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ARTICLE

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 chronic obstructive pulmonary disease (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). The 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 GM-CSF to differentiate into blood monocyte derived macrophages (BMDMs). The BMDMs 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 50 nM. 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.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Airway neutrophil extracellular traps are associated with more severe chronic obstructive pulmonary disease (COPD) but the presence of macrophage extracellular traps (METs) has not previously been reported in COPD airways.

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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 PADs 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.

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 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 (METs) from monocyte-derived macrophages from patients with CF and from alveolar macrophages from Cftr-null mice.[5] Increased NETs 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 METs, 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 institutional review board-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 Global Initiative for Chronic Obstructive Lung Disease (GOLD) score determined by frequency of exacerbations and the COPD Assessment Test score were obtained. Patients provided a whole blood sample at the time of the clinical data collection.

Human peripheral blood monocyte derived macrophages

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.

CRP enzyme-linked immunosorbent assay

Plasma samples from recruited subjects were analyzed for CRP levels by enzyme-linked immunosorbent assay (ELISA; Human CRP ELISA kit; Abcam, ab260058) per manufacturer's instructions.

NE treatments

Differentiated BMDM (~days 10–12) were treated with FITC-NE (Cat# FS563, Elastin Products Company) or NE (Cat# SE563, Elastin Products Company) (50, 100, 200 or 500 nM) or vehicle control for 2 h, 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 1 h. 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 2 h, 37°C, and prepared for DQ-elastin assay by fixation in 2% PFA, permeabilization with 0.1% NP40 for 10 min, 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 the 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 (90–100 K cells/well) were treated with NE (50, 100, 200, or 500 nM) for 2 h. 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 at 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, 100 μL) 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 subjects with COPD 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 4°C 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 1 h. 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 subjects with COPD 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 h 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 analysis of variance (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 (White 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 version 9.4 (SAS Institute). Any $p < 0.05$ 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 (100 nM, 200 nM, or 500 nM) for 2 h 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,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 (100 nM, 200 nM, or 500 nM) for 2 h 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 patients with COPD grown in suspension were treated with control vehicle, or NE 50, 100, or 200 nM for 2 h, 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,e). There was an increase in MET release only at 100 nM NE in women versus 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 subjects with COPD 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 confocal 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, PAD 2 or 4, that catalyze the modification of

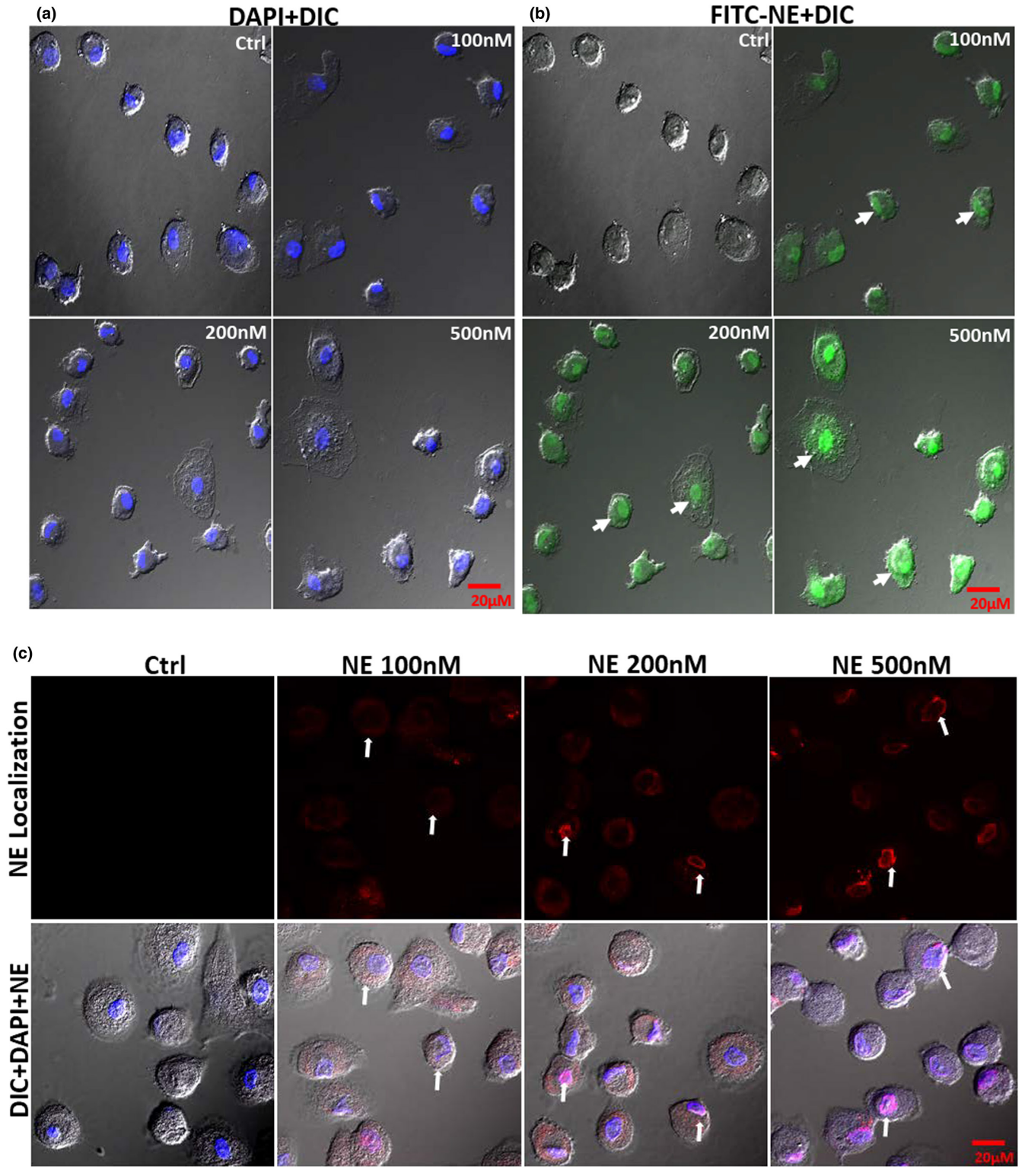

FIGURE 1 NE localization in human BMDM. Human BMDM from subjects with COPD were adhered to coverslips and treated with FITC-NE (100–500 nM) for 2 h at 37°C, followed by DAPI nuclear staining to determine NE localization in the cell. Confocal fluorescence microscopy for DAPI + differential interference 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 are representative of three COPD donors. BMDM, blood monocyte derived macrophage; COPD, chronic obstructive pulmonary disease; NE, neutrophil elastase.

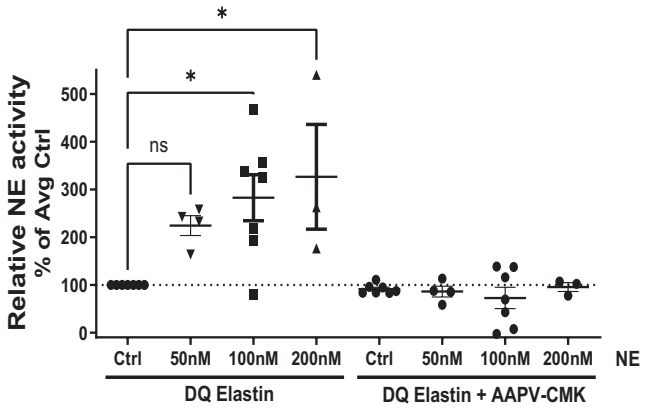

FIGURE 2 NE taken up by BMDM was proteolytically active. Human BMDM from subjects with COPD were treated with NE (0, 50, 100, or 200 nM, 2 h, 37°C) while in suspension. Following treatments, cells were exposed to DQ-elastin. NE inhibitor, AAPV-CMK, was used as a NE-specific, negative control. Relative fluorescence units (RFUs) 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; $^*p < 0.05$. BMDM, blood monocyte derived macrophage; COPD, chronic obstructive pulmonary disease; NE, neutrophil elastase; ns, no significant difference.

arginine to citrulline, we first evaluated PAD2 and 4 protein abundance by Western analysis in total cell lysates of BMDM from subjects with COPD 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 6a–d). We further evaluated whether NE caused increased PAD activity as measured by total protein citrullination. Following NE (200 nM, 2 h) exposure, BMDM total cell lysates from subjects with COPD were separated by SDS-PAGE (4%–20%) and transferred to PVDF membranes. 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 6e,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 NET 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,c) and H4 (Figure 7b,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 (50–200 nM) that are relevant to COPD lung disease. Sputum from stable patients with COPD have mean NE concentrations of 83–114 nM[14,15] whereas 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 CRP 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 subjects with COPD 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,

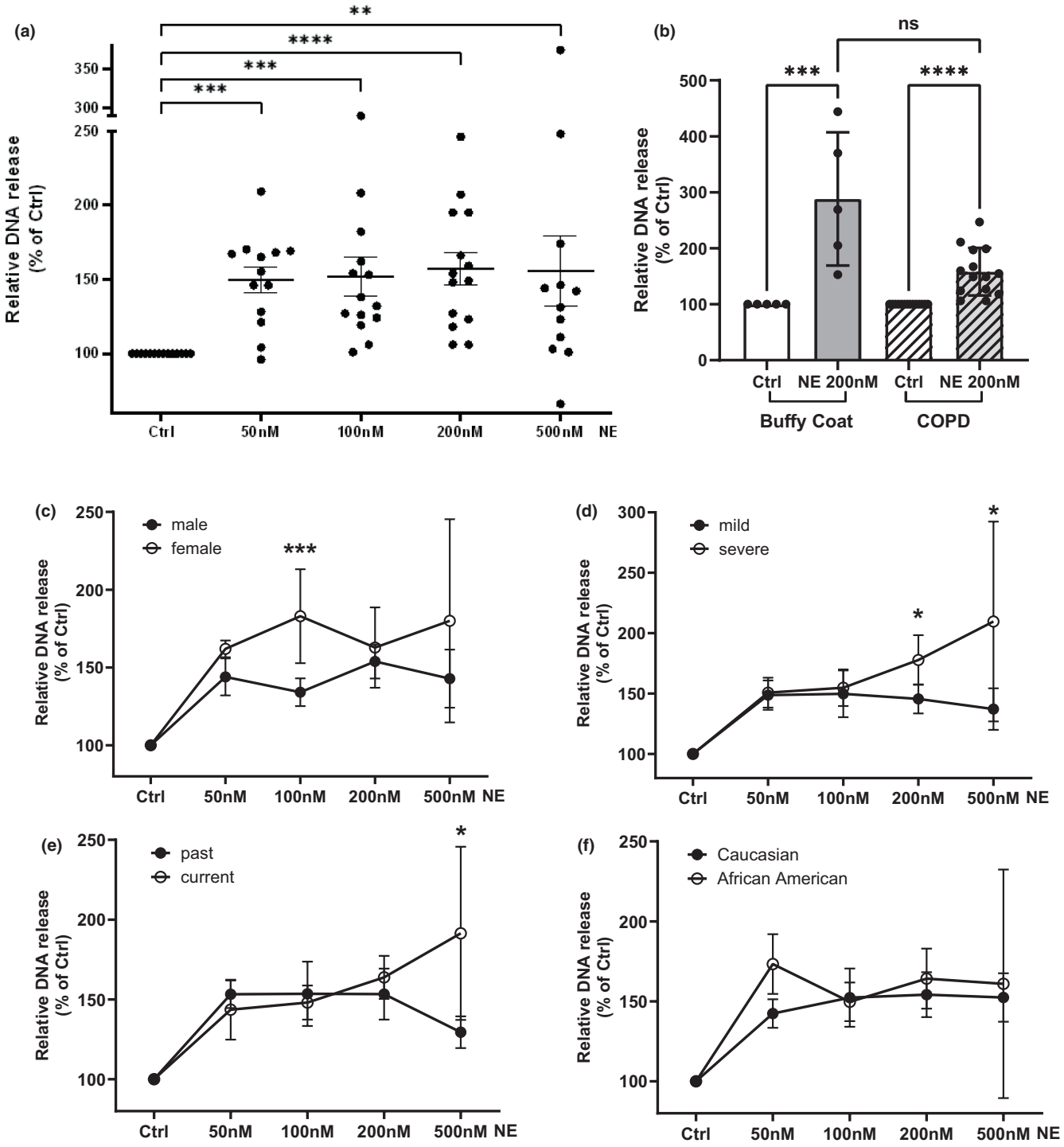

FIGURE 3 NE induced the release of METs as indicated by extracellular (ec)DNA in COPD BMDM. Following NE and control vehicle treatments, 2 h, 37°C, conditioned media was removed and cells were incubated with micrococcal nuclease and the supernatant ecDNA release (MET release) was quantified by Quanti-iT 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 versus NE treatment (200 nM) (b). Comparisons between subjects with COPD: male versus female (c), mild versus severe lung disease (d), past versus current smokers (e), and African American versus White subjects (f) were evaluated. BMDM, blood monocyte derived macrophage; COPD, chronic obstructive pulmonary disease; MET, macrophage extracellular trap; NE, neutrophil elastase; ns, no significant difference.

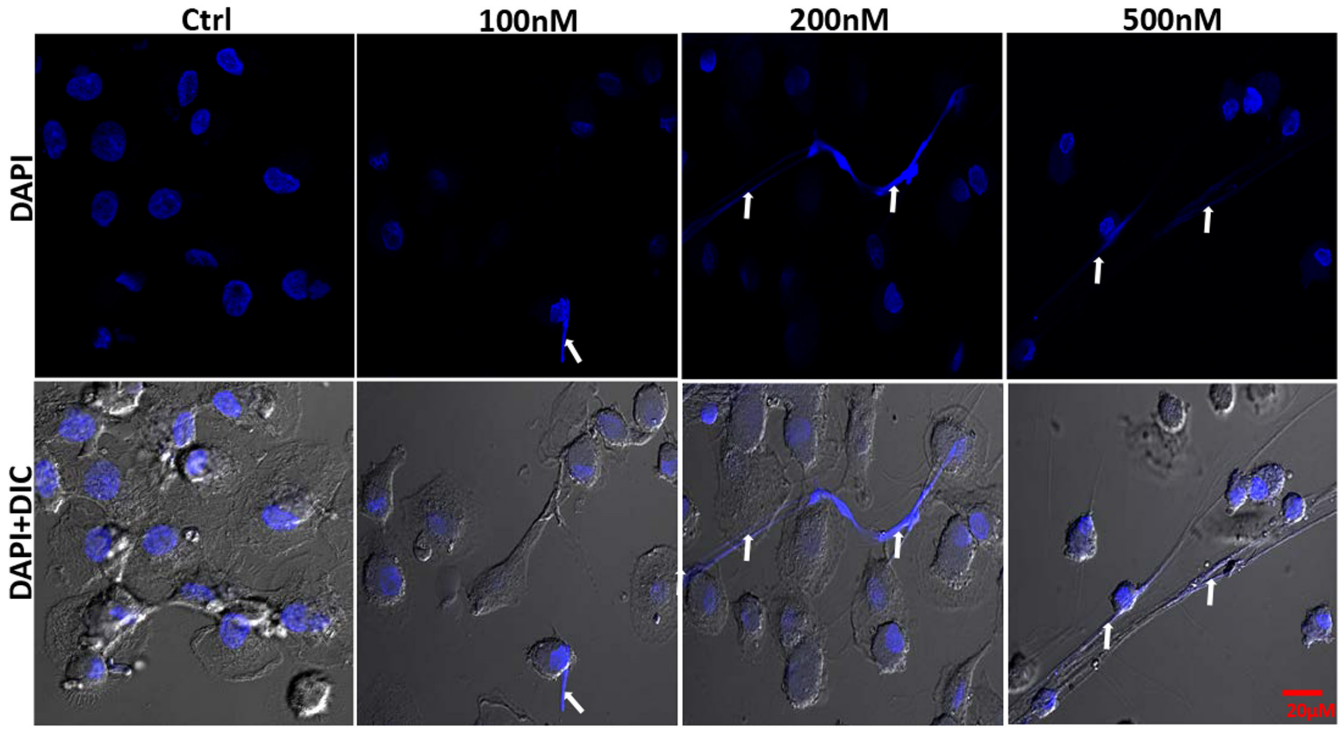

FIGURE 4 NE induced extracellular traps release from COPD BMDM detected by confocal microscopy. Human BMDM were adhered to coverslips and treated with NE (0–500 nM, 2 h, 37°C), fixed, and then stained with DAPI. The control treated slides (Ctrl) had no detectable METs. The NE treated slides all had detectable 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. BMDM, blood monocyte derived macrophage; COPD, chronic obstructive pulmonary disease; MET, macrophage extracellular trap; NE, neutrophil elastase; ns, no significant difference.

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 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. TIMP1 overexpression in a mouse model of liver ischemia and reperfusion injury rescues liver injury in part by inhibition of NET generation in the liver.[24] Furthermore, secretory leukocyte peptidase inhibitor 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

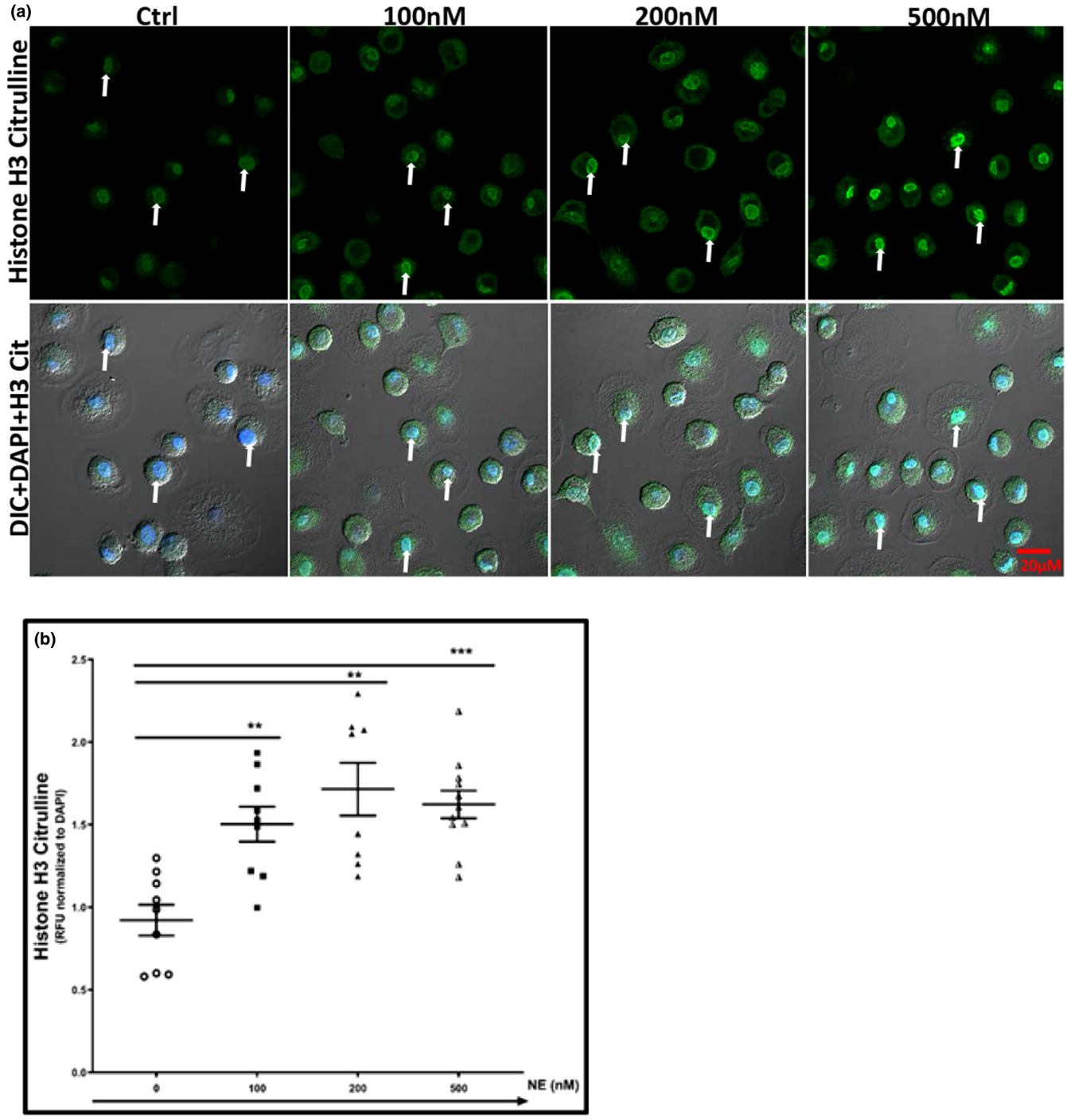

FIGURE 5 NE increased histone H3 citrulline in COPD BMDM detected by confocal microscopy. COPD BMDM adhered to coverslips were treated with NE (100–500 nM) or control vehicle for 2 h, 37°C. After treatment, cells were fixed, permeabilized, and then incubated with rabbit anti-histone H3 citrulline R2-R8-R17. 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 are representative of three experiments using three 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 $^{***}p < 0.001$. BMDM, blood monocyte derived macrophage; COPD, chronic obstructive pulmonary disease; NE, neutrophil elastase; RFU, relative fluorescence intensity.

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 analysis for AMC.[25,26] We did not observe a global increase in citrullination by

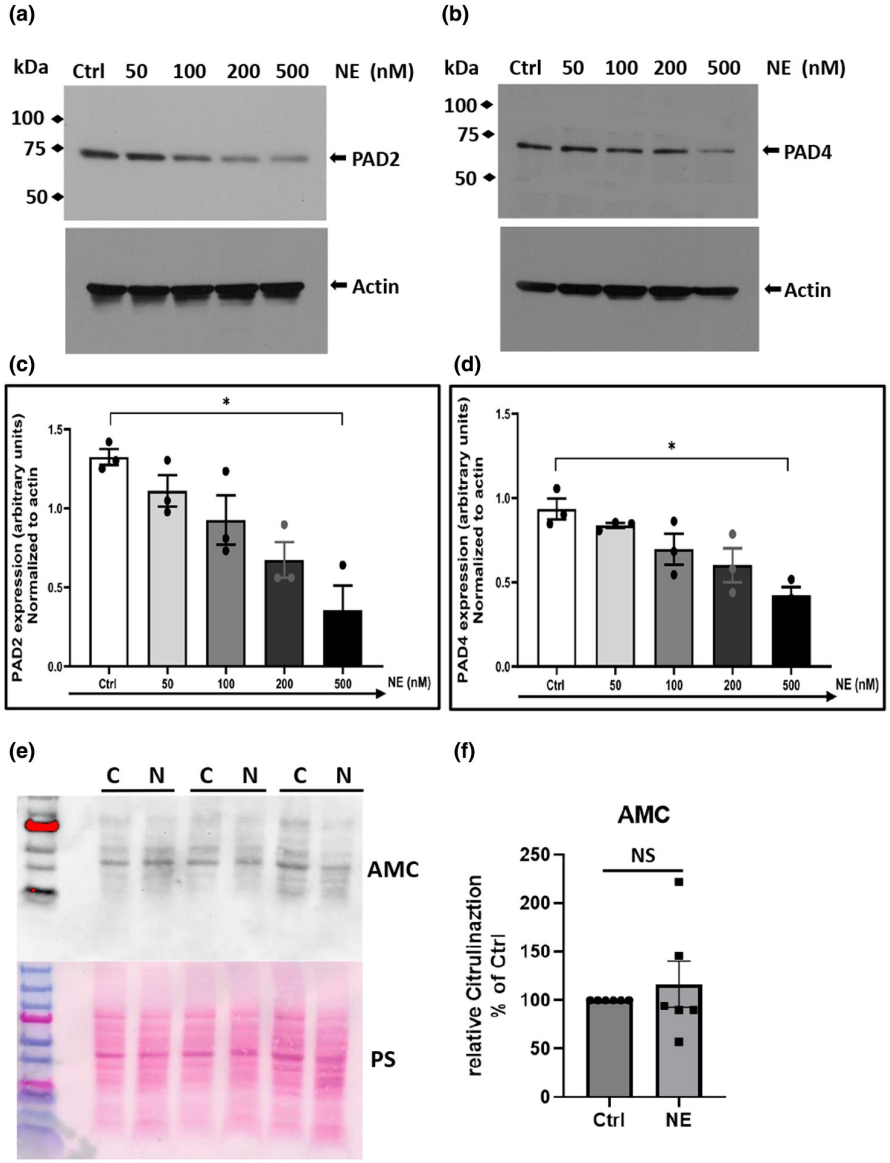

FIGURE 6 NE effect on expression of PAD2 and 4 and total modified citrullination. Following NE treatment (0–500 nM, 2 h, 37°C), total cell lysates were harvested from BMDM and protein lysates (30 μg) were separated on 4%–20% PAGE, and transferred to nitrocellulose membrane. Membranes were incubated with 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 three different donor subjects *$p < 0.05$, versus 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 (50 μg) 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, $n = 6$. AMC, anti-modified citrulline; BMDM, blood monocyte derived macrophage; COPD, chronic obstructive pulmonary disease; NE, neutrophil elastase; NS, no significant difference.

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

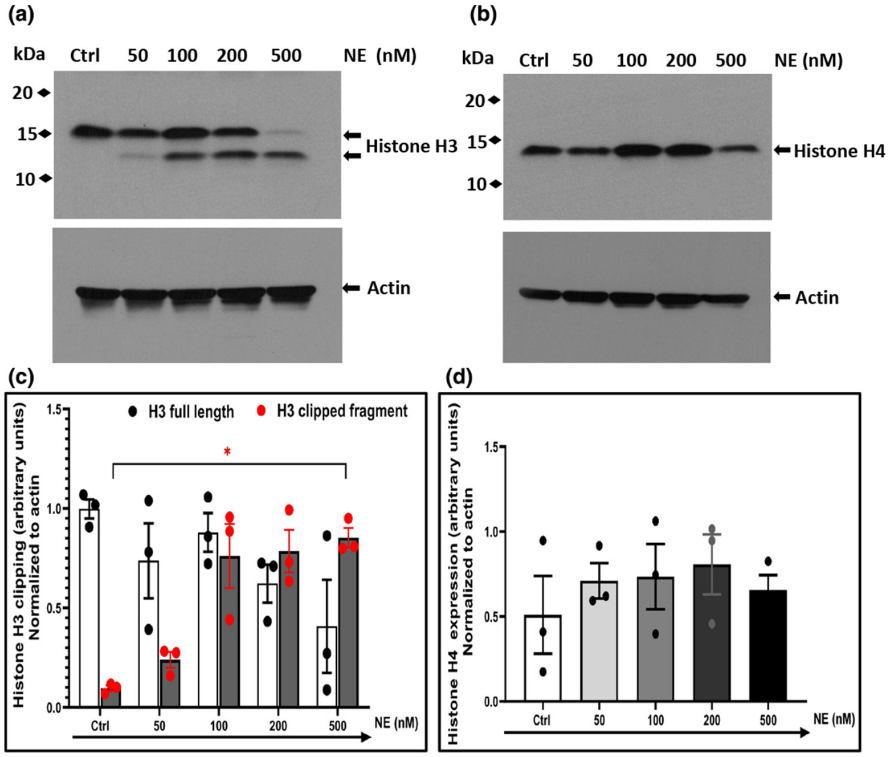

FIGURE 7 NE and histone clipping. Following NE treatment (0–500 nM, 2 h, 37°C), total cell lysates were harvested from BMDM and protein lysates (30 μg) were separated on 4%–20% PAGE, and transferred to nitrocellulose membrane. Membranes were incubated with primary rabbit monoclonal antibody for histone 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 versus 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 three different donor subjects. *$p < 0.05$ versus Ctrl (vehicle control). Arrows indicate location of full-length proteins and clipped histone H3. BMDM, blood monocyte derived macrophage; NE, neutrophil elastase.

modifications were observed after treatment of BMDM with NE for 2 h. Importantly, our results are consistent with the impact of NE treatment for 2–2.5 h or less on other macrophages functions. Hubbard et al. exposed human alveolar macrophages to NE 100 nM, 30 min and demonstrated release of leukotriene B4 and subsequent neutrophil chemotaxis.[27] Ma et al.[28] 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. 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, 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 CF,[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 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 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 because 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 lungs. 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.

AUTHOR CONTRIBUTIONS

S.Z., A.B.K., and J.A.V. wrote the manuscript and designed the research. S.Z., A.B.K., G.B.B., and A.S. performed the research. S.Z., A.B.K., G.B.B., and A.S. performed the research. S.Z., A.B.K., G.B.B., and L.K. analyzed the data.

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CONFLICT OF INTEREST STATEMENT

The authors declared no competing interests for this work.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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