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

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14. ABSTRACT

Neutrophil elastase (NE) impairs phagocytic function. NE is taken up by human blood monocyte derived macrophages (hBMDM) and retains proteolytic activity in the macrophages, leading to cleaving of multiple targets. We discovered that NE clips Histone H3 in hBMDM resulting in chromatin decondensation and release of Macrophage Extracellular Traps (METs). We also discovered that NE cleaves histone deacetylases and sirtuin 1 resulting in unopposed acetyltransferase activity that causes translocation of a major alarmin, High Mobility Group Box 1 (HMGB1) from the nucleus to the cytosol. We have published three papers related to this project and have three additional papers in revision or in preparation about this work this year. We are completing analysis of hBMDM cell lysates from control subjects to determine whether NE increases acetyllysine modifications of Histone H3 or alters other cellular proteins critical for chromatin structure as part of the mechanism of increased inflammation and NE-activated METs release.

15. SUBJECT TERMS

Neutrophil elastase, Macrophage, extracellular traps, HMGB1, histone deacetylase

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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**).

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)

2. **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.

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 localization and protease activity; Cytokine protein expression

3) Significant Results and Key Outcomes:

We have completed NE localization (**Figure 1**) and proteinase activity **(Figure 2**) experiments in human blood monocyte derived macrophages (hBMDM) (1, 2). Human BMDM from subjects with COPD take up NE in a dose-dependent manner and uptake is localized to the nucleus and cytoplasm. We demonstrate this by 2 complementary methods: FITC-NE uptake by confocal microscopy and immunofluorescence with anti-NE antibody. Proteinase activity was measured following NE exposure by a NE proteinase specific assay kit.

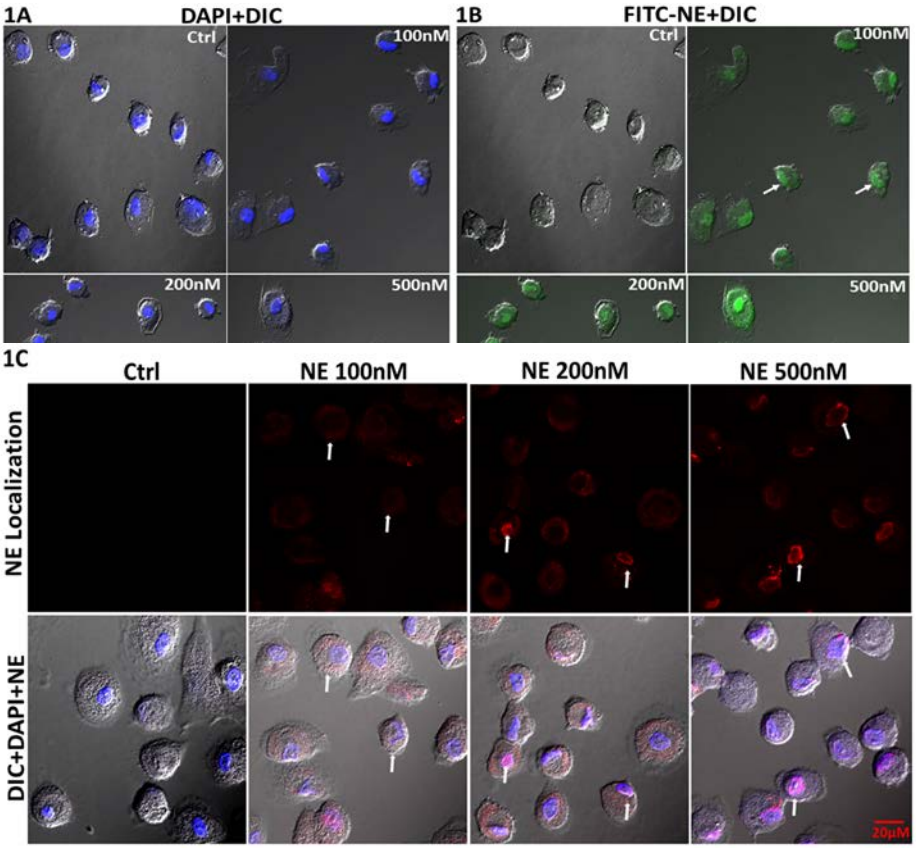


Figure 1. Neutrophil elastase (NE) is taken up by hBMDM in a dose-dependent manner.
A & B. Human BMDM from subjects with COPD were adhered to coverslips and treated with control vehicle (Ctrl) or FITC-NE (100-500 nM) for 2 h, followed by DAPI nuclear staining

C. COPD hBMDM were treated with Ctrl or NE (100 -500 nM), 2 h, and incubated with primary mouse McAb anti-human NE, followed by goat anti-mouse secondary Ab conjugated with AlexaFluor 595. Results representative of 3 COPD donors

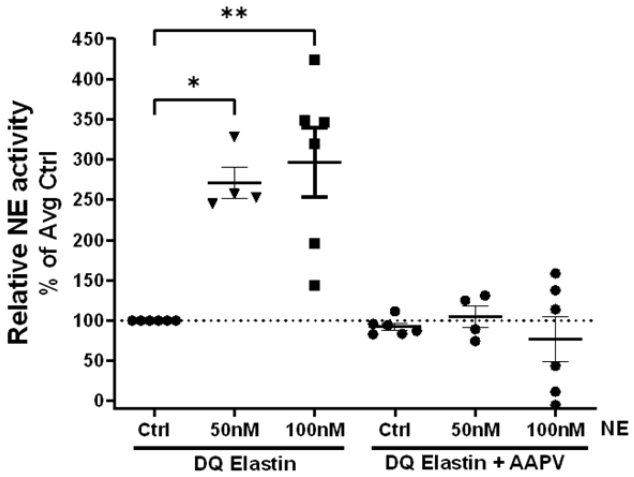


Figure 2. Human BMDM take up NE and retain proteolytic activity intracellularly. Human BMDM from subjects with COPD were treated with NE (0, 50, or 100 nM, 2 h, 37°C) while in suspension. Following treatments, cells were exposed to DQ-elastin. NE inhibitor, AAPV-CMK, was used as a NE-specific, negative control.
N=6 donors, *, $p<0.05$; **, $p< 0.01$.

We recently examined whether hBMDM developed features of M1 or M2 polarization after NE treatment. **We determined that NE treatment caused increased TNFα release from hBMDM consistent with M1**

polarization, but not CCL18, consistent with no M2 polarization (**Figure 3**). In contrast treatment of the hBMDM with IL-4 and IL-13 caused increased CCL18 release but little to no TNFα release, demonstrating that these cells are capable of M2 polarization under control treatment conditions. We also evaluated hBMDM polarization by examining cell surface markers for M1 and M2 by flow cytometry. We determined that hBMDM have persistent CD 68 (a pan macrophage marker) and CD206 consistent with M1 and M2, under all treatment conditions. Following treatment with LPS for 2 days, hBMDM increased expression of CD80, a M1 marker but not CD209, a M2 marker and in contrast, after treatment with IL-4 and IL-13 for 2 days, hBMDM expressed high levels of CD209, a M2 marker, but had no increase in CD80, a M1 marker. We show that with NE treatment for 2.5 h (in contrast to the 2 day treatment with LPS or IL4/ IL-13), there is no significant change in M1 or M2 markers. (**Figure 4**). Therefore, evaluation of hBMDM cytokine release may be more informative of cellular phenotype than the cell surface markers and/ or we will need to broaden our survey of cell surface markers to denote macrophage polarization. These experimental results were combined with data from hBMDM from patients with cystic fibrosis in a recent publication (1).

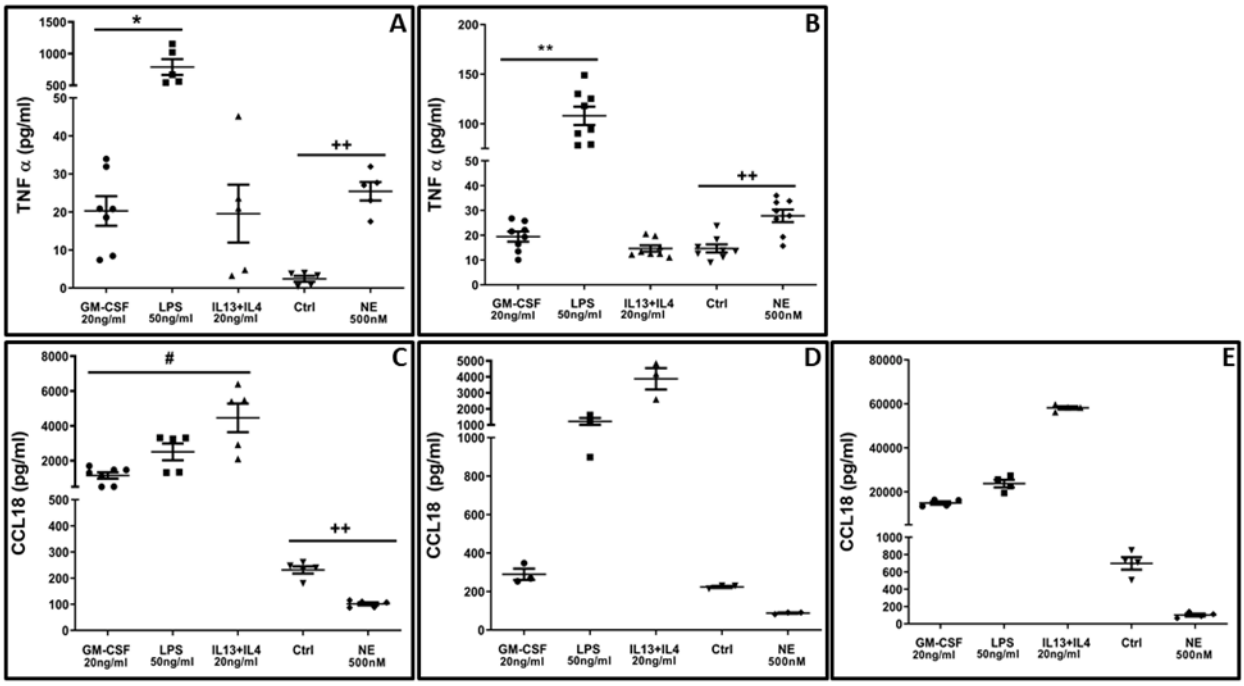
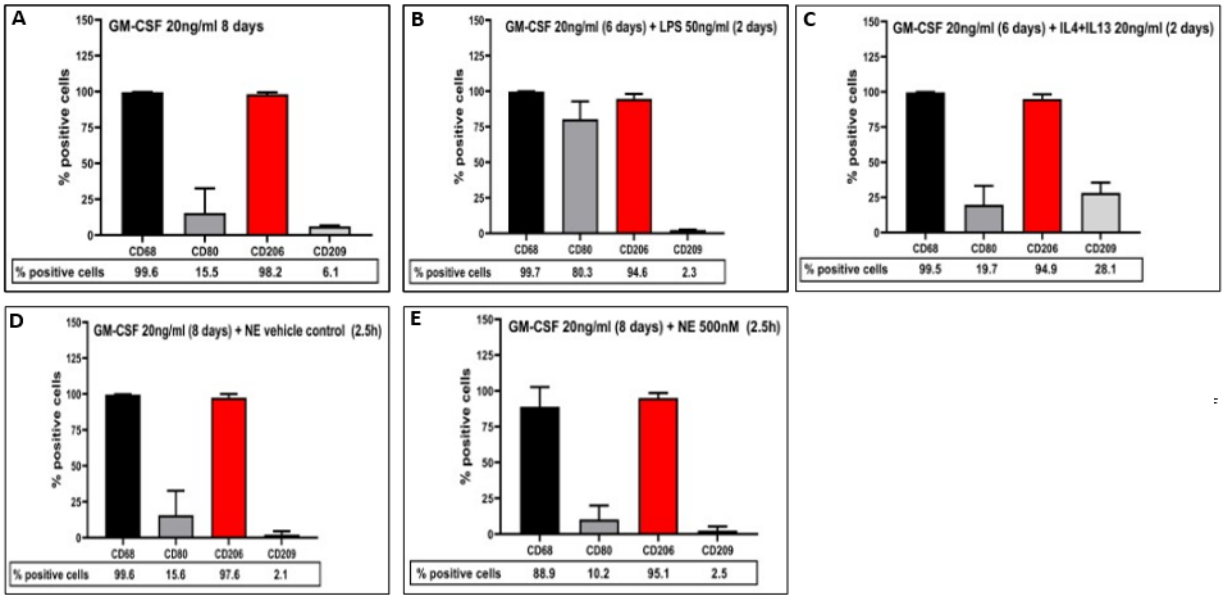


Figure 3. Human BMDM exhibited M1 like phenotype post- NE exposure as determined by cytokine secretion. Monocytes obtained from non-CF and CF donors, were differentiated to macrophages in primary culture with GM-CSF (10 days). These cells alone or after stimulation with NE (500nM) or control vehicle 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**, n= 2 donors) and CF (**B**, n= 2 donors) and M2 cytokine, CCL-18, in the conditioned media obtained from non-CF (**C**, n= 2 donors) and CF (**D & E**, n=2 donors). For positive controls, 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; to generate M2 BMDM. After a media change at 2 days, conditioned media reflected two days of culture for the positive control culture conditions. In contrast, conditioned media from control-treated and NE-treated cells reflected only 2 h treatment. 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.

Figure 4. Flow cytometry of hBMDM cell surface markers for M1 or M2 differentiation. Monocytes from healthy donors were cultured in GM-CSF (20 ng/ml) for 8 days to differentiate into M0 (**A**). To generate positive controls for M1, M0 cells at 6 days were further cultured in LPS (50 ng/ml, 2 days) (**B**) or in IL-4/ IL-13 (20 ng/ml each, 2 days) (**C**). hBMDM cultured for 8 days were also tested for treatment with control vehicle (2.5 h) (**D**) or NE (500 nM, 2.5 h) (**E**). Following these exposures, population frequencies of hBMDM M1 or M2 were assessed by staining with conjugated antibodies against cell surface markers including CD68 (pan-macrophage, M0), CD80 (M1), and CD206 and CD209 (both mannose receptors, M2) for flow cytometry. Bar graphs represent the mean ± SEM of the percentage positive macrophage populations for 2 different donors, with n=4-6 replicates per experiment. There was no change in cell surface markers after NE treatment.



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

3) Significant Results and Key Outcomes: We have a paper in revision evaluating the impact of NE on histone deacetylases and Sirtuins entitled "Neutrophil Elastase degraded Histone Deacetylases and Sirtuin1 resulting in High Mobility Group Box 1 accumulation in the cytoplasm of primary human macrophages". We demonstrated that in hBMDM from healthy individuals, NE causes the degradation of Class I HDACs, HDAC1 and HDAC2 (**Figure 5A**), most class II HDACs (**Figure 5B**) and Sirt1 (**Figure 5C**). Consistent with the loss of HDAC protein abundance, NE treatment caused decreased HDAC activity, but had no significant effect on histone acetyltransferase (HAT) activity (**Figure 6**). NE treatment resulted in increased lysine acetylation of hBMDM cytoplasmic proteins and triggered the translocation of High Mobility Group Box 1 (HMGB1) from the nucleus to the cytoplasm (**Figure 7**), a prerequisite for cell release of HMGB1. Human BMDM from patients with COPD or cystic fibrosis (CF) exposed to NE, have decreased SIRT1 by ELISA (**Figure 8A**) and decreased SIRT activity (**Figure 8B**). NE decreased HDAC4 and HDAC5 (Class II HDACs) in BMDM from subjects with CF and with COPD as determined by western analysis, consistent with our results from NE treatment of healthy BMDM (**Figure 9**). Importantly, pharmacologic inhibition of HDAC activity with a global inhibitor, TSA, increased acetyllysine modifications of hBMDM cytoplasmic proteins from healthy individuals and increased the nuclear to cytoplasmic translocation of HMGB1 at 24hrs (**Figure 10**). Treatment with a Sirt1 specific inhibitor plus TSA significantly increased the translocation of HMGB1 from nucleus to cytoplasm at 2hrs (**Figure 11**). Therefore, NE likely activates acetyllysine modifications and HMGB1 translocation from nucleus to cytoplasm by degrading HDACs and Sirt1. These data are included in a manuscript that is being finalized for submission (2).

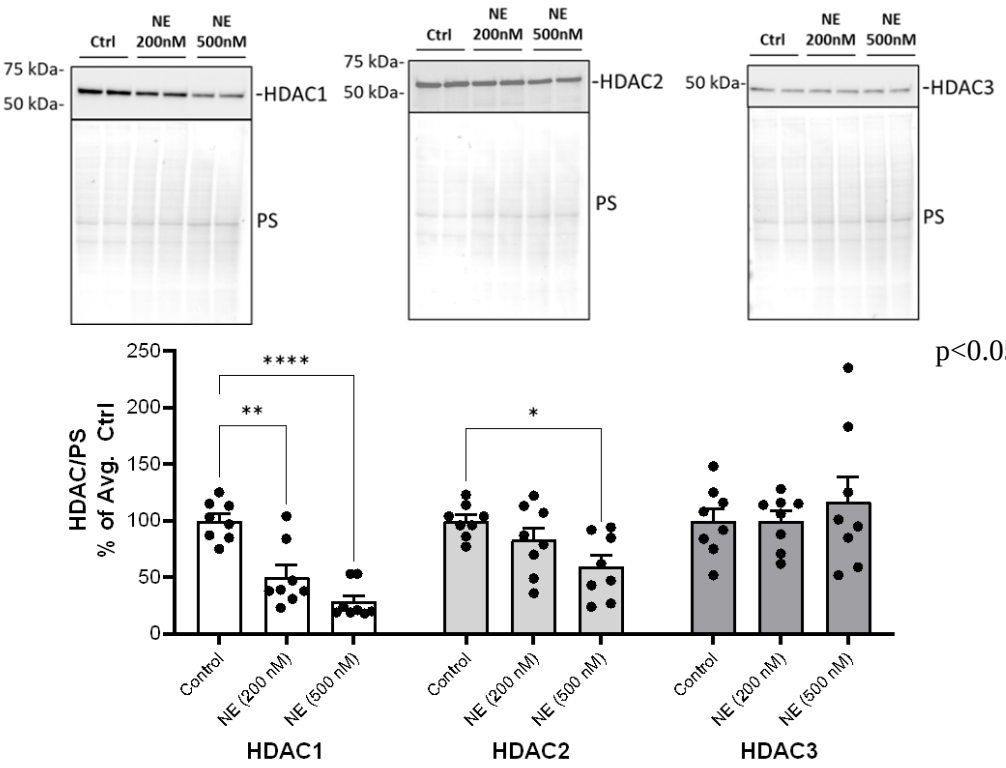


Figure 5A. NE degrades HDAC 1. Healthy hBMDM treated with control vehicle or NE (200 or 500 nM), 2 h
Nuclear Cell fractionation
Nuclear Lysates analyzed by western for Class I HDAC expression normalized to Ponceau S, N=3 donors, 2-3 replicates per donor. *, p<0.05; **, p< 0.01; ****, p< 0.001.

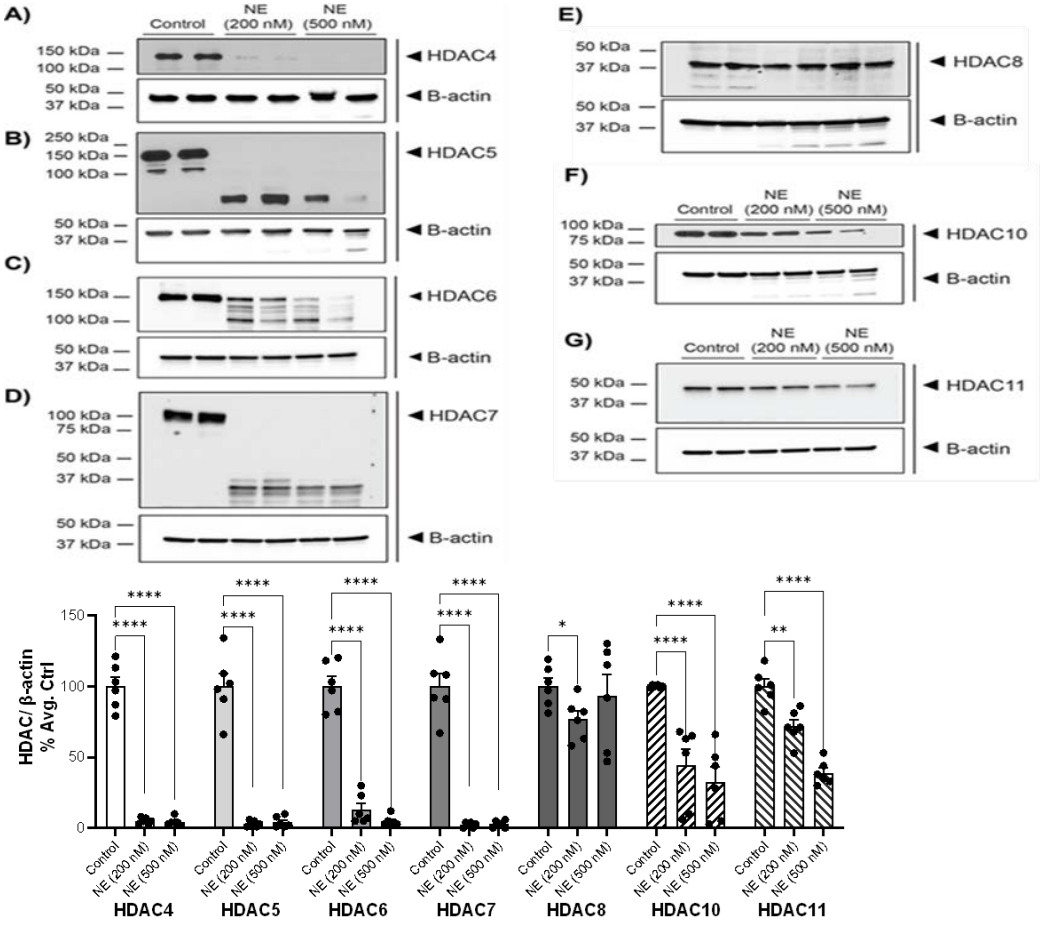


Figure 5B. NE degrades most Class II HDACs. Healthy hBMDM treated with control vehicle or NE (200 or 500 nM), 2 h. Total Cell lysates were analyzed by westerns for Class II HDAC expression, normalized to beta actin and expressed as a percentage of control. Data was summarized for N=3 donors, 2-3 replicates per donor. *, p<0.05; **, p< 0.01; ****, p< 0.001.

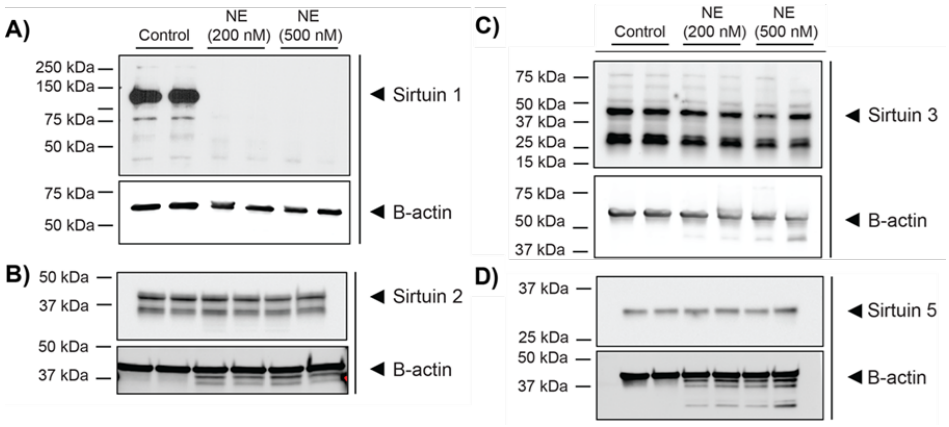
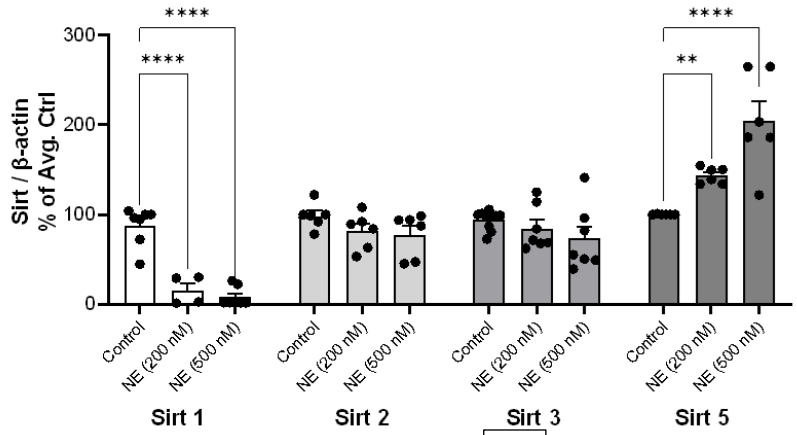


Figure 5C. NE degrades Sirt1 but not other Sirtuins. hBMDM treated with control vehicle or NE (200 or 500 nM), 2h. Total cell lysate harvested and separated by PAGE for western analysis. Data from N-3 donors in 2-3 replicates per donor had bands quantified by Image J, normalized to actin and compared to control concentrations (100%). *, p<0.05; **, p< 0.01; ****, p< 0.001.



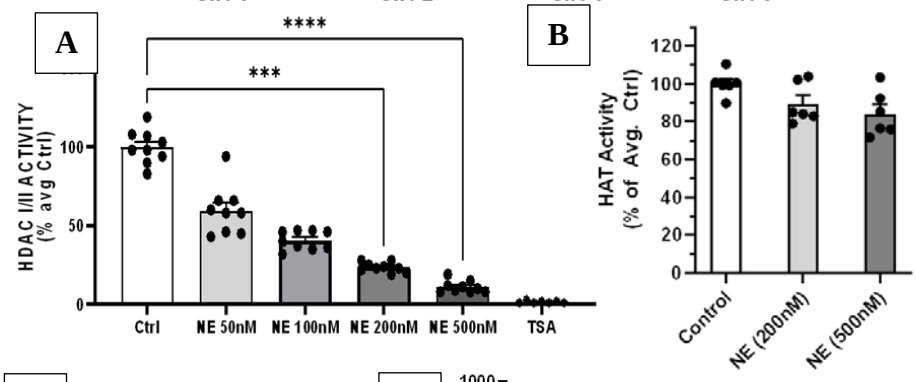


Figure 6. NE degrades HDAC activity but does not affect HAT activity. Healthy hBMDM were treated with control vehicle or NE (50-500 nM) or with trichostatin A (TSA, 10 μM), a global HDAC inhibitor, 2 h. HDAC activity was measured by Promega HDAC I/ II Glo-assay. HAT activity measured in nuclear lysates by Biovision kit. N=3 individuals, triplicate assays per donor, normalized to control treated samples/ cells; ***, p<0.001; ****, p<0.0001.

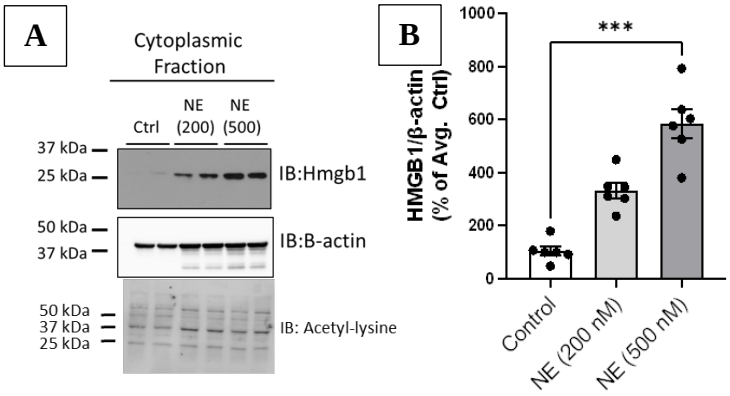


Figure 7. NE treatment increased High Mobility Group Box 1 abundance in the cytoplasm which correlated with increased acetyllysine. Healthy hBMDM were treated with control vehicle or NE (200 or 500 nM, 2 h). Cytoplasmic extracts were prepared (Active Motif kit). **A**. Western analysis of cytoplasmic lysates were prepared for HMGB1, βactin, and total acetyl-lysine (Abcam 21623) **B.** Data summarized from n=3 donors with 2 replicates per treatment condition; ***, p<0.001.

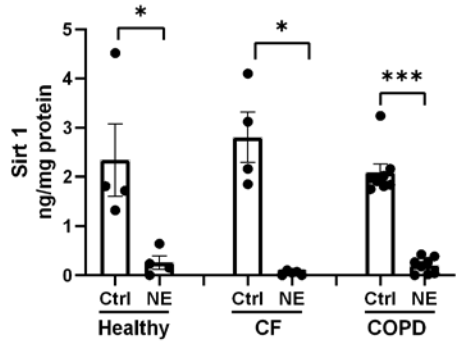


Figure 8A. NE treatment of hBMDM from healthy subjects or subjects with COPD or CF, resulted in degradation of Sirt1. Total cell lysate (TCL) from Ctrl or NE (200nM, 2h) treated hBMDM from Buffy coat samples (healthy), patients with CF, or patients with COPD, were evaluated for Sirt1 protein by ELISA (Abcam, Ab171573). Sirt1 was normalized to total protein levels (ng/mg protein) and data summarized as Mean±SEM. Heathy, n=4; CF, n=4; COPD, n=8. Statistical analysis was performed by Prism, with initial non-parametric ANOVA and post-hoc, Mann-Whitney test, *, p<0.05; ***, p<0.001.

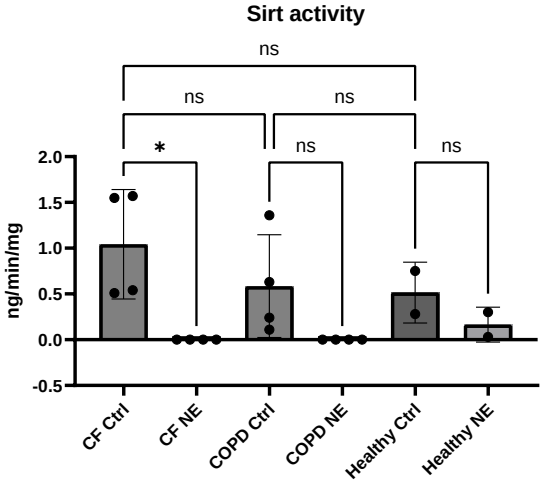


Figure 8B. NE treatment of hBMDM from CF, COPD and healthy subjects decreased SIRT activity. Total cell lysate from hBMDM treated with NE (200 nM, 2 h) or control vehicle were evaluated for SIRT activity. BMDM were from patients with CF (n=4), COPD (n=4) and healthy volunteers (n=2). SIRT1 activity assay kit (Abcam, Ab156915) was used to measure SIRT1 in total cell lysate, and expressed as ng/min/mg protein. *, p<0.05; ns, not significant.

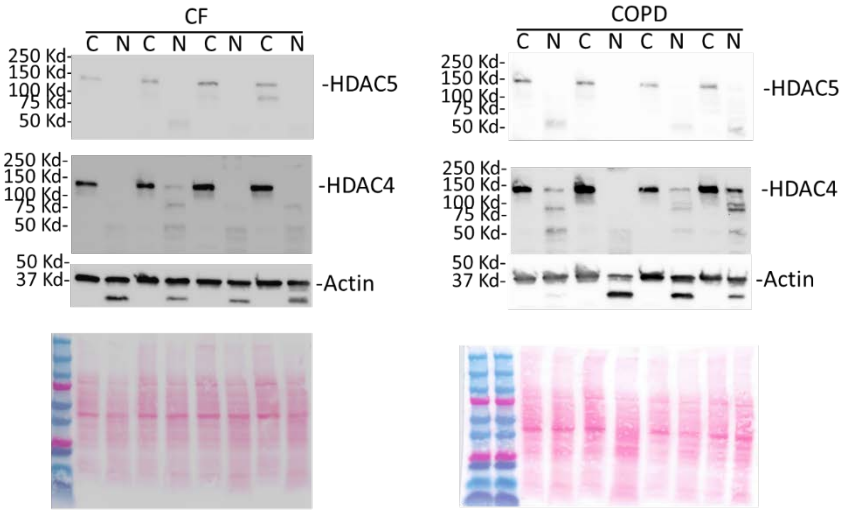


Figure 9. NE degrades HDAC5 and HDAC4 and clips actin in BMDM from subjects with CF and COPD. BMDM from CF (n=4) and COPD (n=4) subjects were treated with NE (N, 200 nM, 2 h) or control vehicle (C). Cell lysates were analyzed by immunoblot for HDAC 5 and HDAC4. HDAC5 antibody (sc-133106, 1:500) and secondary anti-mouse HRP Ab (1:3000) with development using ultra enhanced chemiluminescence (ECL). HDAC4 antibody (CST 7628, 1:1000), and secondary anti-rabbit- HRP Ab and development using ECL. Blots were stained with two controls: actin (Sigma Aldrich A54441,1:3000) and secondary anti-mouse-HRP and ECL. All blots were stained by Ponceau S to evaluate total protein.

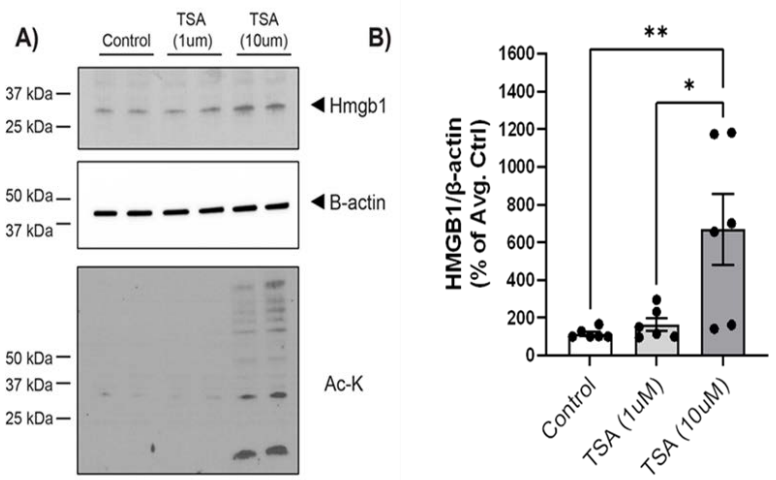


Figure 10. Pharmacologic inhibition of HDACs with Trichostatin A (TSA) for 24 h, increased lysine acetylation and increased HMGB1 translocation from nucleus to cytoplasm. Healthy hBMDM were treated with control vehicle or TSA (1 or 10 μM, for 24h). Cytoplasmic extracts from treated cells were prepared using Active Motif kit
A. Western blot of HMGB1, βactin, and total acetyl-lysine (CST 9814) were performed. HMGB1 abundance was normalized to βactin.
B. Data summarized from n=3 donors with 2 replicates per treatment conditions. *, p<0.05; **, p<0.01

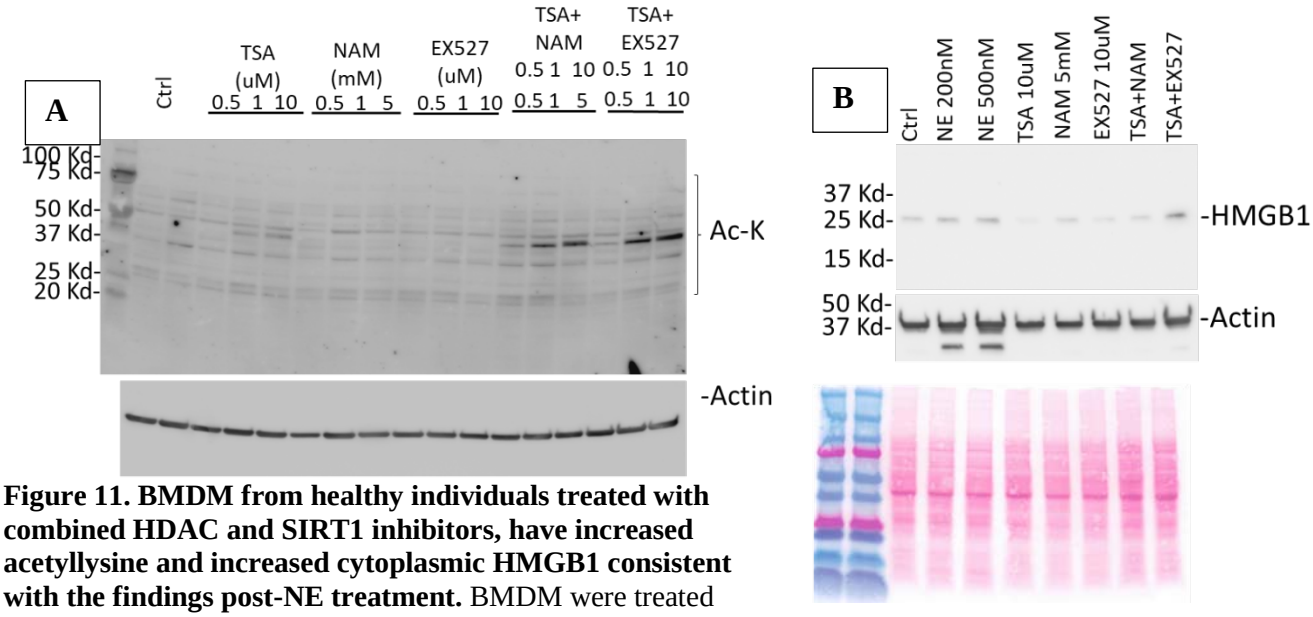


Figure 11. BMDM from healthy individuals treated with combined HDAC and SIRT1 inhibitors, have increased acetyllysine and increased cytoplasmic HMGB1 consistent with the findings post-NE treatment. BMDM were treated with an HDAC inhibitor, Trichostatin (TSA), a general Sirtuin inhibitor, Nicotinamide (NAM), or a specific Sirt1 inhibitor, EX-527, or combined therapy of TSA + NAM or TSA + EX527 for 2 h. Cells were incubated with different doses for 2 h, cytoplasmic fraction was isolated and evaluated by western analysis for acetyllysine (**A**), using anti-acetylysine Ab (Ab21623 Abcam, 1:1000) or HMGB1 (**B**), using anti-HMGB1 Ab, SC56698, 1:1000). NE treatment was a positive control for HMGB1 translocation to the cytoplasm. Beta Actin westerns and Ponceau S staining were loading controls. The combination of TSA + EX527 generated the greatest increase in HMGB1.

Stated Goals not Met: We had proposed to obtain alveolar macrophages from patients with COPD who undergo clinical bronchoscopies. We received IRB approval for clinically indicated bronchoalveolar lavage procedures to obtain fluid to isolate macrophages. However, it was difficult to obtain BAL from interventional bronchoscopy procedures for pulmonary nodule evaluation, because the Interventional Pulmonary and Critical Care physicians do not perform bronchoalveolar lavage during at the time of biopsies for diagnosis of lung nodules. The IRB did not permit us to add bronchoaleolar lavage to the lung biopsy procedures without convening a data safety monitoring board. Therefore, we were unable to obtain BAL samples from COPD patients who were not acutely ill.

We had proposed to use siRNA to inhibit Sirt1 as a complementary approach to determine the requirement for Sirt1 to retain HMGB1 in the nucleus and to prevent lysine acetylation. We had not completed that task due to the need to complete additional experiments for publication of the NE and HDAC degradation manuscript. Instead, we did use a specific inhibitor for Sirt1 which demonstrated that loss of Sirt1 activity was associated with translocation of

HMGB1, an alarmin, from nucleus to cytoplasm. In the future, we will develop siRNA or CRISPR-cas9 methodology to inhibit Sirt1 to evaluate the impact on MDM epigenetic regulation.

We had proposed to use LC-MS to determine post-translational modification/ lysine acetylation of specific targets including HMGB1, Histone H3 and p65/ NFkB. We detected Histone H3 acetyllysine modifications but due to degradation/ clipping of Histone H3, it was not possible to quantify H3 acetyllysine. In future work, we will evaluate for acetyllysine of H3, HMGB1, and p65 by alternative methods including IP and IB for specific 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 macrophage extracellular traps (METs) by LC-MS/MS.

3) Significant Results and Key Outcomes: We determined that NE induced release of METs from COPD BMDM, as quantified by measuring ecDNA by the PicoGreen assay (**Figure 12**). Furthermore, we detected increased MET release with more severe lung disease. We examined the impact of NE on Histone H3 and detected clipping of Histone H3 but not Histone H4 (**Figure 13**), and increased Histone H3 citrullination (**Figure 14**). We performed western analyses of the enzymes required for Citrullination, Peptidyl Arginine deiminase (PAD). We quantified PAD 2 and 4 protein and analyzed total PAD activity (**Figure 15**). There was no increase in PAD activity and no increase in PAD protein levels. Therefore, NE-induced MET release from COPD hBMDM was likely due to Histone H3 clipping. We also completed quantitation of Histone H3 clipping to demonstrate a significant increase in the clipped fragment at the greatest concentration of NE treatment in hBMDM (**Figure 13**). We also quantified total C-reactive protein in the plasma of the 14 subjects with COPD that had METs analyzed and determined that there were no elevated CRP levels in any subjects. These additional data were included in a manuscript in press (Clinical Translational Science (3).

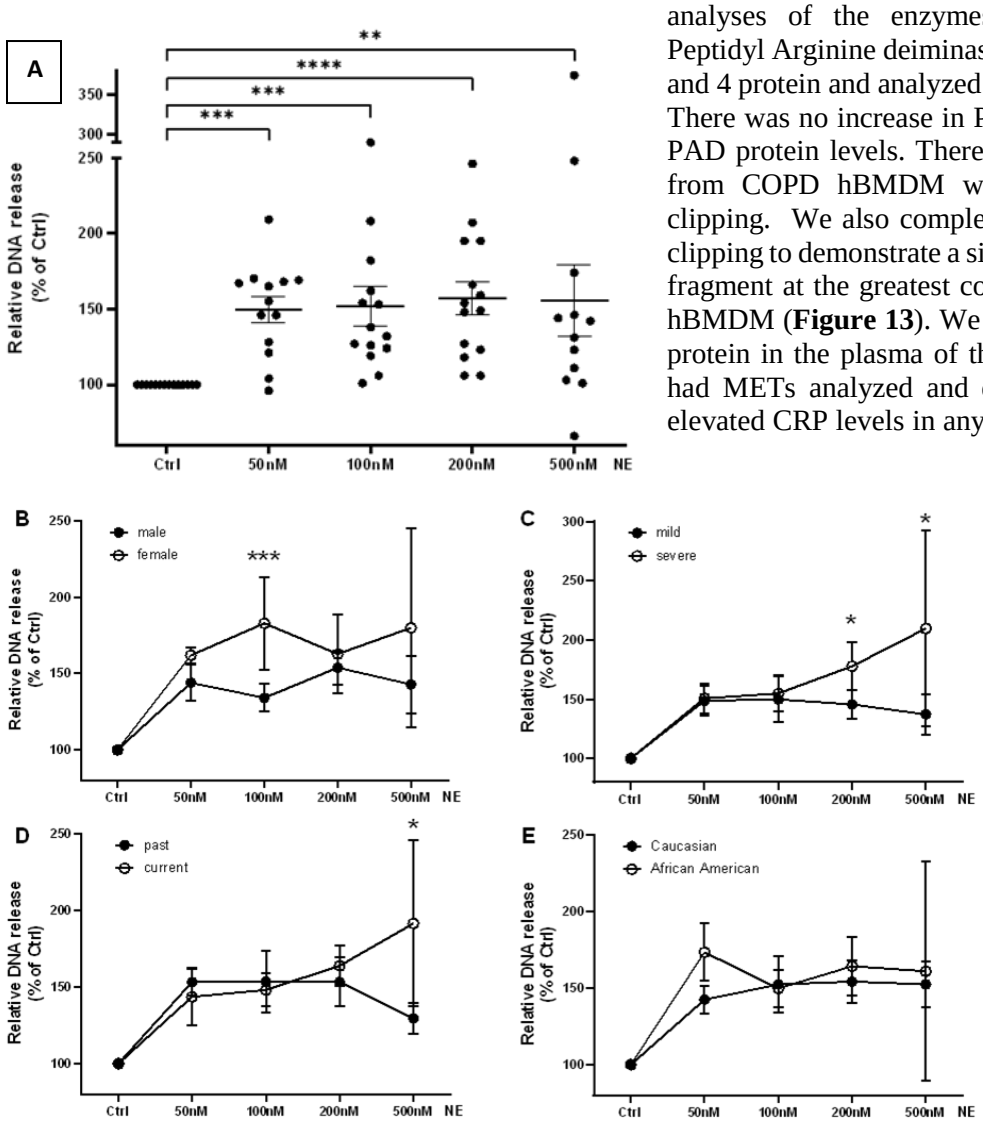


Figure 12. NE increased MET release in COPD BMDM. MET release increase was associated with lung disease severity, female sex, and current vs. past smoking. BMDM from patients with COPD were treated with a dose curve of NE (50-500 nM) or control vehicle for 2 h, and ecDNA that was attached to the cell surface was released into the conditioned media by treatment with micrococcal nuclease and quantified by Pico-Green assay. There was a significant difference in MET release by sex (females > males) **(B)**, by severity of lung disease by GOLD scale (more severe vs. less severe disease (**C**), and by smoking status (current vs. past) (**D**). There was no

difference between Caucasian and African American subjects in MET release over different NE concentrations. N=14, mean ±SEM, *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.

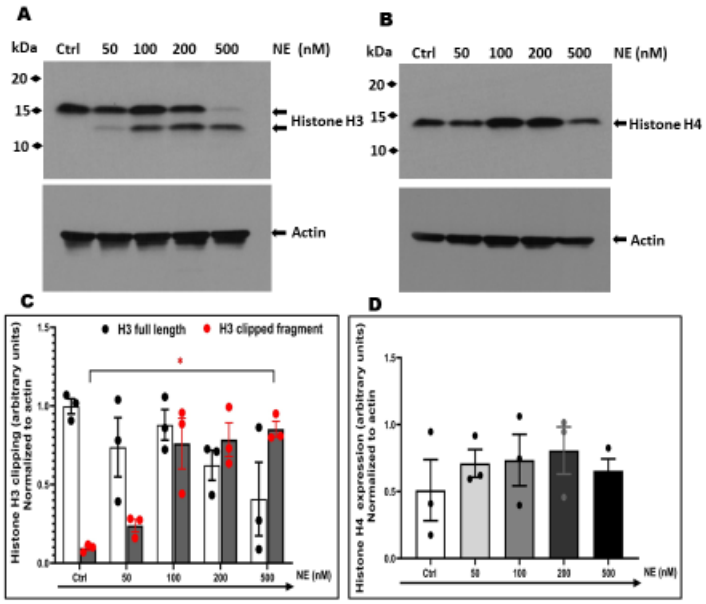


Figure 13. NE clipped Histone H3 but not Histone H4. Following NE treatment (0-500 nM, 2 h, 37°C), total cell lysates were harvested from BMDM and protein lysates (30µg) were separated on 4-20% PAGE, for western analysis for Histone H3 (A) and Histone H4 (B). To confirm equal protein loading and protein normalization, filters were stripped and reprobed for β-actin. Band intensities, quantified by densitometry using ImageJ software, were normalized to actin. Graphs show the band intensities (arbitrary units) of clipped vs full-length Histone H3 as paired columns (C) and the band intensities of full-length histone H4 (D). Data are presented as mean ± SEM; n=3 independent experiments with 3 different donor subjects. * p < 0.05 vs. Ctrl (vehicle control). Statistical analysis was performed by one-way, ANOVA, and post-hoc comparisons by Tukey multiple comparison test. Arrows indicate location of full-length proteins and clipped Histone H3.

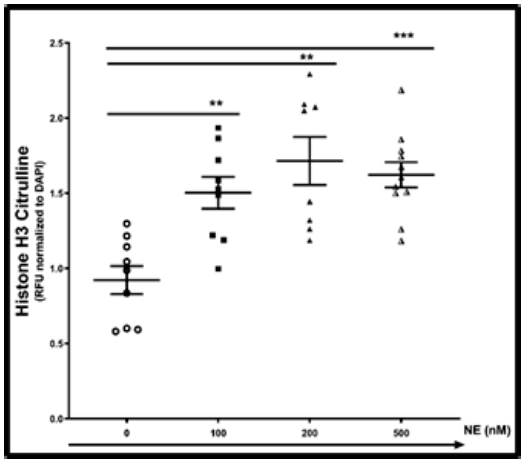


Figure 14. NE increased Histone H3 citrullination. COPD BMDM adhered to coverslips were treated with NE (100-500 nM) or control vehicle for 2 h, 37°C. After treatment, cells were fixed, permeabilized and then incubated with rabbit anti-Histone H3 Citrulline R2-R8-R17. Following PBS washes, coverslips were further incubated with goat anti-rabbit antibody conjugated with Alexa Fluor 488 and counterstained with DAPI before mounting onto glass. Relative abundance of H3-citrulline was evaluated by confocal microscopy. Data shown is representative of 3 experiments using 3 COPD donors Increased Histone H3 citrulline (RFU normalized to DAPI; mean ± SEM) from cells obtained from two COPD donors is summarized, n = 4-5 random images/donor. **, p < 0.01and ***, p < 0.001.

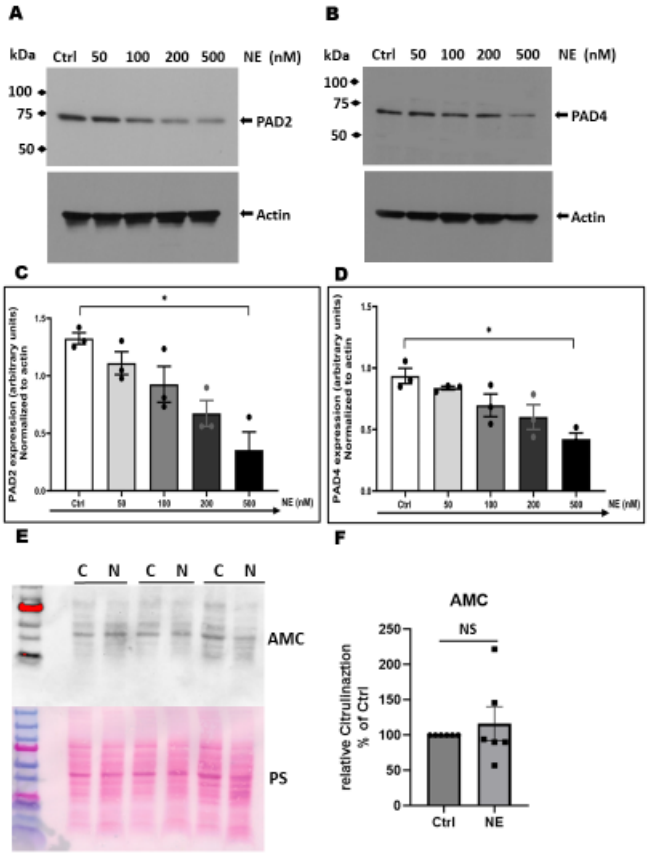


Figure 15. NE effect on expression of PAD2 and 4 and total modified citrullination. Following NE treatment (0-500 nM, 2 h, 37°C), total cell lysates (30µg) were separated on 4-20% PAGE, for western analysis for PAD2 **(A**), and PAD4 (**B**). Filters were stripped and reprobed with mouse monoclonal β-actin primary antibody as a control. Representative westerns are shown. Band intensities, quantified by densitometry using ImageJ software, and normalized to actin are shown for PAD 2 (**C**) and PAD4 (**D**). Data are presented as mean± SEM; n=3 independent experiments with 3 different donor subjects *, $p < 0.05$, NE vs. Ctrl (vehicle control). Statistical analysis was performed by one-way, nonparametric ANOVA and post-hoc comparisons by Tukey multiple comparison test.

To measure total PAD activity, we determined total protein citrullination. Human BMDM from subjects with COPD were treated with control (C) or NE (N) (200 nM, 2 h, 37°C). Total cell lysates were harvested and protein lysates (50µg) were separated on 4-20% PAGE, and transferred to PVDF membrane. Membranes was stained with Ponceau S (PS) and then citrulline residues were modified and detected using Anti-Modified Citrulline kit (**E**). Relative AMC was normalized to Ponceau S first and then normalized to control treated sample (**F**). Data was summarized as mean ± SEM, n=6. NS, no significant difference.

Stated Goals Not Met: We have not yet performed LC-MS analysis to identify proteins associated with METs in conditioned media. We harvested METs from hBMDM from healthy donors, following NE treatment (200 nM, 2 h) or control vehicle treatment and micrococcal nuclease release of METs into conditioned media. We are in the process of quantifying DNA and protein in these preparations. We will then plan LC-MS analysis to determine differences in control treatment vs. NE treatment and MET constitutuents.

Subject Recruitment Table:

Total Recruited	Total COPD	Male: Female	AA#: White	Smokers Current: Past	GOLD Score A: B: D**
77	69*	34:35	30:38:1^	24:45	14:35:20

*, 5 withdrew due to systemic steroids; 2 healthy smokers; 1 never smoker; #, African American^, unknown race; **, No GOLD C patients

References:

1. Kummarapurugu, A. B., Zheng, S., Ma, J., Ghosh, S., Hawkridge, A., Voynow, J. A., Neutrophil Elastase Triggers the Release of Macrophage Extracellular Traps: Relevance to Cystic Fibrosis, 2022, Am. J. Respir. Cell Mol. Biol., 66 (1), 76-85.
2. Gamze B. Bulut[1, 2], Shuo Zheng[1, 2], Apparao B. Kummarapurugu[1], Jonathan Ma[1], Judith A. Voynow[1,3,] Neutrophil Elastase degraded Histone Deacetylases resulting in High Mobility Group Box 1 accumulation in the cytoplasm of primary human macrophages, in revision.
3. Shuo Zheng, PhD[1, 4], Apparao B. Kummarapurugu, PhD[1, 4], Gamze B. Bulut, PhD[1], Aamer Syed MD[2], Le Kang, PhD[3], Judith A. Voynow, MD[1], Neutrophil Elastase activates the release of extracellular traps from COPD blood monocyte-derived macrophages, in press Clinical Translational Science.

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.

Posters were presented for preliminary work:

1. North American Cystic Fibrosis Conference Meeting November 2022, Philadelphia; Neutrophil elastase–mediated degradation of histone deacetylases in macrophages promotes high mobility group box 1 export from nucleus to cytosol, J. Voynow1, G. Bulut2, S. Zheng2, A. Kummarapurugu2.
2. Aspen Lung Conference May 2022, Aspen, CO; NEUTROPHIL ELASTASE ACTIVATES THE RELEASE OF EXTRACELLULAR TRAPS FROM COPD MONOCYTE-DERIVED MACROPHAGES, Apparao B. Kummarapurugu1*, Shuo Zheng1, Adam Hawkridge2, Aamer Syed3, Judith A. Voynow1

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.

Nothing to Report.

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).

The results obtained from this project support the concept that Neutrophil elastase alters the epigenetic landscape of macrophages resulting in transcriptional shift to a pro-inflammatory cell. This is the basis for a new project to evaluate the transcriptome and chromatin structure of monocyte derived macrophages and primary lung macrophages to evaluate the molecular mechanisms that have caused this shift in macrophage phenotype.

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.

We are collaborating with Dr. Jonathan Ma who has discovered that neutrophil elastase also regulates intracellular proteins resulting in phagocytic failure. Our work has propelled his project to evaluate the mechanisms leading to macrophage phagocytic failure.

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:*

We have not yet completed a proteomic analysis of MET protein constituents proposed for Major Task 3, due to the low quantity of METs after NE treatment at 2 h. We did not complete siRNA knockdown of HDACs or Sirt1 to test whether loss of these enzymes is sufficient to induce MET release.

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.

In collaboration with Dr. Adam Hawkridge, we will evaluate the generation of METs using a cell line, THP-1 cultured with PMA to induce a macrophage phenotype and treat these cells with NE over a dose and time course to determine if these cells will have a more robust response of MET release. If this is successful, we will plan to generate a proteomic analysis of the METs and then in future studies evaluate primary blood monocyte derived macrophages and alveolar macrophages for MET associated proteins using the THP1 data as an initial indication of potential associated proteins.

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.

No changes in expenditures.

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

No changes in human subjects recruitment, data collection or biospecimen collection.

Significant changes in use of biohazards and/or select agents

Nothing to Report

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. Voynow, J.A. Shinbashi, M. Neutrophil Elastase and Chronic Lung Disease. Biomolecules 2021, 11, 1065. https://doi.org/10.3390/ biom11081065, Acknowledged federal support-yes
2. Apparao B. Kummarapurugu, Shuo Zheng, Jonathan Ma, Shobha Ghosh, Adam Hawkridge, Judith A. Voynow. Neutrophil Elastase Triggers the Release of Macrophage Extracellular Traps: Relevance to CF. Am J Respir Cell Mol Biol. 2022 Jan;66(1):76-85. doi: 10.1165/rcmb.2020-0410OC. PMID: 34597246, Acknowledged federal support-yes
3. Voynow, JA, Zheng, S, Kummarapurugu, AB, Glycosaminoglycans as Multifunctional Anti-Elastase and Anti-Inflammatory Drugs in Cystic Fibrosis Lung Disease, 2020, Front. Pharmacol., 11: 1011, PMID: 32733248; PMCID: PMC7360816; DOI: 10.3389/fphar.2020.01011, Acknowledged federal support-yes
4. Shuo Zheng, PhD, Apparao B. Kummarapurugu, PhD, Gamze B. Bulut, PhD, Aamer Syed MD, Le Kang, PhD, Judith A. Voynow, MD, Neutrophil Elastase activates the release of extracellular traps from COPD blood monocyte-derived macrophages, Clinical Translational Science, in press. Acknowledged federal support-yes
5. Gamze B. Bulut, Shuo Zheng, Apparao B. Kummarapurugu, Judith A. Voynow, Neutrophil Elastase degraded Histone Deacetylases and Sirtuin1 in human monocyte derived macrophages: implications for CF and COPD, In Preparation, Acknowledged federal support-yes

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".

Name:	Judith Voynow MD
Project Role:	Principal Investigator
Nearest Person month worked	2
No change	
Name:	Shuo Zheng, PhD
Project Role:	Co-Investigator
Nearest Person month worked	6
No change	
Name:	Apparao Kummarapurugu, PhD
Project Role:	Co-Investigator
Nearest Person month worked	6
No change	
Name:	Le Kang, PhD
Project Role:	Co-investigator
Nearest Person month worked	1
Contribution to Project	Statistical analysis of MET release
Name:	Adam Hawkridge, PhD
Project Role:	Co-investigator
Nearest Person month worked	3
Contribution to Project	HPLC-MS/MS analysis of hBMDM lysates and MET proteins
Name:	Erica Memoli
Project Role:	Clinical Research Coordinator
Nearest Person month worked:	4
Contribution to Project:	Screening, recruiting, and enrolling participants.

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.

Nothing to Report.

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.*

biomolecules

MDPI

Review

Neutrophil Elastase and Chronic Lung Disease

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[2] School of Medicine, Virginia Commonwealth University, Richmond, VA 23298, USA; shinbashim@mymail.vcu.edu
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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

Citation: Voynow, J.A.; Shinbashi, M. Neutrophil Elastase and Chronic Lung Disease. *Biomolecules* **2021**, *11*, 1065. https://doi.org/10.3390/biom11081065

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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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ORIGINAL RESEARCH

Neutrophil Elastase Triggers the Release of Macrophage Extracellular Traps

Relevance to Cystic Fibrosis

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Abstract

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. Experiments were performed using human blood monocyte derived macrophages (hBMDM) from patients with and without CF to test specific mechanisms associated with MET release, and MET release by NE was confirmed in alveolar macrophages from *Cftr*-null and wild-type littermate mice exposed to intratracheal NE *in vivo*. Human BMDM were exposed to FITC-NE, and intracellular FITC-NE was localized to cytoplasmic and nuclear domains. Intracellular NE was proteolytically active as indicated by DQ-Elastin substrate cleavage. NE (100 to 500 nM) significantly increased extracellular PicoGreen fluorescence consistent with DNA release/ MET release from hBMDM in the absence of cell death. MET release was further confirmed by confocal microscopy in hBMDM treated with NE, and in alveolar macrophages from *Cftr*-null and wild-type littermate mice that had been exposed to intratracheal NE. NE-triggered MET release was associated with H3 citrullination detected by immunofluorescence assays and with partial cleavage of histone H3 but not H4. 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.

Keywords: neutrophil elastase; macrophage; extracellular traps; histone H3; CF

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 proinflammatory state through

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increased transcription of cytokines IL-6, TNF-α, IL-1β, and sCD14 (4). Macrophage dysfunction may be in part owing 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, IFN-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 (WT) 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).

Methods

Please *see* complete methods in the data supplement.

hBMDM Cultures

Blood samples were obtained from donors with and without CF following Virginia Commonwealth University Institutional Review Board-approved informed written consent. Subject demographics and clinical information are presented in Table 1. Mononuclear cells were isolated and cultured into 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

hBMDM (1×10^5 cells/glass coverslip) were treated with FITC-NE (200 nM) (catalog number FS563; Elastin Products) or control vehicle, 2 or 4 hours, 37°C, then ala-ala-pro-val-chloromethylketone (AAPV-CMK) (NE inhibitor, 10 μM) (catalog number M0398; Sigma), then fixed, counterstained with DAPI (1 μg/ml) (catalog number 9542; Sigma), and evaluated by confocal fluorescence microscopy (Zeiss LSM 700).

Detection of Intracellular NE activity in hBMDM

hBMDM were treated in suspension with control vehicle or NE (200 nM, 2 h, 37°C) (catalog number SE563; Elastin Products), then fixed, permeabilized, and incubated with DQ-elastin (20 μg/ml) (EnzChek Elastase Assay Kit, E-12056), room temperature, overnight. Relative fluorescence intensity was measured (excitation/emission 505/515 nm).

Quantification of NE-induced METs Release by PicoGreen

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

Visualization of NE-induced METs by Confocal Microscopy

hBMDM (1×10^5 cells/coverslip) were treated with NE (200 nM) or control vehicle for 2 hours at 37°C, fixed, counterstained with DAPI, and evaluated by confocal microscopy.

In a Virginia Commonwealth University Institutional Animal Care and Use Committee-approved protocol, *Cftr*-null, FABP human CFTR Tg1/Jaw/J mice (stock number 002364; JAX) and *Cftr* 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 by oropharyngeal aspiration on Days 1, 4, and 7 (21, 22). On Day 8 (24 h following the last dose of NE), BAL macrophages were isolated, stained with DAPI, and evaluated for MET structures by confocal microscopy.

Detection of Histone H3 Citrullination by Confocal Microscopy

hBMDM (1×10^5 cells/coverslip) were treated with NE (200 nM) or control vehicle (2 h, 37°C), fixed, permeabilized, and incubated with histone H3 citrulline (CitH3) rabbit polyclonal antibody (1:100) (catalog number ab5103; Abcam), and secondary Alexa Fluor 488-conjugated goat antirabbit antibody (Invitrogen) for confocal microscopy.

Detection of Histone Cleavage by Western Blot Analyses

hBMDM were treated with NE (200 or 500 nM) or control vehicle (1 or 2 h, 37°C). Cell lysates (30 μg) were analyzed by Western analysis for histone H3 (primary, rabbit IgG, 1:1000) (catalog number 4499; CST) or histone H4 (1:1000) (catalog number 13919; CST), secondary horseradish peroxidase-conjugated goat antirabbit IgG (1:4000) (catalog number 7074; CST), and SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). Westerns for β-actin (1:5000) (catalog number 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 50 nM) 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.

Table 1. Subject Demographics: CF and Non-CF

CF*	FEV^1pp	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	Female	36	W
2	Male	29	A
3	Female	35	H
4	Male	57	AA
5	Female	27	W
6	Male	33	A
7	Female	NR	A
8	Female	41	W
9	Male	33	W
10	Male	32	W
11	Female	26	AA
12	Male	24	A
13	Female	21	H
14	Female	23	AA
15	Female	NR	W

Definition of abbreviations: A = Asian; AA = African American; CF = cystic fibrosis; FEV_1pp = FEV_1% predicted; H = Hispanic; NR = not recorded; W = White.

*Summary demographics for participants with CF: 11 males and 6 females; age range from 6 to 24 years; and racial/ethnic background including 11 White, 4 African American, and 2 Hispanic or not identified individuals.

Results

Cellular Localization of NE in Macrophages

We first sought to determine the intracellular fate of NE taken up by hBMDM from subjects both with and without CF. To assess the localization of NE, macrophages were exposed to FITC-labeled NE (200 nM) or control vehicle for 2 or 4 hours, 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 donors without CF 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 (*see* Figure E1 in the data supplement), whereas 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 (200 nM, 2 h) exhibited significantly increased fluorescence within 16 hours after adding DQ-elastin to the cells (Figure 1D). Coincubation of NE with AAPV-CMK, an 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 subjects both with and without CF.

NE Induced the Release of Extracellular DNA in Macrophages

Although NE is a major airway inflammatory mediator in CF and is

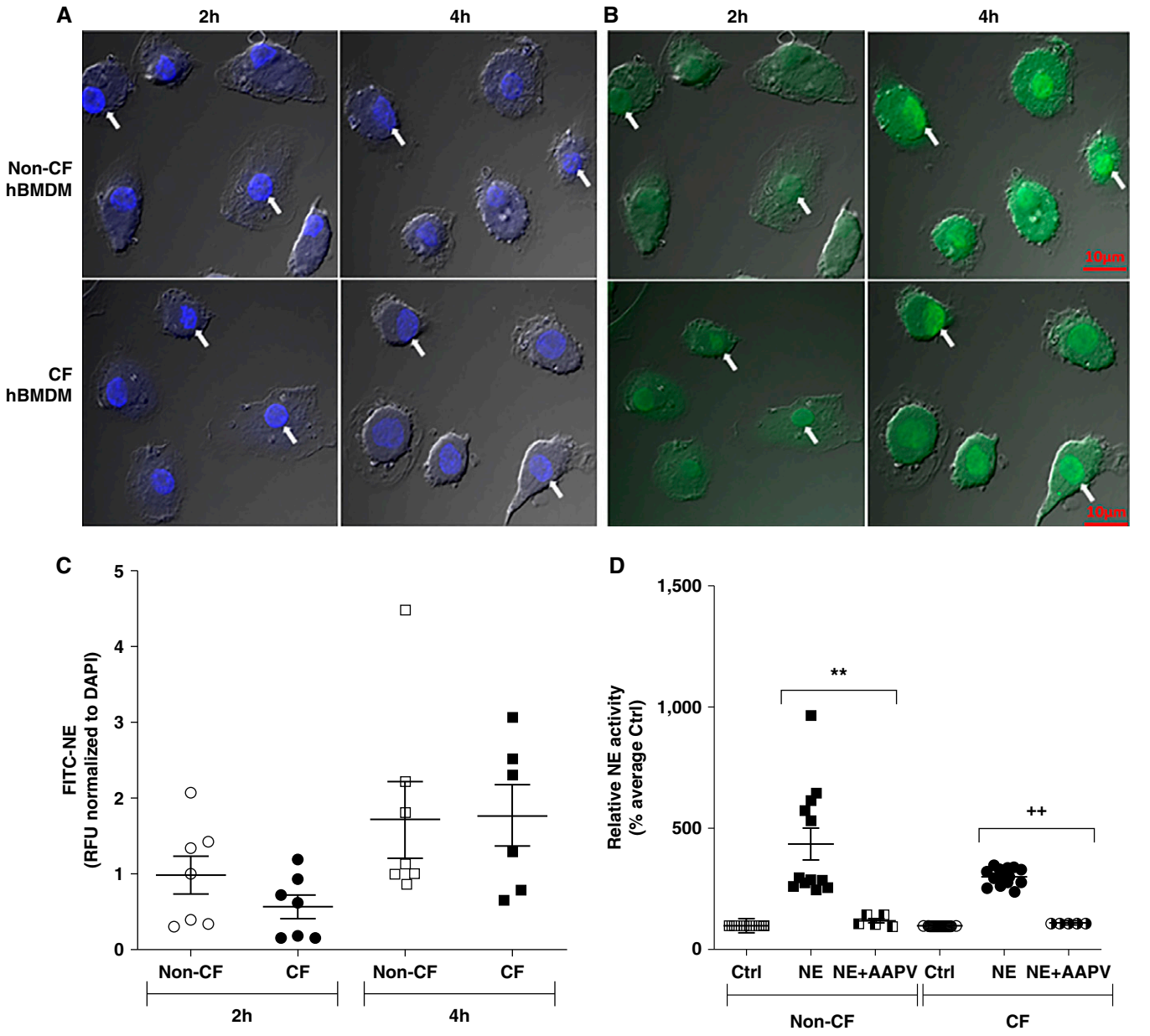

Figure 1. Neutrophil elastase (NE) was internalized in non-cystic fibrosis (CF) and CF human blood monocyte-derived macrophage (hBMDM) cells and exhibited proteolytic activity intracellularly. (*B*) Non-CF and CF hBMDM were exposed to FITC-NE (200 nM, 2 h or 4 h) or vehicle control. (*A*) After the treatments, cells were washed with PBS, fixed, stained with DAPI, and analyzed by confocal microscopy. (*B*) Nuclei are indicated by white arrows. Representative confocal micrographs of FITC-differential interference contrast (DIC) images (63× magnification) for non-CF or CF hBMDM treated with FITC-NE 200 nM for 2 or 4 hours are shown. 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 two experiments using two non-CF and 2 CF donors. Scale bars, 10 μm. (*C*) $n = 3$–4 images per individual. To evaluate NE proteolytic activity intracellularly, hBMDM from patients with and without CF were cultured in suspension for 8–10 days, then treated with control or NE (200 nM) for 2 hours. (*D*) At the end of 2 hours, cells were fixed, permeabilized, and mixed with DQ-elastin overnight to determine NE activity. Ala-ala-pro-val (AAPV)-chloromethylketones 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 two non-CF and two 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 with NE + AAPV in both non-CF and CF hBMDM (Wilcoxon rank sum test, $^{**}P = 0.0002$ or $^{++}P = 0.0002$) (*D*). Ctrl = control.

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 500 nM, 2.5 h) or control vehicle, and DNA

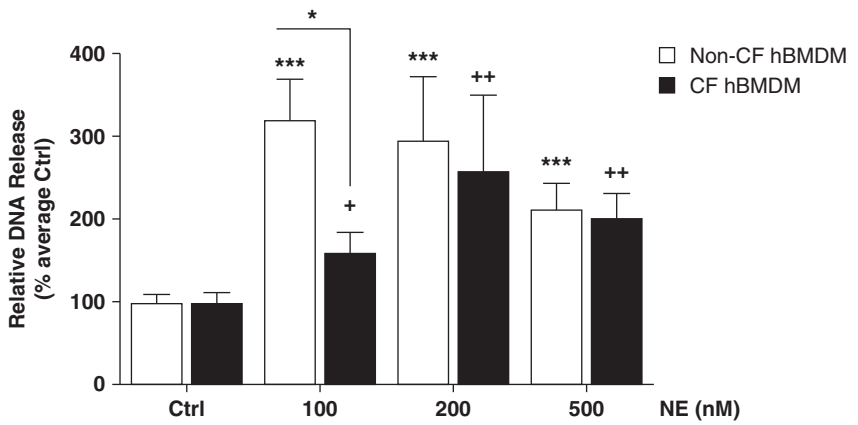

Figure 2. NE-induced extracellular DNA release from non-CF and CF hBMDM. Non-CF hBMDM (white bar) and CF hBMDM (black bar) cultured on 96-well plates were treated with control vehicle (ctrl) or NE (100, 200 or 500 nM, 2 h). At the end of NE treatment, cells were incubated with micrococcal nuclease (4 U, 25 min). At the end of nuclease treatment, a 1:200 dilution of PicoGreen (Thermo Fisher Scientific) 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 four experiments, including four non-CF and three 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 versus non-CF ctrl treated, $^{+}P < 0.05$ CF NE-treated versus CF ctrl treated, $^{++}P < 0.001$ CF NE-treated versus CF ctrl treated, and $^{*}P < 0.012$ non-CF NE (100 nM) versus CF NE (100 nM). There was a significant difference at 100 nM NE concentration between non-CF and CF, but no differences were observed at 200 or 500 nM NE.

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 with control treatment (Figure 2). However, non-CF MDMs released a significantly higher amount of extracellular DNA in culture supernatants compared with CF MDMs at 100-nM 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 (200 nM for 2 h), stained with DAPI, and analyzed by confocal microscopy. No DAPI-stained extracellular DNA structures were found in control vehicle-treated cells at 2 hours' 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* WT littermates were treated with intratracheal human NE (50 μg) or normal saline on Days 1, 4, and 7, and BAL was harvested on Day 8 (21). BAL cells were collected, and alveolar macrophages were enriched by adherence to poly-L-lysine-coated coverslips, fixed with 4% paraformaldehyde, and stained with DAPI (1 μg/ml). No MET structures were observed in macrophages from normal saline-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% to 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 concentrations 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 decondensation of chromatin. In addition, in neutrophils, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activates the cleavage of Histone H4 (24, 25), which is associated with the generation of NETs (26). Based on these insights, we tested whether NE cleaves histones as a potential mechanism for chromatin decondensation and release of extracellular traps in macrophages. Non-CF (Figure 6A) or CF (Figure 6C) hBMDM were exposed to NE (200 or 500 nM for 1 h or 2 h) or control vehicle, and total protein lysates were

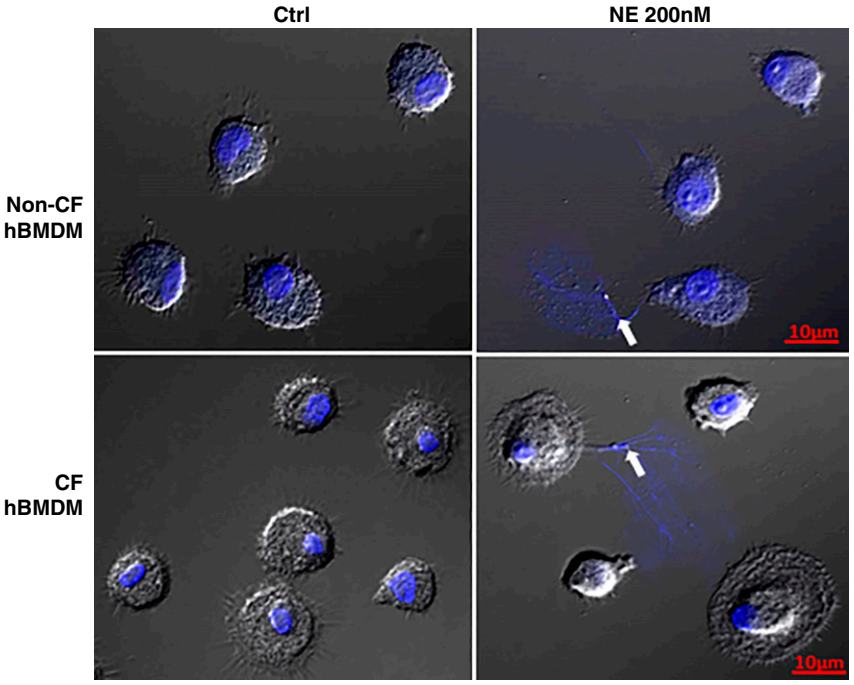

Figure 3. NE-stimulated release of extracellular traps from non-CF and CF hBMDM detected by confocal microscopy. hBMDM from subjects with and without CF were seeded onto poly-L-lysine-coated coverslips and cultured in growth media containing granulocyte-macrophage colony-stimulating factor (GM-CSF) (20 ng/ml) for 8–9 days. Cells were exposed to NE (200 nM) or vehicle control (ctrl) for 2 hours. After NE exposure, NE inhibitor (AAPV-chloromethylketone) 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). NE treatment for 2 hours 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 two experiments with two donors with and two donors without CF. Scale bars, 10 μm.

evaluated by Western analysis for histone H3 cleavage. The results demonstrated that NE treatment resulted in a cleaved fragment for H3, with a 15-kD band representing full-length H3 and a smaller, approximately 13-kD 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 500 nM for 1 h or 2 h) 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 50 nM) for 15 or 30 minutes, 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 a 15-kD band representing full-length H3 and a smaller, approximately 13-kD 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 kD, 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 (27). 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 proinflammatory cytokines and high-mobility group box 1 (HMGB1) (22, 28), 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 proinflammatory molecules, including extracellular DNA, histones, proteinases, myeloperoxidase (17), and high-mobility group box 1 (29). It is important to note that macrophages are the major sentinel leukocyte in the lung and are increased in thelungs of patients with CF (30) and chronic obstructive pulmonary disease (COPD) (31). 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 (classically activated

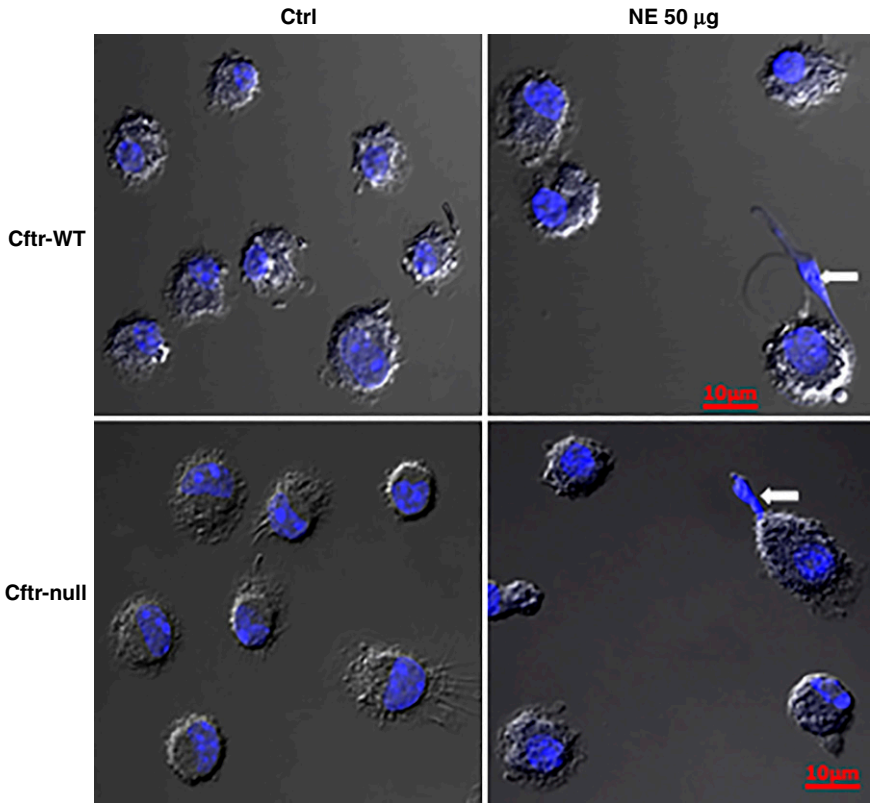

Figure 4. Detection of macrophage extracellular traps (METs) released *in vivo* following intratracheal NE administration in both Cftr-null and Cftr wild-type (WT) mice on the same genetic background. Aged 8–12 weeks old, Cftr-null mice and Cftr WT mice (both male and female) were administered NE (50 μg/40 μl/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 (63×) revealed MET release from macrophages derived from both Cftr-null and WT mice after NE treatment but not following saline treatment (ctrl). Micrographs are representative of four mice per treatment from two different experiments. Scale bars, 10 μm.

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 (alternatively activated macrophages) (23). However, both M1 and M2 macrophages are present in the CF airway (3, 32–34), and there is plasticity with respect to macrophage polarization depending on the airway milieu. it is not yet known whether M1 versus M2 polarization is sufficient to define risk for MET generation. In our report, we demonstrate that both hBMDM 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 owing 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 proinflammatory action whereas other macrophages resolve inflammation.

Several factors have been reported to activate MET release, including bacteria, mycobacteria, yeast, IFN-γ, exposure to neutrophil extracellular traps, phorbol myristate acetate, TNFα, drugs including the antibiotic, fosfomycin, statins (17), and the oxidant, hypochlorous acid (35). MET release is inhibited by superoxide inhibitors, diphenylene iodonium and apocyanin, and by the elastase inhibitor, N-methoxysuccinyl-AAPC-CMK (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 decondensation *in vitro* in neutrophil nuclei (12). Although myeloperoxidase alone is not sufficient to induce NETs, it acts synergistically with NE to increase neutrophil nuclear decondensation (12). Importantly, we and others have reported that NE induces oxidative stress in airway epithelia (36–38), in murine airway surface liquid *in vivo* (39), and in alveolar macrophages via uptake of heme-free iron (37), 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 (40). Neutrophils bind NE via high abundance, low affinity cell surface binding sites composed of chondroitin sulfate and heparan sulfate proteoglycans (41). 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 (42). Breast cancer cells and other tumor cells endocytose exogenous NE (43, 44), mediated in part by neuropilin-1 (NRP1) receptor (45), 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 (43). An alternative mechanism for NE uptake is binding to the cell surface and then internalization by clathrin-pit-mediated endocytosis (46). 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 decondensation (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

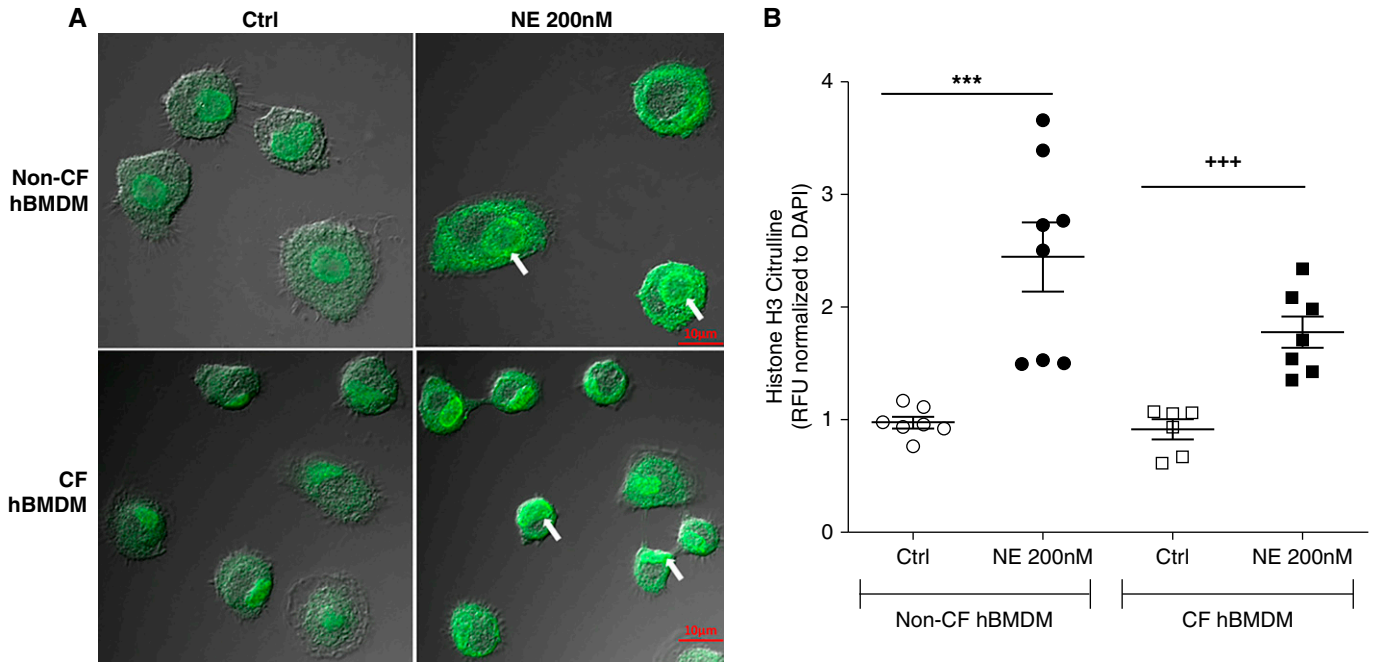

Figure 5. NE increased H3 citrulline (citH3) in both non-CF and CF hBMDM. hBMDM were treated with NE (200 nM, 2 h) or control vehicle (ctrl) on cover slips. Following fixation, cells were incubated with antihistone H3 citrulline R2 + R8 + R17 (1:100 dilution, overnight, Abcam), followed by Alexa Fluor 488-conjugated antirabbit IgG antibody (4 μg/ml, Invitrogen) for 1 hour, room temperature (RT), counterstained with DAPI, and evaluated by confocal microscopy (Zeiss LSM 700). (*A*) Representative confocal micrographs of fluorescein-differential interference contrast (DIC) mode are shown for non-CF and CF hBMDM. Integrated fluorescence intensity of citH3 normalized to nuclear DAPI fluorescence (images not shown) was quantified using ImageJ software. Scale bars, 10 μm. (*B*) Data are summarized as citH3 (RFU normalized to DAPI) (mean ± SEM) from two experiments with two individuals with and two individuals without CF; $n = 7$ random images. Statistically significant differences were determined by ANOVA with *post hoc* comparisons by the Wilcoxon rank sum test; $^{***}P < 0.0002$ and $^{+++}P < 0.0006$.

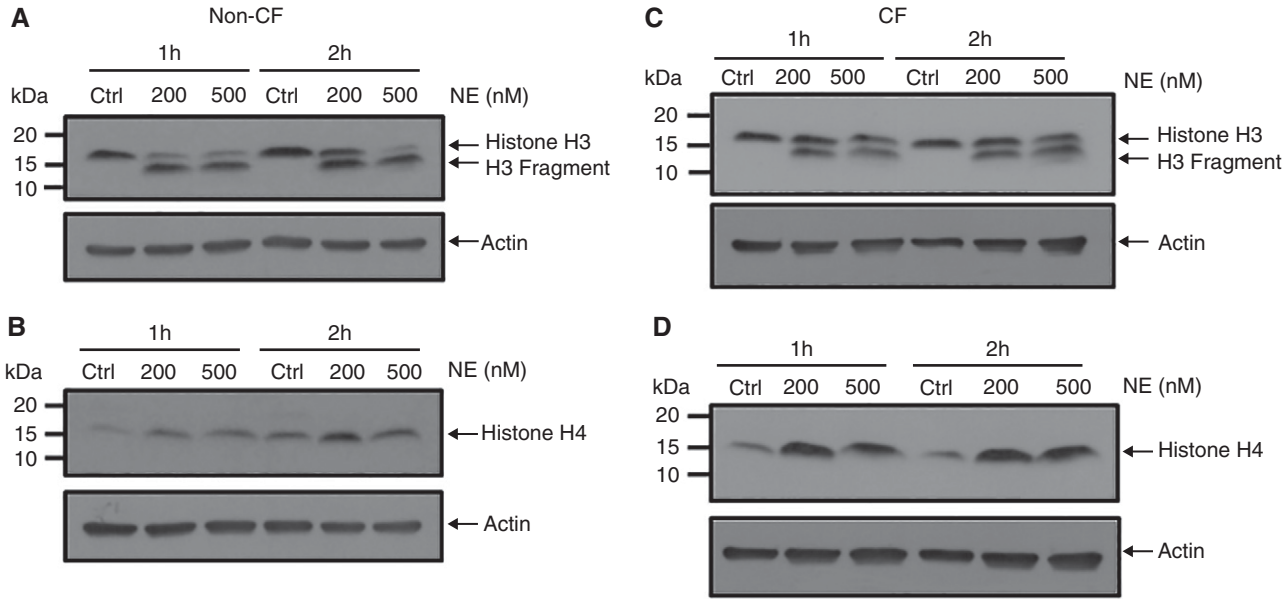

Figure 6. NE induced the cleavage of histone H3 in non-CF and CF hBMDM cells. Non-CF and CF hBMDM were treated with control vehicle (ctrl) or NE (200 or 500 nM) for 1 or 2 hours, and total cell proteins were collected. Total cell lysate protein (30 g) was separated on a 4–20% SDS-PAGE and tested for histone H3 or H4 expression using Western analysis. Blots were probed with primary rabbit monoclonal for anti-H3 or anti-H4 (1:1,000 dilution), secondary horseradish peroxidase-conjugated goat antirabbit IgG antibody (1:5,000 dilution), and development by chemiluminescence (Lightning Ultra ECL; Perkin Elmer). (*A–D*) 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 three donors with and three donors without CF.

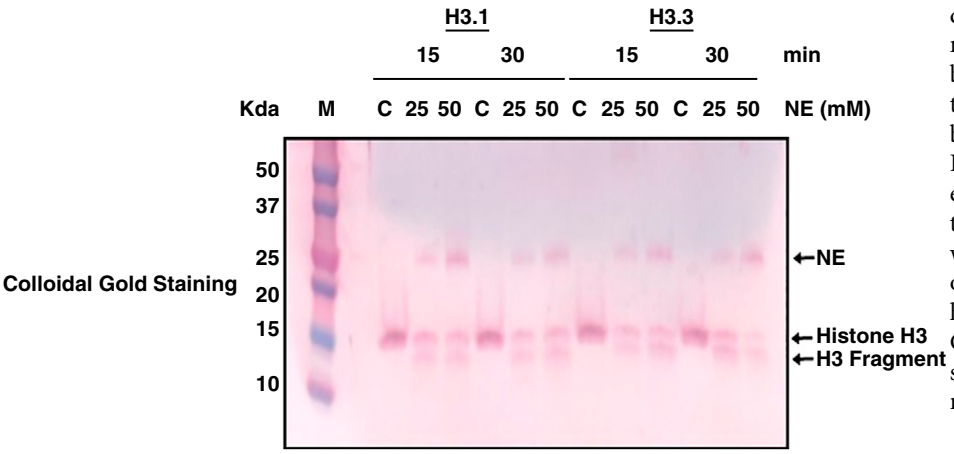

Figure 7. NE cleaved recombinant histone H3.1 and H3.3 isoforms *in vitro*. Recombinant human histone H3.1 and H3.3 were incubated with control vehicle or NE (25 or 50 nM) for 15 or 30 minutes. Equal amounts of reaction products were resolved on 4–20% SDS-PAGE, transferred to membrane, and stained with 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.

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 (47). 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 (24, 25, 48). 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 (25). 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 (25, 48). 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 decondensation, and regulate gene expression (24, 25, 48).

Our study had some limitations. We were not able to match the ages of subjects without CF 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-aged patients. However, despite these limitations, our experimental design, using each subject as their own control to compare treatment with control vehicle versus 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.

Conclusions

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. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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

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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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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

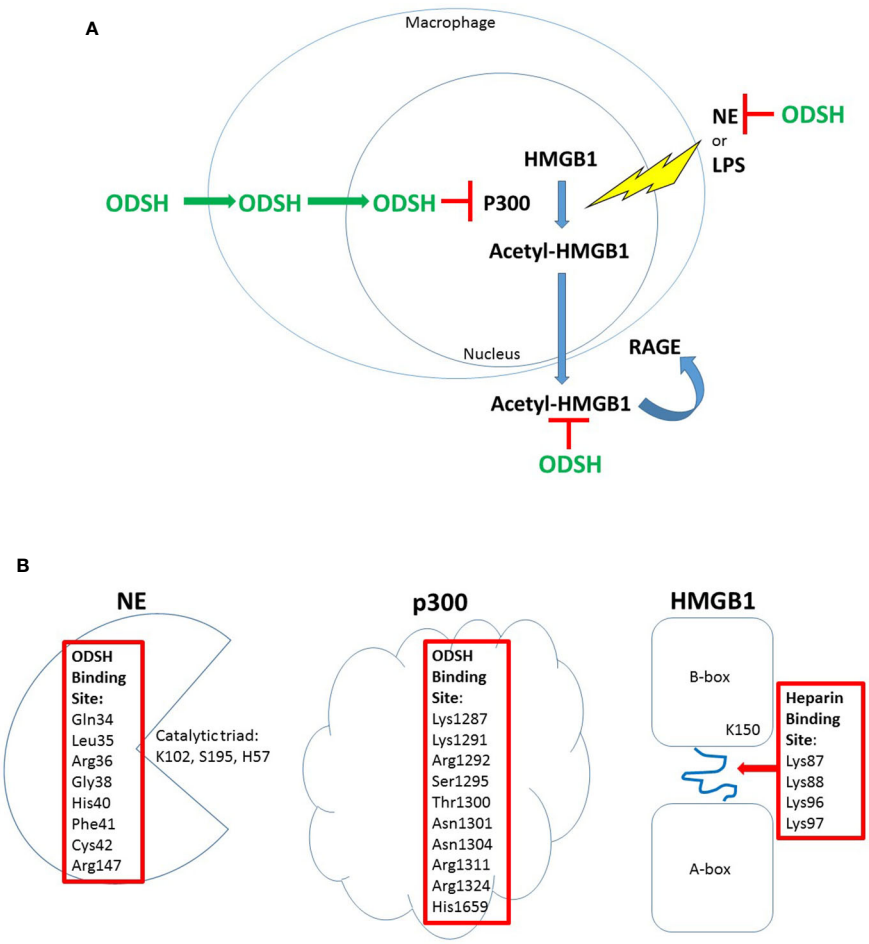

FIGURE 1 | ODSH localization and function in a macrophage cell line. ODSH is taken up by a mouse macrophage cell line (RAW264.7) into the cytoplasm within 2 h and into the nucleus by 24 h (Zheng et al., 2017) **(A)**. ODSH has anti-NE activity and blocks HMGB1-RAGE interaction in the extracellular domain, 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

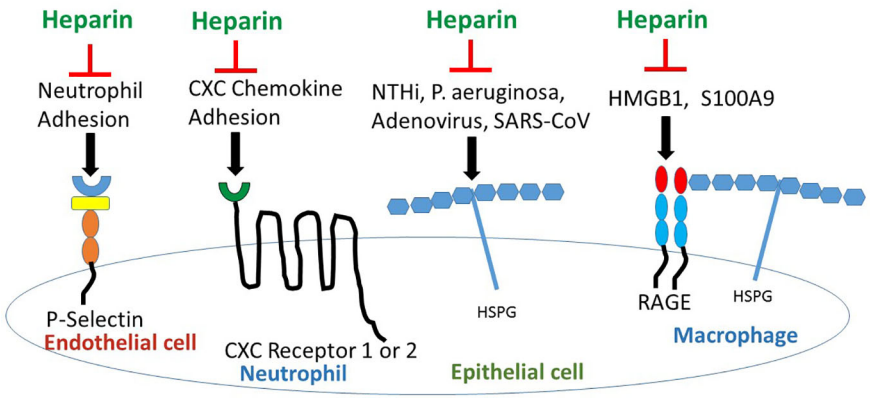

FIGURE 2 | Heparin/ODSH interrupts cell- cell interactions and ligand-receptor binding to block pro-inflammatory pathways. Heparin/ODSH oligosaccharides bind to P- and L-selectins and block neutrophil adhesion and chemotaxis (Nelson et al., 1993; Rao et al., 2010). Heparin inhibits CXCL8/IL-8 and other 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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Title: Neutrophil Elastase activates the release of extracellular traps from COPD blood monocyte-derived macrophages

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Abstract

Neutrophil elastase (NE), a major inflammatory mediator in COPD airways, impairs macrophage function, contributing to persistence of airway inflammation. We hypothesized that NE activates a novel mechanism of macrophage-induced inflammation: release of macrophage extracellular traps (METs). METs are composed of extracellular DNA decorated with granule proteinases and oxidants and may trigger persistent airway inflammation in COPD. To test the hypothesis, human blood monocytes were isolated from whole blood of subjects with COPD recruited following informed written consent. Patient demographics and clinical data were collected. Cells were cultured in media with Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) to differentiate into blood monocyte derived macrophages (BMDM). BMDM were treated with FITC-NE and unlabeled NE to determine intracellular localization by confocal microscopy and intracellular proteinase activity by DQ-Elastin assay. After NE exposure, released extracellular traps were quantified by abundance of extracellular DNA in conditioned media using the Pico Green assay. BMDM cell lysates were analyzed by western analysis for proteolytic degradation of Histone H3 or H4 or upregulation of peptidyl arginine deiminase (PAD) 2 and 4, two potential mechanisms to mediate extracellular trap DNA release. We observed that NE was taken up by COPD BMDM, localized to the cytosol and nucleus, and retained proteinase activity in the cell. NE induced MET release at doses as low as 50nM. NE treatment caused histone H3 clipping but no effect on Histone H4 nor PAD 2 or 4 abundance or activity. In summary, NE activated COPD MET release by clipping Histone H3, a prerequisite for chromatin decondensation.

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by acute exacerbations of bronchitis due to viral or bacterial infections resulting in persistent infection and inflammation (1). In COPD airways, several damage associated molecular patterns (2) including High Mobility Group Box 1 (HMGB1) and calgranulins S100A8/A9, and proteinases including cathepsins, matrix metalloproteinases, and neutrophil elastase (NE) contribute to airway inflammation (3). NE activates airway inflammation by several mechanisms (3): increasing cytokine expression, degrading opsonins and innate immune proteins, generating pro-inflammatory chemokinetic collagen peptides, Proline-Glycine-Proline (4) and triggering the release of extracellular traps (5).

Extracellular traps are web-like DNA structures that contain chromatin-binding proteins and granule proteins that are pro-inflammatory in the airways (6). Extracellular traps released from neutrophils (NETs) have been the predominant extracellular trap studied in cystic fibrosis (CF) and COPD and these are associated with more severe lung disease (6). However, NE also activates release of macrophage extracellular traps from monocyte-derived macrophages from patients with CF and from alveolar macrophages from Cftr-null mice (5). Increased neutrophil extracellular traps have been detected in the airways of patients with COPD (7, 8) and are associated with more severe lung disease (9, 10). Elevated NE abundance in the airway milieu of patients with COPD may significantly increase extracellular traps released from macrophages, resulting in another source for increased inflammatory proteins that contribute to airway inflammation. Therefore, we sought to determine whether NE was sufficient to trigger release of macrophage extracellular traps which have not previously been considered as part of the inflammatory milieu in the COPD airway.

Methods

Patient Recruitment: Subjects with COPD were recruited from the Adult Pulmonary Outpatient Clinic at VCU and provided IRB-approved (HM20015308), informed written consent for participation in the study. The procedures were in accordance with the ethical standards of the responsible committee on human experimentation or with the Helsinki Declaration of 1975 (as revised in 1983). Exclusion criteria were any severe comorbidities such as active uncontrolled cardiac disease or cancer, immunosuppressive therapy including systemic steroids within the past 4 weeks, and age greater than 80 years. Clinical data including GOLD score determined by frequency of exacerbations and the COPD Assessment Test (CAT) score were obtained. Patients provided a whole blood sample at the time of the clinical data collection.

Human peripheral blood monocyte derived macrophages (BMDM): Isolation and culture: Whole blood (20 ml) from COPD donors was processed using the RosetteSep Human Monocyte Enrichment Cocktail (cat #15068, StemCell Technologies) per manufacturer's instructions. The monocytes harvested were cultured in RPMI 1640 medium containing 10% FBS and GM-CSF (20 ng/ml, cat#572903, Biolegend) for 8-12 days to differentiate into macrophages for experiments. Buffy coat from healthy donors were obtained from American Red Cross. Buffy coat was diluted 1:1 with RPMI and then processed using RosetteSep Human Monocyte Enrichment Cocktail.

C-Reactive Protein (CRP) ELISA: Plasma samples from recruited subjects were analyzed for CRP levels by ELISA (Human CRP ELISA kit, Abcam, ab260058) per manufacturer's instructions.

NE treatments: Differentiated BMDM (~Day 10-12) were treated with FITC-NE (Cat# FS563, Elastin Products Company) or NE (Cat# SE563, Elastin Products Company) (50, 100, 200 or 500nM) or vehicle control for 2h, 37°C. NE activity was inhibited by addition of a specific NE

inhibitor, N-(Methoxysuccinyl)-Ala-Ala-Pro-Val-chloromethyl ketone (AAPV-CMK, 10 µM cat# M0398, Sigma) and processed for all the experiments performed for the study.

Localization of FITC-NE in BMDM from COPD by confocal microscopy: BMDM cultured in suspension were adhered to cover slips (100,000 cells /per coverslip), and treated with FITC-NE (100-500 nM) or control vehicle for 2 h at 37°C. Following FITC-NE exposure, cells were fixed with 4% paraformaldehyde (PFA) in PBS for 10 min and stained with 4',6-diamidino-2-phenylindole (DAPI, 1 µg/ml, Cat# D9542, Sigma) and examined for the localization and intensity of FITC-NE signal by confocal fluorescence microscopy (Zeiss LSM 700).

Localization of NE in BMDM from COPD by immunofluorescent confocal microscopy: As a complementary method to determine NE intracellular localization, COPD BMDM were adhered to glass coverslips, and treated with NE (100-500 nM) or control vehicle for 2 h at 37°C. Following treatment, cells were fixed with 4% PFA in PBS for 10 min and permeabilized in ice cold methanol for 10 min. After PBS wash, cells were incubated with mouse monoclonal anti-human NE primary antibody (1:100, clone NP57, Cat# M0752, DAKO), followed by further incubation with goat anti-mouse antibody conjugated with Alexa Fluor 595 (5 µg/ml, Cat# A11020, Invitrogen) for 1h. After PBS wash, cells were stained with DAPI and mounted for confocal immunofluorescence analysis.

Detection of intracellular NE activity in BMDM from COPD: Human BMDM from patients with COPD were harvested using RosetteSep human monocyte enrichment cocktail (Cat#15028, Stem Cell Technologies) and cells were grown in suspension for 8-10 days in RPMI growth medium with GM-CSF. Cells were divided into equal aliquots for treatment with control vehicle or NE (50, 100, or 200 nM) for 2hr, 37°C, and prepared for DQ-elastin assay by fixation in 2% PFA, permeabilization with 0.1% NP40 for 10min, and cell count determination to prepare

suspensions of equal numbers of cells from each treatment group in PBS. Cells were mixed with an equal volume of DQ-elastin (20 µg/ml in reaction buffer, EnzChek® Elastase Assay Kit, (E-12056, ThermoFisher Scientific). Cells/DQ-elastin mixture (100 µl/well) was aliquoted into 96 well black plates. NE inhibitor AAPV-CMK was added to appropriate wells as a NE-specific negative control. PBS/DQ-elastin mixture (1:1) served as a no cell control (blank). The plate was protected from light and incubated at room temperature overnight. Relative Fluorescence Intensity (RFU) was measured by SPARK plate reader (TECAN, Ex/Em 505/515 nm). After correcting for background, relative NE activity was determined by RFU normalized to corresponding average control.

Visualization of NE induced METs by confocal microscopy: Human BMDM from subjects with COPD were adhered to glass coverslips. Following NE treatments (100-500 nM), or control vehicle for 2 h at 37°C, cells were fixed with 4% PFA, DAPI stained (0.1 µl/ml), and the release of extracellular trap DNA as detected by presence of DAPI positive DNA strands in the extracellular space, was evaluated by confocal microscopy.

Quantification of NE-induced METs release by Pico Green Assay: Following NE treatments, extracellular DNA release was quantified with Quant-iT™ Pico Green® dsDNA Assay kit (Cat # P7589, ThermoFisher Scientific). Briefly, COPD BMDM cells seeded on 48 well plates (90-100K cells/well) were treated with NE (50, 100, 200 or 500 nM) for 2 hr. Following NE treatments, NE activity was stopped by AAPV-CMK and conditioned media was removed. Cells were incubated with micrococcal nuclease (MNase, 16 U/mL, cat# M0247S, New England Biolab) for 25 min, 37°C. The reaction was stopped by adding EDTA (final concentration: 5 mM). The MNase conditioned media was collected and an aliquot (100 µl) was transferred to 96 well black plates. PICO green working reagent (1:200 dilution in TE buffer, 100µl) was added to each well. The plate was mixed and incubated in the dark for 5 min. Fluorescent signals were determined using

the SPARK plate reader (TECAN, Ex/Em 485/525). NE-induced MET release by BMDM from healthy donors (Buffy Coat) was evaluated following treatment with control vehicle or NE (200 nM) for 2 h.

Immunofluorescence analysis to evaluate NE regulation of Histone H3-citrulline: Human BMDM from COPD subjects were adhered to glass coverslips (100,000 cells/coverslip). Following NE treatments (100-500 nM), or control vehicle for 2 h at 37°C, cells were fixed with 4% PFA for 10 min and then permeabilized with ice-cold 100% methanol. After PBS wash, cells were incubated with rabbit anti- Histone H3 citrulline (1:100, citrulline residues at positions R2-R8-R17, cat# ab5103, Abcam) at 4ºC for overnight. Following PBS washes, coverslips were further incubated with goat anti-rabbit antibody conjugated with Alexa Fluor 488 (5 µg/ml, Cat# A11070, Invitrogen) for 1h. After a PBS wash, coverslips were counterstained with DAPI (0.1 µg/ml) before mounting them onto glass slides using VECTASHIELD vibrance antifade mounting medium (Cat# H1700, Vector Labs). Relative fluorescence intensities of Histone H3 citrulline normalized to nuclear DAPI fluorescence were quantified using ImageJ software.

Western analysis for Histone H3 and H4 proteinase cleavage and PAD2 and PAD4 protein expression: Human BMDM from COPD subjects seeded onto 6-well plates were treated with NE (100-500 nM), or control vehicle for 2 h at 37°C. Following NE exposure, total cell lysates (30 µg) harvested from BMDM were separated by SDS-PAGE (4–12%). After blocking with 5% milk in PBS-T (0.1% Tween 20 in PBS) for 2 hours at room temperature, membranes were incubated with rabbit anti-Histone H3 (#4499; CST), or anti-Histone H4 (#13919, CST), or anti-PAD2 (#97647, CST) or anti-PAD4 (#684202, Biolegend) (1:1000) antibodies, followed by anti-rabbit-HRP conjugated antibody (1:4000; #7074, CST) or anti-mouse-HRP conjugated antibody (1:4000, #7076, CST), and immunoreactive protein complexes were detected with SuperSignal West Pico Chemiluminescent Substrate (Cat #34580, ThermoFisher Scientific). To confirm

equal loading, blots were stripped and re-hybridized with antibody specific to β-actin (1:8000, A5441, Sigma-Aldrich) and secondary HRP-conjugated anti-mouse IgG. Bands for full length and clipped H3, H4, PAD2 and PAD4 and βactin were quantified by Image J software. Relative Histone and PAD protein band densities were first normalized to β actin and then presented relative to their corresponding control vehicle-treated sample.

Western analysis for anti-modified citrulline (AMC): Human BMDM from COPD subjects seeded onto 6-well or 12-well plates were treated with NE (200 nM), or control vehicle for 2 h at 37°C. Following NE exposure, total cell lysates (50 µg) were separated by SDS-PAGE (4–20%). After transfer to PVDF membrane, membranes were stained with Ponceau S first. After washes to remove Ponceau S staining, citrulline residues on membranes were modified using anti-citrulline (modified) detection kit (Millipore Sigma, Cat#: 17-347B). Modification of citrulline residues is created by a chemical reaction with 2,3-butanedione monoxime and antipyrine in a strong acid solution. This type of citrulline modification ensures the detection of citrulline residues in proteins regardless of neighboring amino acid sequences (11). Detection of the modified citrulline proteins uses a standard immunoblot protocol with an anti-Citrulline (modified) human monoclonal antibody and a goat anti-Human IgG secondary antibody horseradish peroxidase (HRP) conjugate (Millipore Sigma, Cat#: 17-347B). Total AMC and Ponceau S densities in each sample were determined by ImageJ software. Relative AMC in NE samples was first normalized to Ponceau S staining and then normalized to its corresponding control treated sample.

Statistical Analysis: Data (mean ± SEM) for NE activity, NE-induced extracellular (ec)DNA release, NE induced H3 citrullination, clipped histone H3, H4, PAD2 and PAD4 westerns and anti-modified citrulline westerns were analyzed using one-way, nonparametric ANOVA (Kruskal-Wallis) test, followed by Dunn's multiple comparison (Prism, GraphPad). Linear mixed effects

models were used to compare clustered ecDNA release data across different experiment conditions from multiple replicates for each subject. Potentially important covariates such as gender (male vs female), race (Caucasian vs African American), COPD disease severity (mild vs severe) or smoking status (past or current) were adjusted as fixed effects in mixed effects models using PROC MIXED in SAS v9.4 (SAS Institute, Cary, North Carolina). $P<0.05$ was considered statistically significant.

Results

NE was taken up by COPD BMDM:

COPD BMDM cells seeded on glass coverslips were exposed to control vehicle or FITC-NE (100nM, 200nM or 500nM) for 2hr at 37°C, fixed with paraformaldehyde and stained with DAPI. Localization of FITC-NE was examined by confocal fluorescence microscopy. There was a dose-dependent increase in cellular NE uptake in both cytoplasm and nuclear compartments (**Figure 1A and B**). To demonstrate that NE was taken up by BMDM cells using a complementary immunofluorescence method, COPD BMDM cells seeded on glass coverslips were exposed to control vehicle or NE (100nM, 200nM or 500nM) for 2hr at 37°C, fixed with paraformaldehyde and stained with anti-NE antibody. Immunofluorescent microscopy also showed a similar dose -dependent increase of NE uptake in COPD BMDM cells with NE localization to both nuclear and cytoplasmic compartments (**Figure 1C**).

NE retained proteinase activity in the intracellular milieu of BMDM:

To determine if NE taken up by COPD BMDM cells retained proteolytic activity, we performed the DQ-Elastin assay. BMDM from COPD patients grown in suspension were treated with control vehicle, or NE 50, 100, or 200 nM for 2hr, washed, fixed and permeabilized. Equal number of cells from each treatment group were exposed to DQ-elastin overnight. Relative NE

activity was determined by RFU normalized to the corresponding average control vehicle treatment. As a negative control, replicate treatment conditions were treated with AAPV-CMK, the NE-specific inhibitor (**Figure 2**). There was an increasing trend of NE activity in NE treated cells at all doses with significantly increased NE activity at 100 and 200 nM. Assay specificity was demonstrated by loss of proteolytic activity in the presence of the NE specific inhibitor, AAPV-CMK.

NE activated MET release as indicated by PICO Green and by confocal microscopy. Subject demographics and characteristics for the patients who participated in the PICO Green assay to quantitate MET release are shown in **Table S1**. We performed C-reactive protein (CRP) quantitation in the plasma from each subject and noted that all samples had values expected for healthy individuals (CRP levels 0.02-1.05 mg/dL) (12). We observed that at all doses of NE tested (50-500 nM), MET release was significantly increased compared to control treatment (**Figure 3, Figure S1**). However, there was no significant dose-dependent increase in MET release under these treatment conditions (**Figure 3A**). We compared BMDM MET release between healthy individuals and subjects with COPD (**Figure 3B**) and found no significant difference in the relative increase in MET release between these two groups consistent with our prior report that there was no significant difference in MET release between healthy control subjects and subjects with CF(5). Our study was not designed to distinguish dose-dependent susceptibility to NE for MET release between different demographic and characteristic groups. However, based on adjusted mixed effects model analyses, patients with more severe COPD, and current smokers had greater MET release following exposure to NE (**Figure 3D** and **3E**). There was an increase in MET release only at 100 nM NE in women vs. men (**Figure 3C**) and there were no significant differences in MET release related to race (**Figure 3F**). NE-activated release of METs was corroborated by confocal microscopy evidence of DNA strands emanating

from BMDM from COPD subjects treated with NE but was not present in control vehicle treated cells (**Figure 4**).

NE and histone H3 modifications: Histone citrullination is a hallmark of extracellular traps. We evaluated whether NE increased histone H3 citrullination. Following treatment with NE (100-500 nM) or control vehicle, cells were fixed and stained with rabbit anti- Histone H3 (detecting citrulline residues positioned at R2-R8-R17) and relative fluorescent intensity was determined by conforcal microscopy. Although NE-treatment at all concentrations significantly increased Histone H3-citrulline staining compared to vehicle control-treated cells (**Figure 5A**), with ImageJ quantitation of fluorescent intensity, there was no significant concentration-dependent increase in NE induced Histone H3 citrullination (**Figure 5B**).

To determine whether the increase in Histone H3 citrullination was due to NE-induced upregulation of the enzymes, peptidyl arginine deiminase (PAD) 2 or 4, that catalyze the modification of arginine to citrulline, we first evaluated PAD2 and 4 protein abundance by western analysis in total cell lysates of BMDM from COPD subjects after treatment with control vehicle or a dose curve of NE (50-500 nM). We determined that NE did not increase PAD2 or PAD4 protein levels by western analysis (**Figure 6 A-D**). We further evaluated whether NE caused increased PAD activity as measured by total protein citrullination. Following NE (200nM, 2h) exposure, BMDM total cell lysates from COPD subjects were separated by SDS-PAGE (4–20%) and transferred to PVDF membrane. Modified citrulline was detected using a standard immunoblot protocol following membrane treatment with Anti-Modified Citrulline kit reagents, and total bands were quantified by Image J software. BMDM lysate modified citrulline abundance was normalized to Ponceau S stain and NE quantity compared to control treated lysates. NE did not change total protein citrullination abundance in COPD BMDM compared to

control treated lysates from the same individuals (**Figure 6 E, F**), confirming that NE did not increase PAD activity. Thus, NE did not increase PAD protein levels nor PAD activity in BMDM.

We then evaluated whether NE had an alternative mechanism to cause chromatin decondensation and MET release. We investigated whether NE cleaved Histone H3 as we have reported in CF BMDM exposed to NE (5) or cleaved Histone H4 as has been described for neutrophil extracellular trap generation following phorbol myristate acetate (PMA) exposure (13). Using the cell lysates from COPD BMDM exposed to control vehicle or NE (100-500 nM) for western analyses for Histone H3 (**Figure 7A and C**) and H4 (**Figure 7B and D**), we detected NE-induced clipping of H3 but no clipping of H4, consistent with cleaved Histone H3 as a potential trigger for chromatin decondensation and MET release.

Discussion

MET release occurred *in vitro* at NE concentrations (50 nM- 200 nM) that are relevant to COPD lung disease. Sputum from stable patients with COPD have mean NE concentrations of 83 to 114 nM (14, 15) while patients with exacerbations have mean NE concentrations of 217 nM (15). Overall, NE (200 nM)-induced MET release was detected in BMDMs from both healthy subjects and from subjects with COPD, supporting the concept that NE- triggered release of METs from BMDM is not due to specific characteristics of COPD macrophages but may occur in any macrophage exposed to high NE concentrations in the airway milieu. However, when comparing patients with COPD by demographic features, MET release was greater in BMDM from subjects with more severe GOLD scores. Plasma C-reactive protein levels were not elevated in any of the subjects confirming that they were not acutely ill at the time of blood samples were obtained. Thus, MET release due to NE exposure in the airway may be a chronic feature of COPD lung disease that worsens with more severe disease progression. Our results also revealed that at 100 nM NE, BMDM from women with COPD had greater release of METs

than men with COPD. However, at higher NE concentrations, differences in MET levels between women and men were not observed. As women with COPD have more rapidly progressive and more severe lung disease than men (16), further studies to evaluate whether women with COPD have greater lung MET release may be warranted. MET release was greater in BMDM from current smokers than past smokers at 500 nM NE concentration. However, we did not observe differences in NE-induced MET release in COPD subjects related to white or African American race.

Although, we observed MET release at all NE concentrations tested, there was no NE concentration-dependent increase in METs. The lack of a concentration response for NE to induce MET release may be due in part to intracellular alpha-1-antitrypsin activity in BMDM (17) which may inhibit the activity of internalized NE, or possibly due to NE localization to the nucleus where binding to DNA may potentially limit NE activity (18). Interestingly, in THP-1 cells, a macrophage-like cell line, treatment with several mediators including TNFα, hypochlorous acid, Nigericin, a bacterial peptide, and a calcium ionophore, all activate MET release (19), with no significant dose-dependent increases in METs by these stimuli suggesting a possible threshold response for MET release post- exposures. Future studies using lower concentrations of NE will be important to test the threshold hypothesis. MET release is activated by other factors including cigarette smoke, microbial infections, oxidative stress and calcium flux (20, 21) supporting a more common role in airway inflammation in patients with COPD than previously suspected.

Several mechanisms of NE-induced airway inflammation/ macrophage dysfunction in CF and COPD are attributed to extracellular NE proteinase activities (3, 22). However, for MET release, intracellular proteinase activity of NE is likely required. Using COPD BMDM, and previously, using CF BMDM (5), we demonstrated that NE is taken up by BMDM and retains proteinase activity intracellularly. Although macrophage endocytosis and subsequent release of

active NE has been demonstrated (23), there have been no previous reports that intracellular NE proteinase activity is sufficient to cleave macrophage intracellular proteins. In this report we show that NE was localized to both the cytosol and nuclear compartments in BMDM from subjects with COPD, and NE retained its proteinase activity. Furthermore, NE had a specific nuclear target, Histone H3, and not Histone H4. NE proteinase activity is sufficient to clip Histone H3 when evaluated *in vitro* using recombinant Histone H3 incubated with purified NE (5). The biochemical reason for the specificity of the histone target is unknown. Clipping of histone proteins causes chromatin decondensation, a precursor to extracellular trap release, and is the mechanism for phorbol myristate acetate (PMA)- induced neutrophil extracellular trap release (13). It is not known whether other proteinases such as macrophage matrix metalloproteinases or cathepsins may also cleave histones resulting in MET release. However, there is indirect evidence to support the role of other proteinases to activate extracellular trap release. Tissue Inhibitor of Metalloproteinases 1 (TIMP1) overexpression in a mouse model of liver ischemia and reperfusion injury rescues liver injury in part by inhibition of neutrophil extracellular trap generation in the liver (24). Furthermore, Secretory Leukocyte Peptidase Inhibitor (SLPI) blocks PMA-induced NET release via inhibition of proteinase-catalyzed cleavage of histone H4 (13). These observations support the potential for other proteinases including matrix metalloproteinases and other serine proteinases to activate release of extracellular traps. In contrast, although we determined that NE treatment was associated with increased H3 citrullination, we did not detect upregulation of PAD 2 or 4, the PAD enzymes localized to monocytes and macrophages that catalyze this post-translational modification. Previous reports have shown that increased intracellular Ca^{2+} levels activate PAD and cause a global increase in citrullination evidenced by a positive western for AMC (25, 26). We did not observe a global increase in citrullination by western analysis of AMC after NE treatment. Instead, NE clipping of Histone H3 may generate a more susceptible substrate for focal

citrullination that remains to be tested in our future studies. Our results are consistent with prior reports that MET release may be independent of PAD upregulation (21).

All the outcomes reported herein: NE uptake, NE activity, MET release, Histone clipping and citrulline modifications were observed after treatment of BMDM with NE for 2 hours. Importantly, our results are consistent with the impact of NE treatment for 2 – 2.5 hours or less on other macrophages functions. Hubbard et. al. exposed human alveolar macrophages to NE 100 nM, 30 minutes and demonstrated release of leukotriene B4 and subsequent neutrophil chemotaxis (27). Ma et. al. exposed primary human blood monocyte derived macrophages to NE (500 nM, 2.5 h) and demonstrated phagocytic failure associated with loss of intracellular protein components of the phagosome- lysosome machinery (28). Furthermore, we performed an initial characterization of BMDM from control subjects and subjects with CF to determine whether NE altered macrophage polarization (5). We found that NE treatment for 2 h caused BMDM to release TNFα, an indicator for M1 polarization, but not release the C-C motif chemokine ligand 18 (CCL18), a marker for M2 polarization (5). Therefore, although it is possible that with longer duration of NE treatment, MET release may be further increased, the duration of NE treatment chosen in this study was sufficient to test the NE effect on several macrophage functions and to identify potential mechanisms of NE-triggered MET release.

The concentration of extracellular traps in sputum from patients with COPD correlates directly with severe COPD lung disease and worse lung function (9, 10). However, the mechanisms associated with METs that confer increased airflow obstruction are not known. Extracellular traps are associated with increased mucus viscoelasticity (29) due to both increased DNA polymers and oxidized mucin polymers; both factors increase airflow obstruction in cystic fibrosis (30), asthma (31), and COPD (32). Furthermore, extracellular traps have been reported to exacerbate airway inflammation directly (20, 33-35) and this is likely due to the pro-inflammatory properties of extracellular DNA and/or the NET or MET protein constituents. The proteins associated with METs are not yet fully identified. It is likely that different stimuli may

cause additive or synergistic release of METs and may also alter the composition of MET-associated proteins (36).

Importantly, in addition to MET release, NE activates other mechanisms in the airway that contribute to COPD exacerbations including upregulation of mucin expression and increased mucin secretion (37), increased NE concentration on the surface of neutrophil exosomes (38), and release of Pro-Gly-Pro (PGP) chemokine peptides (39), and cytokines (3). Altogether the impact of METs and other NE-activated inflammatory pathways create a broad array of threats to COPD airway homeostasis with no available targeted therapies to mitigate or reverse these pro-inflammatory signals.

There are several limitations with this study. Although we demonstrated an increase in METs with NE, our study was not designed to definitively designate demographic features that are associated with higher risk for MET release and therefore our results supporting differences in MET release associated with sex, severity of disease, and active smoking, will need to be further confirmed using larger cohorts. We limited our evaluation of MET release to 2 h; it is possible that with longer duration of NE exposure, we may observe greater MET release (19). We have demonstrated that NE caused histone H3 clipping and does not increase PAD protein or activity, supporting the role of Histone H3 clipping and subsequent chromatin decondensation as a mechanism for NE-induced MET release. However, another possibility is that NE increases intracellular calcium (Ca^{+}) which has been reported to trigger release of METs (19). This signaling pathway will be the focus of future studies. We have previously reported that alveolar macrophages harvested by bronchoalveolar lavage (BAL) from both *Cftr*-null mice and wild-type littermate mice respond to NE by release of METs (5). In this model, we used human BMDM to test whether NE exposure was sufficient to activate MET release. In chronic lung diseases such as COPD and CF, it is not possible to isolate a single stimulus like NE to test efficacy for MET release since these patients also have chronic airway infections and/ or tobacco smoke

exposure, and these are factors that can induce METs or NETs. Finally, it is not possible to distinguish NETS from METs harvested from patient sputum or bronchoalveolar lavage, because the assays currently available for extracellular traps test for co-expression of DNA and NE, DNA and histone, or NE and Myeloperoxidase; these are extracellular trap components that would be present in both METs and NETs (10). Therefore, new methods will need to be developed to identify which cells are contributing extracellular traps to increase mucus viscoelasticity (29) and to promote inflammation in the COPD airway.

In summary, we demonstrate that NE at concentrations found in the COPD airway is sufficient to trigger release of METs via a proteolytic mechanism of intracellular histone H3 clipping, and this is yet another mechanism by which NE sustains inflammation and injury in the COPD lung. Given the preponderance of macrophages in the airways of patients with COPD, the release of METs may significantly contribute to airway inflammation and airflow obstruction.

Study Highlights:

What is the current knowledge on the topic? Airway Neutrophil Extracellular Traps are associated with more severe COPD but the presence of Macrophage Extracellular Traps (METs) has not previously been reported in COPD airways.

What question did this study address? Is Neutrophil elastase (NE) sufficient to trigger release of METs and how does NE activate release of METs?

What does this study add to our knowledge? NE activates MET release by clipping histone H3 in blood monocyte derived macrophages from patients with COPD. In contrast to other stimuli that trigger extracellular trap release via citrullination of histones, NE does not upregulate the expression or activity of the enzymes required for citrullination, the Peptidyl arginine deiminases (PAD) 2 or 4.

How might this change clinical pharmacology or translational science?

We have identified a novel mechanism by which NE contributes to airway inflammation and mucus viscosity in COPD by increasing MET release into the extracellular milieu. Our findings indicate a new therapeutic target to reduce airway inflammation and mucus obstruction in COPD.

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Figure legends:

Figure 1. NE localization in human BMDM. Human BMDM from subjects with COPD were adhered to coverslips and treated with FITC-NE (100-500 nM) for 2 h at 37°C, followed by DAPI nuclear staining to determine NE localization in the cell. Confocal fluorescence microscopy for DAPI + differential interference contrast (DIC) image (**A**), and for FITC-NE +DIC image (**B**) was performed. White arrows indicate BMDM nuclei. There was a dose-dependent increase in cellular FITC-NE uptake. As a complementary method, NE treatment was performed (100-500 nM) for 2 h at 37°C, followed by incubation with mouse monoclonal anti-human NE primary antibody, goat anti-mouse secondary antibody conjugated with Alexa Fluor 595, stained with DAPI, and fixed (**C**). Red fluorescence was detected in the nucleus (white arrows) in a dose-dependent manner. Scale bars, 20 μm. Data is representative of 3 COPD donors.

Figure 2. NE taken up by BMDM was proteolytically active. Human BMDM from subjects with COPD were treated with NE (0, 50, 100 or 200 nM, 2 h, 37°C) while in suspension. Following treatments, cells were exposed to DQ-elastin. NE inhibitor, AAPV-CMK, was used as a NE-specific, negative control. Relative fluorescence units (RFU) measured by TECAN plate reader (Ex/Em 505/515 nm), indicated NE proteinase activity only in NE-treated cells and was absent in AAPV-CMK-treated cells. N=7 donors; normalized to average control; mean ± SEM; *, $p<0.05$; ns, no significant difference.

Figure 3. NE induced the release of METs as indicated by extracellular (ec)DNA in COPD BMDM. Following NE and control vehicle treatments, 2 h, 37°C, conditioned media was removed and cells were incubated with micrococcal nuclease and the supernatant ecDNA release (MET release) was quantified by Quanti-iT PicoGreen dsDNA Assay kit and compared to control treated cells. N=14; data normalized to average control; mean ± SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$ (**A**). Comparison of MET release from healthy BMDM (n=5) vs. BMDM from subjects with COPD (n=14) following control vs. NE treatment (200 nM)

(**B**). Comparisons between subjects with COPD: male vs. female (**C**), mild vs. severe lung disease (**D**), past vs. current smokers (**E**) and African American vs. Caucasian subjects (**F**) were evaluated.

Figure 4. NE induced extracellular traps release from COPD BMDM detected by confocal microscopy. Human BMDM were adhered to coverslips and treated with NE (0-500 nM, 2 h, 37°C), fixed and then stained with DAPI. The control treated slides (Ctrl) had no detectable METs. The NE treated slides all had detectable METs. Top panel: DAPI only; Bottom Panel: DAPI + DIC. Data shown is representative of 3 experiments; N= 3 COPD donors. Scale bars, 20 µm. White arrows indicate MET DNA strands detected by DAPI positive staining.

Figure 5. NE increased Histone H3 citrulline in COPD BMDM detected by confocal microscopy. COPD BMDM adhered to coverslips were treated with NE (100-500 nM) or control vehicle for 2 h, 37°C. After treatment, cells were fixed, permeabilized and then incubated with rabbit anti-Histone H3 Citrulline R2-R8-R17. Following PBS washes, coverslips were further incubated with goat anti-rabbit antibody conjugated with Alexa Fluor 488 and counterstained with DAPI before mounting onto glass. Relative abundance of H3-citrulline was evaluated by confocal microscopy. Data shown is representative of 3 experiments using 3 COPD donors (**A**). Scale bars, 20 µm. Increased Histone H3 citrulline (RFU normalized to DAPI; mean ± SEM) from cells obtained from two COPD donors is summarized, n = 4-5 random images/donor (**B**). **, $p < 0.01$ and ***, $p < 0.001$.

Figure 6. NE effect on expression of PAD2 and 4 and total modified citrullination. Following NE treatment (0-500 nM, 2 h, 37°C), total cell lysates were harvested from BMDM and protein lysates (30µg) were separated on 4-20% PAGE, and transferred to nitrocellulose membrane. Membranes were incubated with primary rabbit monoclonal antibody for PAD2 **(A**), and primary mouse monoclonal antibody for PAD4 (**B**). To confirm equal protein loading and protein normalization, filters were stripped and reprobed with mouse monoclonal β-actin primary

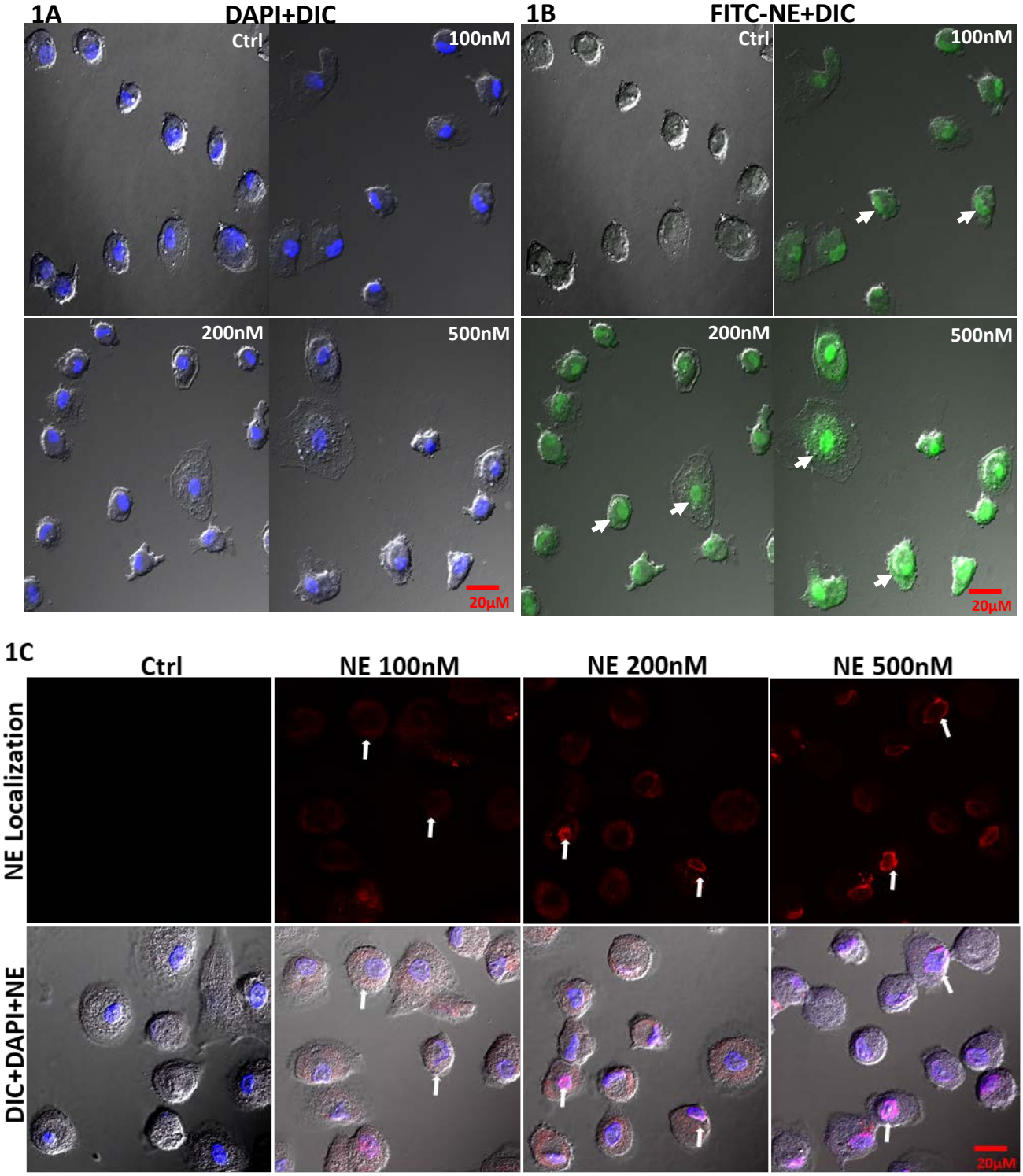
antibody and secondary HRP-conjugated anti-mouse IgG, and developed with SuperSignalWest Pico Chemiluminescent Substrate. Band intensities, quantified by densitometry using ImageJ software, were shown in the graphs. Graphs show the band intensities (arbitrary units) of PAD 2 (**C**) and PAD4 (**D**). Data are presented as mean ± SEM; n=3 independent experiments with 3 different donor subjects *, $p < 0.05$, vs. Ctrl (vehicle control). Western blots shown are representative of N=3 COPD donors. To measure total PAD activity, we determined total protein citrullination. Human BMDM from subjects with COPD were treated with control (C) or NE (N) (200 nM, 2 h, 37°C). Total cell lysates were harvested and protein lysates (50µg) were separated on 4-20% PAGE, and transferred to PVDF membrane. Membranes was stained with Ponceau S (PS) and then citrulline residues were modified and detected using AMC kit (**E**). Relative AMC was normalized to Ponceau S first and then normalized to control treated sample (**F**). Data was summarized as mean ± SEM, n=6. NS, no significant difference.

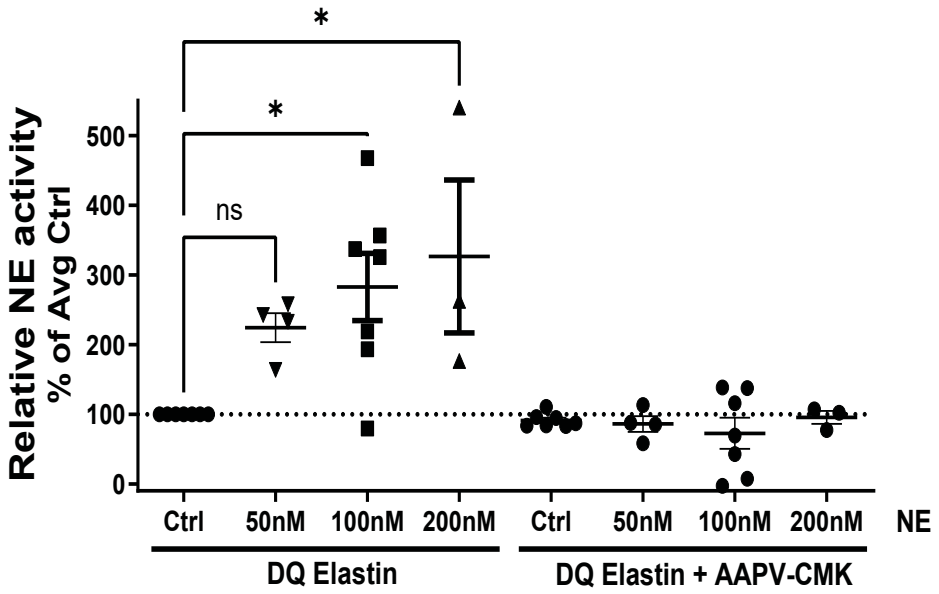
Figure 7. NE and Histone Clipping. Following NE treatment (0-500 nM, 2 h, 37°C), total cell lysates were harvested from BMDM and protein lysates (30µg) were separated on 4-20% PAGE, and transferred to nitrocellulose membrane. Membranes were incubated with primary rabbit monoclonal antibody for Histone H3 (**A**); primary rabbit monoclonal antibody for Histone H4 (**B**). Secondary antibodies included HRP-conjugated anti-rabbit IgG or HRP-conjugated anti-mouse IgG and development with SuperSignal West Pico Chemiluminescent Substrate. To confirm equal protein loading and protein normalization, filters were stripped and reprobed with mouse monoclonal β-actin primary antibody and secondary HRP-conjugated anti-mouse IgG, and enhanced chemiluminescence. Band intensities, quantified by densitometry using ImageJ software, were shown in the graphs. Graphs show the band intensities (arbitrary units) of clipped vs full-length Histone H3 as paired columns **(C)** and the band intensities of full-length histone H4 **(D)**. Data are presented as mean ± SEM; n=3 independent experiments with 3

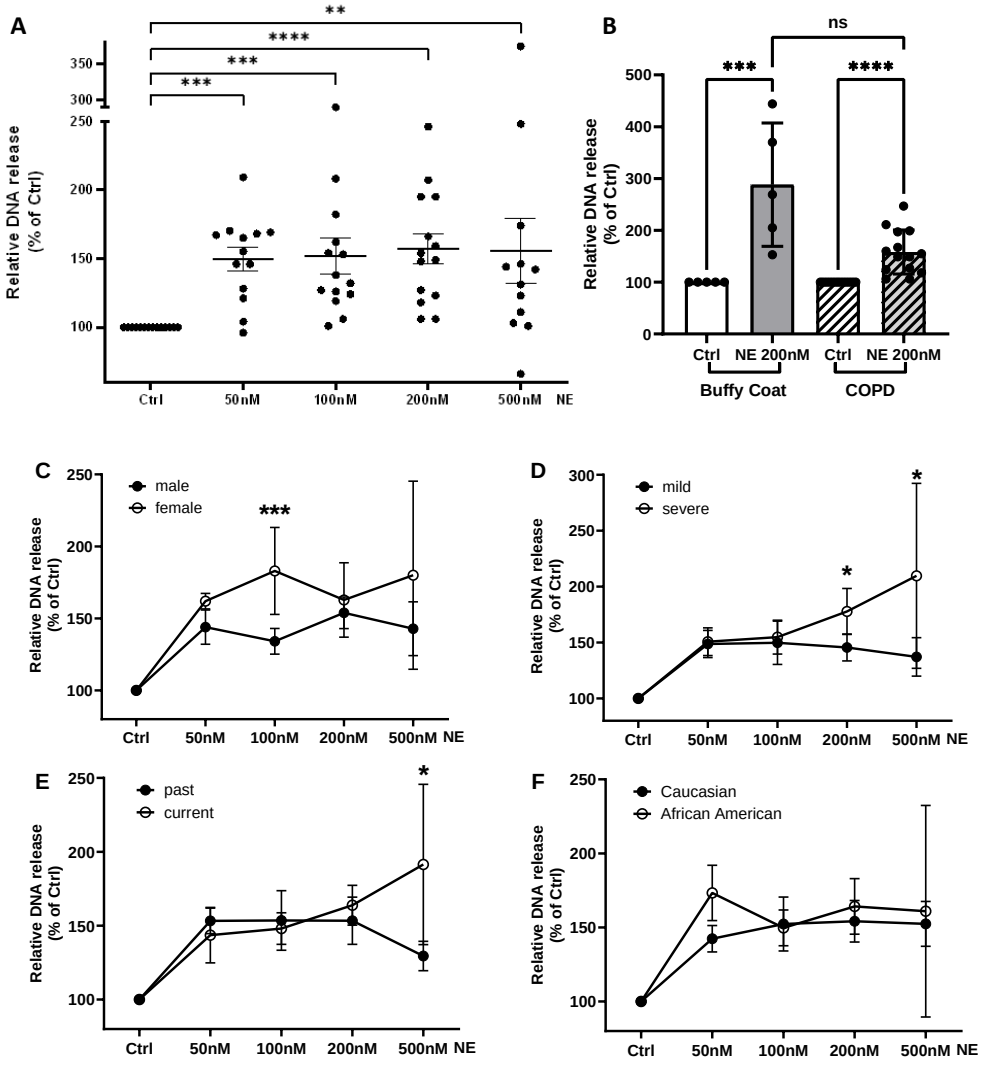
different donor subjects. *, $p < 0.05$ vs. Ctrl (vehicle control). Arrows indicate location of full-length proteins and clipped Histone H3.

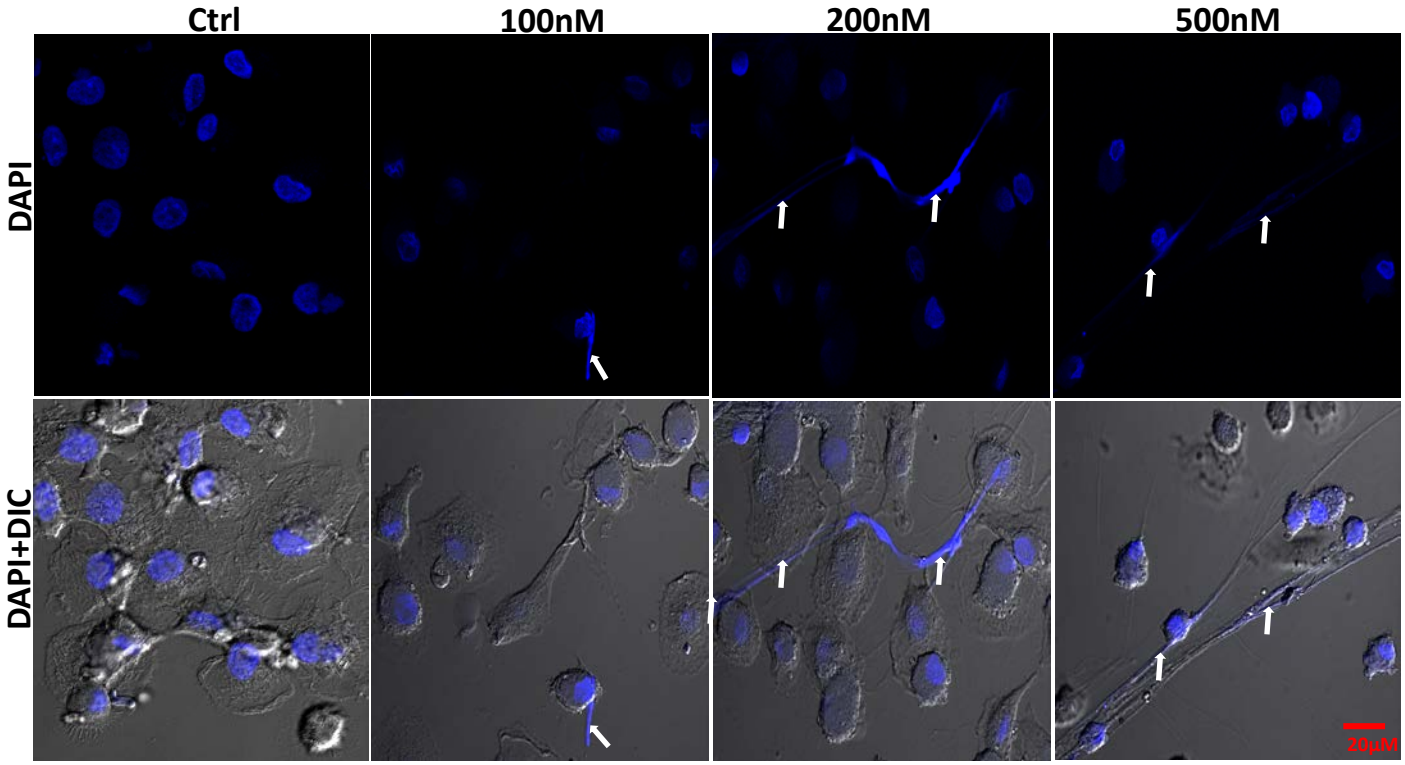
Supplementary Information

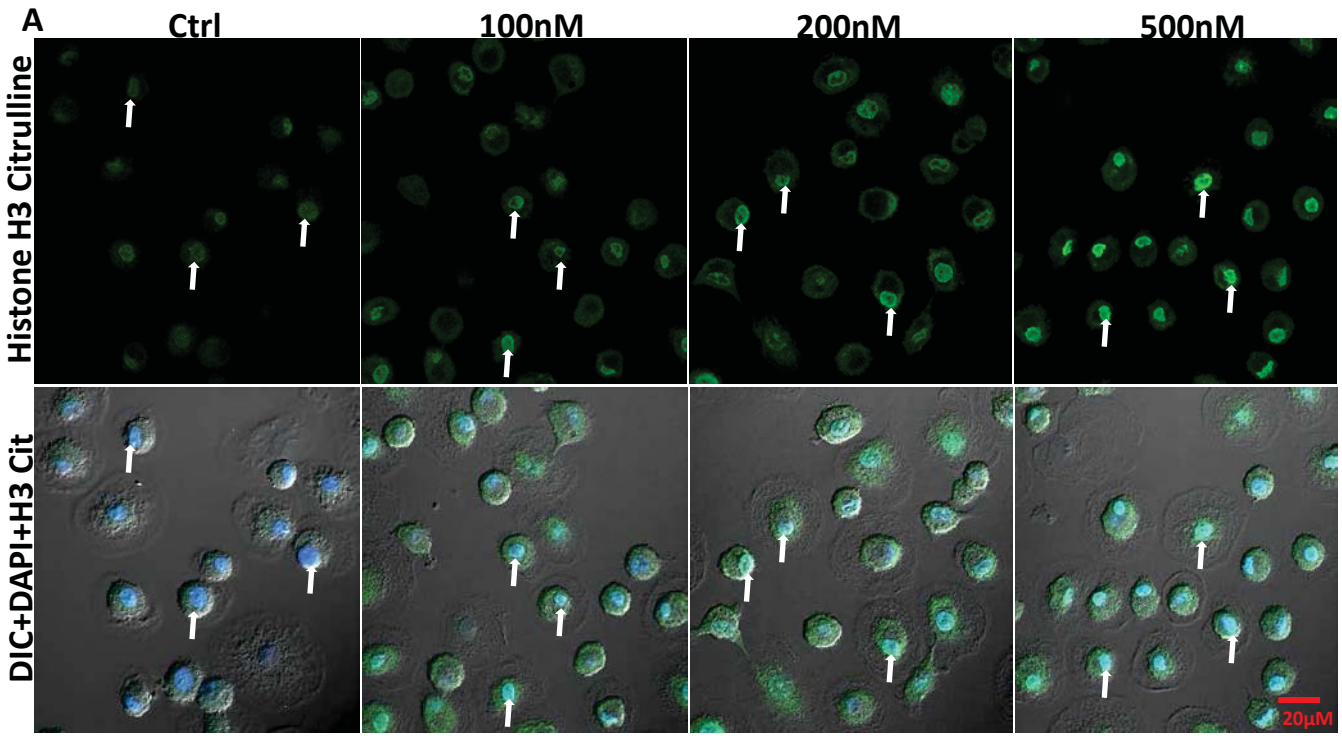
Supplemental Material

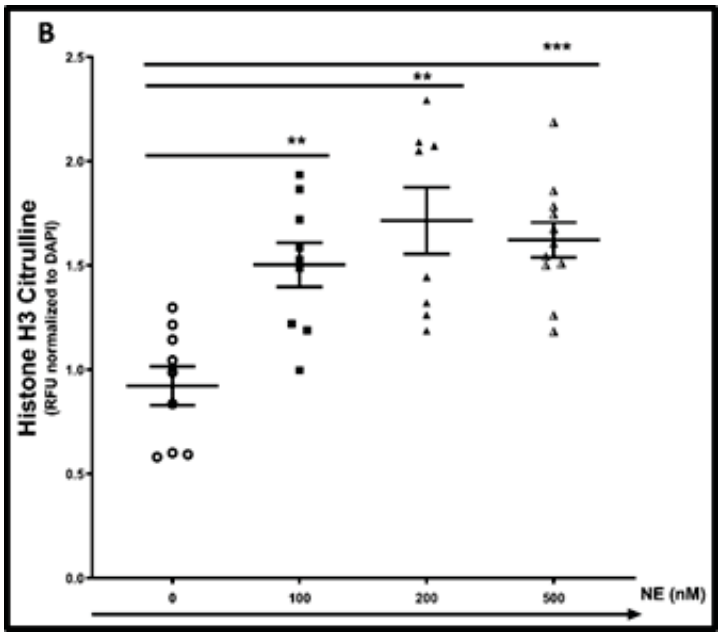

1A
DAPI+DIC
Ctrl
100nM
200nM
500nM
20μM
1B
FITC-NE+DIC
Ctrl
100nM
200nM
500nM
20μM
1C
Ctrl
NE 100nM
NE 200nM
NE 500nM
NE Localization
DIC+DAPI+NE
20μM

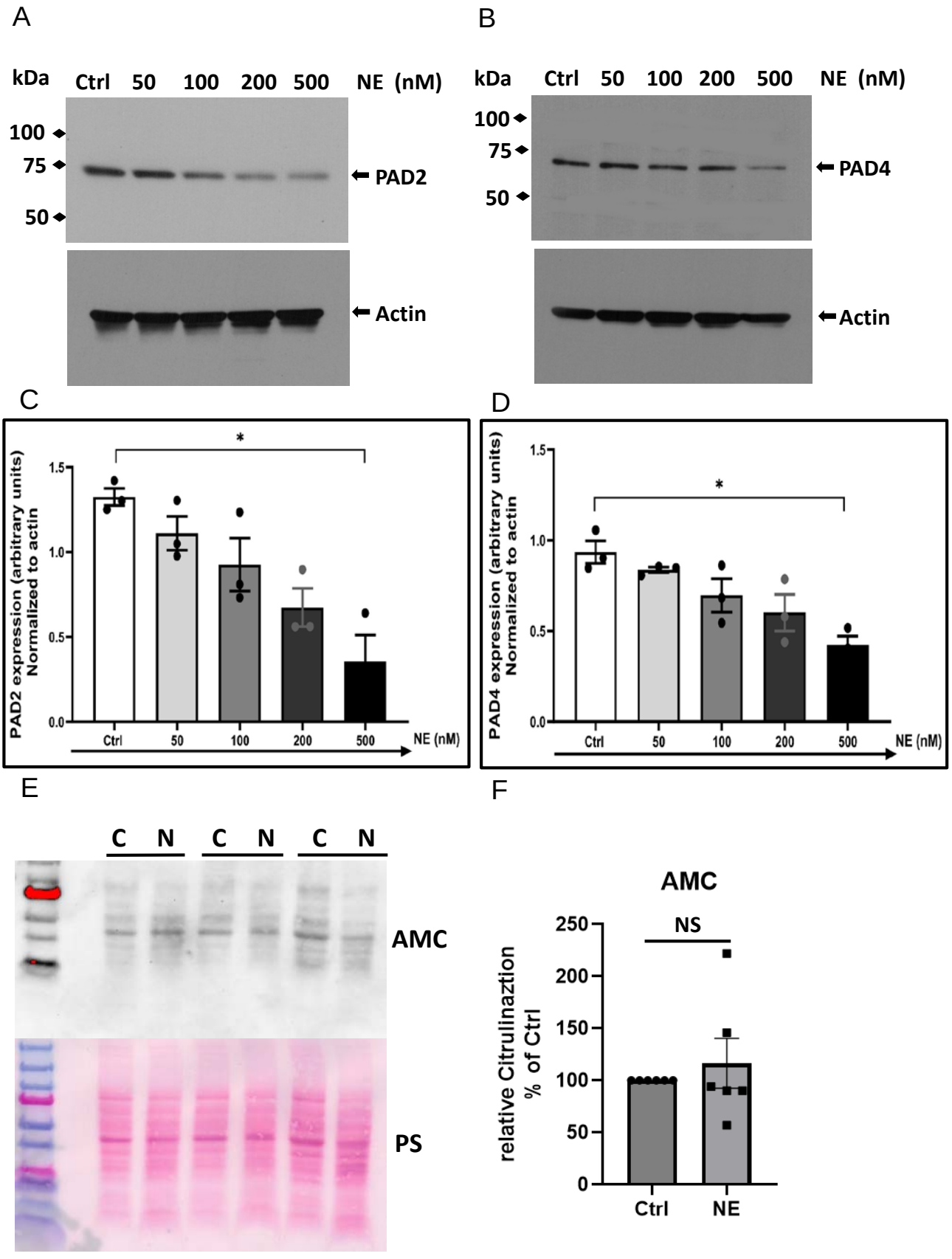

*
*
ns
Relative NE activity
% of Avg Ctrl
500
400
300
200
100
0
Ctrl
50nM
100nM
200nM
Ctrl
50nM
100nM
200nM
NE
DQ Elastin
DQ Elastin + AAPV-CMK

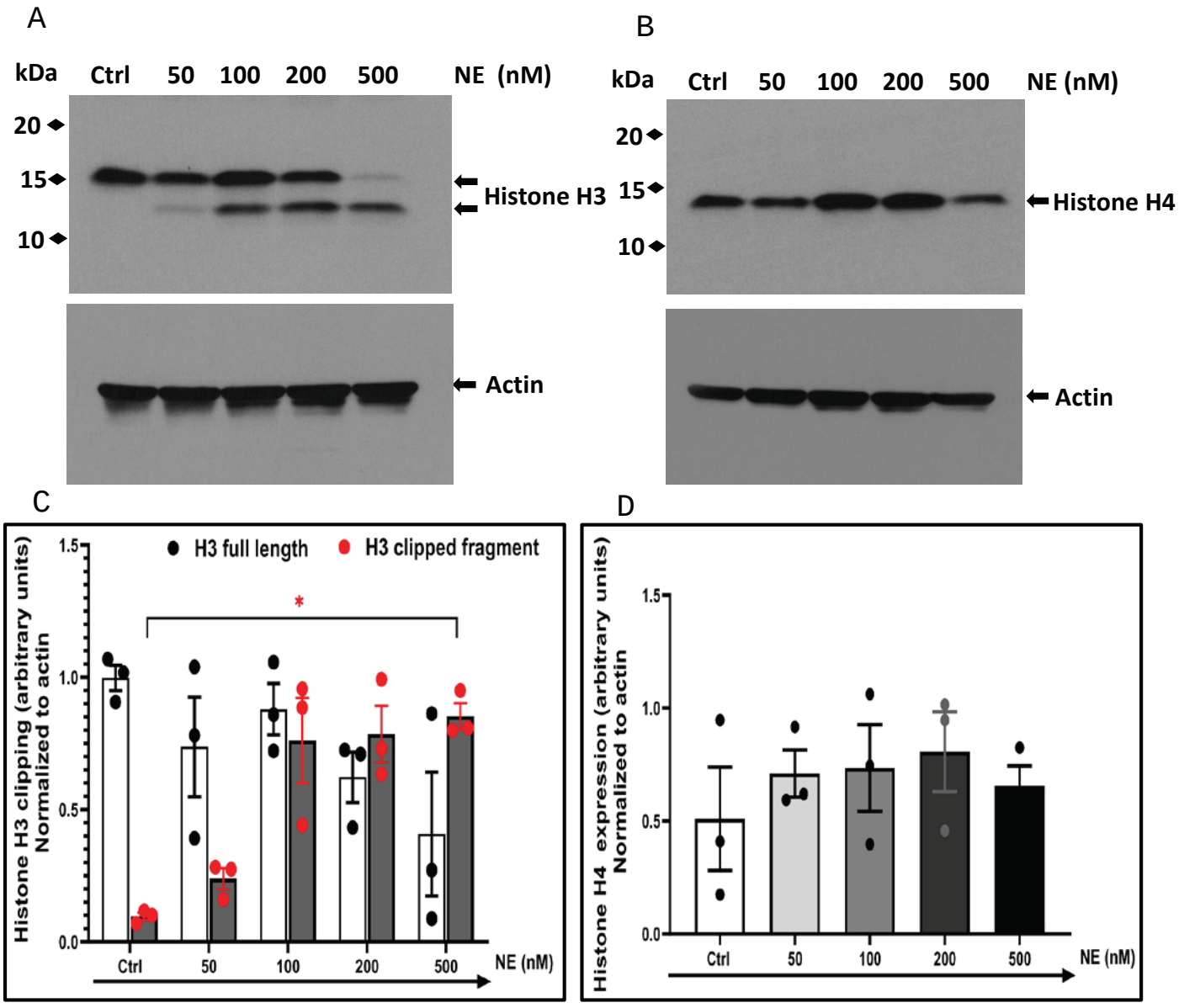

A
Relative DNA release
(% of Ctrl)
**
****
***
***
350
300
250
200
150
100
Ctrl
50nM
100nM
200nM
500nM
NE
B
ns
***
****
500
400
300
200
100
0
Relative DNA release
(% of Ctrl)
Ctrl
NE 200nM
Ctrl
NE 200nM
Buffy Coat
COPD
C
male
female
***
250
200
150
100
Relative DNA release
(% of Ctrl)
Ctrl
50nM
100nM
200nM
500nM
NE
D
mild
severe
*
*
300
250
200
150
100
Relative DNA release
(% of Ctrl)
Ctrl
50nM
100nM
200nM
500nM
NE
E
past
current
*
250
200
150
100
Relative DNA release
(% of Ctrl)
Ctrl
50nM
100nM
200nM
500nM
NE
F
Caucasian
African American
250
200
150
100
Relative DNA release
(% of Ctrl)
Ctrl
50nM
100nM
200nM
500nM
NE


Ctrl
100nM
200nM
500nM
DAPI
DAPI+DIC
20μM


A
Ctrl
100nM
200nM
500nM
Histone H3 Citrulline
DIC+DAPI+H3 Cit
20µM


B
Histone H3 Citrulline
(RFU normalized to DAPI)
2.5
2.0
1.5
1.0
0.5
0.0
0
100
200
500
NE (nM)
**
**
***


A
kDa
Ctrl 50 100 200 500 NE (nM)
100
75
50
PAD2
Actin
B
kDa
Ctrl 50 100 200 500 NE (nM)
100
75
50
PAD4
Actin
C
PAD2 expression (arbitrary units)
Normalized to actin
1.5
1.0
0.5
0.0
*
Ctrl 50 100 200 500 NE (nM)
D
PAD4 expression (arbitrary units)
Normalized to actin
1.5
1.0
0.5
0.0
*
Ctrl 50 100 200 500 NE (nM)
E
C N C N C N
AMC
PS
F
AMC
NS
relative Citrullinaztion
% of Ctrl
250
200
150
100
50
0
Ctrl NE


A
kDa
Ctrl
50
100
200
500
NE (nM)
20
15
10
Histone H3
Actin
B
kDa
Ctrl
50
100
200
500
NE (nM)
20
15
10
Histone H4
Actin
C
H3 full length
H3 clipped fragment
Histone H3 clipping (arbitrary units)
Normalized to actin
1.5
1.0
0.5
0.0
*
Ctrl
50
100
200
500
NE (nM)
D
Histone H4 expression (arbitrary units)
Normalized to actin
1.5
1.0
0.5
0.0
Ctrl
50
100
200
500
NE (nM)