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

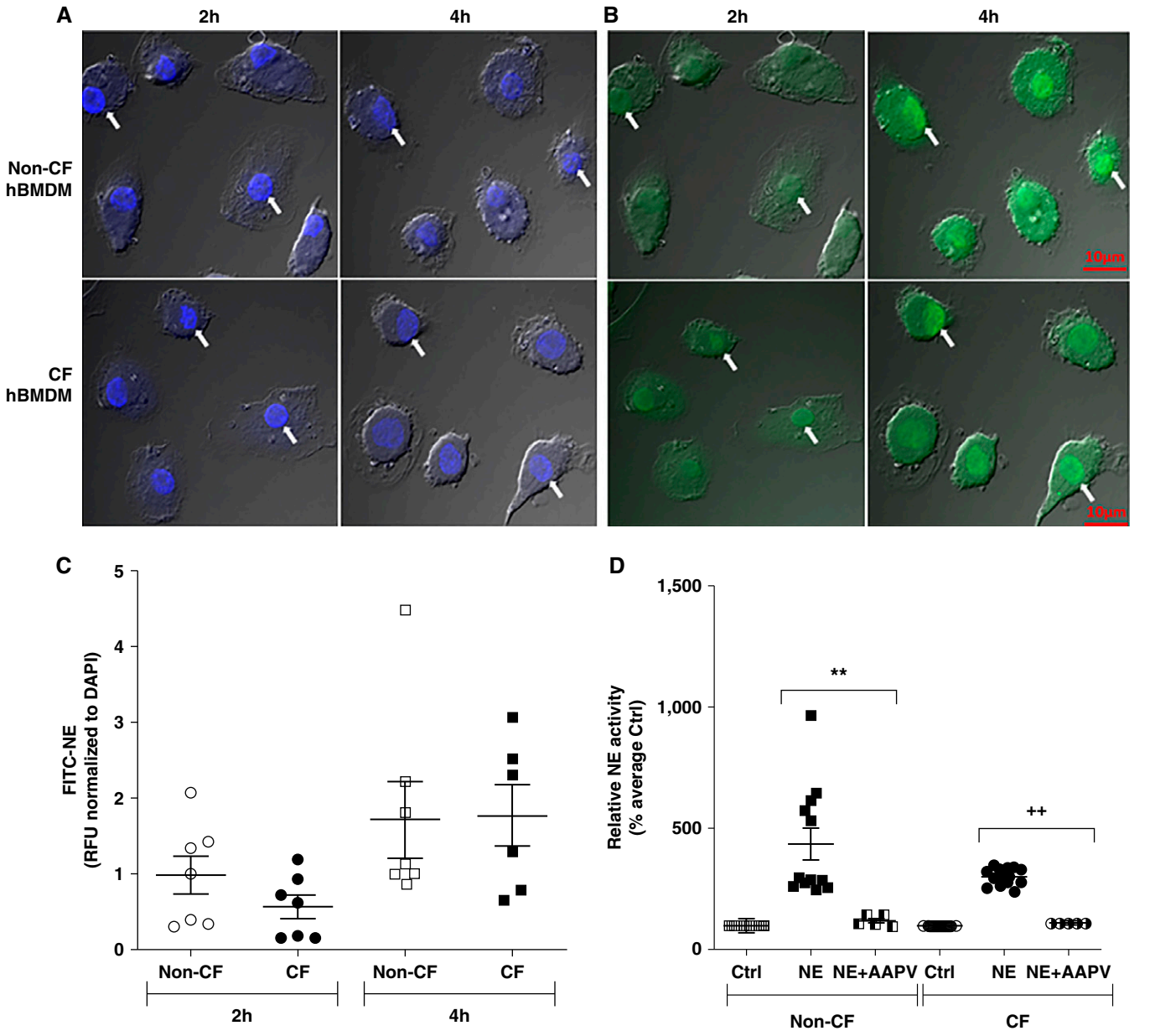

Figure 1. Neutrophil elastase (NE) was internalized in non-cystic fibrosis (CF) and CF human blood monocyte-derived macrophage (hBMDM) cells and exhibited proteolytic activity intracellularly. (*B*) Non-CF and CF hBMDM were exposed to FITC-NE (200 nM, 2 h or 4 h) or vehicle control. (*A*) After the treatments, 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

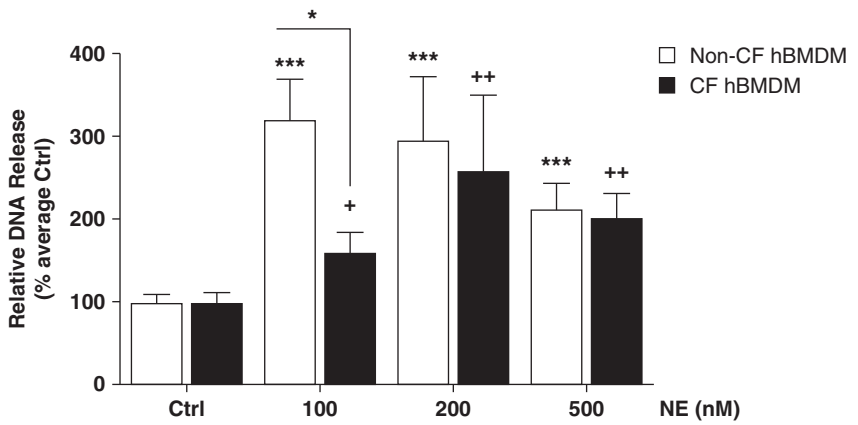

Figure 2. NE-induced extracellular DNA release from non-CF and CF hBMDM. Non-CF hBMDM (white bar) and CF hBMDM (black bar) cultured on 96-well plates were treated with control vehicle (ctrl) or NE (100, 200 or 500 nM, 2 h). At the end of NE treatment, cells were incubated with micrococcal nuclease (4 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

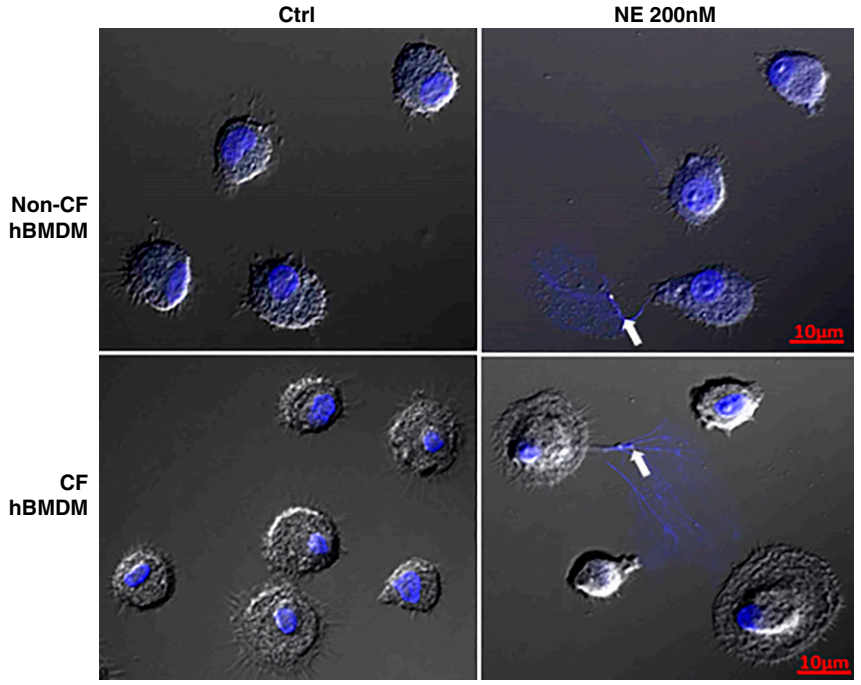

Figure 3. NE-stimulated release of extracellular traps from non-CF and CF hBMDM detected by confocal microscopy. hBMDM from subjects with and without CF were seeded onto poly-L-lysine-coated coverslips and cultured in growth media containing granulocyte-macrophage colony-stimulating factor (GM-CSF) (20 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

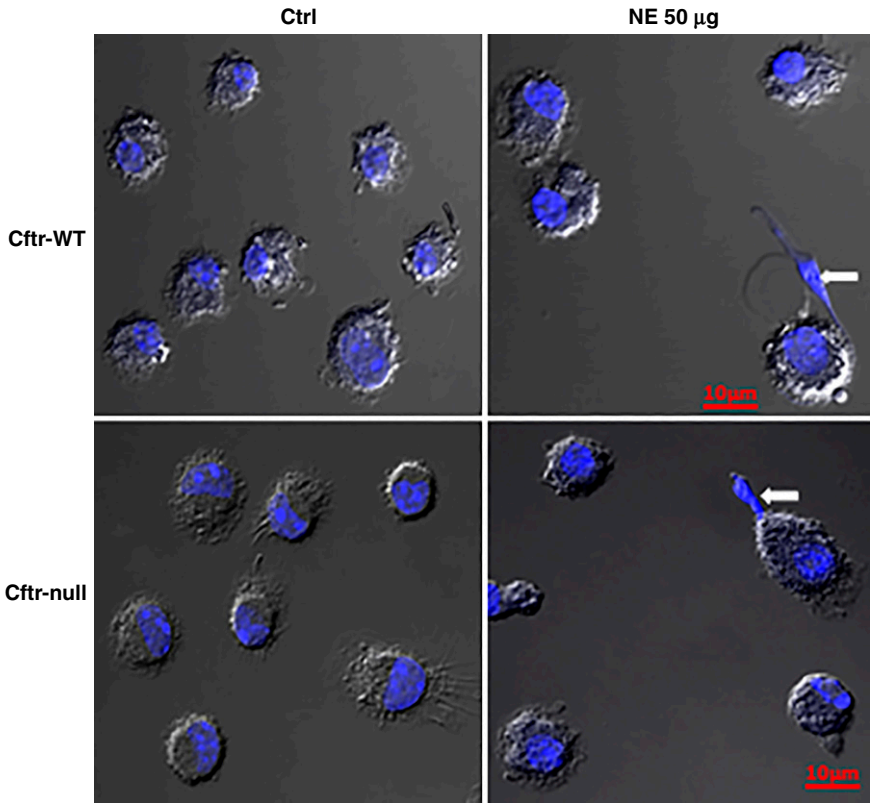

Figure 4. Detection of macrophage extracellular traps (METs) released *in vivo* following intratracheal NE administration in both Cftr-null and Cftr wild-type (WT) mice on the same genetic background. Aged 8–12 weeks old, Cftr-null mice and Cftr WT mice (both male and female) were administered NE (50 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

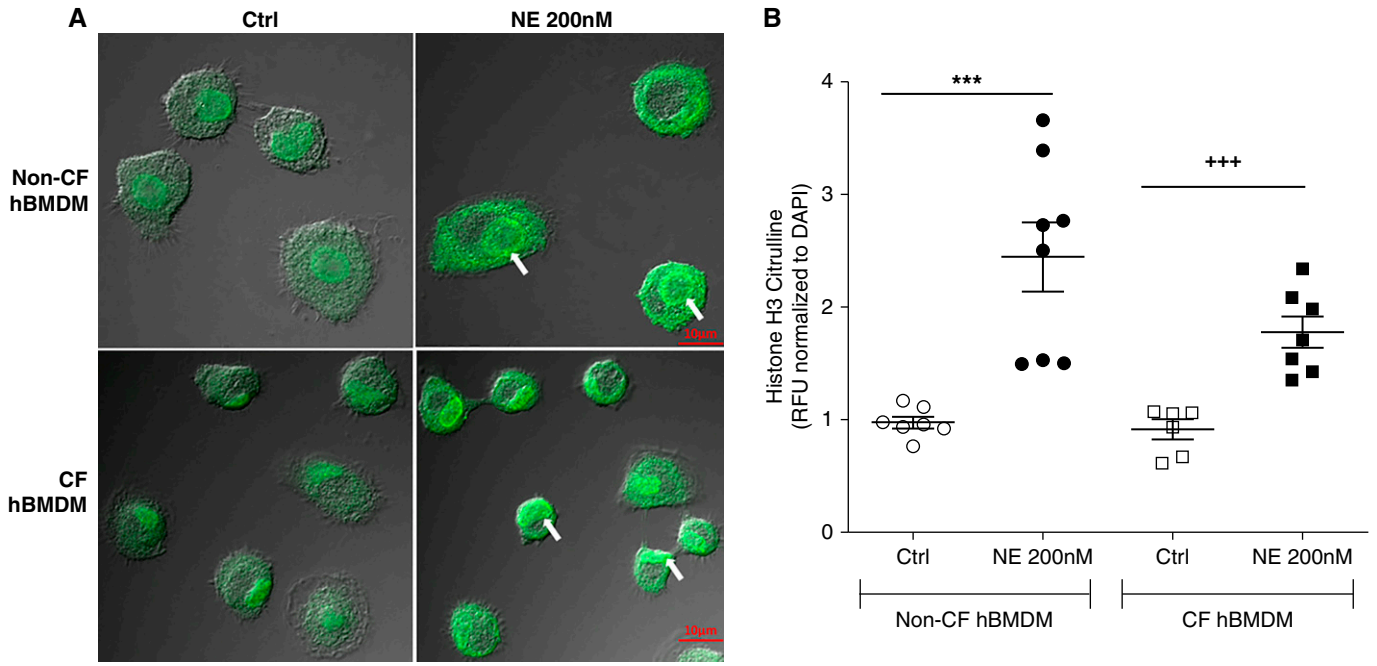

Figure 5. NE increased H3 citrulline (citH3) in both non-CF and CF hBMDM. hBMDM were treated with NE (200 nM, 2 h) or control vehicle (ctrl) on cover slips. Following fixation, cells were incubated with antihistone H3 citrulline R2 + R8 + R17 (1:100 dilution, overnight, Abcam), followed by Alexa Fluor 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 $^{+++}P < 0.0006$.

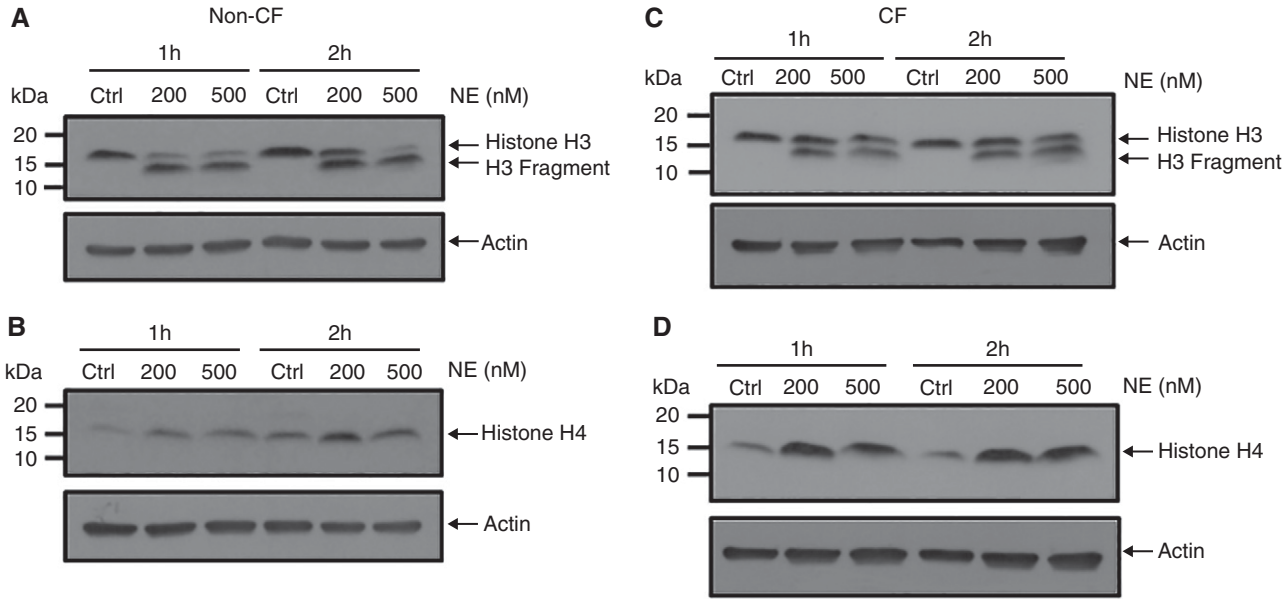

Figure 6. NE induced the cleavage of histone H3 in non-CF and CF hBMDM cells. Non-CF and CF hBMDM were treated with control vehicle (ctrl) or NE (200 or 500 nM) for 1 or 2 hours, and total cell proteins were collected. Total cell lysate protein (30 g) was separated on a 4–20% SDS-PAGE and tested for 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.

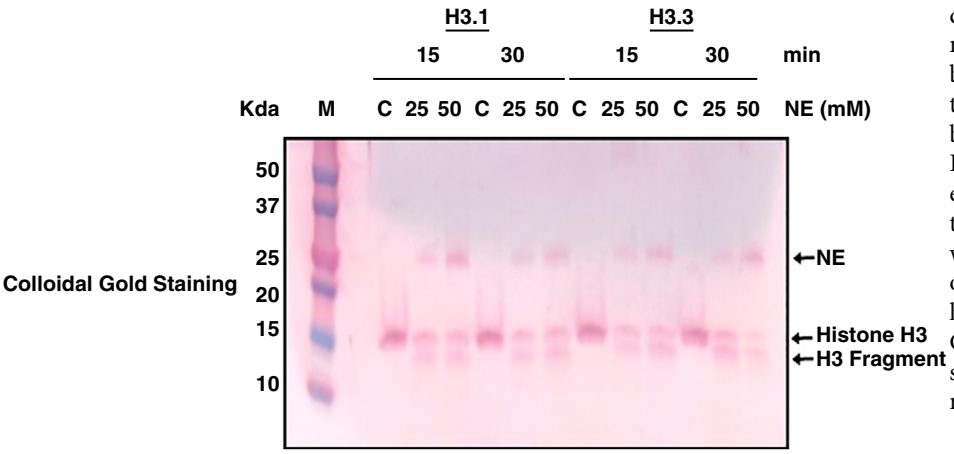

Figure 7. NE cleaved recombinant histone H3.1 and H3.3 isoforms *in vitro*. Recombinant human histone H3.1 and H3.3 were incubated with control vehicle or NE (25 or 50 nM) for 15 or 30 minutes. Equal amounts of reaction products were resolved on 4–20% SDS-PAGE, transferred to membrane, and stained with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