNFα, a marker of M1 polarization, and did not release CCL18, a marker of M2 polarization (23). However, both M1 and M2 macrophages are present in the CF airway (3, 34-36), and there is plasticity with respect to macrophage polarization depending on the airway milieu. it is not yet known whether M1 vs.

M2 polarization is sufficient to define risk for MET generation. In our report, we demonstrate that both human BMDM and murine alveolar macrophages responded to NE exposure with increased MET release, so NE exposure is effective in triggering MET release across two species and two different classes of macrophages. We observed that although all hBMDM endocytosed FITC-NE *in vitro*, not all hBMDM released METs. This may be due to transcriptome variability between MET-releasing macrophages and non- MET-releasing macrophages. Such single cell characterization is beyond the scope of this study but may provide new insights into how some macrophages are fated for pro-inflammatory action while other macrophages resolve inflammation.

Several factors have been reported to activate MET release including bacteria, mycobacteria, yeast, interferon γ, exposure to neutrophil extracellular traps, phorbol myristate acetate, TNFα, drugs including the antibiotic, fosfomycin, statins (17), and the oxidant, hypochlorous acid (37). MET release is inhibited by superoxide inhibitors, diphenylene iodonium and apocyanin, and by the elastase inhibitor, N-methoxysuccinyl-Ala-Ala-Pro-Val chloromethylketone (17). These observations support the concept that both oxidative stress and the proteinase elastase trigger MET release. To our knowledge, no other neutrophil proteinases have been reported to activate release of METs. NE is sufficient to cause chromatin de-condensation *in vitro* in neutrophil nuclei (12). Although myeloperoxidase (MPO) alone is not sufficient to induce NETs, MPO acts synergistically with NE to increase neutrophil nuclear de-condensation (12). Importantly, we and others have reported that NE induces oxidative stress in airway epithelia (38-40), in murine airway surface liquid *in vivo* (41), and in alveolar macrophages via uptake of heme- free iron (39), so NE-activated oxidative stress may contribute to MET formation.

Our results are in agreement with previous reports that NE is taken up by macrophages via binding sites (11) that can be inhibited by a sulfated polysaccharide (42). Neutrophils bind NE

via high abundance, low affinity cell surface binding sites composed of chondroitin sulfate and heparan sulfate proteoglycans (43). It is possible that macrophages have similar sulfated glycosaminoglycan binding sites for NE. Extracellular NE can be taken up by different cell types. NE is taken up by airway epithelial cells into the cytoplasm in response to cigarette smoke exposure, and directly degrades Sirt1 (44). Breast cancer cells and other tumor cells endocytose exogenous NE (45, 46), mediated in part by neuropilin-1 (NRP1) receptor (47), and in breast cancer cells, NE cleaves a nuclear protein Cyclin E which creates an HLA-A2-restricted peptide antigen recognized by cytotoxic T lymphocytes (45). An alternative mechanism for NE uptake is binding to the cell surface and then internalization by clathrin-pit-mediated endocytosis (48). Although our data demonstrated that macrophages were receptive to available NE, how NE is taken up by these cells and targeted to the nucleus is unclear and further investigations are required to understand the mechanism of uptake.

We demonstrated that NE, taken up into the macrophage cytoplasm and nucleus, was proteolytically active and capable of fragmenting histone H3, resulting in chromatin de-condensation (12). We confirmed that NE proteinase activity clipped human H3.1 and H3.3 histones *in vitro*, with a similar clipped size as shown in macrophage cell lysates by western analysis. Interestingly, NE has been reported to clip histones H1 and H4 *in vitro* in assays with neutrophil nuclei (12), and cleave histone H2A in leukocytes (49). Evidence is emerging that proteolytic processing of histones, particularly H3, known as “histone clipping,” is responsible for several cellular processes, such as transcriptional regulation, cell differentiation, and senescence (25, 26, 50). Core histones are packaged into nucleosomes wrapped with DNA leaving the N-terminal tail of all the core histones hanging outside the nucleosome and thus vulnerable to cleavage by proteinases (26). Histone H3 N-terminal tail has numerous sites that are susceptible to proteinases in addition to NE such as Cathepsin L, Cathepsin D, and matrix metalloprotease 9 (26, 50). The biological significance and mechanism of histone clipping are not yet fully

understood, but several studies suggested that clipping regulates the post-translational modifications that alter chromatin structure, induce chromatin de-condensation and regulate gene expression (25, 26, 50).

Our study had some limitations. We were not able to match the ages of non-CF subjects with the ages of subjects with CF. Nine out of 17 of the subjects with CF were on CFTR modulators. However, for Figures 1D and 5, we used two subjects with CF and two subjects without CF and therefore we were not able to assign any difference in outcomes to the use of CFTR modulators. This is a limitation that may be difficult to address in future studies as the most highly effective modulators will be approved for younger age patients. However, despite these limitations, our experimental design, using each subject as their own control to compare treatment with control vehicle vs NE for all outcomes permitted the conclusion that hBMDM from subjects with or without CF, responded to NE exposure via a signaling pathway that resulted in MET release.

In summary, we show that macrophages rapidly took up NE, localized proteolytically active NE to the nucleus, and these events resulted in H3 citrullination and clipped histone H3, precursors to MET release. Although we have identified H3 histone clipping as one potential mechanism for NE-induced MET release, it is possible that NE triggers MET formation through other mechanisms. Investigations are underway to elucidate our understanding of METs in CF and COPD by examining the mechanism of NE-regulated H3 clipping, and whether other proteinases activated by NE such as macrophage metalloproteases may activate MET formation. Furthermore, the macrophages that released METs were alive; and neither the sequelae of MET release nor the function and phenotype of these macrophages are known. These are important areas for future investigation.

Table 1. Subject Demographics: CF and Non-CF

CF*	FEV1pp	Genotype	CFTR Modulators
1	46	F508/ F508	Tezacaftor- Ivacaftor
2	101	F508/ F508	None
3	98	F508/ F508	Tezacaftor- Ivacaftor
4	31	F508/ 1812-1G>A	None
5	74	F508/ F508	None
6	108	F508/ I618T	None
7	91	F508/ F508	Lumicaftor- Ivacaftor
8	89	F508/ 17171G>A	None
9	61	F508/ F508	Lumicaftor- Ivacaftor
10	109	F508/ F508	None
11	92	F508/ G551D	Ivacaftor
12	73	F508/ F508	Tezacaftor- Ivacaftor
13	120	F508/ N1303K	None
14	113	F508/ F508	Elexacaftor/Tezacaftor/Ivacaftor
15	111	F508/ F508	Elexacaftor/Tezacaftor/ Ivacaftor
16	109	F508/ F508	Tezacaftor- Ivacaftor
17	77	F508/ 2307insA	None
Non-CF	Sex	Age (yr)	Race
1	F	36	C
2	M	29	A
3	F	35	H
4	M	57	AA
5	F	27	C
6	M	33	A
7	F	NR	A
8	F	41	C
9	M	33	C
10	M	32	C
11	F	26	AA
12	M	24	A
13	F	21	H
14	F	23	AA
15	F	NR	C

Race: A, Asian; AA, African American; C, Caucasian; H, Hispanic; FEV1pp, FEV1 percent predicted; NR, not recorded. *, Summary demographics for participants with CF: 11 males and 6 females; age range from 6 to 24 years; and racial/ethnic background including 11 Caucasians, 4 African Americans, and 2 Hispanic or not identified individuals.

Figure Legends

Figure 1: Neutrophil elastase was internalized in non-CF and CF hBMDM cells and exhibited proteolytic activity intracellularly. Non-CF and CF hBMDM were exposed to FITC-NE (200 nM, 2 h or 4h) or vehicle control (**B**). After the treatments, cells were washed with PBS, fixed, stained with DAPI (**A**) and analyzed by confocal microscopy. Nuclei are indicated by white arrows. Representative confocal micrographs of FITC-DIC images (63x magnification) for non-CF or CF hBMDM treated with FITC-NE 200 nM, for 2 or 4h, are shown (**B**). Relative integrated fluorescence intensity (RFU) of FITC-NE normalized to nuclear DAPI fluorescence was quantified using ImageJ Software. Data are summarized as FITC-NE (RFU normalized to DAPI); mean ± SEM; from 2 experiments using 2 non-CF and 2 CF donors. n=3-4 images per individual (**C**). To evaluate NE proteolytic activity intracellularly, hBMDM from non-CF and CF patients were cultured in suspension for 8-10 days, then treated with control or NE (200nM) for 2hr. At the end of 2hr, cells were fixed, permeabilized and mixed with DQ-elastin overnight to determine NE activity (**D**). AAPV-CMK were added to negative control wells to block NE specific activity. RFU was measured by TECAN fluorescence microplate reader (excitation/ emission 505/515 nm). Two experiments were performed with a total of 2 non-CF and 2 CF hBMDMs (n=4-5 replicates per subject). Relative NE activity was determined by RFU normalized to corresponding average no cell control. There was a significant increase in NE activity in NE treated wells compared to NE+AAPV in both non-CF and CF hBMDM (Wilcoxon rank sum test, ** or ++, p=0.0002) (**D**).

Figure 2. Neutrophil elastase induced extracellular DNA release from non-CF and CF hBMDM. Non-CF hBMDM (white bar) and CF hBMDM (black bar) cultured on 96 well plates, were treated with control vehicle (Ctrl) or NE (100, 200 or 500nM, 2h). At the end of NE treatment, cells were incubated with micrococcal nuclease (4U, 25 min). At the end of nuclease treatment, a 1:200 dilution of PicoGreen reagent was added to an equal volume of the nuclease treated

culture supernatant. After incubation, samples were analyzed for extracellular DNA by quantifying PicoGreen-derived fluorescence intensities. Fluorescence was quantified at the excitation/ emission wavelengths 480 / 520 nm using an automated plate reader (TECAN). Data are normalized to control vehicle treated cells and results are summarized (mean ± SEM) from 4 experiments including 4 non-CF and 3 CF individuals, n=13-18 replicates. Statistically significant differences were determined by ANOVA with post-hoc comparisons by the Wilcoxon rank sum test. ***, $p<0.001$ non-CF NE treated vs. non-CF Ctrl treated; +, $p<0.05$ CF NE treated vs. CF Ctrl treated; ++, $p<0.001$ CF NE treated vs. CF Ctrl treated; *, $p<0.012$ non-CF NE (100nM) vs CF NE (100nM). There was a significant difference at 100nM NE concentration between non-CF and CF but no differences were observed at 200 or 500 nM NE.

Figure 3. Neutrophil elastase stimulated release of extracellular traps from non-CF and CF hBMDM detected by confocal microscopy. Human BMDM from non-CF and CF subjects were seeded onto poly-L-lysine coated coverslips and cultured in growth media containing GM-CSF (20 ng/ml) for 8-9 days. Cells were exposed to NE (200 nM) or vehicle control (Ctrl) for 2h. After NE exposure, NE inhibitor (AAPV-CMK) was added to all wells, and cells were fixed, and stained with DAPI to detect both nuclear and extracellular DNA. MET structures were examined by confocal microscopy (Zeiss LSM 700) (63x magnification). NE treatment for 2h resulted in DAPI positive chromatin filaments, characteristic of METs, extruded outside of the cell (white arrows) in NE treated cells only. No MET formation was observed when cells were exposed to control vehicle. Micrographs are representative of 2 experiments with 2 non-CF and 2 CF donors.

Figure 4. Detection of macrophage extracellular traps (METs) released *in vivo* following intra-tracheal NE administration in both Cftr-null and Cftr-WT mice on the same genetic background. Eight-12 weeks old, Cftr-null mice and Cftr-WT mice (both male and female) were administered NE (50 µg/40µl/animal) or normal saline (40 µl) by oropharyngeal aspiration

following isoflurane anesthesia on days 1, 4, and 7. On day 8, mice were killed by Euthasol injection and BAL cells were collected and applied to poly L-lysine coated coverslips, fixed, and stained with DAPI for MET detection. Confocal microscopy with a Zeiss LSM 700 (63x) revealed MET release from macrophages derived from both Cftr-null and WT mice post-NE treatment but not following saline treatment (Ctrl). Micrographs are representative of n= 4 mice per treatment from 2 different experiments.

Figure 5. NE increased H3Citrulline (citH3) in both non-CF and CF hBMDM. Human BMDM were treated with NE (200 nM, 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 are shown for non-CF and CF hBMDM (63x magnification) (**A)**. Integrated fluorescence intensity of citH3 normalized to nuclear DAPI fluorescence (images not shown) was quantified using ImageJ Software. Data are summarized as citH3 (RFU normalized to DAPI) (mean ± SEM); from 2 experiments with 2 non-CF and 2 CF individuals, n= 7 random images (**B**). Statistically significant differences were determined by ANOVA with post-hoc comparisons by the Wilcoxon rank sum test; ***, $p < 0.0002$; +++, $p < 0.0006$.

Figure 6. NE induced the cleavage of histone H3, in non-CF and CF hBMDM cells. Non-CF and CF hBMDM 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 was

analyzed in non-CF (**A**) and CF **(C**) hBMDM and Histone H4 was analyzed in non-CF (**B**) and CF (**D**) hBMDM by western analyses. Western blots shown were representative of n= 3 non-CF and 3 CF donors.

Figure 7. NE cleaved recombinant Histone H3.1 and H3.3 isoforms *in vitro*. Recombinant human Histone H3.1 and H3.3 were incubated with control vehicle or NE (25 or 50 nM) for 15 or 30 min. Equal amounts of reaction products were resolved on 4-20% SDS-PAGE, transferred to membrane and stained with colloidal gold total protein stain. The upper arrow indicates NE that was used in the reaction, lower arrows show full length and cleaved fragments of Histone H3.1 and H3.3 following NE treatment. Control vehicle (C) treated had no H3 fragments.

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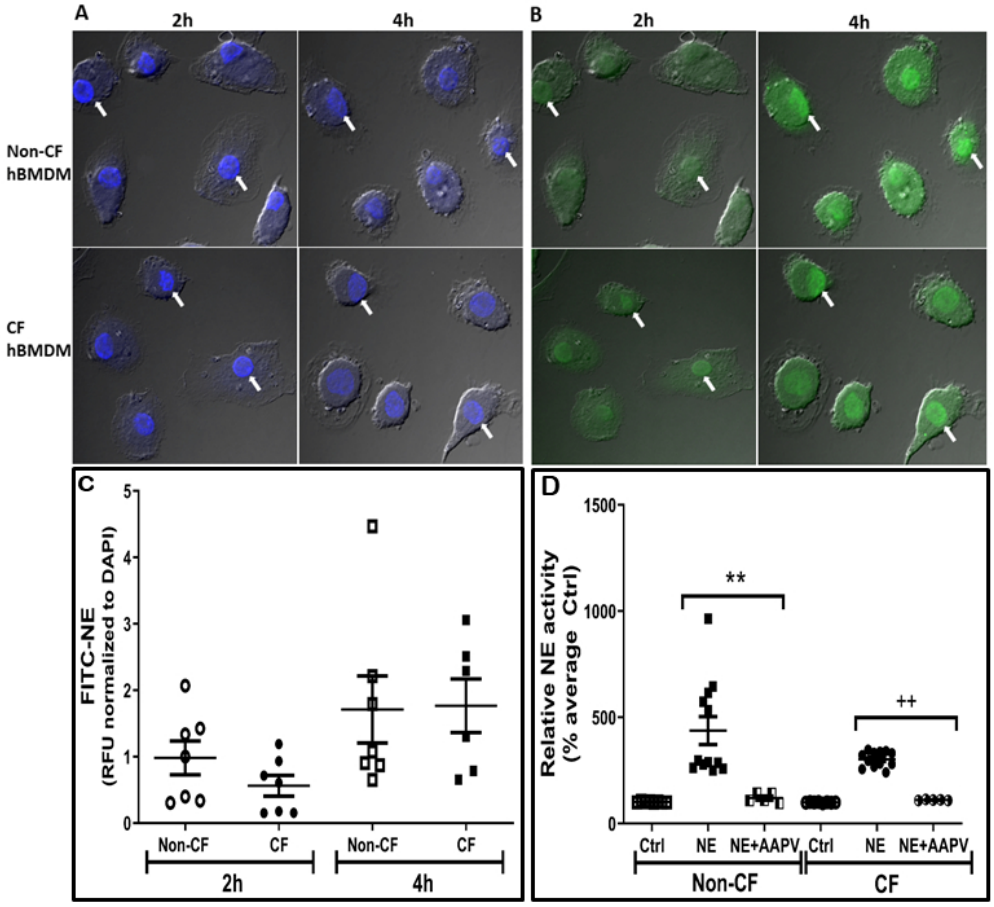
A
2h
4h
B
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C
FITC-NE (RFU normalized to DAPI)
0
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Non-CF
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Non-CF
CF
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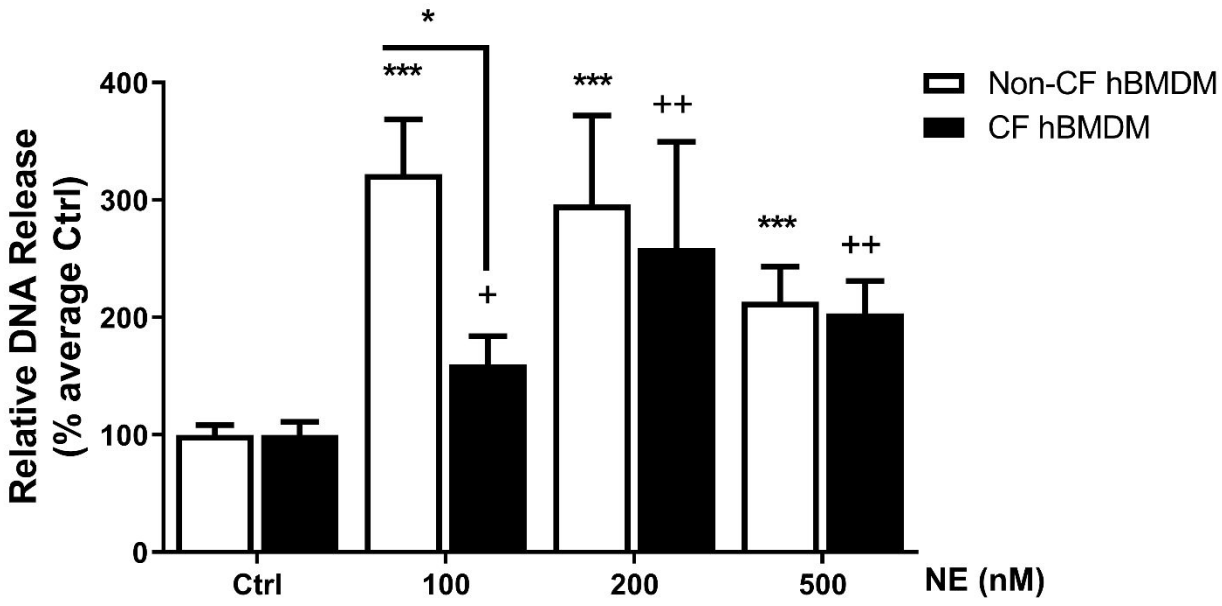
4h
D
Relative NE activity (% average Ctrl)
0
500
1000
1500
**
++
Ctrl
NE
NE+AAPV
Ctrl
NE
NE+AAPV
Non-CF
CF

Figure 1

Figure 2



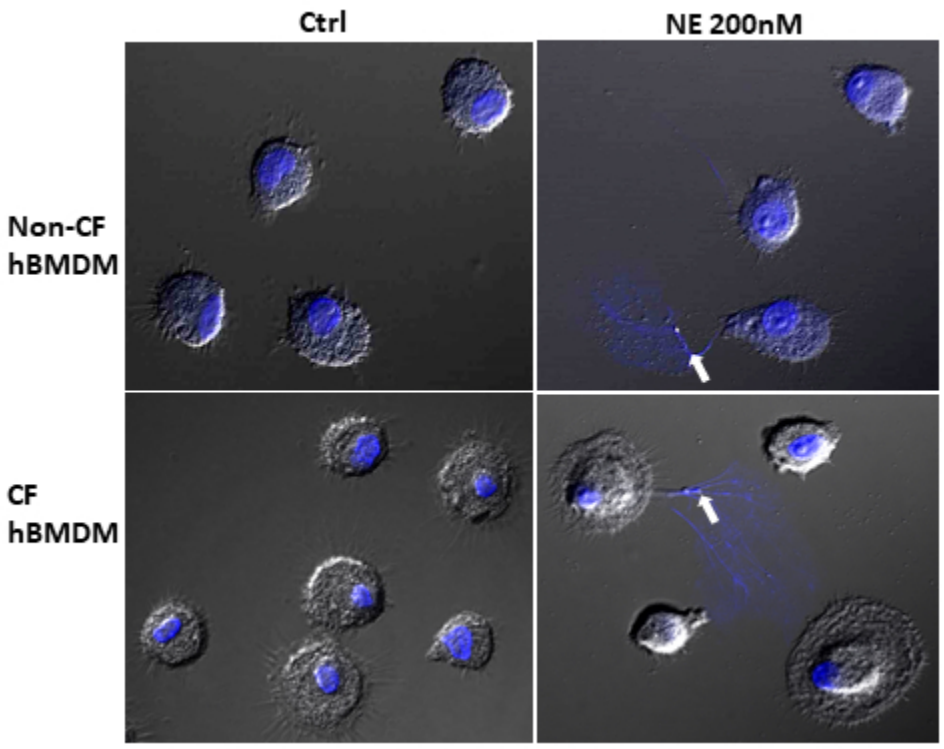
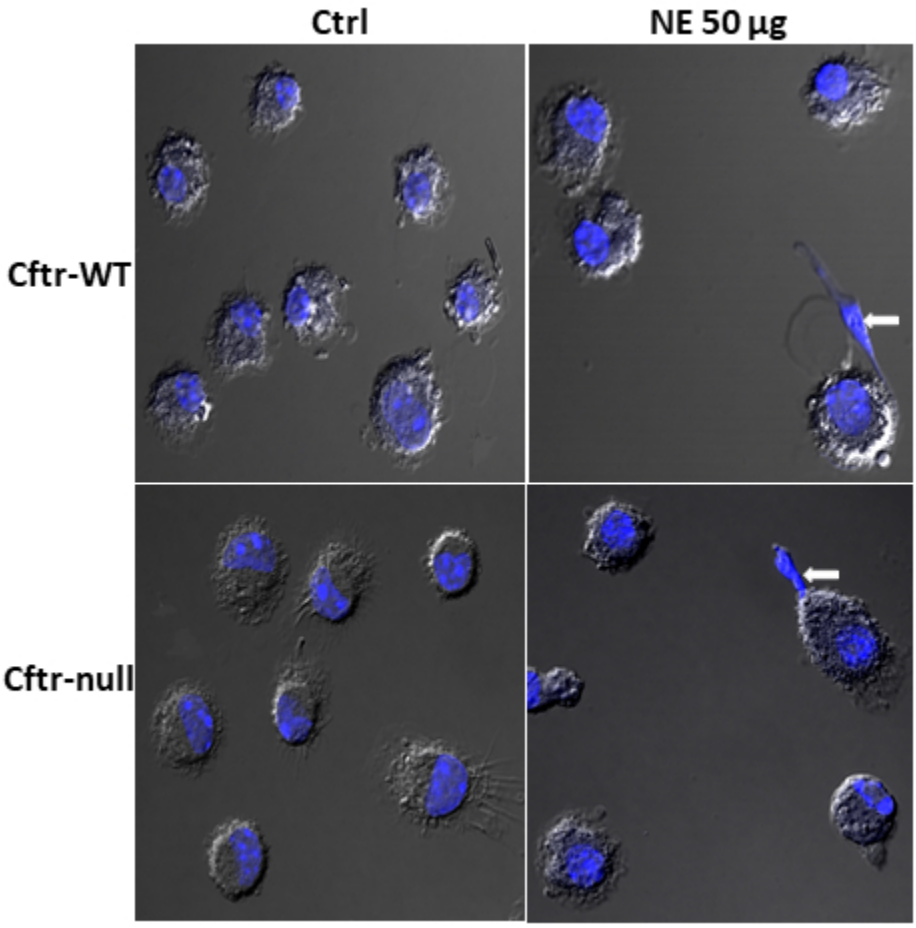


Figure 3



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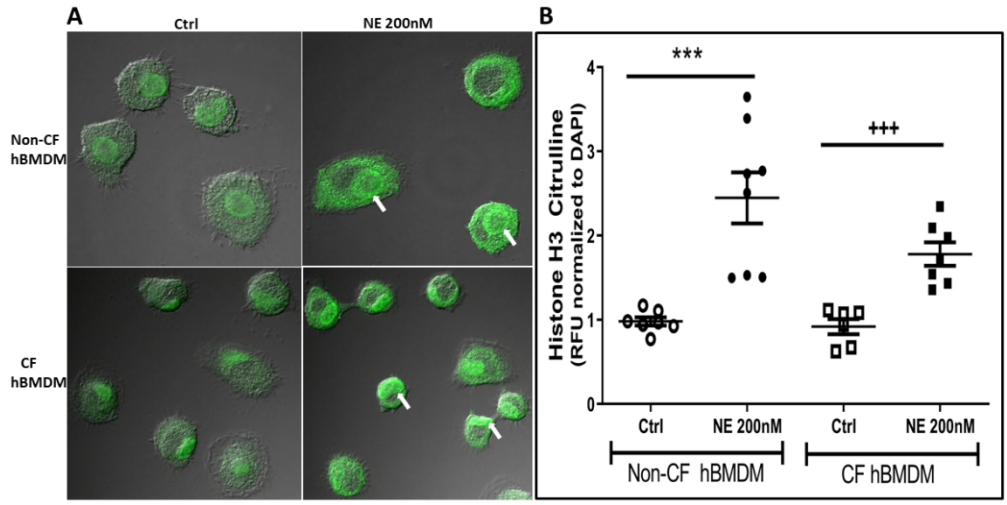

Figure 5

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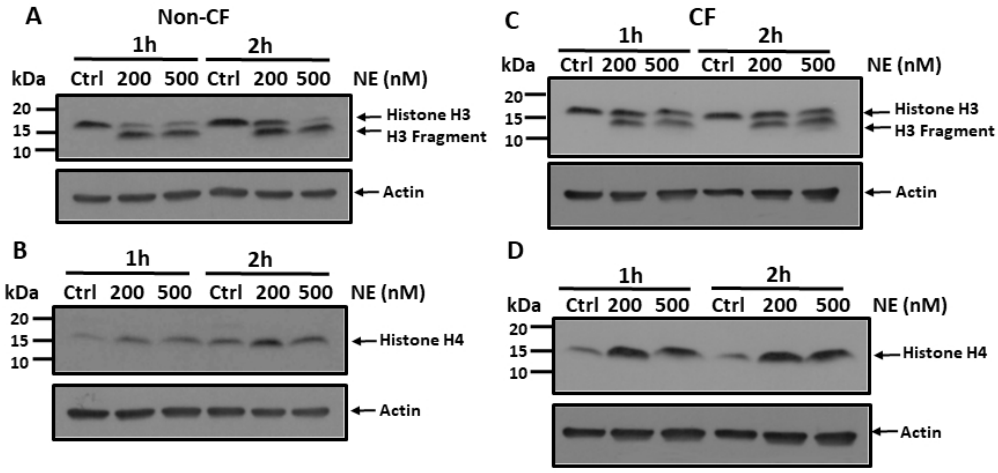
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Figure 6

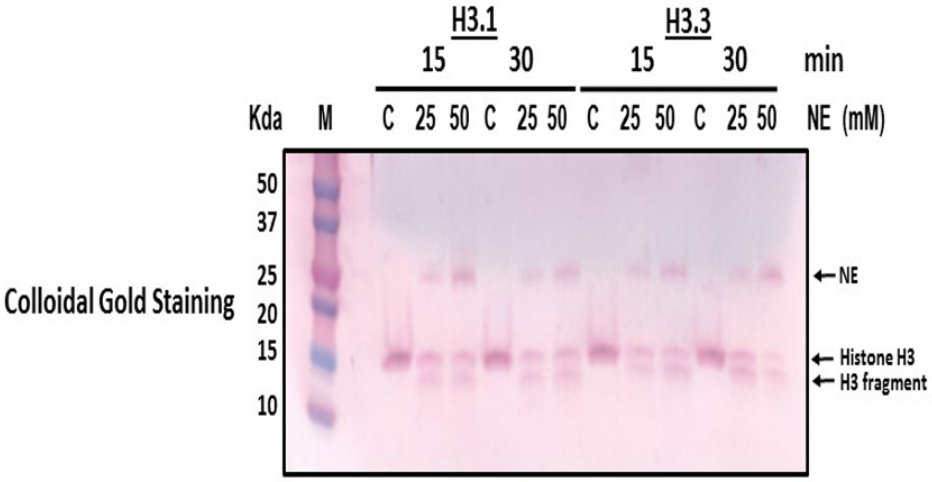

Figure 7

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Supplemental Methods

Materials and Methods

Human BMDM cultures

Blood samples obtained from CF and non-CF donors following VCU IRB-approved informed written consent, were processed as previously described (1). CF patient demographics and clinical information, and non-CF subject sex, age, and race are presented in **Table 1**. Briefly mononuclear cells were isolated from whole blood by negative selection, using the RosetteSep kit (#15068, StemCell Technologies) and Lymphopure (#426202, Biolegend) gradient centrifugation per manufacturer's instructions. The monocyte enriched cells were harvested, and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Life technologies), L-glutamine (2mM), 2.5mM HEPES and penicillin/streptomycin mixture (20 ng/ml 100 U/ml) and differentiated into macrophages by culturing with GM-CSF (20 ng/ml, #572904, Biolegend) for 8-10 days before being used for experiments; culture media was changed every other day. All experiments with hBMDM were performed in serum-free RPMI-1640 medium with no proteinase inhibitors unless otherwise specified.

Treatments of hBMDM with NE

After 8-10 days in culture, hBMDM were stimulated with NE (#SE563) or FITC-NE (#FS563, both from Elastin Products, Owensville, MO) or vehicle control in a serum-free RPMI medium supplemented with L-glutamine (2mM), 2.5mM HEPES and penicillin/streptomycin mixure (20 ng/ml 100 U/ml), for 2-4 h at 37°C for all experiments. At the end of each experiment, NE activity was stopped with NE specific inhibitor [MeOSuc-Ala-Ala-Pro-Val Chloromethyl Ketone (AAPV CMK, 10μM), #M0398, Sigma], and cells were processed for NE uptake, NE activity assays and MET analyses. In separate experiments, after treatments, cell lysates were prepared in lysis buffer (#9803, CST) containing protease (#P8340, Sigma) and phosphatase inhibitors (#P5726,

Sigma) for analysis of histone cleavage by Western analyses. Purity of hBMDM following cultures (~95%) was assessed by flow cytometry following cell labeling with the specific marker CD11b (250ng/1x10^6 cells, #101205, Biolegend), and viability was determined to be >98% by trypan blue dye exclusion. Cellular injury and cytotoxic effects of NE treatment was assessed by measuring the amount of LDH release (#TOX7, In Vitro Toxicology Assay Kit, Sigma) or by staining with Zombie Red Fixable Viability dye (#423109, Biolegend) per manufacturer's instructions.

Localization of FITC-NE in CF and non-CF hBMDM by Confocal Microscopy

Differentiated hBMDM (8-10 days in culture) from CF and non-CF donors were seeded (1x10^5 cells/slide) on poly-L-lysine coated glass coverslips placed in a 6-well plate. On the day of treatments, cells were washed with PBS and exposed to FITC-NE (200 or 500nM) or control vehicle for 2 or 4h. Following exposure, NE activity was stopped with AAPV-CMK and fixed with 4% paraformaldehyde (PFA) in PBS for 10 min room temperature. The cells were counterstaining with 4′,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI; 1µg/ml, #9542, Sigma) in PBS for 1 min and coverslips were mounted on glass slides. As a control for FITC-NE uptake into cells, hBMDM were exposed to fluorescein-labeled E.coli K-12 bio-particles for 1h, followed by washes in PBS, fixed with 4% PFA and stained with DAPI for nuclear staining and analyzed by confocal microscopy. Slides were examined to determine the localization and staining intensities of FITC-NE or fluorescein labeled E.coli bioparticles by confocal fluorescence microscopy (Zeiss LSM 700). Integrated fluorescence intensity of FITC-NE was normalized to nuclear DAPI fluorescence, and relative fluorescence units were quantified using ImageJ Software (Image-processing and analysis software, NIH).

Detection of intracellular NE activity in both CF and non-CF hBMDM. Human BMDM from CF and non-CF donors were harvested and cell aliquots divided into 2 equal parts for treatment with control vehicle, or NE 200nM for 2hr in suspension. At the end of 2 hr, cells were fixed in 2%

PFA, permeabilized with 0.1% NP40 for 10min and resuspended in PBS and cell numbers determined. Equal numbers of cells from each treatment group were diluted in PBS and mixed with equal volume of DQ-elastin (20µg/ml) in reaction buffer (E12056, EnzChek® Elastase Assay Kit). Eight replicates from each donor of cells in DQ-elastin mixture (100µl/well) were incubated in a 96 well black plate for each treatment condition. AAPV-CMK was added to three replicates to inhibit NE as a negative control. In addition, the reaction mixture, PBS/DQ-elastin (1:1), was added to 3 empty wells (100µl/well) to serve as a no-cell control. The plate was protected from light and incubated at room temperature overnight. Relative Fluorescence Intensity (RFU) was measured by TECAN fluorescence microplate reader (excitation/emission 505/515 nm). Relative NE activity was determined by RFU normalized to corresponding average no-cell control.

Quantification of NE induced METs release by PicoGreen

METs were quantified in the culture supernatant with the Quant-iT™ PicoGreen® dsDNA Assay kit (#P7589, Thermo Fisher Scientific) according to the manufacturer's instructions. Human BMDM ($1x10^5$/well) from CF and non-CF donors, were seeded on 96-well plates and after overnight incubation, cells were treated with NE (200 or 500nM) or control vehicle for 2h to induce MET release. Following treatments, micrococcal nuclease (MNase, 16U/mL, #M10247S, NEB) was added to the culture plates and incubated at 37°C for 20 min to release METs tethered to cells, and the reaction was stopped by adding 5mM EDTA. Supernatants containing cell-free DNA were collected and transferred to a 96 well plate for quantification of extracellular dsDNA by adding PicoGreen, (diluted 1:200 in 10 mM Tris/1 mM EDTA buffer, in the dark). Fluorescence was measured with the fluorescent plate reader (TECAN) with excitation/emission at 485/520 nm with optimized gain settings that were used throughout each experiment.

Visualization of NE induced METs by Confocal Microscopy

To visualize METs release by confocal microscopy, differentiated hBMDM from CF and non-CF donors were seeded ($1x10^5$ cells/slide) on poly-L-lysine coated glass coverslips placed in a 6-well

plate. On the day of treatments, cells were washed with PBS and stimulated with NE (200 or 500nM) or control vehicle for 2h. Following treatment, NE activity was stopped with AAPV-CMK and cells were fixed with 4% PFA. Coverslips were stained with DAPI and mounted on glass slides. Strands of extracellular DNA were evaluated by confocal microscopy (Zeiss LSM 700).

Determination of Vital or Suicidal METs

To determine whether cells that released METs were viable, cells were exposed to a fluorescent dye (ZOMBIE RED Fixable Viability Dye) that is taken up only by dead cells. CF and non-CF hBMDM, seeded onto poly-L-lysine glass coverslips, were stimulated with NE (200 or 500 nM, 2 h) or control vehicle, followed by incubation with ZOMBIE RED dye per manufacturer's instructions, washed and fixed. Following fixation, cells were stained with DAPI and visualized by confocal microscopy to determine whether the cells that released METs were viable or dead (ZOMBIE RED positive). As a positive control for cell death, a separate slide of hBMDM was treated with 30% or 20% alcohol for 30 seconds and then stained with ZOMBIE RED.

Visualization of NE-induced in vivo METs in mouse BAL macrophages

At 8-12 weeks of age, *Cftr*-null, FABP human CFTR Tg1/Jaw/J mice (JAX, Stock # 002364) and *Cftr*-wild-type (WT) littermates on the same mixed genetic background (C57BL/6 and 129P2/OlaHsd) were used in this study. The study protocol was approved by the Virginia Commonwealth University Animal Care and Use Committee. Animals were lightly anesthetized with 2% isoflurane and administered human NE (50 µg [43.75 U]/ 40 µl saline) or normal saline (NS) by oropharyngeal aspiration on Days 1, 4, and 7, as previously described (2, 3). On day 8, mice were killed by euthasol injection and bronchoalveolar lavage (BAL) samples (1 ml volume) were obtained for collecting cells. BAL macrophages were enriched by adherence onto poly-L-lysine coated glass coverslips (90 min), washed to remove non-adherent cells and fixed with 4% PFA. Following staining with DAPI (0.1µg/ml) in PBS for 1 min, coverslips were mounted on glass slides. Strands of extracellular DNA were evaluated by confocal microscopy (Zeiss LSM 700).

Quantification of BAL macrophages undergoing MET formation

To determine the percentage of macrophages that participate in the release of extracellular DNA, mice with *Cftr*-null, FABP human CFTR Tg1/Jaw/J mice (JAX, Stock # 002364) (CFKO) and *Cftr*-wild-type (WT) littermates on the same mixed genetic background (C57BL/6 and 129P2/OlaHsd) were used in this study. Mice (both males and females at 8-12 weeks of age) were killed by euthasol injection and lungs were subjected to bronchoalveolar lavage fluid collection for collecting cells. Within each group, BALs were pooled and macrophages were enriched by adherence onto poly-L-lysine coated glass coverslips (90 min), washed to remove non-adherent cells and further incubated overnight in growth media. The next day, cells were washed with PBS and exposed to NE (100, 200 or 500nM) or control vehicle for 2h. Following exposure, all cells were treated with AAPV-CMK and fixed with 4% PFA in PBS for 10 min at room temperature. The cells were then counterstaining with DAPI (1µg/ml) in PBS for 1 min and coverslips were mounted on glass slides. The strands of extracellular DNA were evaluated by confocal microscopy (Zeiss LSM 700). To determine the percentage of macrophages undergoing MET formation, confocal images (n=32 for WT & n= 42 for CFKO) were obtained from non-overlapping fields for each treatment condition, MET counts were performed by an observer (JAV) who did not know the treatment conditions. Cells exhibiting intact DNA strands were considered extracellular trap producing cells and included in the analyses. In total, 347 cells for WT and 424 cells for CFKO were counted for the analyses.

Detection of Histone H3 Citrullination by Confocal Microscopy

Histone H3 citrullination (citH3) in CF and non-CF hBMDM was evaluated by immunofluorescence and confocal microscopy. Human BMDM seeded on poly-L-lysine glass coverslips in a 6-well plate were treated with NE (200 or 500 nM) or control vehicle for 2h. Following exposure, coverslips were fixed with 4% paraformaldehyde for 10 min and then permeabilized with ice-cold

100% methanol. After washing, coverslips were blocked with 5% normal serum for 60 min followed by incubation with Histone H3 (citrulline residues at positions R2-R8-R17) rabbit polyclonal antibody (1:100, #ab5103 Abcam) for overnight at 4°C. Following PBS washes, coverslips were further incubated with goat anti-rabbit antibody conjugated with Alexa Fluor 488 (4mg/ml, Invitrogen) for 1h and then counterstained with DAPI (0.1µg/ml) before mounting them onto glass slides using VECTASHIELD vibrance antifade mounting medium (Vector Labs). Positive staining was evaluated by confocal microscopy (Zeiss LSM 700). Five random fields were captured for each donor. Intracellular citH3 expression was quantified by measuring integrated fluorescence intensity and normalizing to nuclear DAPI fluorescence (images not shown) using ImageJ Software.

Detection of Histone cleavage by Western Blot analyses

Cleavage of histone H3 or H4 in CF and non-CF hBMDM was evaluated by western analyses. Human BMDM were treated with NE (200 or 500nM) or control vehicle for 1 or 2h. Following exposure, total cell protein was isolated with Lysis Buffer (#9803, Cell Signaling Technology, (Danvers, MA) with 1x concentrations of protease inhibitors and phosphatase inhibitors (Sigma-Aldrich). The concentration of protein in the supernatants was determined by DC protein assay (Bio-Rad, Hercules, CA). Equivalent amounts of protein (30 µg) were separated by SDS-polyacrylamide gel electrophoresis (4–12%) and then transferred to a nitrocellulose membrane. After blocking, membranes were probed with primary antibody against Histone H3 (1:1000, #4499, CST) or Histone H4 (1:1000, #13919, CST) for overnight at 4°C. After washing, the membranes were further incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (1:4000, #7074, CST) and the immunoreactive protein complexes were detected using SuperSignal West Pico Chemiluminescent Substrate (#34580, Thermo Fisher Scientific). To confirm equal loading, blots were re-hybridized with antibody specific to β-actin (1:5000; #A5441, Sigma-Aldrich).

In-vitro Histone H3 Cleavage assay

Direct cleavage of histone H3 by NE was evaluated using human recombinant histone H3.1 and H3.3 isoforms. Purified H3.1 (#M2503S) or H3.3 (#M2507S, both from NEB, 500ng) was incubated in a reaction buffer (10mM HEPES, pH 6.0, 1mM DTT) containing NE (25 or 50nM) for 15 or 30 min at 37°C and the reaction was stopped by adding by 2x SDS loading buffer. Equal amounts of reaction products post NE incubations were analyzed by 12% SDS-PAGE gel electrophoresis and then transferred to a nitrocellulose membrane. Following transfer, membranes were washed with TBS-T (Bio-Rad) followed by 3 washes (5 min each) with distilled water before staining with colloidal gold stain (#170-6527, Bio-Rad). The length of the incubation varied depending on the protein amount loaded onto the gel, 30-60 min.

ELISAs to evaluate protein levels, M1 (TNFα) and M2 9(CCL-18) in culture supernatants

Human blood monocytes obtained from non-CF and CF donors were cultured for macrophage polarization as described earlier (4). Briefly, monocytes were cultured in growth media containing GM-CSF (20ng/ml) for 6 days in suspension before seeding them into 12-well plates for further polarization. One aliquot of cells were cultured with GM-CSF (20ng/ml) for an additional 4 days. On day 10, cell were washed and treated with NE (500nM) or vehicle control in a serum free RPMI media for 2h and the conditioned media was harvested from these culture conditions. As positive controls for M1 polarization, hBMDM were exposed to LPS (20ng/ml) in serum containing growth media and for M2 polarization, cells were exposed to IL4 (20ng/ml) and IL13 (20ng/ml) for 4 days with media change once at 48 h. On day 10, conditioned media was collected from each group and used as cytokine secretion controls. To enrich the secreted protein concentrations in the conditioned media, the samples were concentrated by 10- fold using Amicon Ultra Centrifugal Filters (cat# UFC500396, Ultracel, 3K, Millipore) and the concentrated conditioned media was stored at -80°C until further ELISA use. The levels of TNF-α were determined using ELISA kit

obtained from Biolegend (Cat # 430204) and CCL18 using ELISA kit from R&D (Cat # DY394-05), according to the manufacturer's instructions.

Statistical Analysis

Statistical analyses were performed using Statistix 8.0 (Analytical Software, Tallahassee, FL). Data, mean $\pm$ SEM, was analyzed using a one-way, nonparametric ANOVA (Kruskal-Wallis) test, followed by post hoc comparisons using the Wilcoxon rank sum test. $P < 0.05$ was considered statistically significant.

Table E1. The percentage of mouse alveolar macrophages positive for METs release

Genotype	NE (nM)			
	0	100	200	500
WT	0% (0/140 cells)	9.3% (7/75 cells)	11.1% (4/36 cells)	9.4% (9/96 cells)
CF KO	0% (0/76 cells)	7.3% (9/124 cells)	3.4% 3/87 cells)	5.8% (8/137 cells)

Supplement Figure Legends

Figure E1. Fluorescein-labeled (FITC) E.coli bio-particles taken up by non-CF hBMDM cells were localized in the cytoplasm, not the nucleus. Non-CF hBMDM grown on glass coverslips were exposed to fluorescein-labeled E.coli K-12 bio-particles for 1h. After incubation, cells were washed with PBS, fixed, stained with DAPI for nuclear staining and analyzed by confocal microscopy. Fluorescein-labeled bio-particles localized in cytoplasm are indicated by white arrows. Representative confocal image (3 separate experiments) (63x magnification) showing the presence of green bioparticles in the cytoplasm by merging the FITC conjugated bio-particles (FITC-BP), the DAPI nuclear stain with differential interface contrast (DIC).

Figure E2. Neutrophil elastase exposure caused no cytotoxicity as determined by lactate dehydrogenase (LDH) release. Non-CF hBMDM (A) and CF hBMDM (B) were exposed to control vehicle or neutrophil elastase (200 or 500nM) in a serum-free media for 2 to 4h. Conditioned media was collected and assayed for lactic dehydrogenase release, expressed as % total LDH release. Data shown are summarized (mean ± SEM) from 4 experiments including 3 non-CF (n= 6-10 replicates) and 1 CF individual (n= 4 replicates).

Figure E3. Neutrophil elastase induced METs released by hBMDM are viable cells as determined by the absence of Zombie Red staining. Non-CF hBMDM were exposed to 30% alcohol for 30s **(A)**, 20% alcohol for 30s**(B)**, vehicle control **(C)** and neutrophil elastase (200nM for 2h) **(D)**. Following exposure, cells were washed and stained with Zombie Red as well as DAPI for nuclear staining and analyzed by confocal microscopy. Representative images showing zombie red positive cells as dead cells. White arrows indicate positive Zombie Red+ cells and the arrowhead indicate cells with METs.

Figure E4. Human BMDM exhibited M1 like phenotype post- NE exposure as determined by cytokine secretion. Monocytes obtained from non-CF and CF donors, were differentiated to macrophages in primary culture with GM-CSF (10 days). These cells alone or after

stimulation with NE (500nM) or control vehicle for 2h were evaluated for cytokine expression in the conditioned media to determine M1 or M2 polarization. Quantitation of cytokines was performed by ELISAs: M1 cytokine, TNFα, in the conditioned media obtained from non-CF (**A**, data combined from 2 donors) and CF (**B**, data combined from 2 donors) and M2 cytokine, CCL-18, in the conditioned media obtained from non-CF (**C**, data combined from 2 donors) and CF (**D & E**, data from 2 individual donors). To generate positive controls for M1 and M2 macrophage differentiation, monocytes were cultured for 6 days with GM-CSF, and then treated with LPS (50ng/ml) for 4 days to generate M1 BMDM, or treated with IL-4 (20ng/ml) + IL-13 (20ng/ml) for 4 days; with media changes after 2 days. Conditioned media reflected two days of culture due to the treatment conditions from GM-CSF -differentiated BMDM cells, and the positive control cells for M1 (LPS treated) and for M2 (IL-4 and IL-13 treated). In contrast, conditioned media from control-treated and NE-treated cells reflected only contribution from 2 h of culture. NE treated cells had increased TNFα compared to control treated cells, but lower CCL-18 compared to control treated cells, consistent with M1 phenotype. The results are summarized (mean ± SEM) from 2 experiments including 2 non-CF and 2 CF individuals, n= 6-8 replicates. Statistically significant differences between NE and control treatment were determined by ANOVA with post-hoc comparisons by the Wilcoxon rank sum test: ++, $p< 0.008$ vs Ctrl treated. Statistically significant differences between M1-polarized and M2-polarized cells vs. GM-CSF treated cells for TNFα expression (*, $p<0.025$, **, $p<0.0002$); and for CCL-18 expression (#, $p <0.02$, vs GM-CSF treated) are shown.

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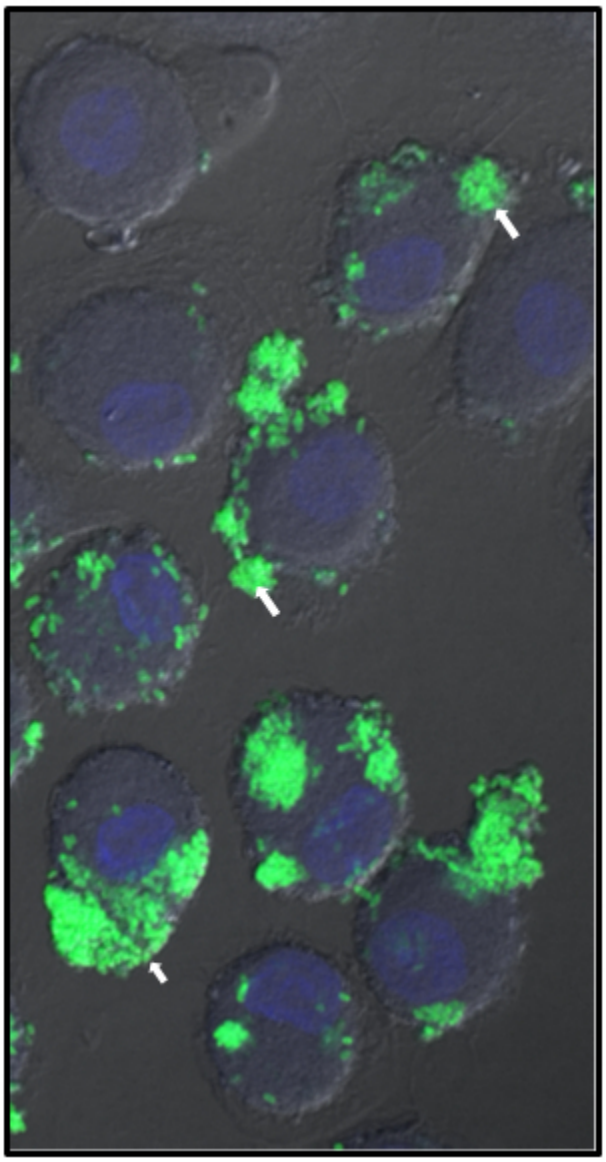

Figure E1

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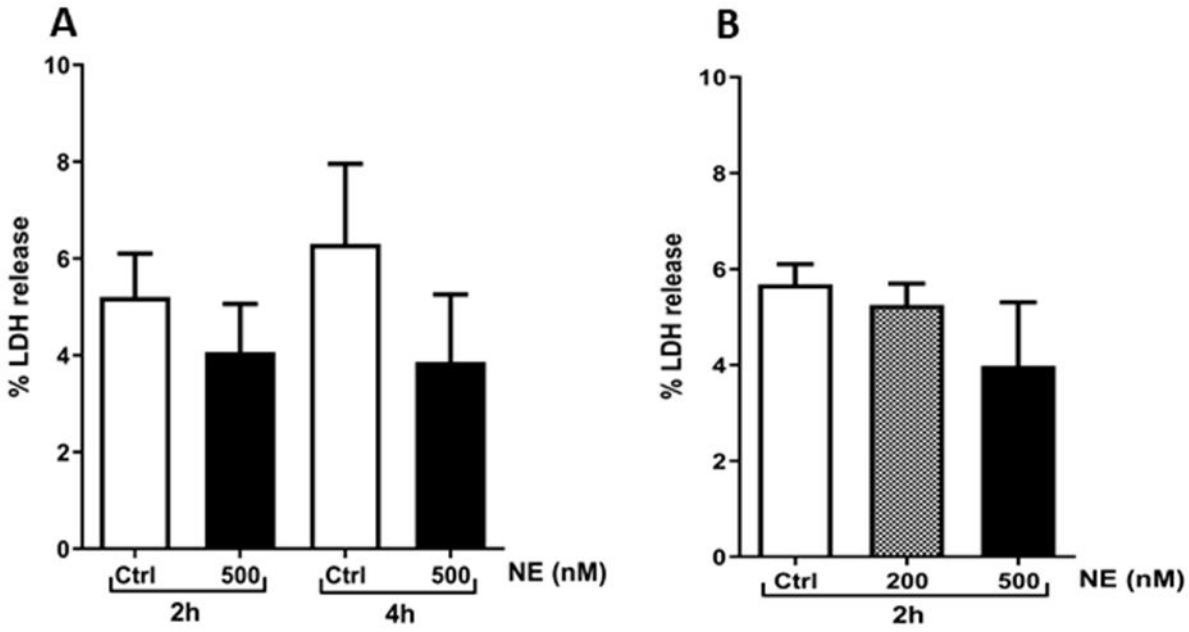
Figure E2



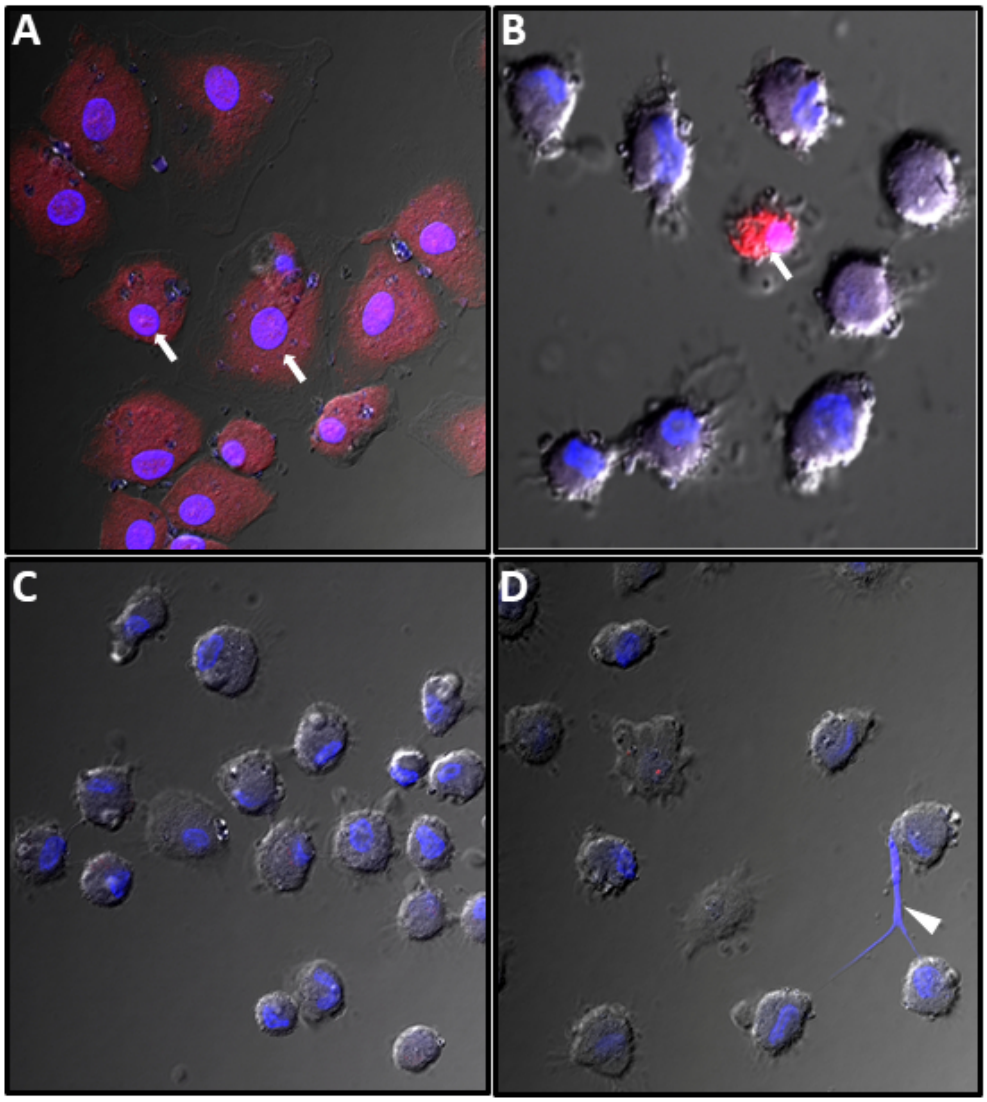


Figure E3

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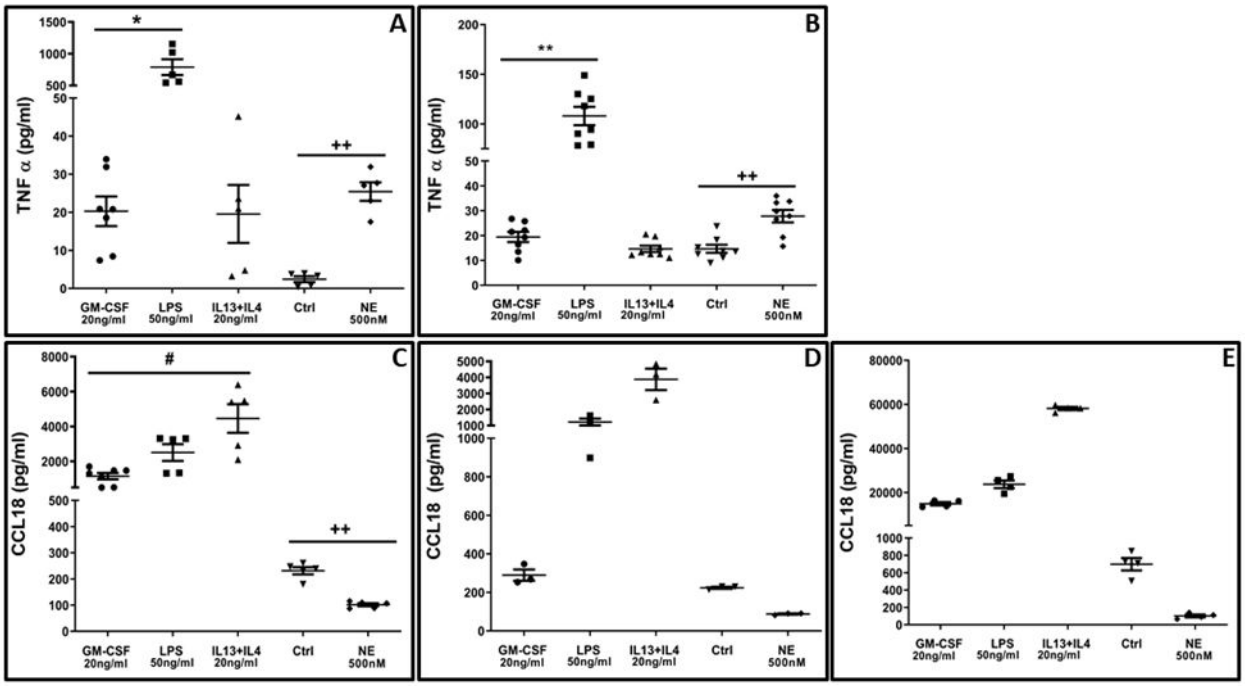
Figure E4



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RESEARCH ARTICLE

Translational Physiology

Neutrophil elastase-regulated macrophage sheddome/secretome and phagocytic failure

Jonathan Ma,[1] Apparao B. Kummarapurugu,[1] Adam Hawkridge,[2] Shobha Ghosh,[3] Shuo Zheng,[1] and Judith A. Voynow[1]

[1]*Department of Pediatrics, Children's Hospital of Richmond at Virginia Commonwealth University, Richmond, Virginia;* [2]*Department of Pharmaceutics/Department of Pharmacotherapy & Outcomes Science, Virginia Commonwealth University School of Pharmacy, Richmond, Virginia; and* [3]*Department of Internal Medicine, Virginia Commonwealth University School of Medicine, Richmond, Virginia*

Abstract

Patients with cystic fibrosis (CF) have defective macrophage phagocytosis and efferocytosis. Several reports demonstrate that neutrophil elastase (NE), a major inflammatory protease in the CF airway, impairs macrophage phagocytic function. To date, NE-impaired macrophage phagocytic function has been attributed to cleavage of cell surface receptors or opsonins. We applied an unbiased proteomic approach to identify other potential macrophage targets of NE protease activity that may regulate phagocytic function. Using the murine macrophage cell line, RAW 264.7, human blood monocyte-derived macrophages, and primary alveolar macrophages from Cftr-null and wild-type littermate mice, we demonstrated that NE exposure blocked phagocytosis of *Escherichia coli* bio-particles. We performed liquid chromatography-tandem mass spectroscopy (LC-MS/MS) proteomic analysis of the conditioned media from RAW264.7 treated either with active NE or inactive (boiled) NE as a control. Out of 840 proteins identified in the conditioned media, active NE upregulated 142 proteins and downregulated 211 proteins. NE released not only cell surface proteins into the media but also cytoskeletal, mitochondrial, cytosolic, and nuclear proteins that were detected in the conditioned media. At least 32 proteins were associated with the process of phagocytosis including 11 phagocytic receptors [including lipoprotein receptor-related protein 1 (LRP1)], 7 proteins associated with phagocytic cup formation, and 14 proteins involved in phagocytic maturation (including calpain-2) and phagolysosome formation. NE had a broad effect on the proteome required for regulation of all stages of phagocytosis and phagolysosome formation. Furthermore, the NE sheddome/secretome included proteins from other macrophage cellular domains, suggesting that NE may globally regulate macrophage structure and function.

calpain-2; cystic fibrosis; macrophage; neutrophil elastase; proteomics

INTRODUCTION

Cystic fibrosis (CF) lung disease is marked by the failure to resolve airway infections and recurrent cycles of bronchitis with inflammation (1). In the CF airway, a major component of this innate immune failure is defective macrophage phagocytosis and efferocytosis (1). Phagocytosis is a complex physiologic process, vital for protecting the airway against potential pathogens and toxins. Macrophages from cystic fibrosis patients have defective phagocytosis attributed at least in part to loss of cystic fibrosis transmembrane conductance regulator (CFTR) function (2).

However, there is growing evidence to suggest that CFTR dysfunction alone cannot fully explain CF macrophage phagocytic failure in the airways which may be due to persistent inflammation. It has been shown that exposure to mucopurulent sputum from the cystic fibrosis lung is able to increase markers of inflammation in both healthy and CF macrophages (3). Furthermore, non-CF macrophages that are treated with bronchoalveolar lavage (BAL) fluid from CF patients have reduced expression of phagocytic receptors mCD14 and HLA-DR (4). Neutrophil elastase (NE) is present in micromolar concentrations in the CF airway (5) and it independently contributes to impaired macrophage innate immunity (6). NE degrades cell surface receptors required for both neutrophil and macrophage phagocytosis including complement receptors CR1 and CR3, neutrophil Fcγ receptors (7), neutrophil opsonins and opsonin receptors (8), macrophage phosphatidylserine receptor (9), and macrophage mCD14 and monocyte human leukocyte antigen-DR isotype (HLA-DR) (4). However, the mechanisms of NE-induced macrophage phagocytic dysfunction are not completely

Correspondence: J. A. Voynow (judith.voynow@vcuhealth.org).
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known. We hypothesize that NE has a broader impact on macrophage function beyond degradation of cell surface receptors. NE preferentially cleaves proteins at valine, alanine, and isoleucine (the NE extracellular sheddome) (10) and NE also upregulates gene expression, translation, and secretion (the NE secretome) (11). The secreted and cleaved proteins following NE exposure may provide new insights into how NE abrogates macrophage phagocytic function. To investigate this possibility, we utilized a mouse macrophage cell line, RAW264.7, and evaluated the impact of active NE exposure on the cellular secretome and sheddome using a proteomic-based approach. We confirmed our findings in the conditioned media from NE-treated human monocyte-derived macrophages (hMDM) and in CF bronchoalveolar lavage specimens.

MATERIALS AND METHODS

RAW Cells and NE Treatment

RAW 264.7, a murine macrophage cell line (ATCC TIB-71) was cultured to 80% confluence (10^7 cells) in DMEM + 10% fetal bovine serum + 100 IU/mL penicillin-100 μg/mL streptomycin media. After washing cells to remove serum, cells were exposed to either active NE (NE+, 500 nM) or inactive/boiled NE (NE−) for both phagocytosis assay and to generate the conditioned media for LC-tandem mass spectrometry (LC-MS/MS) analysis. Supplemental methods are provided in the Supplemental Material (https://doi.org/10.6084/m9.figshare.11310629).

Human Blood Monocyte-Derived Macrophages Culture

Whole blood (10–20 mL) from healthy participants was used to isolate monocytes for differentiation to human blood monocyte-derived macrophages (hMDM) by the method of Winkler et al. (12) for hMDM phagocytosis assays. For hMDM Western blot analyses, buffy coat isolated from healthy volunteers was obtained from the American Red Cross and used to isolate monocytes for differentiation to hMDM by the method of Aksoy et al. (13).

Subject Recruitment

A total of six pediatric subjects with CF (Table 1) were recruited for BAL samples at the time of clinically indicated bronchoscopy at Children's Hospital of Richmond at Virginia Commonwealth University. The diagnosis of CF was confirmed by sweat test after newborn screening and by CFTR genotype. This study was approved by the Virginia Commonwealth University Institutional Review Board for human research (IRB Protocol No. HM20011759), and written informed consent was obtained. Control subject bronchoalveolar lavage fluid (BALF) samples were collected from non-CF healthy volunteers after written informed consent at the Environmental Protection Agency under a University of North Carolina-Chapel Hill Institutional Review Board-approved research protocol (PI, Dr. Andrew Ghio, M.D.). Consent was obtained prior to potential collection and use of BAL fluid by IRB-approved protocol.

Table 1. *Subject demographics*

Subject	Sex	Race	Age, yr	Mutation
BCF1	M	White	9	*Phe508del/Phe508del*
BCF2	M	White	11	*Phe508del/Phe508del*
BCF3	M	White	11	*Phe580del/c.1117-G->A*
BCF4	F	White	18	*Phe508del/Phe508del*
BCF5	M	White	9	*Phe508del/Phe508del*
BCF6	F	White	5	*Phe580del/R553X*
HC1485	F	White	26	N/A
HC1487	M	Black	34	N/A
HC1489	M	Asian	39	N/A
HC1491	M	Black	40	N/A
HC1497	M	Black	40	N/A
HC1500	M	White	21	N/A

F, female; M, male.

Murine Alveolar Macrophage Collection

A well-characterized CF model, *Cftr*-null, gut-corrected mice (JAX Cftrtm1Unc Tg(FABPCFTR)1Jaw/J; Stock # 0022364), and their wild-type congenic littermates were used to harvest resident alveolar macrophages for phagocytosis assays under an IACUC-approved protocol at Virginia Commonwealth University. At 6 to 8 wk of age, 6–8 mice of each genotype were euthanized and bronchoalveolar lavage (BAL) was performed by instilling 1 mL of sterile normal saline and extracted for a total of two aliquots. The BAL fluid was pooled for each genotype and collected by centrifugation (500 *g*, 10 min) to harvest the macrophage cells for phagocytosis assays.

Human Bronchoalveolar Lavage Fluid Collection

BALF was obtained from six pediatric subjects with CF (2 female, 4 male) who underwent clinically indicated bronchoscopy to obtain respiratory cultures. The mean age was 10.5 yr and four were *Phe508del* homozygous and two were *Phe508del* heterozygous. Patients were anesthetized with intravenous propofol and inhaled sevoflurane and underwent laryngeal mask airway placement. Topical 2% lidocaine was applied to the vocal cords and a fiberoptic bronchoscope was passed sequentially to each lobe for lavage with normal saline in 1 mL/kg aliquots (up to 20 mL). A portion of BAL fluid (2 mL) was collected under IRB approval, centrifuged (500 *g*, 10 min) to pellet cellular debris, and aliquoted and frozen at −80°C. Consent was obtained before potential collection and use of BAL fluid by IRB-approved protocol.

Bronchoalveolar lavage fluid was obtained from healthy, nonsmoking adult volunteers who underwent research fiberoptic bronchoscopy with bronchoalveolar lavage as described in Ghio et al. (14), and provided to us from Dr. Ghio as deidentified BALF aliquots.

Confocal Microscopy Phagocytosis Assay

RAW 264.7 cells (10^5 cells), hMDM (4×10^5 cells), *Cftr*-null, and *Cftr*-wildtype murine alveolar macrophages (10^5 cells) harvested from bronchoalveolar lavage as described in Karandashova et al. (15) were treated with NE (500 nM), or vehicle control at 37°C, for 4 h (RAW cells) or 2.5 h (hMDM, murine alveolar macrophages). Cells were incubated with fluorescein-labeled *E. coli* K-12 bio-particles (200 μL of 6×10^8 particles/mL, Thermo Fisher Scientific, V-6694), 37°C, 1 h, fixed with paraformaldehyde, stained with

4′,6-diamidino-2-phenylindole (1.43 mM DAPI, Thermo Fisher Scientific, D1306), and sealed with mounting media. Confocal microscopy images were obtained with Zeiss LSM 700. Mean integrated fluorescence intensity of phagocytosed bio-particles was determined using ImageJ Software and data presented as total fluorescein: 4′,6-diamidino-2-phenylindole (DAPI) ratio.

Proteomics Sample Preparation and LC-MS/MS Analysis

Detailed proteomics sample preparation and LC-MS/MS analysis conditions are described in the online supplement. Briefly, the active and inactive NE-treated RAW 264.7 conditioned media (20 mL) was concentrated using 10 kDa molecular mass cutoff filters (MWCO) and subsequently prepared for

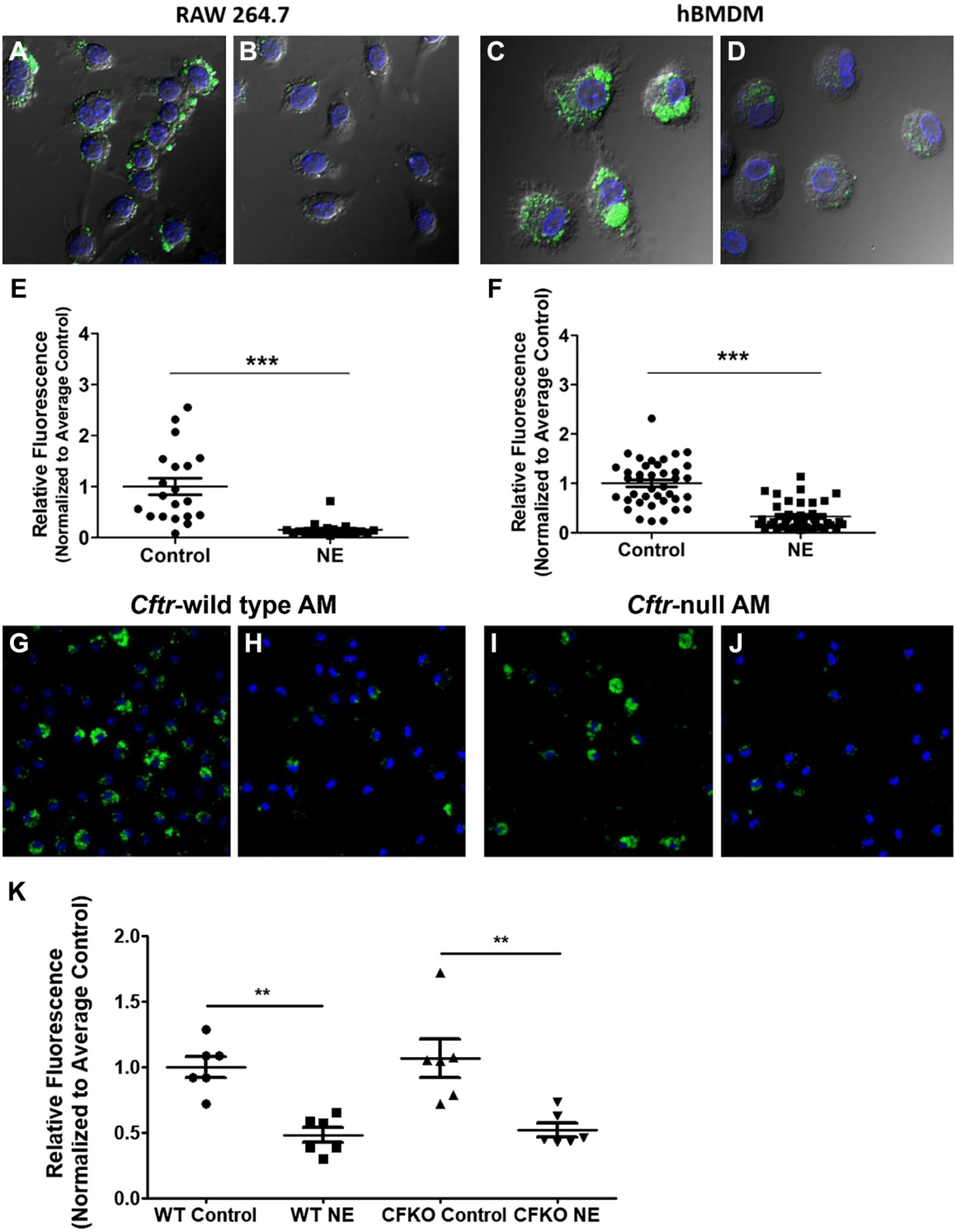


Table 2. *Summary statistics of the LC-MS/MS and database searches*

	Summary Statistic
LC-MS/MS	
Number of fractions	2 conditions, 3 replicates (2 × 3)
Search engine	Andromeda
Total number of acquired spectra	51,209
Number of identified peptides	5,459
Number of identified proteins	1,482
Number of proteins present in ≥ 2 of 6 MS/MS runs	840
GO annotation	
Plasma membrane	186
Exosome	12
Mitochondria	70
Nucleus	454
Cytosol	390
Cytoskeleton	135
Phagosome	9

GO, gene ontology; LC-MS/MS, liquid chromatography tandem-mass spectrometry.

shotgun proteomics analysis using a modified filter-assisted sample preparation (FASP) protocol (16). Shotgun proteomics samples were analyzed in technical triplicate by data-dependent LC-MS/MS which consisted of an Eksigent 415 nanoflow HPLC system (Sciex, Framingham, MA) coupled to a Q-Exactive Orbitrap tandem mass spectrometer (Thermo Fisher Scientific, San Jose, CA).

Proteome Data Analysis

Proteomic data sets were processed in MaxQuant (v. 1.6.0.16; Max Planck Institute of Biochemistry, Martinsried, Germany) and searched using the Andromeda search algorithm against the Uniprot Murine Proteome FASTA database (download: 1/22/2018) and Human Neutrophil Elastase P08426 FASTA file. Perseus (Max Planck Institute of Biochemistry, Martinsried, Germany) was used for relative label-free quantification (LFQ). For functional analysis, String Database 11.0 (https://string-db.org/) (17) and DAVID Bioinformatics 6.8 (https://david.ncifcrf.gov/) analysis (18) was performed.

Western Analysis of Conditioned Media to Confirm Proteomic Targets

RAW 264.7 macrophage cells (equal seeding onto Thermo Fisher Nunc P100 dish and treated at 80% confluence) or hMDM macrophage cells (equal seeding onto Thermo Fisher Nunc P100 dish, treated at 40%–50% confluence), were treated with NE (500 nM), or vehicle control for 2 h at 37°C, and 7 mL conditioned media was collected, concentrated, and separated by 4%–20% PAGE for Western analysis. Similarly, 38 μL of BALF from six subjects with CF and six non-CF controls was separated by 4%–20% PAGE for Western analysis. Western analyses were performed for Calpain-2 (Calpain 2 Large Subunit Ab, 1:1,000, Cat. No. 2539, Cell Signaling Technology), low-density lipoprotein receptor-related protein 1 (LRP1, anti-LRP1 Ab, 1:1,000, Cat. No. ab168454, Abcam), or Histone 1.4 (anti-histone H1 Ab AE4, 1:1,000, Cat. No. sc-8030), three of the highest enriched proteins identified by LC-MS/MS in the NE+ conditioned media. Following incubation with secondary antibodies (anti-rabbit IgG, HRP-linked Ab, 1:8,000, Cat. No. 7074, Cell Signaling Tech or horseradish peroxidase-conjugated sheep anti-mouse (1:6,000–10,000, Cat. No. NA931; GE Healthcare), Western blots were developed with enhanced chemiluminescence (ECL, GE). Western band densities were quantitated using Image-J (19).

Statistical Analysis

For the phagocytosis assays, statistical differences between treatment conditions were determined by Mann-Whitney test for RAW264.7 and hMDM experiments and one-way ANOVA with post hoc testing for the murine alveolar macrophage experiments. For Western analyses, the relative abundance of Calpain-2, LRP1, and Histone 1.4 between treatment conditions or between CF and non-CF BAL was compared by Mann-Whitey test.

RESULTS

Neutrophil Elastase Impaired Macrophage Phagocytic Function

We investigated the effect of NE on macrophage phagocytic function. We found that RAW 264.7 macrophage cells had significantly decreased phagocytosis post-NE treatment (500 nM, 4 h) compared with control vehicle treatment (Fig. 1, *A* and *B*) as determined by mean integrated fluorescence intensity of nonopsonized fluorescein-labeled *E. coli* K-12 bio-particles in the cytoplasm (Fig. 1*E*). Similarly, we found that hMDM from healthy individuals had significantly decreased phagocytosis when exposed to 500 nM NE for 2.5 h when compared with control vehicle-treated hMDM (Fig. 1, *C* and *D*), quantified by mean integrated fluorescence intensity of fluorescein: DAPI (Fig. 1*F*). We also found that resident alveolar macrophages from both *Cftr*-null (Fig. 1, *I* and *J*) and *Cftr*-wildtype (Fig. 1, *G* and *H*) littermates both had significantly decreased phagocytosis when exposed to

Figure 1. Neutrophil elastase impaired macrophage phagocytic function. RAW 264.7 macrophage cells (*A*, *B*), human peripheral blood monocyte-derived macrophages (hMDM, *C*, *D*), or murine alveolar macrophages (AM, *G*, *H*, *I*, *J*) seeded on glass coverslips were treated with NE (500 nM) or vehicle control for 4 h (RAW) or 2.5 h (hMDM, AM) followed by incubation with fluorescein-labeled *Escherichia coli* K-12 bio-particles for 1 h. After incubation, cells were fixed with paraformaldehyde, stained with 4′,6-diamidino-2-phenylindole (DAPI for nuclear staining), and sealed with mounting media. Confocal microscopy images were obtained with Zeiss LSM 700. Representative images are shown of RAW 264.7 (*A*, *B*), hMDM (*C*, *D*), *Cftr*-wildtype AM (*G*, *H*), or *Cftr*-null AM (*I*, *J*) treated with control vehicle (*A*, *C*, *G*, *I*) or NE (*B*, *D*, *H*, *J*). Control vehicle-treated cells revealed the presence of opsonized fluorescein-labeled (FITC) *E. coli* K-12 bio-particles (green) in the cytoplasm which was markedly decreased in the NE-treated cells. Morphology is shown by merging the FITC green fluorescence, the DAPI blue nuclear stain + differential interference contrast (DIC). The phagocytic index was determined by the ratio of green fluorescence intensity divided by the DAPI fluorescence to normalize phagocytic activity per cell. Graphical representation of the mean integrated fluorescence intensity of phagocytosed bio-particles, determined using ImageJ Software [RAW264.7, $n = 20$ cells/group from 3 experiments (*E*); hMDM, $n = 40$ cells/group, from 4 donors (*F*); murine AM, $n = 6$ replicates/group from 3 experiments (*K*)]. Results are presented as means ± SEM and significant differences between NE treated vs. vehicle control as determined by Mann-Whitney test for RAW264.7 and hMDM and one-way ANOVA with posthoc testing for murine AM. Conditions are noted by $^{**}P < 0.01$, $^{***}P < 0.001$. DAPI, 4′,6-diamidino-2-phenylindole; NE, neutrophil elastase.

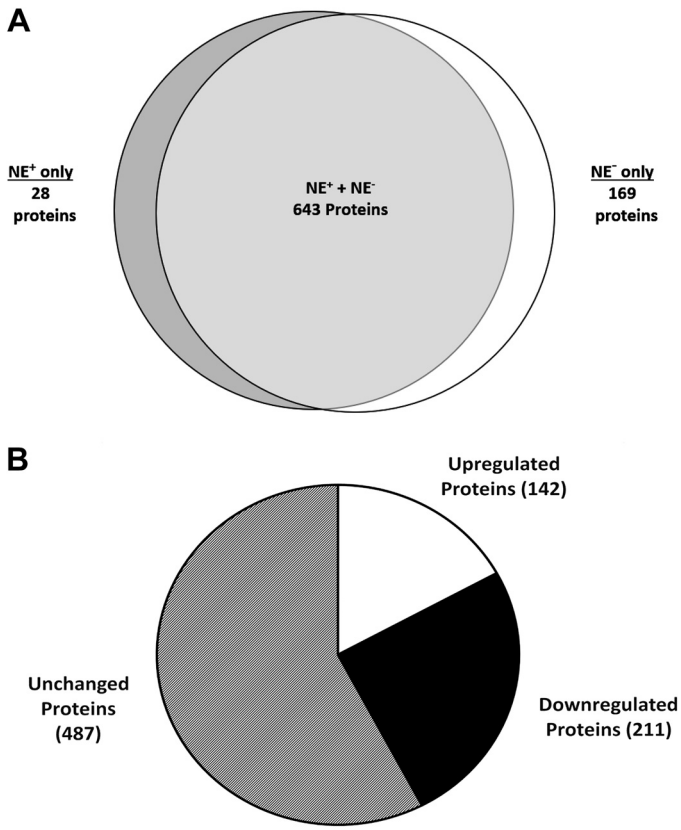


Figure 2. RAW264.7 media proteome following active NE vs. inactive NE (Control) treatments. The Venn diagram shows the comparison of NE+ and NE− (control) culture media proteomes. Of the 840 proteins identified present in at least 2 of 6 LC-MS/MS runs, 28 were found in only the NE+ fractions, 169 in only the NE− fractions, and 643 in both fractions (*A*). The majority (57.3%) of proteins identified were not significantly changed by NE treatment (the magnitude of log2 fold NE+/NE− < 1.00). Of the 840 total identified proteins, 142 (17%) were upregulated by NE treatment (log2 fold NE+/NE− > 1.00) and 211 proteins (25%) were downregulated by NE treatment, and the remaining proteins were not changed in abundance between NE+ and NE− treatment conditions (*B*). LC-MS/MS, liquid chromatography-tandem mass spectroscopy; NE, neutrophil elastase.

500 nM NE for 2.5 h compared with control vehicle treated (Fig. 1*K*).

Proteomic Landscape of RAW 264.7 Culture Media Proteome

The effect of NE on macrophage phagocytosis function is likely to involve many different processes including NE-triggered protein secretion and NE-cleaved proteins shed from the cell surface and extracellular domain. To catalog NE-related changes in the proteome of the extracellular milieu and identify potentially novel mechanisms by which NE effects phagocytic function, we employed shotgun proteomics to examine the conditioned media of macrophages exposed to either active or inactivate (i.e., boiled) NE. A total of 1,483 proteins were identified in the LC-MS/MS proteomics analysis of these two proteomes from RAW cells (Table 2 and Supplemental Table S1; https://doi.org/10.6084/m9.figshare.14785983). We then filtered out low abundant proteins by accepting only those proteins that were found in at least two of the six MS/MS runs for quantitative analysis. A total of 840 proteins remained after applying this filter, of which 643 were found in both the NE+ and NE− fractions, 28 were present only in the NE+ media, and 169 were present only in the NE− media (Fig. 2*A*). NE upregulated 142 proteins (log_2 fold-change NE+/NE− >1.00) consistent with increased shedding or secretion and possibly upregulation of expression, and NE downregulated (log_2 fold-change NE+/NE− < −1.00) 211 proteins consistent with NE degradation or transcriptional and or translational downregulation (Fig. 2*B*).

Analysis of the data using Gene Ontology of the 840 proteins found in at least 2 of 6 MS/MS runs revealed 186 plasma membrane proteins, 12 extracellular exosome proteins, 135 cytoskeletal proteins, 70 mitochondrial proteins, and 454 nuclear proteins, and 390 cytosolic proteins (Table 2 and Supplemental Table S1).

The Global Effect of Neutrophil Elastase on Macrophage Phagocytosis and Immune Regulation

Of the total sheddome and secretome generated, we were particularly interested in 32 proteins involved in

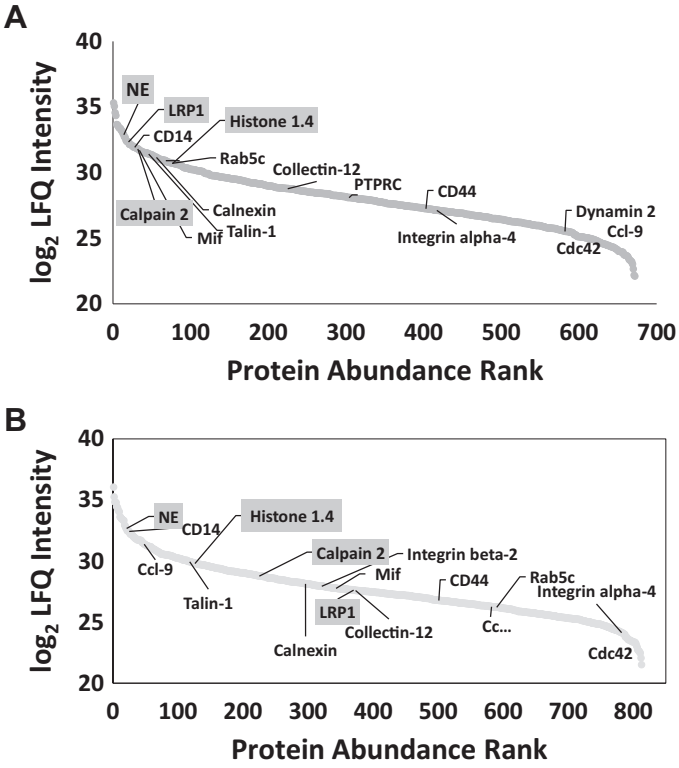


Figure 3. Rank abundance curve of proteins identified in NE-treated and control-treated media. Rank abundance curves were constructed based on rank of abundance vs. protein abundance determined by label-free quantitation (LFQ) separately for the NE+ (*A*) and NE− (control) (*B*) fractions. In total, there were 672 proteins with a detectable LFQ intensity in the NE+ fractions and 813 proteins in the NE− fractions. Selected phagocytosis proteins are displayed by their relative abundance and rank. The relative levels of human NE protein (flagged in grey) are similar between active and inactive NE-treated conditioned media consistent with the introduction of the same total protein for each treatment condition. Three of the most enriched proteins in the NE-active treatment condition, Calpain-2, Histone 1.4, LRP1 (flagged in grey) are highlighted to demonstrate their decrease in rank order of abundance in the NE-inactive (control) media. LRP1, lipoprotein receptor-related protein 1; NE, neutrophil elastase.

Table 3. *Phagocytic receptor proteins*

Gene	Protein	Relative Abundance [log2 (+NE/−NE)]	Function
Lrp1	Low-density lipoprotein receptor-related protein	4.71	Efferocytosis of apoptotic cells
Ptprc	Protein tyrosine phosphatase, receptor type, C	3.97	Efficient FcR-mediated phagocytosis
Itgb2	Integrin β-2	2.35	Receptor for C-lectin, complement C3
Itga4	Integrin α-4	2.24	Receptor for IgG3 in FcR-mediated phagocytosis
Colec12	Collectin-12	1.72	Scavenger receptor
H-2 K-1	H-2 class I histocompatibility antigen, K-D alpha chain	1.25	MHC class I complex
H2-L	H-2 class I histocompatibility antigen, L-D α-chain	1.00	MHC class I complex
Cd44	Extracellular matrix receptor III	0.47	Receptor for hyaluronic acid
C3	Complement C3	0.27	Positive regulation of phagocytosis
Cd74	H-2 class II histocompatibility antigen γ-chain	−0.24	MHC class II complex
Cd14	Monocyte differentiation antigen CD14	−0.50	Coreceptor for bacterial lipopolysaccharide

phagocytosis (Fig. 3), including cell surface receptor proteins for phagocytic and apoptotic cell targets (Table 3), and proteins required for phagocytic cup formation, and phagosome generation and maturation (Tables 4 and 5). Importantly, the relative concentration of NE (denoted by the ELANE gene product) was not different between treatment conditions ($\log_2$ NE/-NE), consistent with the same concentration of NE protein in each treatment condition. NE has been shown to modulate the levels of cytokines and chemokines released by macrophages (6, 20). In our proteomic analysis, NE increased the release of macrophage inhibitory factor protein (Mif), which is a major macrophage and monocyte chemokine (21) that is increased in those with cystic fibrosis and that may contribute to reduced innate defense against *Pseudomonas aeruginosa* and increased airway inflammation (22). NE decreased levels of macrophage inflammatory protein-1β (MIP-1β or Ccl-4) and macrophage inflammatory protein-1γ (MIP-1γ or Ccl-9), which is congruent with the findings in a recent investigation of the effect of NE on reduction of MIP-1β a Pneumococcal pneumonia model (6). The top 10 ranked enriched functional pathways by Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were generated for the NE upregulated proteins (Fig. 4). The most highly enriched KEGG pathway in the NE upregulated proteins was phagocytosis. Several other top categories involved protein processing, secretory pathways, proteasomal processing, and leukocyte transendothelial migration. However, other unexpected protein pathways regulated by NE were identified including proteins associated with Alzheimer's disease, the ribosome, and oxidative phosphorylation. A summary list of the differentially expressed proteins in the media is summarized in Supplemental Table S1.

Western Analysis Confirmation of Differentially Expressed Macrophage Proteins

To confirm proteomic results, we selected three proteins that were most significantly upregulated in conditioned media post-NE treatment compared with inactive NE by MS/MS analysis and used Western analysis to confirm that their protein expression was increased in NE-treated conditioned media. We demonstrated that for both RAW264.7 cells and hMDM cells, NE increased secretion/release of Calpain-2, a protease that is responsible for phagocytic cup formation and macrophage motility (Fig. 5*A*) and LRP1, a receptor for efferocytosis (Fig. 5*B*), confirming the relative increase in abundance of these proteins in conditioned media by proteomic analysis. We also found that NE increased the release of Histone 1.4, a linker histone, (Fig. 5*C*) for both RAW264.7 and hMDM cells. Interestingly, the size of Calpain-2 (MW ~75 kDa) and Histone 1.4 (MW 30 kDa) revealed an intact protein, whereas LRP1 (MW 515 kDa) was digested to a smaller size (~70 kDa for RAW264.7, and ~75 kDa and ~120 kDa for hMDM in the conditioned media, and a smaller size (~50 kDa) in CF BALF. Consistent with the LRP1 size by Western analysis (~75kDa for RAW264.7 and ~120kDa and ~75kDa for hMDM), NE proteolysis map (Fig. 6) revealed cleavage sites in LRP1 that would generate roughly 75 kDa peptides by cleavage between amino acids 246 and 952 and assuming an average amino acid mass of 110 Da as observed by Western analysis and would be detected by the primary antibody with an amino terminus epitope. Likewise, a 120 kDa peptide as seen in hMDM media could be generated by cleavage between amino acids 246 and 1,350. The identification of only ~55kDa and ~40kDa fragments of LRP1 in the CF BALF fluid suggests the presence of additional proteases in addition to neutrophil elastase to account for the smaller fragments.

Table 4. *Phagocytic cup formation proteins*

Gene	Protein	Relative Abundance [log2 (+NE/−NE)]	Function
Rab5c	Ras-related protein Rab-5C	5.32	GTPase initiating actin remodeling in FcR-mediated phagocytosis
Capn2	Calpain-2 catalytic subunit	4.70	Protease, selected cleavage of cytoskeletal phagocytic machinery
Canx	Calnexin	3.02	Chaperone
Dnm2	Dynamin II	2.36	GTPase regulating membrane scission
Cdc42	Cell division control protein 42 homolog	1.44	GTPase initiating actin remodeling in FcR-mediated phagocytosis
Myh9	Myosin-9	1.31	Phagosome cup closure in FcR-mediated phagocytosis
Anax1	Annexin A1	0.61	Actin remodeling

Table 5. *Phagocytic maturation and phagolysosome proteins*

Gene	Protein	Relative Abundance [log2 (+NE/−NE)]	Function
Rab14	Ras-related protein Rab-14	3.90	Regulatory GTPase
Nrp2	Neuropilin-2	3.57	Regulates Rab5c levels for early phagosome maturation
Anln	Actin-binding protein anillin	2.08	Cytoskeletal-plasma membrane dynamics
Atp6v1c1	V-type proton ATPase subunit C 1	1.83	Phagosomal acidification
Sep7	Septin-7	1.47	Actin cytoskeleton regulation
Tln1	Talin-1	1.47	Cytoskeletal-plasma membrane dynamics
Flna	Filamin-A	1.42	Cytoskeletal-plasma membrane dynamics
Dync1h1	Cytoplasmic dynein 1 heavy chain 1	1.39	Phagosome transport
Ctsd	Cathepsin D	1.31	Protease
Msn	Moesin	1.15	Cytoskeletal-plasma membrane dynamics
Actn1	α-Actinin-1	0.93	Actin cytoskeleton regulation
Rac1	Ras-related C3 botulinum toxin substrate 1	−0.76	Actin cytoskeleton regulation
Rab7A	Ras-related protein Rab-7A	−0.95	Regulatory GTPase
Stx7	Syntaxkin-7	−1.24	Fusion of endosomes and lysosomes with phagosome

Similarly, we detected significantly increased Calpain-2 (Fig. 5*A*), LRP1 (Fig. 5*B*), and Histone 1.4 (Fig. 5*C*) in CF BALF compared with non-CF BALF.

DISCUSSION

To investigate potential mechanisms of phagocytic failure in the CF airway induced by NE, we employed an unbiased proteomic approach to study the changes in the extracellular milieu of a macrophage cell line, RAW264.7, exposed to active versus inactive NE. To our knowledge, this is the first analysis of the impact of NE on a macrophage proteome. There were dramatic alterations in the shed and/or secreted proteome of macrophage RAW264.7 conditioned media following NE exposure. We identified cell surface proteins that were cleaved and released as well as proteins that were secreted.

Previous reports focused on the impact of NE on neutrophil phagocytic function. NE sheds or releases phagocytic receptors from neutrophils including CR1 and CR3, Fcγ receptors (5), opsonins and opsonin receptors (8), phosphatidylserine receptor (9), and HLA-DR (9) as part of a mechanism to abrogate neutrophil phagocytosis. In addition, NE cleaves complement receptor CR3 from the neutrophils of patients with CF, resulting in an "opsonin-receptor mismatch" (8). Our report demonstrates that NE also cleaved macrophage receptors required for phagocytosis, suggesting that NE exposure impairs both neutrophil and macrophage phagocytic function by a similar mechanism.

We also report that NE caused the upregulation or secretion of several proteins required for the generation and maturation of the phagosome, cytoskeletal dynamics, and macrophage motility (such as regulatory GTPases and calpain-2), all important functional proteins that have not previously been linked with inflammation-induced phagocytic impairment. NE has been reported to have many cellular functions including transcriptional and posttranscriptional upregulation and activation of signaling molecules. Upregulation of mucins MUC5AC (11, 23), MUC4 (24), MUC1 (25) by NE has been reported in epithelial cells. NE cleavage activates ion channels, for example the epithelial sodium

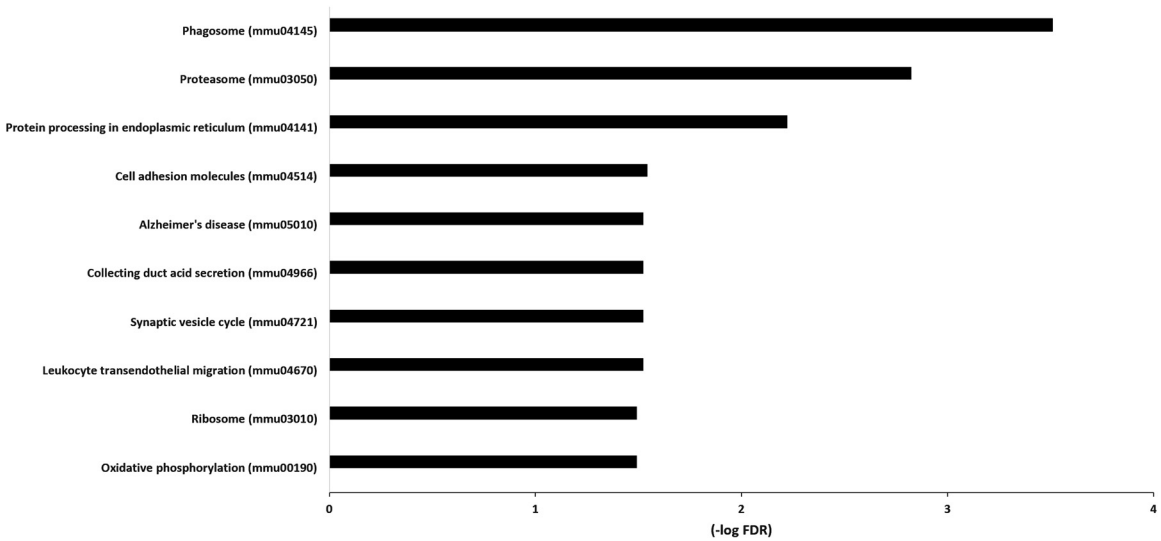
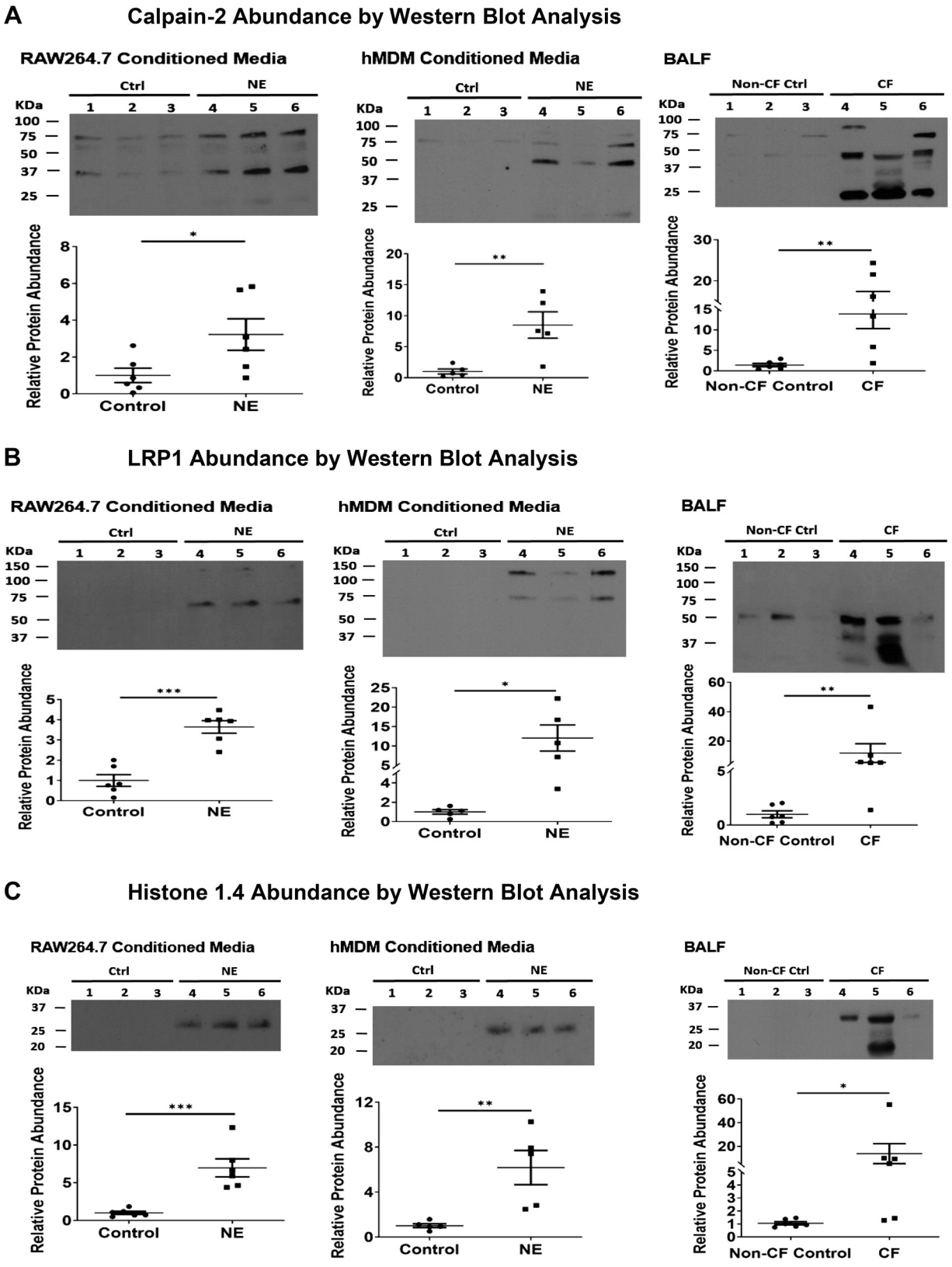


Figure 4. Top 10 functional pathways upregulated by NE treatment. Bar graphs show the top 10 enriched functional pathways upregulated by NE treatment identified by KEGG analysis. The pathways are labeled on the y-axis with the KEGG pathway ID in parenthesis. The bars on the x-axis show the −log of FDR (false discovery rate), with higher values representing higher probability of enrichment of that pathway. KEGG, Kyoto Encyclopedia of Genes and Genomes; NE, neutrophil elastase.

channel (ENaC) (26) activates signaling receptors including TNF alpha-converting enzyme in epithelial cells (27). Thus, NE may regulate phagocytosis-related proteins by several potential mechanisms.

We additionally found increased plasma membrane proteins that were intact in the conditioned media following NE treatment that orchestrate cytoskeletal rearrangements required for phagolysosome formation. One of these proteins



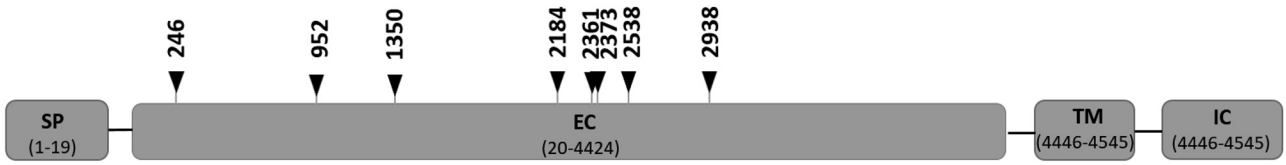


Figure 6. Identification and mapping of NE-specific cleavage sites of low receptor protein 1. Nontryptic peptides corresponding to LRP1 were identified and those that were consistent with the cleavage pattern of neutrophil elastase were mapped. Eight NE-specific cleavage sites were identified, and all were localized to the extracellular domain. The primary antibody epitope is to the amino terminus. LRP1, lipoprotein receptor-related protein 1; NE, neutrophil elastase.

is calcium-regulated calcium ion-dependent papain-like cysteine protease-2 (calpain-2), which is a protease associated with leukocyte migration, adhesion, and phagocytosis. Interestingly, calpain-2 was found as an intact protein of greater abundance in NE-treated conditioned media versus control (Fig. 5). Although we do not yet know the effect of increased extracellular calpain-2, we will investigate whether calpain-2 cleaves other proteins required for phagocytosis. Furthermore, we do not yet know the mechanism of NE upregulation of calpain-2 and will need to investigate whether NE upregulates calpain-2 messenger RNA (mRNA) and or protein expression in future studies.

We identified the extracellular domain peptide of low-density lipoprotein receptor-related protein 1 (LRP1) in the NE + conditioned media. LRP1 is a major receptor for efferocytosis and inhibition or loss of LRP1 on macrophages blunts neutrophil efferocytosis (28); this may result in increased airway inflammation. In addition, soluble LRP1 in the extracellular space is itself a proinflammatory mediator (29). Increased LRP1 has been reported in the bronchoalveolar lavage fluid from patients with acute respiratory distress syndrome (30).

Interestingly we also identified the nuclear protein Histone 1.4 as a full-length protein that was enriched in the NE-treated macrophage cell media and CF BALF. Histone 1.4 does not have a direct role in phagocytosis but has been found to be a component of neutrophil extracellular traps (NETs) (31) and NETs-mediated extracellular histone release has been associated with decreased phagocytosis (32) and efferocytosis (33). In addition, extracellular histone release by NE treatment raises some intriguing questions about its significance as well as the impact of NE on macrophage function and structure beyond phagocytosis.

Our results suggest that NE imparts a more global repertoire of protein dysregulation with complementary and nonredundant effects to block macrophage phagocytic function. Mitochondrial and nuclear proteins were detected in the NE-treated conditioned media even though cell viability was intact and cells had not undergone necrosis (no change in LDH abundance in NE vs. control media; data not shown). These results imply that NE may be internalized by macrophages and retain protease activity, further wreaking havoc on the macrophage cell. Alternatively, we speculate that NE may result in extracellular vesicle release with both mitochondrial and nuclear protein cargo. Therefore, NE may cause significant macrophage functional changes ranging from cell surface proteolytic cleavage to alterations in proinflammatory and innate immune proteins (5).

The limitations of this study are that although we were able to show that NE blocked both hMDM and murine alveolar macrophage phagocytosis, we were not able to harvest enough resident murine alveolar macrophages to confirm that the secretome and sheddome changes observed after NE exposure in RAW264.7 and hMDM were also present for alveolar macrophages. Although we demonstrate enrichment of the NE-activated macrophage sheddome/secretome proteins in CF bronchoalveolar lavage fluid and not in healthy non-CF BALF, we cannot assign which cells contribute these proteins to the BALF and they may be released from macrophages, but also from neutrophils and airway epithelial cells as well. Nonetheless, it is interesting that these same NE-increased proteins in the macrophage cell culture media are also increased in the CF airway, an airway environment enriched in NE activity especially during pulmonary exacerbations.

In this report we demonstrated that NE treatment induced significant changes in the shed/secreted proteome in RAW 264.7 and hMDM cells. At least three proteins increased by NE activity in the proteome have been confirmed by Western analyses in separate experiments. Calpain-2 and LRP1 proteins have functional properties consistent with phagocytic activity. Histone 1.4 may be a component of an extracellular trap or exosome released by NE activity (34). Our results shift the current paradigm of NE-impaired phagocytic function to emphasize that NE function extends beyond the clipping and loss of plasma membrane receptors required for phagocytosis. We propose that NE alters intracellular proteins in several macrophage organelles potentially resulting in alternative pathways leading to macrophage phagocytic failure.

Figure 5. Western analyses of Calpain-2, LRP1, Histone 1.4 to confirm relative proteomic abundance. RAW 264.7 or hMDM cultured to 80% confluence and treated with NE (500 nM, 2 h) or control media in serum-free media. At the end of the treatment period, an NE inhibitor, AAPV-CMK (10 μM) was added to all cultures. Media for all treatment conditions was harvested and concentrated 14-fold (7 mL to 0.5 mL) using an Amicon filter. Concentrated conditioned media (38 μL) or neat BALF (38 μL) were separated on a 4%–20% PAGE and following transfer to nitrocellulose, filters were incubated with primary antibodies to Calpain-2 (*A*), LRP1 (*B*), or Histone 1.4 (*C*). Following incubation with secondary antibodies and Western blot development with ECL, band densities were quantified by ImageJ and data summarized as means ± SEM for each experiment ($n = 6$ from 2 experiments for RAW 264.7, $n = 5$ from 2 experiments for hMDM, and $n = 6$ for BALF). Data were normalized to control-treated cells for RAW264.7 and hMDM, and to non-CF control for BALF. Significant differences between treatment conditions are noted by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. BALF, bronchoalveolar lavage fluid; CF, cystic fibrosis; ECL, enhanced chemiluminescence; hMDM, human monocyte-derived macrophages; LRP1, lipoprotein receptor-related protein 1; NE, neutrophil elastase.

SUPPLEMENTAL DATA

Supplemental methods: https://doi.org/10.6084/m9.figshare.11310629; Supplemental Tables S1: https://doi.org/10.6084/m9.figshare.14785983.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.M., A.H., and J.A.V. conceived and designed research; J.M., A.B.K., A.H., and S.Z. performed experiments; J.M., A.B.K., A.H., and S.Z. analyzed data; J.M. and A.B.K. interpreted results of experiments; J.M. prepared figures; J.M. drafted manuscript; J.M., A.B.K., A.H., S.G., S.Z., and J.A.V. edited and revised manuscript; J..M., A.B.K., A.H., S.G., S.Z., and J.A.V. approved final version of manuscript.

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Review

Neutrophil Elastase and Chronic Lung Disease

Judith A. Voynow [1,*] and Meagan Shinbashi [2]

[1] Division of Pediatric Pulmonology, Children's Hospital of Richmond at Virginia Commonwealth University, Richmond, VA 23298, USA
[2] School of Medicine, Virginia Commonwealth University, Richmond, VA 23298, USA; shinbashim@mymail.vcu.edu
* Correspondence: judith.voynow@vcuhealth.org

Abstract: Neutrophil elastase (NE) is a major inflammatory protease released by neutrophils and is present in the airways of patients with cystic fibrosis (CF), chronic obstructive pulmonary disease, non-CF bronchiectasis, and bronchopulmonary dysplasia. Although NE facilitates leukocyte transmigration to the site of infection and is required for clearance of Gram-negative bacteria, it also activates inflammation when released into the airway milieu in chronic inflammatory airway diseases. NE exposure induces airway remodeling with increased mucin expression and secretion and impaired ciliary motility. NE interrupts epithelial repair by promoting cellular apoptosis and senescence and it activates inflammation directly by increasing cytokine expression and release, and indirectly by triggering extracellular trap release and exosome release, which magnify protease activity and inflammation in the airway. NE inhibits innate immune function by digesting opsonins and opsonin receptors, degrading innate immune proteins such as lactoferrin, and inhibiting macrophage phagocytosis. Importantly, NE-directed therapies have not yet been effective in preventing the pathologic sequelae of NE exposure, but new therapies are being developed that offer both direct antiprotease activity and multifunctional anti-inflammatory properties.

Keywords: neutrophil elastase; cystic fibrosis; chronic obstructive pulmonary disease; bronchiectasis; bronchopulmonary dysplasia; antiprotease; glycosaminoglycan

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1. Introduction

Neutrophil elastase (NE) is a major proteinase in primary granules in neutrophils that participates in microbicidal activity [1–3]. NE (Leukocyte elastase, EC 3.4.21.37), a serine endopeptidase, is characterized by serine in the active tripeptide catalytic site: Asp, His, Ser [4]. NE is the most abundant of four serine proteases present in neutrophils, which also include proteinase 3, cathepsin G, and neutrophil serine protease 4 (NSP4) [5]. NE is a 29.5 kD protein stored in mature form in the azurophilic granules of neutrophils and is present at high concentrations per azurophilic granule—approximately 67,000 molecules (~5 mM) per granule. NE localization to azurophilic or primary granules requires serglycin, a proteoglycan with chondroitin sulfate residues, which bind to the basic amino acid residues of NE [6]. NE is also localized to the cell surface after neutrophil activation by binding to highly abundant but low-affinity chondroitin sulfate and heparan sulfate proteoglycans [7]. NE activity at the neutrophil surface occurs in quantum proteolytic bursts attributed to the high local concentration of NE, which overwhelms local antiprotease concentrations for the initial seconds of activity [8]. NE cleaves neutral, non-aromatic dipeptides, and thus, has a broad array of substrates (reviewed in [9,10]). Several factors modulate NE airway protease activity, including the abundance and localization of NE, the concentrations of lung antiproteases, α_1-antitrypsin, α_2-macroglobulin, or secretory leukocyte proteinase inhibitor (SLPI), at the site of enzyme activity [8], and even the target protein O-linked glycosylation [11]. Although NE proteinase activity is critical for normal innate immune function, release of NE into the airway milieu contributes to lung

disease progression. In this review, we will discuss the pathogenesis of dysregulated NE release into the airways in chronic lung diseases, and the mechanisms by which NE alters airway and lung parenchymal structures, and modulates innate immune processes and inflammation, leading to worsening disease. We will review the status of clinical trials to target NE in chronic lung diseases, and alternative strategies to control NE-mediated lung injury.

2. Neutrophil Elastase Is Required for Microbial Clearance

Exposure of neutrophils to cytokines, chemokines, or bacterial products activates granule fusion with the plasma membrane and NE localization at the cell surface for chemotaxis to the site of infection. During neutrophil phagocytosis, NE plays a critical role in microbial killing by fusing primary granules with phagolysosomes and releasing NE, which digests the microorganisms in concert with microbicidal peptides and reactive oxygen species (ROS) generated by NADPH oxidase and myeloperoxidase [4]. NE antimicrobial activity is required for clearance of Gram-negative bacteria [2]. Specifically, NE cleaves the *E. coli* outer membrane protein A (OMP A), resulting in bacterial death. NE-knockout mice exposed to *E. coli* have greater mortality than wild-type mice, with uncontrolled infection and death [1]. NE also participates in extracellular microbial killing by neutrophil extracellular traps (NETs). NETs are extracellular DNA webs released by neutrophils. They are composed of DNA-adherent pro-inflammatory proteins including NE, myeloperoxidase, histones and High Mobility Group Box 1, that together with DNA, adhere to and destroy microbes [12].

However, when NE extracellular release is dysregulated, NE can remodel the airways and lung parenchyma, promote sustained inflammation, and impair the innate immune system; together, these actions contribute to lung disease pathogenesis (summarized in Table 1).

Table 1. Extracellular neutrophil elastase: mechanisms for pathogenesis of chronic lung disease.

Airway Remodeling	Pro-Inflammatory Effects	Impaired Innate Immunity
Upregulates airway mucins, MUC5AC, MUC4, and MUC1 [13–16]; Triggers mucin secretion [17]	Activates TLR4 and upregulates IL-8 [18]	Degrades transferrin, lactoferrin [19] and midkine [20]
Stimulates goblet cell metaplasia [a] [21]	Activates IL-1α, IL-33, IL-36α, IL-36γ [22]	Cleaves phosphatidyl serine receptor resulting in efferocytosis failure [23]
Inhibits ciliary motility and injures cilia [24]	Activates High Mobility Group Box 1 [HMGB1] release [25]	Cleaves opsonins- C3bi [26], IgG [27], SP-D [28] SP-A [29]; Cleaves opsonin receptors [26]
Degrades CFTR [30] and activates ENaC [31] to promote airway dehydration	Increases cellular oxidative stress by releasing heme-free iron for uptake [32]	Cleaves lymphocyte receptors CD2, CD4, CD8 [33]; Cleaves neutrophil CXCR1 receptor [34]
Increases epithelial apoptosis [35]	Upregulates ceramide in vivo which mediates airway inflammation [a] [36]	Impairs macrophage phagocytic function [37]
Transiently down-regulates ErbB2 and suppresses epithelial proliferation [38]	Activates other proteases meprin alpha [16], matrix metalloprotease (MMP)-2 [39], MMP-9 [40], calpain-2 [30], Cathepsin B [39]	Impairs neutrophil *E. coli and S. aureus* killing [22]
Promotes epithelial cell cycle arrest [41] and senescence [42]	Degrades TIMP1 [40] and SLPI [43]	Blocks dendritic cell maturation [44]
Degrades extracellular matrix [b] [45]	Triggers NETs [46] and exosome release [47]	Cleaves fibrin degradation products that are chemotactic for PMN [48]

All results reported in human epithelial cells except the following: a, mouse; b, dog.

3. NE-Dependent Mechanisms Inducing Airway Mucus Obstruction

The major macromolecular component of airway secretions is airway mucins (for a detailed review, see reference [49]). Mucins are large polymeric glycoproteins. Some mucins are cell-associated and are required to maintain airway hydration homeostasis for normal ciliary motility [50], while secreted gel-forming mucins, released from submucosal glands and goblet cells, are critical for clearing microbes and pollutants from the airway via the mucociliary escalator. However, in chronic lung diseases, gel-forming mucin expression is upregulated by viruses, bacteria, pollutants, and neutrophil mediators including reactive oxygen species (ROS) and NE, resulting in hyperconcentrated airway mucus, a condition associated with mucostasis and airway mucus obstruction [51].

NE upregulates the gene expression of one of the major gel-forming, secreted mucins in the airway, *MUC*5AC, by both transcriptional and post-transcriptional mechanisms [13]. *MUC*5AC expression is upregulated by intracellular signals activated by NE-including ROS [52–54], NADPH quinone oxidoreductase 1 (NQO1) [53] and epidermal growth factor receptor (EGFR) activation [55]. NE facilitates transforming growth factor α (TGFα) - induced EGFR ligation by a coordinated mechanism: 1) releasing cell surface TGFα, and 2) increasing epithelial permeability by degrading Zona occludins-1 [56] and E-cadherin [57] junctional proteins, thereby permitting TGFα, a basolateral ligand, access to activate EGFR, an apical receptor [58]. In addition to upregulation of MUC5AC, NE also activates secretion of mucins from primary human bronchial epithelial cells [59], which contributes to mucus obstruction of airways. Finally, NE increases goblet cell metaplasia, which alters the epithelial composition in the airway and perpetuates increased mucin production and secretion. NE induces goblet cell metaplasia in a murine model [21] via Nqo1 [60] and tumor necrosis factor (TNF) alpha converting enzyme (TACE) [61]. In addition to NE, goblet cell metaplasia is increased by cytokines including IL-13, IL-1β, IL-6, IL-17, IL-9, TNF-α, and increased by microbes and microbial products, including lipopolysaccharide (LPS), *M. pneumoniae*, *P. aeruginosa* [62], rhinovirus [63] and non-typeable *H. influenza* [63]. The presence of cigarette smoke [63] or ozone [64] further enhances goblet cell hyperplasia (reviewed in [49,65,66]).

To generate effective mucociliary clearance requires normal mucin abundance, sufficient airway surface liquid hydration, and a healthy ciliated epithelium. NE interferes with each of these components required for normal mucociliary clearance. In addition to increasing mucin abundance in the airway, NE impairs airway surface liquid hydration by degrading cystic fibrosis transmembrane conductance regulator (CFTR) [30], an apical chloride channel, and activating ENaC [31], an apical epithelial sodium channel, resulting in perturbed ionic regulation of airway hydration. In addition, NE decreases ciliary motility and injures ciliary structures [24,67]. The failure of ciliary motility in concert with airway surface liquid dehydration and increased mucin production results in mucus obstruction of airways, a hallmark of chronic inflammatory airway diseases such as cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), and asthma.

4. NE Alters Cellular Differentiation and Cellular Fate

In addition to degrading mucociliary clearance and contributing to airway mucus obstruction, NE protease activity degrades extracellular matrix (ECM) proteins, particularly elastin, which is critical for alveolar structure [68,69]. NE-mediated ECM degradation causes direct pulmonary alveolar injury, resulting in emphysema, and subepithelial fibrosis [70] following the release of the pro-fibrotic factor, transforming growth factor β1 (TGF-β1), from the ECM [71,72]. Thus, NE activity contributes to the progression of COPD with emphysema, and CF and asthma with increased basement membrane thickening.

NE injures epithelial cells and the severity of the insult influences epithelial cell fate. After NE exposure and loss of epithelial cells, DNA synthesis is transiently inhibited and this is associated with degradation of ErbB2, an EGFR receptor [38]. However, NE also upregulates MUC4 [14], a ligand for ErbB2, and activation of ErbB2 increases epithelial proliferation and differentiation [38].

NE exposure activates at least three different epithelial cell fates. NE upregulates the cyclin kinase inhibitor p27 kip1, which interrupts cell cycle progression, causing cell cycle arrest, and a state called quiescence, which is reversible [41]. NE induces epithelial apoptosis via protease-activated receptor 1 (PAR1) activation [73], triggering phagocytosis by macrophages, which detect the apoptotic cell surface marker, phosphatidyl serine, to clear these cells, a process called efferocytosis. However, in the lungs of patients with CF or bronchiectasis, high concentrations of NE also cleave the macrophage phosphatidylserine receptor, resulting in efferocytosis failure and increased burden of apoptotic cells that undergo necrosis in the lung [23]. NE also activates senescence markers in airway epithelia [42], which are present at greater levels in CF airway tissue than in non-CF airway tissue. Cellular senescence also occurs in COPD and may be activated by oxidative stress and DNA injury [74]. Senescence due to cellular stress is associated with three genetic indicators: (1) upregulation of p16 ^{INK4a}, a cyclin-dependent kinase 4 inhibitor, (2) markers of DNA injury phospho-histone 2AX (γ-H2A.X) and phospho-checkpoint kinase 2 (p-Chk2) and (3) telomerase shortening [42]. Importantly, senescent cells activate NF-κB, resulting in the senescence-associated secretory phenotype (SASP) [75]. The SASP is characterized by the release of proinflammatory cytokines, IL1β, IL-6, TNF-α, chemokines, CXCL1, CXCL8, CCL2, matrix metalloproteinases, MMP-2, MMP-9, and growth factors, TGF-β [75]. Thus, NE propagates a vicious cycle of pro-inflammatory signaling and accumulation of pro-inflammatory senescent cells in CF and COPD [75].

5. NE Activates Pro-Inflammatory Signaling

NE upregulates epithelial expression of CXCL8 (IL-8), a major neutrophil chemokine, via TLR4 signaling, which activates downstream signals MyD88, IL-1 receptor-activated kinase (IRAK), and tumor necrosis factor receptor-activated factor-6 (TRAF-6), leading to NFκB translocation into the nucleus [76]. NE also upregulates CXCL8 via activation of meprin α, a metalloprotease, that activates EGFR by releasing the EGFR ligand TGFα [16]. Thus, NE activates both TLR4 and EGFR to increase CXCL8 gene regulation and protein production. In addition to CXCL8, extracellular NE activates IL-1α, IL-33 and IL-36α and γ [22] from epithelial cells, and TNFα and IL-1β from macrophages [37], resulting in increased neutrophil chemotaxis and airway inflammation. NE activates the release of High Mobility Group Box 1 (HMGB1), a damage-associated molecular pattern, from macrophages [25], which is associated with more severe lung disease in CF [77,78] and COPD [79]. NE protease activity also generates fibrin degradation products that are chemotactic for neutrophils [48]. By all these mechanisms, NE proteolytic activity creates airway signals that perpetuate neutrophilic inflammation.

NE also increases airway inflammation by other mechanisms. NE increases the abundance of a pro-inflammatory sphingolipid, long chain ceramide, in murine airways by upregulating serine palmitoyl transferase long chain subunit 2 (SPTLC2), the enzyme that catalyzes the rate-limiting step for ceramide generation. Inhibition of SPTLC2 prevents NE upregulation of ceramides and blocks the release of an inflammatory cytokine, keratinocyte-derived chemokine (KC, the murine analogue of CXCL8), and HMGB1 [36]. In CF sputum, NE concentration correlates with increased ceramide abundance [80]. NE generates an outside-to-inside signal for oxidative stress by degrading heme-containing proteins in the extracellular space, resulting in heme-free iron, which is taken up by cells and creates intracellular oxidative stress [32]. NE is a master regulator of protease activation and antiprotease destruction in the airway (for review, see [81]). Free NE activates pro-MMP-9, which is associated with bronchiectasis in CF [82]. NE degrades tissue inhibitor of matrix metalloprotease-1 (TIMP-1), and degrades the anti-elastase inhibitor, secretory leukoprotease inhibitor [43], resulting in sustained protease activity.

NE stimulates the release of neutrophil extracellular traps (NETs) [46], DNA web-like structures that have attached chromatin and granule proteins including NE, myeloperoxidase, HMGB1 and antimicrobial proteins. NE activates NET release through a highly orchestrated signaling pathway, requiring myeloperoxidase and hydrogen peroxide for

release from the azurophilic granule into the cytosol, association with F-actin during this transit, then degradation/release of actin for NE entry into the nucleus [83]. Once in the nucleus, NE clips Histone H4, which is required for chromatin decondensation and NET release [83]. Although NETs have been assigned as antimicrobial structures [12], they can also activate the inflammasome [84].

Neutrophil exosomes are nm-diameter vesicular structures released into the extracellular milieu that share membrane phospholipids derived from neutrophil endosomes and plasma membrane [47]. Exosomes carry cargo including both membrane and cytosolic proteins, lipids, metabolites, RNA and DNA [85]. Neutrophil exosomes are released spontaneously from cells and, under control circumstances, have no protease on the exosome surface [47]. However, activated neutrophil exosomes treated with FMLP or harvested from inflamed airways, including bronchoalveolar lavage fluid (BALF) of patients with COPD, harbor high concentrations of surface NE [47]. When instilled into mouse lungs, both activated neutrophil exosomes and COPD and bronchopulmonary dysplasia (BPD) BALF exosomes cleave ECM and are sufficient to cause emphysema [47]. Thus, neutrophil exosomes harbor high NE concentrations at the membrane surface, which are protected from airway antiprotease inhibition.

6. NE Impairs Innate Immunity

Although extracellular NE activity promotes inflammation, which should result in clearance of microbes, this antimicrobial function is thwarted when NE activity is dysregulated and overcomes the normal antiprotease capacity in the lung. The reason for this failure is that excessive NE interferes with innate and adaptive immune mechanisms. NE cleaves antimicrobial proteins, lactoferrin [86] and midkine [20], important for bacterial clearance. NE cleaves several opsonins including the surfactant protein (SP) collectins SP-D [28] and SP-A [29], complement C3bi and its receptor CR1 [26], and pseudomonas-specific IgG [27]. Altogether, the cleavage and loss of opsonins and phagocytic receptors due to NE activity [37] result in failure of both neutrophil and macrophage microbial killing and clearance. NE degrades monocyte CD14, a major monocyte receptor for bacterial lipopolysaccharide (LPS) [87]. NE also impairs maturation of monocyte-derived dendritic cells (mDC) by cleaving cell surface receptors, CD40, CD80, and CD86, resulting in failure of mDC to prime lymphocyte proliferation or cytokine production in response to antigen [88]. In CF airways, apoptotic neutrophils display the "eat-me" signal of increased plasma membrane phosphatidylserine, but these cells fail to be phagocytosed and cleared by macrophages due to NE-mediated degradation of the macrophage phosphatidylserine receptor [23]. Failure of apoptosis contributes to inflammation, as apoptotic cells undergo necrosis, releasing DNA and granular inflammatory mediators.

7. NE and Cystic Fibrosis Lung Disease

Although CF affects many organ systems, the major cause of morbidity and mortality is chronic lung disease due to infection and inflammation of the airways, which leads to bronchiectasis and respiratory failure [89]. Mutations in the CF Conductance Regulator (CFTR) gene are the primary defect in CF with loss of function of this anion channel. This defect causes abnormal mucus, which is adherent to airway epithelia [90,91], causing mucociliary clearance failure, and recurrent bronchitis. Although exacerbations of bronchitis lead to neutrophilic inflammation, the primary defect associated with airway mucus obstruction is sufficient to induce sustained neutrophilic inflammation, even in the absence of infection/presence of antibiotics [92]. There is strong evidence that NE participates in many of the pathogenic events that lead to chronic lung disease in CF. NE is present very early in the airway in CF infants in bronchoalveolar lavage fluid (BALF) and is associated with computer tomographic (CT) evidence of bronchiectasis in children with CF [93]. Over the first 6 years of life in children with CF, detection of bronchial lavage fluid NE correlates more closely with progressive structural lung damage (bronchiectasis

and mucus obstruction of airways on CT) than infections [94]. The amount of free NE in sputum has also been shown to correlate inversely with FEV_1 in children with CF [95].

NE impairs mucociliary function and innate immune function and increases inflammation in the CF lung by several mechanisms. NE upregulates the gel-forming mucin, MUC5AC. NE activates the apical epithelial sodium channel [31], which increases sodium uptake from the airway surface liquid (ASL) and contributes to ASL dehydration and airway mucus obstruction. NE upregulates epithelial expression of CXCL8 [18,96], a chemokine that increases NE release from CF neutrophils, resulting in a self-perpetuating cycle of neutrophil inflammation and overabundant NE in ASL [97]. In addition, NE degrades secretory leukoprotease inhibitor (SLPI) [43], activates the neutrophil metalloprotease gelatinase (MMP-9), and degrades tissue inhibitor of metalloprotease-1 (TIMP-1), further sustaining overwhelming proteolytic inflammation [40]. The abundance of airway NE is also associated with airway remodeling with increased airway basement membrane thickness [71], and premature epithelial senescence [42]. Excess NE impairs both the innate and adaptive immune systems by degrading antimicrobial proteins in the ASL, cleaving opsonins and opsonin receptors [98], and generating oxidative stress in airway epithelia [99]. Finally, NE as well as myeloperoxidase, bacteria, ROS and other stimuli activate release of NETs [46] that add DNA to the airway, increasing the viscoelasticity of airway mucus [100]. NETs contain cargo including NE and other pro-inflammatory proteins that contribute to persistent inflammation in the CF airway and the progression of lung disease [99].

8. NE and Chronic Obstructive Pulmonary Disease

COPD is the third leading cause of death globally [101]. It is commonly associated with cigarette smoking, exposure to biomass fuel combustion and air pollution, and is characterized by persistent inflammation and progressive airflow limitation. As with CF, neutrophilic inflammation is a notable feature of COPD. Exposure to irritants such as cigarette smoke and pollutants triggers the release of a cytokine network that promotes neutrophil recruitment, resulting in a protease–antiprotease imbalance [102] and establishing a vicious cycle of inflammation and airway remodeling [103]. Sputum neutrophil counts have been shown to correlate with the rate of lung function decline [104] and peripheral airway dysfunction [105]. Acute exacerbations of bronchitis due to bacterial or viral infections are the major cause of morbidity and mortality in COPD [106] and are associated with elevated NE levels [107]. NE and other proteases cooperate to regulate the protease–antiprotease activity in the COPD airway. For example, there is compelling evidence that MMP-12/Macrophage elastase is required for emphysema after smoke exposure [108]. NE activates MMPs and cysteinyl cathepsins that induce emphysema, and NE sustains MMP activity by degrading TIMP-1, a major inhibitor of MMPs [109]. Reactive oxygen species oxidize and inactivate α-1-antitrypsin, resulting in unrestrained NE activity [110]. NE and MPO activate the release of NETs into the airway milieu that propagate NE and neutrophil granule proteolytic and pro-inflammatory activities [47]. NET abundance in the COPD airway is associated with decreased lung function, increased exacerbations, and with diminished neutrophil phagocytosis in the COPD airway [111]. The effects of NE are especially prominent in α1- antitrypsin deficiency, where decreased amounts or complete loss of α1-antitrypsin result in the unopposed actions of NE and subsequent destruction of the alveolar matrix [112]. There is synergy between NE and MMP-12 (macrophage elastase) to promote tobacco smoke-induced COPD lung pathology, as MMP-12 degrades the NE inhibitor, α-1 antitrypsin [108] and NE degrades the MMP-12 inhibitor, TIMP1 [40], resulting in unrestrained protease activities.

9. NE and Bronchiectasis

Bronchiectasis is a disease that is defined by permanent and abnormal airway widening with mucus obstruction [51] and subsequent airflow obstruction [113]. Bronchiectasis may be due to inherited diseases such as primary ciliary dyskinesia or primary immunodeficiencies. However, bronchiectasis may also be caused by mechanical airway obstruction,

recurrent insults such as aspiration, secondary immunodeficiency, or severe bacterial or viral pneumonia and subsequent airway injury [114]. Although the pathogenesis of non-CF bronchiectasis is not fully understood, there is increasing evidence that neutrophils are associated with its progression. For example, bronchiectatic airways have higher levels of neutrophil infiltration in the lamina propria compared to control airways [115]. One commonly proposed hypothesis for pathogenesis is that an initial bacterial infection of the lower respiratory tract triggers an exaggerated and uncontrolled neutrophilic airway inflammatory response [116]. This results in damaged airways with impaired mucociliary clearance and increased susceptibility to severely damaging pathogens such as *P. aeruginosa*, leading to further inflammation [117]. Another hypothesis for pathogenesis is that an insult causes the initial event of mucus obstruction of the airway sufficient to activate both macrophage and epithelial signaling and lead to neutrophil activation [51]. NE proteolytic activity has been shown to correlate with decreased lung function [118] and increased susceptibility to airway bacterial colonization [119], indicating NE as a potential biomarker of disease severity. Furthermore, sputum NE activity in patients with bronchiectasis is associated with an increased risk and frequency of exacerbations, hospitalizations, and mortality [120]. This is the rationale for a phase 2 study of AZD9668, an oral NE inhibitor, which was tested in patients with bronchiectasis in a randomized, double-blind, placebo-controlled trial over 4 weeks [121]. Although AZD9668 was not associated with adverse side effects, efficacy was modest, with only a small increase in FEV_1 (100 mL) and no significant changes in sputum NE or IL-8 or patient symptoms survey results [121]. The lack of efficacy of AZD9668 may be due to the small number of subjects enrolled and subject variability. Therefore, it is still not clear whether targeting NE activity alone will be a successful therapeutic strategy for bronchiectasis. However, NE may still be a useful marker for the clinical assessment of patients with bronchiectasis and may also identify patients at the highest risk of disease progression. The potential that airway NE concentrations may serve as a biomarker for disease activity would be an important advance, given that there are currently no gold standards for measuring inflammation in bronchiectasis [122]. A point-of-care sputum NE activity assay is established [123]; however, future studies will be necessary to evaluate whether sputum NE measurements will translate into improved outcomes for bronchiectasis.

10. NE and Bronchopulmonary Dysplasia

Bronchopulmonary dysplasia (BPD) is a chronic lung disease that occurs in premature infants and is defined by the requirement for supplemental oxygen at 36 weeks post-gestational age [124,125]. BPD is attributed to an arrest in lung development and is the end result of a complex process where factors including gestational age, birth weight, ventilatory support, and oxygen toxicity compromise normal lung development [126]. This leads to a sustained reduction in lung function with airspace enlargement and altered capillary development. Inflammation is a key component in the pathogenesis of BPD, as chorioamnionitis and postnatal sepsis are associated with this disease [127,128]. There is also increasing evidence that NE is a key mediator in BPD, as NE is elevated in BPD airways [129] and has increased enzymatic activity on the surface of neutrophil exosomes obtained from tracheal aspirate of infants with BPD [47]. A recent study using a transgenic mouse model for NFκB activation in the airway found that sublethal inflammation from NE instillation during the saccular stage of lung development, but not during the alveolar stage of development, resulted in a BPD-like lung phenotype of enlarged simplified alveoli, while neutrophil-depleted mice showed normal alveolar structure [130]. NE was also found to be elevated in airways of the mice with lung disease, strongly suggesting that excess NE proteolytic activity leads to aberrant lung development. Culturing lung fibroblasts from these mice revealed that NE or neutrophil exosomes from tracheal aspirate of infants with BPD downregulate the mRNA expression of elastin assembly genes, further implicating the involvement of NE in the pathogenesis of BPD. Finally, the NE-exposed mice had aberrant lung structure that persisted into adulthood and resembled emphysema. Future studies

should investigate the role of NE during this critical period and whether anti-NE therapies can reduce the risk of developing BPD and subsequent COPD in adult life.

11. NE Inhibitors and Mechanisms of Action

Given the increasing evidence for NE playing a major role in the pathogenesis of chronic lung diseases, there is a need for developing NE-targeted therapies. One potential strategy is to directly address the protease–antiprotease imbalance seen in these diseases by increasing antiprotease function. Replacement therapy has been approved for patients with α1-antitrypsin deficiency based on evidence of NE inhibition. In three large multicenter placebo-controlled, randomized, double-blind trials, α1-antitrypsin infusion stops progression of emphysema, as determined by CT scores over a 2-year time course [131–133]. However, there is no evidence that α1-antitrypsin replacement therapy affects risk for exacerbations or improves lung function in patients without genetic α1-antitrypsin deficiency. At the time of this review, studies have yet to find a significant effect of α1-antitrypsin augmentation on improving lung function, reducing exacerbation frequency, or on morbidity or mortality in patients with other chronic lung diseases. A recent study in patients with α1-antitrypsin deficiency suggested that this lack of clinical efficacy may be due to suboptimal dosing and that doubling the standard dose could further slow the loss of lung function [134]. Inhaled α1-antitrypsin therapy has also been evaluated in patients with CF. An initial open label study of inhaled α1-antitrypsin [135] showed that the therapy reduced NE abundance in BALF and that neutrophils added to BALF from post-treatment subjects were effective in killing *P. aeruginosa* compared to bacterial killing by neutrophils added to pretreatment BALF. However, although subsequent trials of inhaled α1-antitrypsin in CF have demonstrated that therapy is safe and tends to decrease NE in airway BALF or sputum, these studies have yet to show any improvement in lung function or in decreasing rates of exacerbations [136,137].

Other antineutrophil elastase therapies have been studied in clinical trials. Silvelestat is the only NE synthetic inhibitor approved for clinical use and is exclusively used in Japan and Korea to treat acute lung injury and respiratory distress syndrome. However, studies testing Silvelestat treatment for acute respiratory distress syndrome in the US were stopped early by the Data Safety Monitoring Board providing oversight for the study, due to increased long-term mortality for subjects on Silvelestat [138]. AZD9668 is a reversible and selective NE inhibitor that was tested for efficacy in COPD [139], CF [140], and bronchiectasis [121] in randomized, double-blind, placebo-controlled trials. Although AZD9668 decreased sputum measures of inflammation, these trials did not demonstrate significant improvement in lung function or symptom scores for any of these three protease-dominant diseases. These studies of well-characterized and potent antiproteases which failed to demonstrate a robust impact on clinical outcomes, suggest that the strategy of focusing solely on anti-NE activity alone will not be sufficient to block the unremitting inflammatory milieu in the airways to change the trajectory of clinical outcomes. Instead, a new strategy employing combination therapy and/or multi-function drugs that have antiprotease and anti-inflammatory properties may be a more successful strategy.

Flavonoids are polyphenolic compounds derived from plants. They have been investigated over the past two decades for antiprotease and anti-inflammatory activity [141]. Several flavonoid glucuronide derivatives at 1 μM inhibited NE release by 30–50% and at 10 μM, decreased ROS release by 50–70% from activated neutrophils [142]. Several modified flavonoids also have anti-elastase activity, with IC50 in the micromolar range [141]. However, these compounds have not yet been tested in chronic lung diseases characterized by neutrophil predominant inflammation.

Polysulfated glycosaminoglycans (GAGs) are potent anti-elastase drugs with multiple anti-inflammatory properties [143], and the prototypical drug in this class, heparin, has a strong record of safety and efficacy when administered for other lung disease indications such as asthma, acute lung injury, and smoke inhalation in humans [143]. In one double-blind, placebo-controlled pilot study, inhalation of unfractionated heparin as a therapeutic

for COPD resulted in improved lung function, underscoring the significant promise of GAG therapy for chronic lung diseases [144]. Polysulfated GAGs, including 2-O, 3-O desulfated heparin (ODSH) [145], a polysulfated hyaluronan (GM-1111) [146], and non-saccharide glycosaminoglycan mimetic (NSGM) (G32) [147], are potent anti-elastase drugs that have minimal anticoagulant activity and, therefore, may be advantageous for chronic inhalation. We have demonstrated by in silico modeling and Michealis–Menten kinetics that ODSH functions by an allosteric mechanism, binding to basic amino acid residues outside the NE catalytic domain [145] and competing with sputum DNA for access to that site. G32 also binds in part to the allosteric domain but also interacts with Histidine in the catalytic domain [147], so G32 has a dual mechanism of action. ODSH, GM-1111, and G32 inhibit NE in CF sputum supernatant treated with dornase alfa and hypertonic saline, the current mucolytic and mucokinetic therapies for the CF airway. GM-1111 has also been effective to resolve chronic allergic rhinosinusitis in a mouse model [148].

Importantly, polysulfated GAGs also have multifunction anti-inflammatory properties. ODSH inhibits histone acetyltransferase activity, blocking acetylation of HMGB1 and preventing NE-triggered release of HMGB1 by macrophages in vitro [149]. Heparin and ODSH inhibit NFκB activation, block L- and P-selectin binding, and interfere with HMGB1–receptor for advanced glycation end-products (RAGE) interactions and S100A9/calgranulin–RAGE interactions [150,151]. Polysulfated hyaluronan resolves allergy-mediated and LL-37-mediated rhinosinusitis in a mouse model, supporting a broader anti-inflammatory activity for this novel drug [148,152]. Future comprehensive studies to explore how GAGs can be developed and implemented to treat chronic inflammatory airway diseases are warranted.

12. Summary

NE is critical for the host immune response to infection, but NE is also a major instigating factor for inflammation and airway injury in chronic inflammatory lung diseases. Because of the pleiotropic impact of NE activity, it is unlikely that inhibition of NE activity alone will resolve or reverse chronic inflammatory lung diseases. Instead, we propose a strategy of targeting multiple proteases and signaling pathways activated by NE to successfully inhibit inflammation and facilitate airway repair. GAGs may provide a cornerstone therapy in this new therapeutic strategy.

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REVIEW
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Glycosaminoglycans as Multifunctional Anti-Elastase and Anti-Inflammatory Drugs in Cystic Fibrosis Lung Disease

Judith A. Voynow, Shuo Zheng and Apparao B. Kummarapurugu*

Department of Pediatric Pulmonology, Children's Hospital of Richmond at VCU, Richmond, VA, United States

Neutrophil elastase (NE) is a major protease in the airways of patients with cystic fibrosis (CF) that activates airway inflammation by several mechanisms. NE stimulates epithelial toll like receptors (TLR) resulting in cytokine upregulation and release, upregulates MUC5AC, a major airway mucin, degrades both phagocytic receptors and opsonins resulting in both neutrophil and macrophage phagocytic failure, generates oxidative stress *via* extracellular generation and uptake of heme free iron, and activates other proteases. Altogether, these mechanisms create a significant inflammatory challenge that impairs innate immune function and results in airway remodeling. Currently, a major gap in our therapeutic approach to CF lung disease is the lack of an effective therapeutic strategy targeting active NE and its downstream pro-inflammatory sequelae. Polysulfated glycosaminoglycans (GAGs) are potent anti-elastase drugs that have additional anti-inflammatory properties. Heparin is a prototype of a glycosaminoglycan with both anti-elastase and anti-inflammatory properties. Heparin inhibits NE in an allosteric manner with high potency. Heparin also inhibits cathepsin G, blocks P-selectin and L-selectin, hinders ligand binding to the receptor for advanced glycation endproducts, and impedes histone acetyltransferase activity which dampens cytokine transcription and High Mobility Group Box 1 release. Furthermore, nebulized heparin treatment improves outcomes for patients with chronic obstructive pulmonary disease (COPD), asthma, acute lung injury and smoke inhalation. However, the anticoagulant activity of heparin is a potential contraindication for this therapy to be developed for CF lung disease. Therefore, modified heparins and other GAGs are being developed that retain the anti-elastase and anti-inflammatory qualities of heparin with minimal to no anticoagulant activity. The modified heparin, 2-O, 3-O desulfated heparin (ODSH), maintains anti-elastase and anti-inflammatory activities *in vitro* and *in vivo*, and has little residual anticoagulant activity. Heparan sulfate with O-sulfate residues but not N-sulfate residues blocks allergic asthmatic inflammation in a murine model. Polysulfated hyaluronic acid abrogates allergen- triggered rhinosinusitis in a murine model. Finally, nonsaccharide glycosaminoglycan mimetics with specific sulfate modifications can be designed to inhibit NE activity. Altogether, these novel GAGs or GAG mimetics hold significant

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***Correspondence:**
Judith A. Voynow
judith.voynow@vcuhealth.org

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promise to address the unmet need for inhaled anti-elastase and anti-inflammatory therapy for patients with CF.

Keywords: neutrophil elastase, cystic fibrosis, glycosaminoglycans, heparin, hyaluronic acid, High Mobility Group Box 1

INTRODUCTION

Cystic fibrosis (CF) lung disease is marked by recurrent exacerbations of acute bronchitis with an overexuberant inflammatory response and markedly high airway concentrations of neutrophil elastase (NE). A major gap in current therapy for patients with CF is the lack of anti-protease and anti-inflammatory therapies to inhibit NE and NE-activated sequelae. In this review, we will discuss the impact of NE on CF lung biology, review the current landscape of anti-protease and anti-inflammatory therapies for CF lung disease, and then discuss the biology and pharmacology of glycosaminoglycans (GAGs) as potential anti-protease and anti-inflammatory therapies for CF.

Neutrophil Elastase and Cystic Fibrosis Lung Disease

The primary defect in CF, an autosomal recessive disorder, is the loss of function of the Cystic Fibrosis Transmembrane Conductance Regulator protein, which results in abnormal airway mucus (Stoltz et al., 2015; Boucher, 2019). CF airway mucus is tethered to submucosal ducts and airway epithelia (Ostedgaard et al., 2017; Ermund et al., 2018) with subsequent mucus stasis and failure to clear infections. Thus, recurrent cycles of infection and inflammation are established and neutrophils are recruited to the airway. In addition, mucus stasis alone may be sufficient to increase neutrophilic inflammation (Rosen et al., 2018), possibly by generating airway hypoxemic stress which triggers IL-1β and IL-1α cytokine release (Chen et al., 2019). The CF airway milieu, characterized by viscous sputum containing microbes and pro-inflammatory cytokines, further impairs neutrophil function and clearance (Voynow et al., 2008). Ultimately, in the CF airways, neutrophils release extracellular traps (Gray et al., 2018) or undergo necrosis (Vandivier et al., 2002), and release DNA and granule contents including proteases. The most abundant protease released into the CF airway is neutrophil elastase (NE).

NE is present in the bronchoalveolar lavage (BAL) fluid in infants with CF, and BAL NE concentrations are directly associated with lung disease progression starting in infancy (Sagel et al., 2012; Sly et al., 2013; Rosenow et al., 2019). NE accelerates the progression of CF lung disease by several mechanisms (Voynow et al., 2008; McKelvey et al., 2019). First, NE contributes to altered ion flux in the CF airway by activating the epithelial sodium channel (Caldwell et al., 2005) and degrading CFTR *via* an endogenous proteinase, calpain (Le Gars et al., 2013). These NE actions further aggravate altered ion and water flux across the CF airway. Second, NE activates signaling pathways that promote abnormal epithelial structure and repair. NE upregulates mucin expression and goblet cell metaplasia (Voynow et al., 2004; Park et al., 2013); and triggers epithelial apoptosis (Suzuki et al., 2009) and/or premature senescence (Fischer et al., 2013), which impair epithelial proliferation and restoration following injury. Third, NE employs several mechanisms to promote airway inflammation (Voynow et al., 2008; Bruscia and Bonfield, 2016; Roesch et al., 2018). NE amplifies inflammation by upregulating neutrophil chemokines, e.g. IL-8 (Cosgrove et al., 2011), proteolytically activating chemokines such as IL-1α or IL-33 (Clancy et al., 2018), and releasing damage associated molecular pattern proteins such as High Mobility Group Box 1 (HMGB1) (Griffin et al., 2014) which binds to the Receptor for Advanced Glycation End-products (RAGE) or facilitates ligand binding to TLR2 and TLR4 (Lotze and Tracey, 2005). NE further contributes to airway inflammation by increasing the expression of pro-inflammatory long chain ceramides (Karandashova et al., 2018; Horati et al., 2020); these lipids impact plasma membrane structure and receptor clustering. NE degrades innate immune proteins including lactoferrin and surfactant proteins A and D, and cleaves both complement and complement receptors causing impaired neutrophil and macrophage phagocytic activity (Voynow et al., 2008). NE increases the activity of other proteases; NE activates matrix metalloproteinase 9 (MMP 9) by cleavage of its prodomain and by degradation of its inhibitor, Tissue inhibitor of metalloprotease-1 (Jackson et al., 2010). In addition, the protease load is further exaggerated by the loss of endogenous anti-proteases. Anti-NE capacity is depleted in the CF airway due to NE degradation of elafin (Guyot et al., 2008), secretory leucoprotease inhibitor (Weldon et al., 2009; Twigg et al., 2015) and both oxidation and protease degradation of alpha-1- protease inhibitor (A1-PI) (Twigg et al., 2015). Finally, NE generates oxidative stress in epithelial cells and macrophages by degrading heme-containing proteins and releasing heme-free iron which is taken up by cells (Fischer et al., 2009); this process occurs in the airways of patients with CF (Ghio et al., 2013) and with COPD (Fischer et al., 2009). NE has a broad repertoire of activities that increase inflammation, impair host immunity and result in airway remodeling. Although NE appears to be a central regulator of inflammation in CF lung disease, NE actions are amplified by ligand-receptor interactions, oxidative stress, and the presence of other active proteases that contribute to a complex pro-inflammatory milieu. This may be one reason why the strategy of therapy for a single target, NE activity, in the CF airway, has not yet been successful.

Status of Current Anti-Proteases and Anti-Inflammatory Therapies for Cystic Fibrosis

Currently, there are two anti-inflammatory therapies approved for CF: azithromycin for patients with *Pseudomonas aeruginosa* infections (Nichols et al., 2020) and ibuprofen high dose oral

therapy (Konstan et al., 1995). These therapies blunt the rate of decline of lung function over time; however, they do not resolve the high airway protease load that is associated with progression of bronchiectasis and lung injury. Many anti-protease candidate drugs have been tested in the CF airway (reviewed in (Voynow et al., 2008) and (Twigg et al., 2015)). An oral neutrophil elastase inhibitor, AZD9668, was tested in a Phase II randomized, double-blind, placebo-controlled trial in patients with CF (Elborn et al., 2012). Although AZD9668 treatment was associated with decreased urine desmosine, a marker of NE activity, and decreased sputum IL-6 and Regulated on Activation, Normal T Expressed and Secreted (RANTES), there was no improvement in sputum NE activity, sputum neutrophil counts, or measures of quality of life. A recent Phase IIa randomized, placebo-controlled clinical trial of inhaled alpha1 proteinase inhibitor (A1-HC) (Gaggar et al., 2016) revealed that the treatment group had increased sputum concentrations of A1-HC, but there was no significant change in lung function, quality of life measures, or sputum NE activity or sputum cytokine levels. Recently, an inhaled anti-NE therapy, POL6014, was studied in a Phase I trial using an ascending dose schedule in both healthy volunteers and participants with CF (Barth et al., 2019). A single inhaled dose was safe in both healthy volunteers and subjects with CF. Sputum active NE levels were reduced by greater than 1-log at 3 h after treatment at all doses. Therapy with POL6014 for subjects with CF is currently being evaluated in a Phase IIa/IIb randomized, placebo-controlled, double-blind study (NCT03748199). This initial report of POL6014 activity is promising; however, there is still a compelling need to develop drugs with multifunctional anti-protease and anti-inflammatory activities that are resistant to protease degradation or oxidation.

GAGs: Structure and Function

GAGs are polymers composed primarily of disaccharides which consist of a D-glucosamine bound to either uronic acid (D-glucuronic acid or L-iduronic acid) or galactose (Morla, 2019). The composition and linkage of monosaccharides and addition of modifications define the four major classes of GAGs: heparin/heparan sulfate (HS), chondroitin sulfate, dermatan sulfate, and hyaluronan. The uronic acid has a carboxylic acid unit and both monosaccharides are decorated with N- and O-linked sulfate residues that together confer a negative charge to the polymers. Native hyaluronan is not sulfated. In the CF lung, there are high levels of chondroitin sulfate and hyaluronan. Chondroitin sulfate proteoglycans contribute to turbidity and the mass of insoluble pellet in CF sputum; these qualities are relieved by depolymerization with chondroitinase ABC (Khatri et al., 2003). Low molecular weight hyaluronan may contribute to inflammation in the CF lung *via* TLR2 and TLR4 signaling and downstream NK-κB signaling (reviewed in (Reeves et al., 2011)). However, GAG structures can be modified to alter sulfation which plays a critical role in mediating biological effects. Moreover, GAG mimetics are being generated to achieve optimal drug characteristics while minimizing adverse properties. Heparin and HS proteoglycans bind to predicted basic amino acid-rich domains (Cardin and Weintraub, 1989; Hileman et al., 1998). GAGs have many biological effects that impact coagulation, infection, inflammation, cell adhesion, metastasis, cell matrix structure, and tissue differentiation and repair (Lima et al., 2017; Morla, 2019). Importantly, heparin can be taken up by cells and localized to cytoplasm and nucleus (Richardson et al., 2001; Raman et al., 2013) (**Figure 1A**). The localization of administered heparin to both extracellular and intracellular domains permits a wide array of anticipated functions including enzyme inhibition and interference with cell- cell receptor interactions, microbe- cell interactions, and HS proteoglycan pro-inflammatory activities. In this review, we will focus on GAG properties that impact CF and other chronic lung diseases.

Modified Non-Anticoagulant Heparins and Anti-Inflammatory Activity

Heparin is well known for its anticoagulant activity, but in addition, heparin has a broad repertoire of anti-inflammatory functions including anti-NE and anti-cathepsin G activity, inhibition of NF-kB, blockade of L- and P-selectin binding, and interference with HMGB1 release and interaction with its receptor, RAGE (Morla, 2019; Mulloy, 2019). At least three modified heparins have been developed to reduce anti-coagulant activity but retain anti-inflammatory activity: glycol-split heparin, sulfated-non-anticoagulant Low Molecular Weight Heparin (S- NACH) and 2-O, 3-O desulfated heparin (ODSH). Glycol-split heparin, generated by periodate oxidation of porcine mucosal heparin, is characterized by a cleavage between C2 and C3 of the nonsulfated uronic acid residue (Naggi et al., 2005). Glycol split heparin, administered subcutaneously to mice daily starting 10 days after establishment of chronic *P. aeruginosa*-agar bead pneumonia, decreases inflammatory cytokines, BAL neutrophil counts, and bacterial lung burden at 28 days (Lore et al., 2018). S-NACH is a purified fraction of low molecular weight heparin isolated to select drug with minimal anti-coagulant activity (Shastri et al., 2015). In a murine asthma model generated by ovalbumin (OVA)-sensitization and challenge, S-NACH intraperitoneal administration following OVA challenge blunted BAL inflammation by eosinophils, macrophages, and neutrophils, blocked goblet cell metaplasia, and blocked T2 cytokine expression in serum and BAL (Ghonim et al., 2018).

Of the modified non-anticoagulant heparins tested for anti-inflammatory efficacy, there is the most experience with 2-O, 3-O, desulfated heparin (ODSH). Fryer et al. (1997) lyophilized heparin under alkaline condition to produce ODSH. ODSH has substantially reduced anticoagulant activity compared to heparin as determined by activated partial thromboplastin time (APTT) and anti-Xa clotting assays. But the anti-neutrophil protease activities, including anti-NE and anti-cathepsin G activities, are largely unchanged in ODSH compared to heparin. ODSH also retains the pharmacological properties of heparin *in vivo*, including inhibition of bronchial hyperreactivity after antigen challenge, and prevention of airway smooth muscle cell proliferation (Fryer et al., 1997). Importantly, ODSH does not bind to platelet factor 4 and thus doesn't trigger heparin-induced thrombocytopenia (Rao et al., 2010). ODSH interrupts ligand-receptor interactions, blunting pro-inflammatory signaling

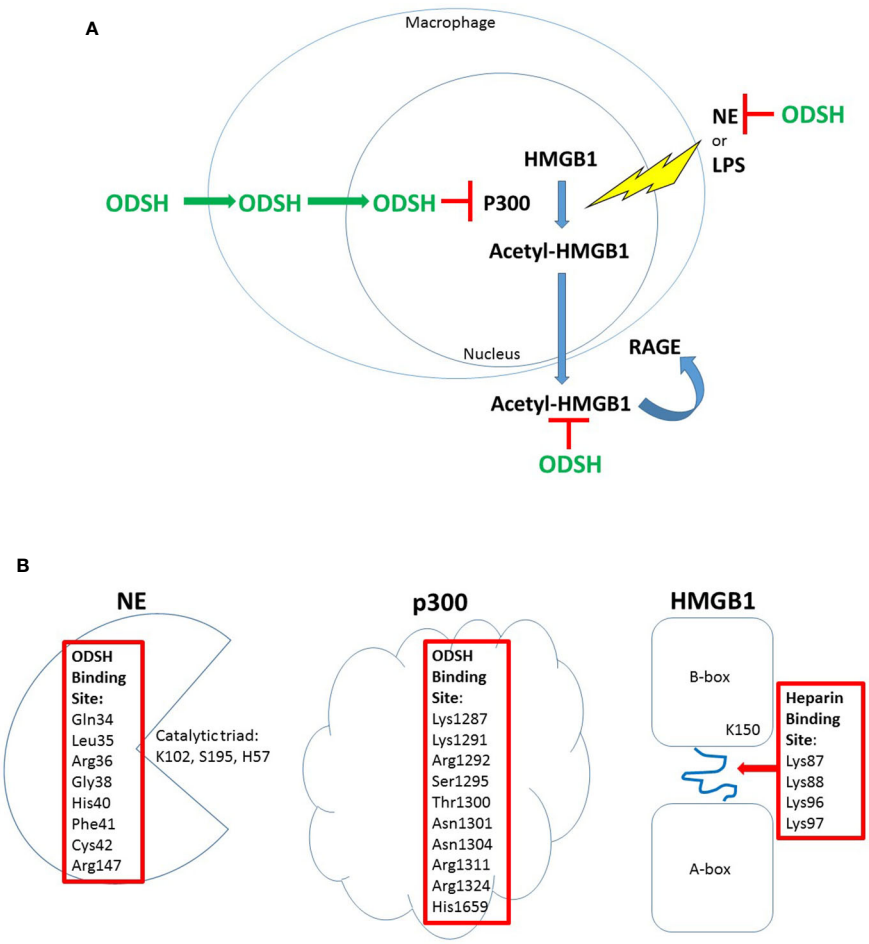

FIGURE 1 | ODSH localization and function in a macrophage cell line. ODSH is taken up by a mouse macrophage cell line (RAW264.7) into the cytoplasm within 2 h and into the nucleus by 24 h (Zheng et al., 2017) **(A)**. ODSH has anti-NE activity and blocks HMGB1-RAGE interaction in the extracellular domain, and inhibits p300 lysine acetyltransferase activity in the nucleus **(A)**. ODSH inhibits NE activity by binding to an allosteric inhibitory site (Kummarapurugu et al., 2018), and ODSH inhibits p300 enzyme activity by binding to the acetyl-CoA binding site in the catalytic domain (Zheng et al., 2017) **(B)**. In contrast, ODSH binds to the loop connecting the A-box and B-box of HMGB1, blocking interaction with heparan sulfate proteoglycans required for HMGB1 ligation of the RAGE receptor (Xu et al., 2011) **(B)**. Amino acid residues required for ODSH or heparin inhibitory activity are shown (Red Box).

cascades. Both heparin and ODSH inhibit RAGE- HMGB1 interaction and RAGE-S100A9/calgranulin interaction *in vitro* (Rao et al., 2010). The ODSH concentrations (IC_{50}) to inhibit NE activity (0.14 μg/ml), and to block HMGB1-RAGE binding (0.23 μg/ml) are similar, supporting the concept that ODSH (MW approximately 10 kD) achieves both anti-protease and anti-inflammatory activities within a nanomolar concentration range.

Heparin and ODSH Anti-HMGB1 Activity

HMGB1 is recognized as a major inflammatory mediator in CF plasma and sputum that is strongly associated with lung disease progression (Liou et al., 2012; Chirico et al., 2015). Therefore, HMGB1 is likely to be an important target for CF anti-inflammatory therapy. HMGB1 has two major functions; it is a nuclear non-histone chromatin binding protein that facilitates transcriptional regulation, and it is an extracellular damage associated molecule pattern or alarmin that is secreted by activated macrophages as a delayed mediator of inflammation (Lotze and Tracey, 2005). HMGB1 release is triggered by HMGB1 lysine acetylation which is activated following exposure to microbial products (LPS), cytokines (TNFα) (Lotze and Tracey, 2005), or NE (Griffin et al., 2014). HMGB1 can also be released from necrotic cells. HMGB1 has been reported to transduce cellular signals by interacting with at least three receptors: RAGE, TLR2 and TLR4 (Park et al., 2004; Sharma et al., 2014). Binding of HMGB1 to RAGE activates NF-κB and the ERK/p38 pathway which promotes cytokine production (TNF, IL-6, and IFN-γ). Binding of HMGB1 to TLR2/TLR4 leads to NF-κB activation through a MyD88 (myeloid differentiation primary-response protein 88)-dependent mechanism. Importantly, ODSH blocks both HMGB1 release and HMGB1 ligation of receptors both *in vitro* and *in vivo*. Intratracheal HMGB1 in a mouse model induces significant pulmonary inflammation with increased BAL total

cells, neutrophils, and TNF-α levels at 24 hr. Simultaneous intratracheal ODSH administered with HMGB1 decreased all of these BAL measures, indicating that ODSH can inhibit HMGB1-RAGE- induced inflammatory responses *in vivo* (Rao et al., 2010). A summary of glycol split heparin, S-NACH, and ODSH activities *in vivo* in preclinical models relevant to CF is summarized in **Table 1**.

ODSH is effective in preclinical models of infection and inflammation to blunt these pathologic processes. In a *P. aeruginosa* (PA)-induced murine pneumonia model, intranasal ODSH decreases BAL HMGB1 levels, reduces pulmonary bacterial burden, ameliorates PA-induced lung injury, and improves survival (Sharma et al., 2014). In a murine model of intratracheal NE-induced lung inflammation and remodeling, ODSH pretreatment blocks NE-induced neutrophil influx, upregulation of KC, and release of HMGB1 into BAL (Griffin et al., 2014). To investigate the mechanism of ODSH inhibition of HMGB1 release, the impact of fluorescein-labeled (FITC)-ODSH on NE- or LPS-treated mouse macrophage cells (RAW264.7) was investigated. ODSH is taken up by RAW264.7 cells, and is localized to the cytoplasm and nucleus (Zheng et al., 2017). The sulfation pattern of modified heparins influence intracellular uptake and localization that is specific for different cell types (Raman et al., 2013). In RAW264.7 cells treated with NE or LPS, ODSH blocks HMGB1 lysine-acetylation in a dose-dependent manner, by inhibiting P300 histone (lysine) acetyltransferase (HAT) activity. Spectrofluorometry reveals that ODSH binding to p300 results in a conformational change in p300, and further tightens ODSH-p300 binding; this mechanism is supported by a complementary approach of in silico modeling with combinatorial virtual library screening of interactions between p300 and ODSH (Zheng et al., 2017) (**Figure 1B**). Importantly, heparin also interacts directly with HMGB1, changing its conformation and reducing its affinity for RAGE which interrupts the HMGB1-RAGE signaling cascade (Ling et al., 2011). Furthermore, heparin and ODSH bind to NE and inhibit its activity.

Heparin and ODSH Anti-NE Activity in *Ex Vivo* CF Sputum

High concentrations of NE released by neutrophils are found in CF sputum. Importantly NE, a cationic serine protease, binds to the copious polyanionic polymers in sputum including DNA (Gray et al., 2015); mucins (Nadziejko and Finkelstein, 1994) and actin filaments (Broughton-Head et al., 2007; Kater et al., 2007). Dornase alfa (Fuchs et al., 1994) and 7% hypertonic saline (HTS) (Elkins et al., 2006), the mainstay mucoactive therapies for patients with CF, improve pulmonary function, and decrease the frequency of pulmonary exacerbations. However, both therapies have been reported to increase NE activity in CF sputum (Cantin, 1998; Chen et al., 2006). ODSH is a robust inhibitor of NE activity *in vitro* with a low IC_{50} (Griffin et al., 2014; Kummarapurugu et al., 2018), but in CF sputum, both ODSH and heparin inhibition of NE activity requires DNA depolymerization by DNase-1 (Kummarapurugu et al., 2018). This observation suggests that anionic DNA polymers compete with anionic ODSH for binding to NE. When these interactions were investigated, it was discovered by both pharmacokinetic studies and by combinatorial virtual library screening, that both DNA and ODSH bind to the same allosteric domain on NE that is required for inhibition (Kummarapurugu et al., 2018) (**Figure 1B**). Furthermore, inhibition of NE activity in sputum by heparin or DNA is chain length dependent, with a requirement for a larger size than approximately 15 monosaccharides for heparins (Spencer et al., 2006; Kummarapurugu et al., 2018) or 12-mer for DNA oligomers (Kummarapurugu et al., 2018). Neither fondiparinux, a heparin pentasaccharide (1.8 kDa) nor a DNA hexamer have anti-NE activity, confirming that a threshold length is necessary for heparin and DNA to bind to NE and exert anti-elastase activity (Kummarapurugu et al., 2018). Interestingly, unfractionated heparin releases soluble DNA from sputum that is available for dornase alfa cleavage (Broughton-Head et al., 2007). Thus, heparin enhances DNase activity.

Novel Glycosaminoglycan Therapeutics as Anti-Protease, Anti-Microbial, and/or Anti-Inflammatory Therapies

Glycosaminoglycans have a broad array of functions both in native tissues and when modified to be used as competitors for endogenous heparan sulfate proteoglycans or for their properties to bind to cationic proteins and modify activities. Development of small synthetic non-saccharide glycosaminoglycan mimetics (NSGMs) offer modifiable alternatives for polysaccharide GAGs. NSGM 32 (Morla et al., 2019) has robust anti-elastase activity *in vitro* and has a mixed allosteric and orthosteric mechanism of action. However, NSGM 32 requires DNA depolymerization for anti-elastase activity in CF sputum, and is less potent than ODSH (Kummarapurugu et al., 2018). It was speculated that NSGM 32 binds to other positively charged moieties in CF sputum and therefore higher concentrations of drug are required for inhibition of NE activity (Morla et al., 2019). A sulfated synthetic lignin, sulfated dehydropolymer caffeic acid (CDSO3), inhibits the development of emphysema in a VEGFR-inhibitor-induced rat model *via* multiple functions including anti-oxidant activity, and prevention of epithelial and endothelial cell death *via* iron-chelation- induced stabilization of HIF-1α and VEGF signaling (Truong et al., 2017). These two compounds illustrate the exquisite target specificity due to sulfation patterns incorporated into small synthetically produced GAG mimetics. Another advantage of synthetic sulfated lignins is that they are homogeneous compounds that do not require porcine or bovine bioproducts for production.

Polysulfated hyaluronan is a modified hyaluronic acid which has potent anti-inflammatory properties (Zhang et al., 2011). Low molecular weight polysulfated hyaluronan blocks LPS-stimulated macrophage release of cytokines including TNFα, IL-6, IL-12, MCP-1, and increases expression of antioxidants, superoxide 2 and 3 (Jouy et al., 2017). In a murine model of second hand smoke induced lung disease, a polysulfated hyaluronan administered by intraperitoneal (i.p.) injection inhibits release of

TABLE 1 | *In vivo* models of chronic lung diseases treated with modified or non-saccharide GAGs.

Animal Model	Treatment (Dose and Administration)	Outcome Measures	Reference
Balb/c mice: NE airway inflammation model NE (o.a.) ± **ODSH** (o.a.)	Days 1, 4, 7: NE (44 μM) or NS **ODSH** (635 μM) or NS o.a. Day 8: BAL/lung harvest	NE induces BAL cells & PMN, KC, HMGB1 **ODSH**+NE: decreases total cells and PMN; decreases KC and HMGB1	Griffin et al. (2014)
C57BL/6 mice: *P. aeruginosa* (PA01) pneumonia model PA01 (i.n.) PA01 (i.t.) ± **ODSH** (s.c.)	Day 1: PA01 i.n. **ODSH** (8.3- 75 mg/kg) or NS s.c. q 12 h x 2 Day 2: BAL/lung harvest Day 1: PA01 i.t. **ODSH** (75 mg/kg) or NS s.c. 12 h x 4 Day 3: survival	**ODSH** decreases PA01 CFU; decreases lung protein content and edema; decreases total and PMN cell count; decreases BAL HMGB1; inhibits TLR2 and TLR4 binding **ODSH** improves mouse survival	Sharma et al. (2014)
C57Bl/6N *P.aeruginosa* pneumonia model (PA) CF isolate AA43- embedded in agar beads (i.t.) ± **glycol split LMWH, C3gs20 vs. N-acetyl LMWH, C23** s.c.	Day 1: PA- agar beads (1-2 x 10^6) vs. sterile beads i.t. Day 1-14: **C3gs20 or C23** (30 mg/kg/d) or vehicle s.c. Day 14: BAL and lung harvest Day 1: PA- agar beads (1-2 x 10^6) vs. sterile beads i.t. Day 10-28: **C3gs20 or C23** (30 mg/kg/d) or vehicle s.c. Day 28: BAL and lung harvest	**C23** decreased BAL total cells and PMN; No significant change in PA CFU. **C3gs and C23** decreased BAL total cells and PMN, decreased total PA CFU, and decreased IL-17A **C3gs20** decreased IL-1β, IL-12pp40, G-CSF, and KC	Lore et al. (2018)
C57BL/6J mice Allergic Asthma model OVA i.p. sensitization and challenge with Ova ± **sulfated non-anticoagulant LMWH (S-NACH) i.p.**	Wk 1: Alum/Ova i.p.once per wk x 2 Wk 2-4: Ova 3% inhaled 3x per week **S-NACH** (10 mg/kg) or NS i.p. Week 5: BAL and lung harvest	**S-NACH** decreased Ova-triggered eosinophils, macrophages, lymphocytes in BAL, decreased goblet cell metaplasia, decreased lung tissue hydroxyproline, decreased BAL and serum T2 cytokines, decreased Ova-IgE.	Ghonim et al. (2018)
C57BL/6 mice: LL-37- induced rhinosinusitis model LL37 i.n.± **polysulfated HA (GM-0111)** or HA i.n.	Day 1: LL-37 (115 μg) **GM-0111** or HA (800μg) Day 2: sinus harvest	LL-37 increases Mast cells, MPO, lamina propria (LP) thickening and cell death **GM-0111**+LL-37: Decreased Mast cells, MPO, LP thickening and cell death **GM-0111** more effective than HA	Pulsipher et al. (2017)
BALB/c mice: Aspergillus chronic rhinosinusitis (CRS) model A.fumigatus extract ± **polysulfated HA (GM-1111) or PBS** i.n. 3 Groups: 1. 1. PBS 2. 2. A fumigatus+ PBS 3. 3. A. fumigatus+ GM-1111	Week 0: All groups sensitized with Alum + PBS or *A.fumigatus* i.p. Weeks 1-8: PBS or *A.fumigatus* extract (20,000 PNU i.n.) 3 x per wk. Weeks 5-8: PBS or GM-1111 (600 μg) i.n.5x per wk Week 9: Collect blood and sinonasal tissue	**GM-1111**+ *A.fumigatus* (Af) extract decreased Af-induced CRS symptoms, mucosal edema and injury, goblet cells, TLR2 and TLR4, T2 cytokines, and IgE	Alt et al. (2018)
C57BL/6 mice Second hand smoke model of lung disease ± **sulfated semisynthetic HA GAG ethers (SAGEs)**	SHS vs. Rm air nasal inhalation 10 min/day x 5 d/wk 4 weeks exposure **SAGE** (30 mg/kg) i.p. for 3 d/wk Collect BAL and lung RNA and protein	**SAGEs** effect on SHS exposure: Blocked lung RAGE expression Blocked BAL protein, total cells, and cytokines: IL-α, IL-2, TNFα	Tsai et al. (2019)
Sprague Dawley rats Rat Emphysema Model with SU51416 (VEGFR inhibitor)±	Day 1: SU5416 (20 mg/kg) s.c. ± Day 1–Day 21:	**CDSO3** prevented SU5416-induced emphysema, improved rat exercise endurance, decreased oxidative stress,	Truong et al. (2017)

(Continued)

TABLE 1 | Continued

Animal Model	Treatment (Dose and Administration)	Outcome Measures	Reference
polysulfated dehydropolymer of caffeic acid (CDSO3) 3 Groups: Untreated healthy SU5416 + NS SU5416 + **CDSO3**	**CDSO3** (60 μg/kg) or NS inhaled 3x per week	and increased VEGF and HIF-1α, and decreased cleaved caspase-3	

BAL, bronchoalveolar lavage; HIF-1α, hypoxia inducible factor- 1α; i.n., intranasal; i.p., intraperitoneal; i.t., intratracheal; KC, keratinocyte chemoattractant; LMWH, low molecular weight heparin; MPO, myeloperoxidase; PMN, neutrophil; PNU, protein neoantigen units; o.a., oropharyngeal aspiration; RAGE, receptor for advanced glycation end products; s.c., subcutaneous; SHS, second hand smoke; TNFα, tumor necrosis factor α; VEGF, vascular endothelial growth factor.
The bolded text are to emphasize the stimuli for the model type and the drugs used to treat this model.

BAL TNFα, IL-1α, and IL-2, and decreases BAL inflammation and lung permeability (Tsai et al., 2019). A sulfated semisynthetic low molecular weight hyaluronan, GM-1111, (molecular weight 5.5 kD), has been tested for anti-inflammatory properties. In a mouse model of rhinosinusitis generated by intranasal administration of a cathelicidin fragment, LL37, GM-1111 blocks neutrophil and mast cell mucosal infiltration and significantly decreases epithelial apoptosis (Pulsipher et al., 2017). In vitro, in nasal epithelial cells, LL37 stimulates inflammation and cell death; another GM compound, GM-0111, inhibits IL-6 and IL-8 release and blocks Caspase-1- and Caspase-8 -induced cell death (Thomas et al., 2017). In an *A.fumigatus*- intranasal allergen-sensitization mouse model of chronic rhinosinusitis, intranasal GM-1111 introduced 3 weeks after *A. fumigatus* sensitization, significantly inhibits goblet cell metaplasia and mucosal T2 inflammation, and decreases TLR2 and TLR4 expression (Alt et al., 2018). In addition, in a periodontitis model, GM-0111 suppresses the growth of *P. gingivalis* and *A. actinomycetemcomitans* and biofilm formation, demonstrating antimicrobial activity (Savage et al., 2016). A summary of polysulfated hyaluronan activities *in vivo* in preclinical models of chronic lung disease is summarized in **Table 1**.

Heparan sulfate (HS) is expressed widely on many cell types as a proteoglycan. HS proteoglycans regulate inflammation by binding to ELR (Glu Leu Arg)- CXC chemokines at conserved His, Lys, Arg residues, controlling chemotactic gradients in the extracellular and pericellular matrices (Rajarathnam KaD, 2020). However, in the CF lung, endogenous HS proteoglycans have pro-inflammatory properties (Reeves et al., 2011); HS stabilizes cytokine and chemokine ligands, preventing protease digestion, thus increasing CXCL ligation to CXCR1 and 2 to upregulate inflammation (Rajarathnam KaD, 2020). HS enables RAGE hexamer formation for more efficient intracellular signaling (Xu et al., 2013), and binds L-selectin to promote neutrophil slowing and diapedesis across endothelial cells into tissues (Farrugia et al., 2018). HS also serves as a cell receptor for microbe adhesion and invasion (Rostand and Esko, 1997) (**Figure 2**). Bacteria, *P. aeruginosa* (Paulsson et al., 2019) and *nontypable H. influenza* (NTHi) (Su et al., 2019), and viruses, adenovirus (Dechecchi et al., 2001) and Severe Acute Respiratory Syndrome-Coronavirus (SARS-CoV) (Lang et al., 2011) and SARS-CoV-2 spike protein (So Young Kim et al., 2020) all bind to HS proteoglycans. Importantly, exposure to heparin competes with and inhibits binding to HS proteoglycans (**Figure 2**) resulting in inhibition of binding of *P. aeruginosa* (Paulsson et al., 2019) and NTHi (Su et al., 2019) to laminin, a major component of the basal lamina in the airway, and inhibition of binding of adenovirus (Dechecchi et al., 2001) and SARS-CoV (Lang et al., 2011) to epithelia, and inhibition of SARS-CoV-2 (So Young Kim et al., 2020) spike protein to HS as detected by surface plasmon resonance. Treatment with synthetic HS or heparin inhibits cytokine/chemokine binding to G-protein coupled receptors and blocks neutrophil interaction with endothelial selectins resulting in decreased neutrophil influx (Lore et al., 2018) (**Figure 2**). Altered sulfation of HS affects pro- vs anti-inflammatory behavior; increased N- and 6-O-sulfation increase cytokine ligation and neutrophil recruitment while increased 2-O-sulfation blunts neutrophilic inflammation (Axelsson et al., 2012). HS is also required for HMGB1-RAGE receptor binding; heparin can compete with HS and interrupt RAGE ligation by binding to HMGB1 (Xu et al., 2011).

Clinical Trials Using GAGs for Respiratory Diseases

Unfractionated heparin is the only GAG used in clinical trials to date. Inhaled heparin was tested in healthy volunteers and is safe and well tolerated. When delivered by nebulization, approximately 8% of the nebulized dose of heparin is delivered to the lower respiratory tract (Bendstrup et al., 1999). Importantly, inhaled doses up to 400,000 IU, did not affect lung function, but did increase circulating anti-Factor Xa activity and activated partial thromboplastin time (APTT) (Bendstrup et al., 2002). BAL fluid was tested for anti-coagulant activity in the presence of control plasma and by this method, the half-life of inhaled heparin was determined to be 28 h (Markart et al., 2010).

Inhalation of unfractionated heparin has been tested as a therapeutic for severe COPD (Shute et al., 2018), asthma (Yildiz-Pekoz and Ozsoy, 2017), smoke inhalation (Miller et al., 2014), and acute lung injury (ALI) (Dixon et al., 2010; Tuinman et al., 2012; Juschten et al., 2017), but the number of randomized, double-blind, placebo-controlled studies for these indications is limited (**Table 2**). There is one randomized, double- blind, placebo-controlled, crossover trial of twice daily inhaled heparin (50,000 IU per dose) for 2 weeks in adults with CF, which demonstrates a good safety profile, but does not show any significant improvement in lung function, sputum inflammatory markers or mucus clearance (Serisier et al., 2006). In contrast, a

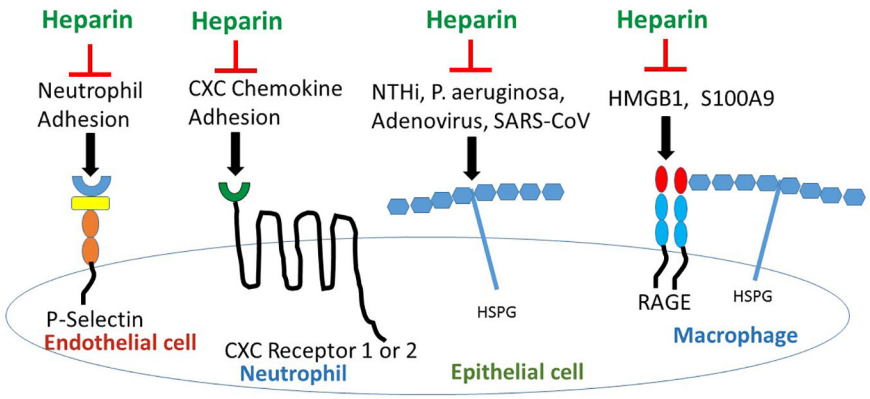

FIGURE 2 | Heparin/ODSH interrupts cell- cell interactions and ligand-receptor binding to block pro-inflammatory pathways. Heparin/ODSH oligosaccharides bind to P- and L-selectins and block neutrophil adhesion and chemotaxis (Nelson et al., 1993; Rao et al., 2010). Heparin inhibits CXCL8/IL-8 and other ELR (Glu Leu Arg)-CXC chemokines from binding to G-protein coupled receptors CXCR1 and CXCR2 (Rajarathnam KaD, 2020). Heparin competes with HSPG for binding to microbial proteins which prevents bacterial or viral-epithelial adhesion and invasion (Rostand and Esko, 1997). Heparin/ODSH bind to HMGB1 and S100A9 and interrupt RAGE ligation (Rao et al., 2010). HSPG, heparan sulfate proteoglycan; NTHi, non-typeable H. influenza; S100A9, calgranulin; SARS-CoV, Severe acute respiratory syndrome- corona virus.

randomized, double-blind, placebo-controlled single site study for COPD using twice daily inhaled heparin (150,000 IU per dose) in addition to inhaled twice daily salbutamol & beclomethasone and airway clearance for 21 days reveals that heparin improves lung function including FEV_1, 6 minute walk distance, and Borg dyspnea score (Shute et al., 2018). The contrast between the COPD study (Shute et al., 2018) and the previously cited CF study (Serisier et al., 2006) suggests that possible reasons for the failure of heparin to improve lung function in patients with CF were an insufficient dose of heparin and/or a limited trial duration to observe clinically significant changes in pulmonary function.

SUMMARY

There are many challenges for developing anti-protease and anti-inflammatory drugs for patients with CF. The innate immune

TABLE 2 | Clinical trials using heparin for chronic lung diseases*.

Disease	Trial design	Drug: dose and administration mode	Outcomes compared to placebo	Reference
Cystic Fibrosis	R, PC, DB- 2 weeks; CF adults; moderate to severe lung disease;N=18	Heparin (50,000 U) inhaled every 12 h	No change in FEV_1 serum CRP sputum IL-8, MPO, NE, TCC, sputum volume	Serisier et al. (2006)
COPD	R, PC, DB- 3 weeks; COPD- GOLD II- IV; N=40	Heparin (75,000 or 150,000 IU) Inhaled twice per day	Adherence 56% Improved FEV_1 Improved 6MWD Increased SpO2	Shute et al. (2018)
Asthma	R, PC, DB crossover; Allergic to dust mite; N=10	Heparin (20,000 U) inhaled 10 min before inhaled dust mite extract bronchoprovocation challenge	Heparin increased the Log_2 provocation dose of dust mite protein nitrogen units causing 20% fall in FEV_1	Bowler et al. (1993)
Asthma	R, PC, DB crossover; Allergic to dust mite; N=8	Heparin (1000 U/kg/dose) inhaled: 90 min and 30 min pre-dust mite inhaled challenge, and 2, 4, 6 h post-dust mite inhaled challenge	Heparin blunted the severity of FEV_1% decrease in late asthmatic responses compared to placebo	Diamant et al. (1996)
Asthma EIB	R, PC, SB, cross-over—5 days; Asymptomatic; N = 12	Day 1: baseline PFT and exercise challenge; Day 3-5: Heparin (1000 U/kg) or cromolyn (20 mg) or placebo inhaled followed by exercise challenge	Heparin blocks post-exercise decrease in SGaw	Ahmed et al. (1993)
Asthma EIB	R, PC, DB, cross-over -7 days; Asymptomatic; N=13	Day 1: baseline PFT and exercise challenge; days 3–7: inhaled Heparin (80,0000 U) or Enoxaparin (0.5, 1, 2 mg/kg) or placebo 45 min before baseline PFTs and then serially post-exercise	Decrease in FEV_1 was blocked by heparin and enoxaparin	Ahmed et al. (1999)

**Only trials with randomized, double or single blind, placebo controlled design were included. 6MWD, 6 minute walk distance test; CRP, C-reactive protein; DB, double-blind; EIB, exercise-induced bronchospasm; FEV_1, Forced expiratory volume at 1 sec; MPO, myeloperoxidase; NE, neutrophil elastase; PC, placebo controlled; R, randomized; SB, single-blind; SGaw, Specific conductance of the airways; SpO2, oxyhemoglobin saturation; TCC, terminal complement complex.*

response is impaired for both viral (Zheng et al., 2003; Berkebile et al., 2020) and bacterial infections. The CF airway milieu is typified by high concentrations of several proteases including neutrophil serine proteases: NE, proteinase 3, Cathepsin G; lysosomal proteases: Cathepsins B, L, and S; and matrix metalloproteases: MMP-9, MMP-8 and MMP-12 (McKelvey et al., 2019) which stimulate downstream signaling cascades that perpetuate oxidative stress and inflammation. The strategy of directing therapy to one target is unlikely to be successful to control inflammation and prevent lung injury. Instead, we propose that GAGs can be developed and harnessed as multi-functional anti-elastase and anti-inflammatory therapies and serve an important function as part of the armamentarium for CF lung disease.

AUTHOR CONTRIBUTIONS

All authors wrote the text and edited the text. JV designed the figures.

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