PSEUDOMONAS AERUGINOSA-INDUCED FORMATION OF HUMAN NEUTROPHIL EXTRACELLULAR TRAPS: MECHANISM AND RELEVANCE IN CYSTIC FIBROSIS AIRWAY DISEASE

by

DAE-GOON YOO

(Under the Direction of Balázs Rada)

ABSTRACT

Cystic fibrosis transmembrane conductance regulator (CFTR) deficiency, the cause of cystic fibrosis (CF), leads to unbalanced anion transport across the airway epithelium, limiting mucociliary clearance and resulting in chronic bacterial lung infections. The main CF pathogen, *Pseudomonas aeruginosa* stimulates neutrophil recruitment and activation in the lung. In CF, neutrophils are incapable of eliminating bacteria and undergo excessive activation, causing tissue damage and contributing to lung failure. Neutrophil extracellular traps (NETs) are one of the crucial innate immune, antimicrobial responses of neutrophil granulocytes. NET formation requires *superoxide production* and extrusion of DNA associated with citrullinated histones (citH4) and neutrophil granule proteins such as myeloperoxidase (MPO) and human neutrophil elastase (HNE). However, uncontrolled NET release offers a potential mechanism for *excessive inflammatory response in CF airways*. Thus, understanding the involvement of NET formation in CF airway pathophysiology is of clinical importance. We aimed to characterize *P. aeruginosa*-

induced NET formation and its potential relevance in CF airways. Our data show that *P. aeruginosa* initiates robust release of extracellular DNA from human neutrophils. MPO, HNE and citH4 co-localize with extracellular DNA upon *P. aeruginosa* stimulation. The superoxide generating NADPH oxidase and MEK/ERK signaling contribute to NET formation. NET formation was quantified by our newly developed ELISA assays detecting MPO-DNA and HNE-DNA complexes. By comparing 'early' and 'late' bacterial isolates obtained from the same CF patient, we found that early isolates promoted more significant NET release than late isolates. This result suggested that release of *P. aeruginosa*-induced NETs is an important mechanism for release of neutrophil-derived CF inflammatory markers, and that decreased induction of NETs is required for adaptation and long-term survival of *P. aeruginosa* bacilli in CF airways. Correlation was performed between CF inflammatory markers and NET markers. We found that CF sputa contain significant amounts of NET markers whose levels correlate with lung disease severity. In summary, this work supports our hypothesis that *P. aeruginosa*-induced NET formation has deleterious effects to CF pulmonary airways.

INDEX WORDS: Cystic Fibrosis, Myeloperoxidase, Human Neutrophil Elastase, *Pseudomonas aeruginosa*, Neutrophil Extracellular DNA traps, Superoxide, MPO-DNA, HNE-DNA.

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DAE-GOON YOO

BS, Mokwon University, Republic of Korea, 2005

MS, Chungnam National University, Republic of Korea, 2007

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2015

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DAE-GOON YOO

Major Professor:	Balázs Rada
Committee:	Frederick Quinn Russell Karls Wendy Watford Robert Jeff Hogan

Electronic Version Approved:

Julie Coffield
Interim Dean of the Graduate School
The University of Georgia
May 2015

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEWS

Cystic Fibrosis

Cystic Fibrosis (CF) is an inheritable genetic disorder caused by the recessive gene of the CFTR protein [1-3]. CFTR is a member of the ATP-binding cassette (ABC) transporter superfamily [127]. It mainly regulates the transport of chloride ions and antioxidants such as glutathione (GSH) and thiocyanate (SCN^-) [1]. It is expressed on chromosome 7 and encodes a 1480 amino acid long protein [60]. There are about 900 mutations identified in CFTR, however, the most common mutation is ΔF508-CFTR [3]. Approximately 70% of CF individuals have a homozygous ΔF508-CFTR mutation, and up to 90% of the patients have at least one ΔF508-CFTR allele [4]. The ΔF508 mutation leads to misfolded mutant proteins, and CFTR is degraded through the ER quality control mechanism [4,70]. ΔF508 CFTR does not reach its final cellular destination, the apical plasma membrane in bronchial epithelial cells, which leads to excessive sodium reabsorption, dehydration of the outer surface of epithelial cells and adhesive mucus [5,6]. These mucus layers serve as a niche for bacterial pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* [3,7-10]. During respiratory infection, *P. aeruginosa* bacilli undergo chronic changes resulting in its adaptation. As seen in figure illustrating CF airway inflammation below (Figure S.1), inhaled bacteria expressing pili, flagella and a type 3 secretion system (T3SS) accumulate and form biofilms in the CF

airways. Within the biofilm, bacteria downregulate flagella, pili and T3SS, whereas they upregulate alginate production, release CpG DNA and express or modify a variety of virulence factors that promote immune evasion [1]. *P. aeruginosa* bacilli release outer membrane vesicles containing Cif that inhibits the CFTR recycling [1]. LPS also shifts its structure by adding palmitate and aminoarabinose, resulting in increased antibiotic resistance, and promotes IL-8 production from host immune cells [1]. In CF airways, IL-10 secretion is relatively low [107], and lipoxin production is also diminished [108-110]. This leads to failed resolution of the acute inflammatory response by limiting transition of the inflammatory process from neutrophil predominance to monocytes [111,112]. This finally causes the hyper-inflammatory state characteristic for CF airways [2,5,28,86]. Thus, the main clinical problem leading to morbidity and mortality of CF patients is inflammation driven by chronic bacterial infections [2,5,7-10].

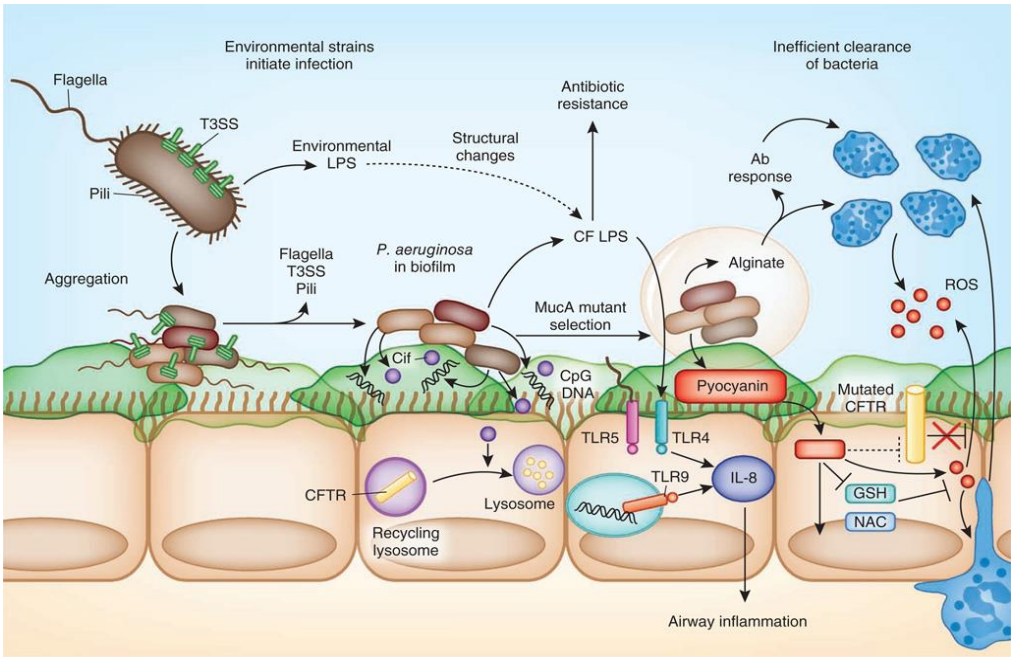

Figure S.1 CF airway inflammation. Reprinted from reference 1 with permission

Pseudomonas aeruginosa

Pseudomonas aeruginosa is the most common opportunistic infectious pathogen in CF [7-10], Ecthyma Gangrenosum [98] and Chronic Obstructive Pulmonary Disease (COPD) [99]. *P. aeruginosa* exists in two strikingly different phenotypes [11,75]. Motile bacteria (planktonic forms) are present when nutrients are available in sufficient amounts. In this form, they display an exponential growth pattern. Free swimming *P. aeruginosa* bacilli have a motile flagella and pili [71,72]. These are important for motility and immunogenicity in the host [71,72]. The flagellum-deficient mutant, Δ*fliC*, displays a decreased level of virulence and adherence of the bacilli to human airway epithelial cell lines including 1HAEo-cells [71]. The mutant strain PA103, which expresses no pilin, does not activate the inflammasome in infected macrophages [72]. These strains emphasize the pivotal role of the flagellum and pili in *Pseudomonas* pathogenesis. *P. aeruginosa* has five major types of secretion systems: Type I, II, III, V and VI [73,74]. Among them, the Type III secretion system (TTSS) mainly releases virulence factors such as Exoproteins (ExoS, T, U and Y) [73,74]. ExoS and ExoT have a GTPase activating protein (GAP) domain that contribute to disrupt the actin cytoskeleton. ExoY has an adenylate cyclase activity that increase intracellular cyclic AMP to indirectly disrupt actin cytoskeleton [73]. ExoU is known as a critical virulence factor involved in rapidly lysing host cells during early *P. aeruginosa*-induced pneumonia [74].

Biofilms represent the other characteristic form of *P. aeruginosa* cultures [75]. Biofilms have the advantage of protecting the bacterial population from attacks by protozoa, antibiotics and the host immune defense [11]. These specific characteristics of biofilms lead to chronic infection and represent the main problem in CF patients [7-9]. It

is known that only about 1% of genes significantly participate in these two types of growth patterns [76]. About half of the genes are downregulated, whereas the remainder are upregulated during biofilm formation [76]. A process called quorum sensing is responsible for initiating *P. aeruginosa* bacterial growth prior to biofilm formation [11]. In this process, the acyl-homoserine lactone (HSL) is utilized as a chemical communication mechanism [11]. Bacteria first produce the exopolysaccharide alginates: Psl and Pel [12]. The initial trail guide Psl encourages continuous attachment of bacteria to form a microcolony at the initial attached site, like molecular glue [12]. ΔPsl bacilli have shown ineffective adherence and mobility [12]. Once the population grows enough to select the *mucA* (anti-sigma factor) mutant, they continuously select that mutant to induce the release of sigma factor AlgU [13]. The AlgU protein induces activation of the alginate biosynthesis operon through AlgU-dependent AlgR expression [13]. This mechanism inhibits the production of Vfr (acute virulence factors) but promotes alginate production causing the biofilm to extend through a positive feedback mechanism [13]. Simultaneously, bacteria in the biofilm lose their pili, flagella and T3SSs but release CpG DNA and Cif (a protein to prevent recycling of CFTR in the host) [1]. The bacteria modify their LPS to result in antibiotic resistance in CF [1]. The majority of *P. aeruginosa* bacilli found in sputa of CF patients are mutated to *mucA* (29/35 mucoid strains, 82% mutation) [61]. Interestingly, the *mucA* mutation is also induced at high frequency upon exposure to estradiol leading to a deletion of the nucleotide adenine at position 60 of *mucA* (E20D), which results in a premature stop codon of the *mucA* gene that induces transitioning from the planktonic phenotype to the mucoid phenotype [62].

Current research present that dendritic cells overexpressing CD40L were able to promote humoral IgG production in response to infection by *P. aeruginosa* clinical isolate PA514 in immunized mice. These immunized mice could survive by cross-protection against lethal dose challenges of both the PA514 clinical strain and laboratory strain PAO1, but not against an *E. coli* control [100]. This result suggests that clinical isolates and laboratory strains of *P. aeruginosa* share overall similarities with each other but not with other bacterial genera. However, based on the genetic adaptability of *P. aeruginosa* such as the *mucA* deletion and AlgU activation to become mucoidic, clinical isolates of *P. aeruginosa* showed better resistance against bactericidal activity of antibiotics by formation of biofilm than planktonic bacteria in the logarithmic growth phase [101]. Clinical isolates of *P. aeruginosa* can evade bactericidal activity better than exponential planktonic bacteria by not only biofilm formation [11,101] but also by extended β-lactamase production [102]. Clinical isolates are generally well-adapted to the environment such as in CF and COPD. Accordingly, clinical isolates readily reach stationary phase and form biofilms from the planktonic stage [7-9]. In contrast, the PA14 laboratory strain is more virulent than clinical isolates because of the mutation in the *ladS* gene that is an important cascade gene promoting biofilm formation but repressing the T3SS [103]. For these reasons, we analyzed both clinical and laboratory strains for their ability to generate NET formation in human neutrophils.

Neutrophil activation & Neutrophil Extracellular DNA Traps (NETs)

Neutrophils typically engulf and kill pathogens by fusing their antimicrobial granules with phagosomes. [21,65] However, in 2004, a new mechanism of neutrophil response to invading pathogens was found: NET formation [14]. During NET formation,

neutrophils release their antimicrobial granule proteins and histones linked to extracellular DNA [14,15,17,25]. NET formation is generally known to be beneficial for the host immune response against invading pathogens [14-18]. Whether the antimicrobial mechanism of NETs manifests in direct killing of the pathogen or only exposes them to other toxic agents by entrapping them is still under debate. Several studies have shown that NETs directly kill HIV-1, *Pseudomonas* and *Leishmania amazonensis* [15-17]. In contrast, NETs were suggested to promote biofilm growth of nontypeable *Haemophilus influenzae* and to activate macrophages in response to *Mycobacterium tuberculosis*, respectively [18,19]. NET formation has both positive and negative consequences. Although the main function of NETs is to entrap microbes, uncontrolled NET release has been shown to contribute to disease pathologies [63,64,66]. NETs and their components enhance macrophage activation through NLRP3 inflammasome activation in the presence of LL-37 in lupus [20]. MPO and proteinase 3 (PR3) are the main targets of antineutrophil cytoplasmic antibodies (ANCA) in Wegener's granulomatosis [21], and bactericidal/permeability-increasing protein (BPI-ANCA) is elevated in CF [22]. Furthermore, neutrophilia and increased IL-8 cytokine levels were reported as the main pathogenic phenomena in CF patients [23]. Neutrophils residing in CF sputum samples showed increased expression of conventional neutrophil activation markers, such as CD16, CD14 and CD63, as well as unconventional neutrophil activation markers, such as CD80, MHCII and Th2-associated receptor CD294, relative to blood neutrophils. This finding indicates that neutrophils homing to the airways of CF patients cooperatively function with T cells during chronic inflammation [24]. Additionally, MPO and HNE (main markers of CF airway pathophysiology) have also been linked to NET formation. MPO and HNE

bound to NETs were found in CF sputum samples [63,64]. HNE enhanced-sputum solubilization by histone proteolysis was found in NET formation [64]. Figure S.2 explains the mechanism of NET formation. In brief, neutrophil activation by different stimuli such as LPS, IL-8, PMA, complement receptors or growth factors results in activation of the NADPH oxidase and production of reactive oxygen species (ROS). As a result, the activated cells form NETs through chromatin decondensation followed by PAD4-mediated citrullination of histones [106]. NETs contain granule components, modified histones and antimicrobial peptides [106]. A more detailed description of each component is provided in the following sections.

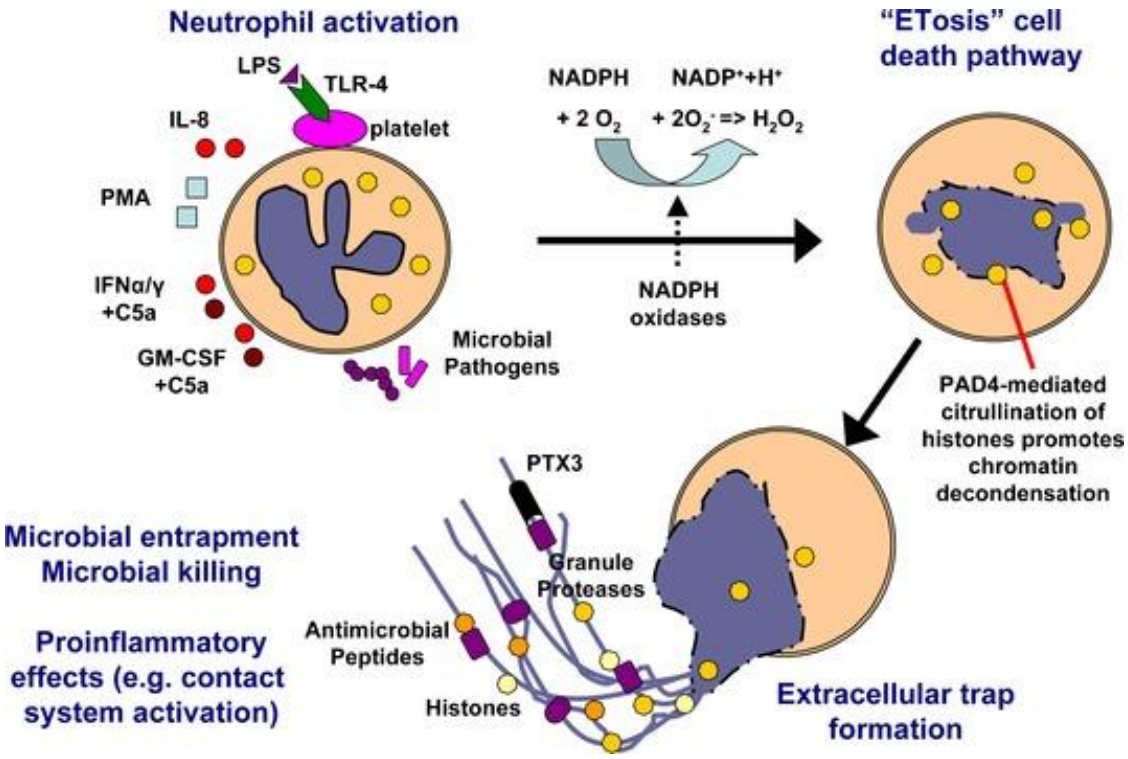

Figure S.2 The mechanism of NET formation. This figure was reprinted from reference 106 under open access terms and conditions as Creative Commons Attribution Noncommercial License.

NADPH oxidase, Chronic Granulomatous Disease (CGD) and NETs

NADPH oxidase is the professional multicomponent phagocyte oxidase (Phox) complex comprising several catalytic subunits: $gp91^{phox}$, $p47^{phox}$ $p67^{phox}$ and $p40^{phox}$ [77]. NADPH oxidase-independent ROS production is typically no more than a few percent of total ROS produced in an activated neutrophil [77]. This crucial superoxide producer is mutated in CGD patients [68,78,79]. The most common genetic subgroup, approximately 70% of the CGD patients, is X-linked CGD caused by a mutation of $gp91^{phox}$ [79]. Because of NADPH oxidase dysfunction, neutrophils from CGD patients have limited superoxide production and formation of NETs in response to bacteria and bacterial virulence factors, such as pyocyanin produced by *P. aeruginosa* [68]. However, CGD neutrophils can release NETs following treatment with hydrogen peroxide and hypochlorous acid, the product of myeloperoxidase [49].

MPO and NETs

MPO is the main peroxidase of neutrophils located in the azurophilic granules [21,65]. MPO has the ability to convert H_2O_2 to hypochlorous acid (HOCl) by using one H_2O_2 and one Cl^- ion to produce one H_2O [49,80]. The potent oxidizer HOCl can be further converted to chloramines, a hydroxyl radical or singlet oxygen [80]. MPO is typically released into the phagosome to kill phagocytosed pathogens [67]. In addition to this canonical degranulation process, MPO has been linked to NET formation during antimicrobial activity of neutrophils [25,26]. NET-linked MPO protein is active and mediates bacterial killing in the presence of hydrogen peroxide [25]. Another report revealed MPO is required for NET formation [26]. Patients completely deficient in MPO

were unable to make NETs, and this defect was not rescued by exogenous MPO [26]. This indicates that MPO and the cells act autonomously to promote formation of NETs [26]. In contrast to its antimicrobial activity, MPO has also been linked to certain types of diseases such as COPD, CF and atrial fibrosis [27-31]. Of particular interest, increased levels of active MPO directly correlates with airway obstruction in both COPD and CF patients [27,28]. As we found previously, pyocyanin-triggered extracellular DNA also contains MPO [68].

HNE and NETs

HNE is another main protein of the azurophilic granules of neutrophils. [21,65] HNE is a potent serine proteinase that has catalytic triad (His-Asp-Ser) [69]. It is typically known for its antimicrobial activity by cleaving bacterial proteins and virulence factors in phagosomes [32]. It is also known that NET formation is mediated by HNE activity and action in the nucleus [33]. Upon activation of neutrophils, HNE translocates from the granule to the nucleus [33]. The active HNE cleaves histones, promoting chromatin decondensation [33]. After this process, MPO arrives and binds to the chromatin backbone to further enhance chromatin decondensation, increases cell permeability and induces release of the NET [33]. On the contrary, HNE activation that is disadvantageous for the host has been also reported. In COPD and CF patients, increased HNE levels were shown in sputum samples and were inversely correlated with lung function measured as % value of Forced Expiratory Volume in 1 second (FEV_1) defined as the volume of air maximally forced out in one second after forced exhalation [27,34-36]. This indicates that HNE activity and release are tightly linked with the pathophysiological progression of those diseases.

Citrullination of Histones and NETs

Histones are alkaline proteins that tightly pack DNA [104,105]. A 147 bp DNA segment is packed by a core histone octamer (two of each H3, H2B, H2A and H4) to form a compact particle called the nucleosome [37]. Histones are very important not only for the orderly compaction of DNA but also for contribution to cell signals [38]. Histones can be modified by acetylation, methylation or even by phosphorylation [37,38]. Another form of histone modification, citrullination of arginine residues (Arg) to citrulline (Cit) by peptidylarginine deiminases (PADs) is known as the main mechanism for chromatin decondensation and formation of NETs [37,39] in a calcium dependent manner [40]. Once neutrophils are activated by calcium ionophores, they increase their citrullinated histones (H4cit3) and release long, stretched decondensed chromatin fibers [37]. In PAD4-deficient mice, neutrophils lack histone deimination and do not release NETs [39]. Histone citrullination has also been linked to autoimmunity in a variety of diseases such as rheumatoid arthritis (RA). In RA, anti-citrullinated protein/peptide antibodies (ACPA) are very specifically and sensitively diagnosed, but do not exclude other autoimmune diseases including systemic lupus erythematosus (SLE), Sjögren's syndrome and psoriatic arthritis [41]. In CF, citrullinated histone H3 is found in CF sputum samples [63]. Another report from our lab also demonstrated that only activated neutrophils formed NETs that contain citrullinated histone H4 [43].

Neutrophils in CF

In CF, impaired bacterial clearance leads to chronic airway inflammation and continuous recruitment of immune cells [10,113-116]. During chronic inflammation, the first recruited cells, neutrophils, are activated and retained in the site of inflammation for

unusually long time compared to acute respiratory distress syndrome (ARDS) or common respiratory pneumonia [113,115]. Paradoxically, the large number of neutrophils present in CF airways is not capable of clearing colonizing bacteria [10, 113]. Instead, FEV_1 % values inversely correlate with an increase of total neutrophils [34]. As a consequence of this neutrophilia, CF neutrophils release significantly greater amounts of HNE compared with neutrophils from control subjects and bronchiectasis patients [113]. HNE is known to degrade a series of cell surface receptors: CD2, CD4 and CD8 of lymphocytes, phagocyte receptors of neutrophils such as complement receptor 1 and antigen presenting receptors of dendritic cells in CF patients [113,117,118]. In addition, HNE degrades CFTR and epithelial sodium channel (ENaC), thereby increasing mucus production [113]. The increased level of mucus in response to neutrophilia in CF suggests that the huge influx of neutrophils may have adverse effects against resolving infection and aggravate CF airways. Therefore, we investigated which mechanism of neutrophil dysfunction affects CF airway disease. As mentioned above, NETs have multitasking ability: they not only clear microbes but also influence inflammatory responses [15,18,21,43,113,119,122,123]. NET formation is a very dynamic process as shown by live imaging [120,121]. Based on these sporadic observations, we focused on studying NET formation in CF airways and to correlate it with lung function of CF patients. As a result, we found that laboratory strains and clinical isolates of *P. aeruginosa* isolated from CF patients trigger NET formation that can be in excess in CF airways, contributing to severe CF lung function decline.

Specific Aims

The objective of this study is to investigate whether NETs and their components are involved in CF pathophysiology. The central hypothesis of this study is that *formation*

of NETs is the main mechanism for release of neutrophil-derived CF airway inflammatory mediators in the presence of Pseudomonas aeruginosa, and that NET formation impairs CF airway function. The rationale for this study is that once we analyze the clinical importance and the mechanism of NET formation associated with CF, it will provide crucial information to understand the mechanism of CF airway inflammation and provide an opportunity to control CF disease progression. To test our central hypothesis, we have focused our efforts on these specific aims:

Specific Aim 1: Identify the cellular mechanism of *Pseudomonas aeruginosa*-triggered NET formation in human neutrophils.

For this aim, we isolated human neutrophils from donated blood of healthy volunteers. *P. aeruginosa* laboratory strain PA14 was used as the bacterial stimulus. We characterized a broad range of inhibitors to study their effects on *P. aeruginosa*-initiated NETs *in vitro*. We assessed the roles of important proteins in phagocytosis, production of reactive oxygen species, and histone modifications in the process. Our working hypothesis is that *P. aeruginosa* induces NET formation through bacterial engulfment, MAPK signaling pathways, superoxide production and citrullination of histones.

Specific Aim 2: Detect NET markers such as MPO-DNA and HNE-DNA complexes, triggered by CF clinical isolates of *Pseudomonas aeruginosa*.

We hypothesized that CF clinical isolates of *P. areruginosa*-stimulated NETs can be measured by MPO-DNA and HNE-DNA complexes in neutrophil supernatants. By developing the method to detect NET-associated protein-DNA complexes, we can quantify the amount of NETs in clinical samples. This will be a good diagnostic tool to quantify and

analyze how NET formation relates to CF disease severity. Thus, we have developed new assays to detect both MPO-DNA and HNE-DNA complexes, and firstly applied them in CF clinical isolates.

Specific Aim 3: Determine the correlation between NET markers and clinical parameters in CF sputum samples.

We hypothesized that a direct correlation exists between levels of NET markers in CF sputum samples and CF clinical measures, supporting *P. aeruginosa*-initiated NET formation as the main mechanism of neutrophil activation in CF airways. For this study, we measured levels of NET/neutrophil markers (extracellular DNA, HNE, MPO, histones, MPO-DNA, HNE-DNA complexes and active PAD4) in sputum samples of CF patients. FEV_1% was correlated with NET markers to investigate whether NET markers are related to CF lung function decline.

CHAPTER 2

RELEASE OF CYSTIC FIBROSIS AIRWAY INFLAMMATORY MARKERS FROM *PSEUDOMONAS AERUGINOSA*-STIMULATED HUMAN NEUTROPHILS REQUIRES THE NADPH OXIDASE, THE CYTOSKELETON AND PARTIAL CONTRIBUTION OF THE MEK1/2-ERK1/2 SIGNALING PATHWAY[1]

[1]Dae-goon Yoo, Matthew Winn, Lan Pang, Samuel M. Moskowitz, Harry L. Malech, Thomas L. Leto and Balázs Rada. 2014. *The Journal of Immunology*. 192:4728-4738.

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Abstract

We first aimed to understand the mechanism of NET formation stimulated by the opportunistic bacterial pathogen *P. aeruginosa.* Our data show that *P. aeruginosa* PA14 stimulates robust release of extracellular DNA from human neutrophils. H4cit3 and primary granule components MPO and HNE co-localized with extracellular DNA in *P. aeruginosa*-stimulated NETs. Using inhibitors of NADPH oxidase (DPI), MEK1/2 (U0126, MEK162 and PD98059) and actin polymerization (Cytochalasin-D, cytoD), we demonstrated that cytoskeletal movement and NADPH oxidase-induced superoxide generation are critically important to NET formation and that the MEK1/2-ERK1/2 pathway partially contributes to this mechanism.

Results

1-1 Human neutrophils release extracellular DNA in response to *Pseudomonas aeruginosa*.

First, we investigated whether neutrophils can release DNA in response to laboratory *P. aeruginosa* strain PA14 (Figure 1-1 A and B). PA14-induced DNA release occurs at a time point similar to that of induction by the positive control, phorbol myristate acetate (PMA) (Figure 1-1 A). PA14 alone failed to induce detectable extracellular DNA release. DNA release from adherent human neutrophils is induced in a dose-dependent manner by the *P. aeruginosa* strain PA14 (Figure 1-1 B). The average released DNA from 10^4 neutrophils was 3.01 ng/ml upon PA14 exposure (Figure 1-1 C). We next sought to estimate the proportion of live bacteria attached to NETs after 4 hr incubation. Supernatants of *P. aeruginosa*-activated neutrophils carefully removed after incubation, did not contain NETs and were henceforth referred to as ‘NET-free’ (Figure 1-1 D). An

equal volume of new assay medium containing DNAseI was added back; this treated supernatant is henceforth referred to as 'NET-linked'. As seen in Figure 1-1 D, we found that 41.3±3.4% of live bacteria were associated with NETs following neutrophil stimulation with 10 MOI (Multiplicity of Infection) PA14, and 29.7±13.6% following stimulation with 50 MOI. This suggests that NETs are able to ensnare large amounts of *P. aeruginosa* bacilli *in vitro*.

1-2. MPO, HNE and citrullinated histone H4 co-localize with extracellular DNA in NETs.

Next, we aimed to confirm that PA14 stimulates NET formation by human neutrophils. We first addressed whether the released DNA is linked to NET formation or associated with neutrophil necrotic cell death. We did this via immunostaining and ELISA, the latter of which allowed us to quantify our results. As shown in Figure 1-2, resting neutrophils did not release detectable amounts of extracellular DNA, MPO, HNE or H4cit3. Following stimulation with PA14, neutrophils released detectable extracellular DNA consisting of MPO, HNE and H4cit3 (Figure 1-2). PMA was used as a positive control. The precise signaling cascade of *P. aeruginosa*-induced NET release is not well-defined. However, several groups found that citrullination of histones, especially H3 and H4, and MPO and HNE release are associated with the process of NET formation [25,26,33,37,39,43,68,82]. Thus, we also investigated whether PA14 stimulates neutrophils to release NETs in complex with these aforementioned components. As seen in Figure 1-2, MPO, HNE and citrullinated histones co-localized with extracellular DNA, confirming the release of NETs following stimulation with PA14. In Figure 1-2 B (inset), we observed that resting neutrophils stored HNE in granules, while neutrophils activated

to release NETs displayed HNE associated with NETs. These results confirm our hypothesis that *P. aeruginosa* triggers NET formation by activated neutrophils.

1-3. Neutrophils stimulated by *Pseudomonas aeruginosa* secrete active MPO and HNE.

To verify the release of major neutrophil granule proteins MPO and HNE in response to *P. aeruginosa* challenge, we measured MPO and HNE by ELISA. As shown in Figure 1-3 A, MPO was detected at 176.0±14.5 ng/ml in supernatants of PA14-stimulated neutrophils. The basal level of MPO release by resting neutrophils was at 29.0±1.7 ng/ml. Peroxidase activity was determined via the Amplex red oxidation assay. The basal level of oxidation occurring within resting neutrophils was 144.7±29.9 μmole/L, while PA14-stimulated human neutrophils displayed an increased oxidation level of 388.4±59.3 μmole/L (Figure 1-3 B). HNE release was also significantly increased by approximately 2.7-fold in response to PA14-stimulated neutrophils, as compared to a basal level (447.2± 40.4 ng/ml compared to 165.1±19.8ng/ml, respectively) (Figure 1-3 C).

1-4. *Pseudomonas aeruginosa* activates the neutrophil respiratory burst.

Activation of NADPH oxidase is known to be critically important for NET formation by neutrophils [42,44,68]. However, the requirement of NADPH oxidase for *P. aeruginosa*-stimulated NET formation by human neutrophils has not yet been studied. Thus, we examined whether PA14 can induce superoxide production in activated neutrophils. Adherent human neutrophils were activated by 10 MOI PA14 (Figure 1-4 A) in the presence or absence of the flavoenzyme inhibitor, diphenyleneiodonium (DPI). As seen in Figure 1-4 A, PA14 induced superoxide production in human neutrophils was inhibited by the NADPH oxidase inhibitor DPI. Dose-dependent stimulation by PA14 is

reported in Figure 1-4 B. To compare whether this superoxide production affects NET formation and release, we employed the Sytox orange-based assay to assess extracellular DNA release. As seen in Figure 1-4 C, extracellular DNA release was significantly reduced in the DPI-treated group. These results support the idea that activity of the NADPH oxidase is required for release of NETs [89]. MPO, HNE and peroxidase activities were confirmed by ELISA and Amplex red oxidation (Figure 1-4 D-F). DPI pretreatment greatly diminished PA14-induced MPO release. MPO release was 146.8±14.8 ng/ml in the PA14-stimulated group but decreased by 77.5% (33.2±7.0ng/ml) in the DPI-treated group (Figure 1-4 D). Peroxidase activity was also decreased by 63.6% in the DPI-treated group (243.6±34.7 to 88.6±21.6 μmole/L) (Figure 1-4 E). The PA14-induced secretion of total HNE (282.1±29.4 ng/ml) was also suppressed by 61.5% with DPI pretreatment (108.7±23.2 ng/ml). Our results demonstrate that the NADPH oxidase is largely involved in *P. aeruginosa*-stimulated NET formation, and this process is primarily responsible for the release of MPO and HNE from *P. aeruginosa*-challenged neutrophils.

1-5. Cytoskeletal movement is required to induce the respiratory burst and NET formation by neutrophils in response to *Pseudomonas aeruginosa* challenge.

Neutrophil phagocytosis following bacterial challenge is a well-known process for eliminating bacteria [32,65]. However, the requirement of cytoskeleton movement for NET formation remains elusive. Thus, we stimulated neutrophils with PA14 in the presence of actin polymerization inhibitor, cytochalasin D (cytoD), and then measured superoxide production and NET release. Both production of superoxide and NET formation were greatly inhibited by cytoD treatment as 59.2±10.74% and 74.9±16.55%, respectively.

(Figure 1-5 A and B). This result demonstrates that the cytoskeleton play a crucial role to produce superoxide and NETs.

1-6. The role of MEK1/2-ERK1/2 signal transduction pathway in *Pseudomonas aeruginosa*-induced NET formation.

The involvement of MEK/ERK signaling pathway in the process of PMA-induced NET formation was previously described [42]. To test whether this signaling pathway is also involved in the process of NET formation by human neutrophils in response to *P. aeruginosa* stimulation, we used U0126 as a selective MEK inhibitor [42,45]. As seen in Figure 1-6 A, extracellular DNA release, superoxide production, MPO and HNE release and peroxidase activity were all significantly suppressed by this inhibitor. However, it was concerning that the U0126 treatment resulted in a complete block of peroxidase activity, whereas others demonstrated only a partial inhibition (67.0% decline in superoxide production, and a 42.6% suppression in MPO release, Figure 1-6 A). This raised the question of whether or not this inhibitor may have off-target effects. Thus, we measured peroxidase activity by using active MPO (500 μM) and the previously described Amplex red oxidation method in a cell-free *in vitro* system with U0126 and two other known MEK1/2 inhibitors, MEK162 and PD98059. As shown in Figure 1-6 B, cell-free MPO activity was surprisingly inhibited by the U0126 inhibitor only, while the other two inhibitors (MEK162 and PD98059) failed to demonstrate any inhibitory effect. Thus, we confirmed that U0126 has strong inhibitory effect on MPO activity. Through these experiments, we determined peroxidase activity and the release of DNA, MPO, and HNE. As seen in Figure 1-6 C, the release of DNA was minimally but significantly inhibited by MEK162 and PD98059 (33% and 38%, respectively). Both inhibitors failed to influence

the release or activity of MPO, but MEK162 had a notably significant inhibitory effect on HNE release. These results indicate that the MEK/ERK signaling cascade has only minor effects on contributing to DNA release and does not generally influence release of MPO and HNE.

CHAPTER 3

NET FORMATION STIMULATED BY CLINICAL ISOLATES OF *PSEUDOMONAS AERUGINOSA* DETECTED AS RELEASE OF MPO-DNA AND HNE-DNA COMPLEXES[2]

[2]Dae-goon Yoo, Madison Floyd, Matthew Winn, Samuel M. Moskowitz, Balázs Rada. 2014. *Immunology letters*. 160:186-194. Reprinted here with permission of the publisher.

Abstract

NET formation produced by activated neutrophils upon exposure to laboratory strain *P. aeruginosa* PA14 was confirmed in Chapter 2. In Chapter 3, we investigated whether clinical isolates of *P. aeruginosa* can also trigger NET formation by human neutrophils. Based on our results, citrullinated histone H4, MPO and HNE co-localized with released DNA. To quantitate NET release, we modified the previously employed MPO-DNA ELISA detection method and established a new method to detect HNE-DNA complexes [59]. Together, MPO-DNA and HNE-DNA ELISA assays provide much more pertinent evidence about NET complex formation by *P. aeruginosa*-stimulated human neutrophils.

Results

2-1. Clinical isolates of *Pseudomonas aeruginosa* trigger superoxide production by human neutrophils.

Ten clinical isolates obtained from Dr. Samuel Moskowitz in Massachusetts General Hospital were tested for induction of human neutrophil superoxide production with a superoxide-specific Diogenes chemiluminescence assay. Two laboratory strains of *P. aeruginosa*, PA14 and PAO1, were also assayed. Ten MOI was used for all strains. We incubated them with healthy human neutrophils for 2 hrs to detect superoxide production. As seen in Figure 2-1, all clinical isolates triggered neutrophils to produce slightly higher or similar superoxide levels, as compared to PA14, PAO1 or the chemical inducer PMA. As expected, DPI treatment dramatically reduced generation of superoxide by all experimental groups. Our results clearly demonstrate that healthy human neutrophils

respond to *P. aeruginosa* clinical isolates with NADPH oxidase-mediated superoxide generation.

2-2. Extracellular DNA released from human neutrophils stimulated by *Pseudomonas aeruginosa* clinical isolates co-localizes with MPO and HNE.

To verify whether superoxide production in response to *P. aeruginosa* clinical isolates is related to NET release, immunostaining was performed. Clinical isolates #035 and #155 were used as representatives of the 10 clinical isolates. As shown in Figure 2-2, clinical isolates #035 and #155 both clearly induced NET release by activated neutrophils. The main markers of NET formation, MPO, HNE and DNA, were stained and confirmed. These results were comparable to those obtained with the PA14 laboratory strain. Thus, our results show that clinical isolates induce NET formation by neutrophils.

2-3. Quantification of MPO-DNA and HNE-DNA complexes as NET markers.

We already investigated and confirmed that *P. aeruginosa* is a major trigger for NET formation by healthy human neutrophils. However, those data illustrated effects of separate parameters: citrullinated histone H4, MPO, HNE and DNA release. Here, we measured two new parameters: MPO-DNA and HNE-DNA complexes specifically indicative of NET formation. For this assay, we modified a previously described MPO-DNA ELISA method [59] and developed an HNE-DNA ELISA method. We used rabbit anti-human MPO or rabbit anti-human elastase capture antibodies, followed by sample application and horse radish peroxidase conjugated anti-dsDNA detection antibody, as seen in Figure 2-3. After reaction with TMB substrate solution and stopping solution (1 *N* HCl), we detected MPO-DNA and HNE-DNA complex levels as absorbance at optical density OD650 nm or OD450 nm. In our experiments, we typically incubated neutrophils

with PMA or *P. aeruginosa* bacilli for 3-4 hrs, allowing enough time to generate NETs. We then added DNaseI to cleave DNA later detected as NETs.

2-4. Optimization of MPO-DNA and HNE-DNA detection.

In our experience, collection of NETs was not sufficiently reliable. To optimize this process, we examined whether dose-dependent DNA digestion may generate a dose-dependence including a dosage at which DNA is small enough to be harvested but large enough to be caught by capture antibodies (Figure 2-3). Based on our 0.8% agarose gel electrophoresis, PMA-induced release of DNA was detectable within the 0.3-3.0 μg/ml treatment range of DNaseI. The DNaseI treatment demonstrated a dose-dependent increase of DNA yield and decrease of segment size. As seen in Figure 2-4 A and B, the optimal DNaseI concentration was determined to be 1 μg/ml. In Figure 2-4 C, we verified that PMA and PA14 treatments both induced a detectable OD value at the 1 μg/ml DNase concentration. The higher 30 μg/ml dose clearly decreases the detectable OD value. These results illustrate that digestion with 1 μg/ml concentration of DNaseI is optimum to generate detectable DNA complexes for this NET-specific ELISA, hereby referred to as a 'NET-ELISA'.

2-5. Human neutrophils challenged by 'early' phase clinical isolates of *Pseudomonas aeruginosa* showed significant increases in release of MPO-DNA and HNE-DNA complexes compared to corresponding 'late' phase clinical isolates.

After optimization of MPO-DNA and HNE-DNA detection methods, we evaluated MPO-DNA and HNE-DNA complexes released from neutrophils incubated with clinical isolates. As seen in Figure 2-5 A and B, MPO-DNA and HNE-DNA complexes were detectable, and these complexes proved to be directly correlated to one another (Figure 2-

5 C) (r= 0.96 with $p<0.001$). These results suggest that MPO-DNA and HNE-DNA complexes are physically linked. “Early” phase clinical isolates were collected from cystic fibrosis (CF) patients between three months to eleven years of age after onset of infection. “Late” phase clinical isolates were collected from those same CF patients between 5 to 20 years after the corresponding early isolates were collected. A total of 5 patients were examined in these assays. All clinical isolates were stored in Luria-Bertani broth with 16% glycerol at -80°C until used. Interestingly, “early” phase isolates demonstrated significant increases in detected amounts of MPO-DNA and HNE-DNA complexes compared to “late” phase isolates (Figure 2-5 D). These results indicate that the observed decrease in induction of NET release may be related to an adaptation process initiated by *P. aeruginosa* bacilli in CF airways. This conclusion is consistent with the previous finding that late CF isolates are killed less efficiently by NETs in suspension than are early CF isolates [16], which also suggests that reduced induction of NET formation is at least partially associated with resistance of CF clinical isolates in CF airways.

CHAPTER 4

CORRELATION STUDY OF NET MARKERS AND CLINICAL PARAMETERS IN CF SPUTUM SAMPLES[3]

[3] Dae-goon Yoo and Balázs Rada. To be submitted to *the Journal of Immunology*.

Abstract

In Chapters 2 and 3, we found that *P. aeruginosa* bacilli are able to induce NET formation through the NADPH oxidase and that the MEK1/2-ERK1/2 intracellular signaling pathway partially contributes to this mechanism. *P. aeruginosa* clinical isolates demonstrated a very strong direct correlation between NET markers (i.e., MPO, HNE and DNA). In Chapter 4, we investigated whether NET markers present in CF sputum samples are also linked with CF disease progression. Based on our hypothesis detailed in Aim 3, we measured DNA, MPO and HNE concentrations and corresponding activity levels of MPO and HNE. We assayed extracellular DNA, MPO, HNE, MPO-DNA, HNE-DNA, total histone H3 and PAD4 activity in CF patient sputum. As the results, clinical parameters were directly correlated with NET markers' expression and both were associated with decline of CF airways lung function.

Results

3-1. Association of extracellular DNA and FEV_1 value with acute pulmonary exacerbation (APE) stage.

CF patients generally suffer from chronic pulmonary worsening called acute pulmonary exacerbation (APE) [83]. Patients experiencing APE frequently exhibit an array of symptoms, including, but not limited to: recurrent cough, increased sputum production, chest pain, shortness of breath, loss of appetite, loss of weight and lung function decline [83]. Because more severe disease is typically accompanied by high amounts of extracellular DNA, we tested whether extracellular DNA and FEV_1 value correlate during the acute pulmonary exacerbation stage. Pulmonary exacerbations were categorized into one of three stages: pre-APE, APE or post-APE. Samples were obtained from 9 patients at

each of these three stages from the Emory+Children's Pediatric Research Center CF Discovery Core at the Emory University (Atlanta, GA) lead by Dr. Nael McCarty. APE stages were defined as the increase in symptoms such as cough, sputum production, dyspnea, fatigue, hemoptysis, fever, sinus symptoms as well as decrease in FEV_1 % greater than or equal to 10%, weight loss and/or change in physical examination of the chest. Pre-APE indicates the stage before onset the aforementioned symptoms and post-APE stage is later stage after treatment of antibiotics or hospitalization. First, we analyzed FEV_1 value in terms of the three stages and generated a box and whisker plot. As seen in Figure 3-1 A, pre- and post-APE stages exhibited a 75^{th} percentile with a broader range of FEV_1 values (Q3 to median, 17% and 19% respectively), as compared to the APE stage. In contrast, the APE stage demonstrated only a 2% difference between median and 75^{th} percentile. These results support the fact that samples in the APE stage exhibited worse lung function. Pre-APE stage patient sputa had lower DNA concentrations than the other two stages (Figure 3-1 B). Most APE stage patients' sputum ranged between 135 to 148 ng/µl, with only a 13 ng/µl difference among the 75^{th} percentile group (Figure 3-1 B). The pre-APE and post-APE stages had a 54.5 and 53.4 ng/µl difference, respectively, between the median and 75^{th} percentile (Figure 3-1 B). These results suggest that APE stage patients exhibit more consistent release of DNA. All three stages displayed an inverse correlation between extracellular DNA and FEV_1 value (Figure 3-1 C-F). This illustrates that high DNA concentration directly correlates with worsening CF pulmonary function.

3-2. MPO and HNE have been inversely correlated with FEV_1.

MPO and HNE are the main azurophilic granules that function in antimicrobial activity. However, abnormal expression and secretion of them often aggravates certain

disease, such as CF [28-30, 34-36]. Thus, we tested whether CF sputum exhibits a pattern or tendency based on stage difference (i.e., pre-APE, APE, and post-APE). We examined whether MPO and HNE concentrations differ amongst the three stages. As seen in Figure 3-2, MPO and HNE concentrations demonstrate no significant differences across the three exacerbation stages (Figure 3-2, A and B). Even though HNE activity revealed a considerable association with diseases progression, MPO and HNE activities show no significant differences amongst the groups (Figure 3-2 C and D). However, once analyzed in terms of FEV_1 value, MPO and HNE activities demonstrated a considerable association with disease progression (R= -0.32, n=27) and a significant inverse correlation (R= -0.38, n=27) with lung function, respectively (Figure 3-2 E and F). These results suggest that active MPO and HNE influences CF lung function.

3-3. MPO-DNA and HNE-DNA complexes were measured and correlated.

To investigate whether MPO-DNA and HNE-DNA complexes were also detectable in CF sputum samples, we employed the aforementioned NET-ELISA protocol. As seen in Figure 3-3 A and B, APE stage sputum samples exhibited slightly increased levels of MPO-DNA and HNE-DNA, as compared to samples categorized as either pre-APE or post-APE. Furthermore, HNE-DNA and MPO-DNA measurements generated a very tight direct correlation (R= 0.93) for APE stage sputum samples – notably tighter than the correlations observed for pre-APE (R= 0.75) and post-APE (R= 0.78) stage sputum samples (Figure 3-3 C-E). These results suggest a link between MPO-DNA and HNE-DNA complexes and efficient NET production, regardless of exacerbation stage classification (i.e., pre-APE, APE, or post-APE). This is supported by the fact that all three stages demonstrated no less

than a 0.7 Pearson correlation coefficient. Moreover, all three Pearson coefficients were found to be statistically significant.

3-4. MPO-DNA, and HNE-DNA complexes were inversely correlated with FEV_1, while PAD4 activity was directly correlated with histone H3 concentrations.

In Figure 3-3, we determined that MPO-DNA and HNE-DNA are significantly correlated. Next, we considered whether the increased level of each NET-associated marker correlates with CF lung function. To test this, we examined Pearson correlation statistics between MPO-DNA or HNE-DNA and FEV_1 value. As shown in Figure 3-4 A and B, MPO-DNA and HNE-DNA levels exhibit significant inverse correlation with FEV_1, respectively (MPO-DNA vs FEV_1, R= -0.43, n=22; and HNE-DNA vs FEV_1, R= -0.27, n=22). Based on these data, we hypothesized that expression of NET-associated markers inversely correlates with CF pulmonary function. To support this hypothesis, we further analyzed PAD4 activity level and histone H3 concentration in CF sputum samples. PAD4 is a peptidylarginine deiminase believed to act as the core enzyme in histone modification, conversion of arginine residues of histone to citrulline [37,39,96]. It has previously been shown that high active PAD4 is detected in severe disease state such as acute renal injury after ischemia/reperfusion [84] and neurotrauma [85]. Accordingly, active PAD4 has also been linked with disease progression [84,85,97]. Therefore, we investigated whether CF sputum samples exhibit different PAD4 activities depending on their histone levels. As seen in Figure 3-4 C, significant direct correlation between histone H3 expression and PAD4 activity was found (R= 0.43, n=23). Correspondingly increased levels of histone H3 concentration and PAD4 activity suggest that MPO-DNA and HNE-DNA were not simply

secreted from neutrophils undergoing necrotic cell death. Instead, it suggests these DNA complexes originated as a consequence of NET formation by neutrophils in CF.

3-5. CF sputum alone promotes induction of neutrophil extracellular DNA traps.

Next, we investigated whether CF sputum cultured with human (non-CF) neutrophils alone is sufficient to induce NET formation. CF sputum samples were co-incubated with healthy human neutrophils for 3-4 hrs as release of extracellular DNA was measured via the previously described Sytox Orange assay. CF sputum samples, blind tested as a cohort of 18 total samples, induced a 1.5 to 2-fold enhanced release of extracellular DNA, as compared to that detected for the assay buffer treated control, from healthy human neutrophils (Figure 3-5 A). These data indicate that CF patient sputum alone is able to induce release of extracellular DNA from human neutrophils. Corresponding kinetics data for this assay showed that release of DNA differed, from assay buffer treated control, from 90 min onward following neutrophil activation (Figure 3-5 B). This finding suggests that an environmental buildup of CF sputum successfully triggers NET formation and may influence CF pulmonary dysfunction.

CHAPTER 5

FIGURES AND LEGENDS

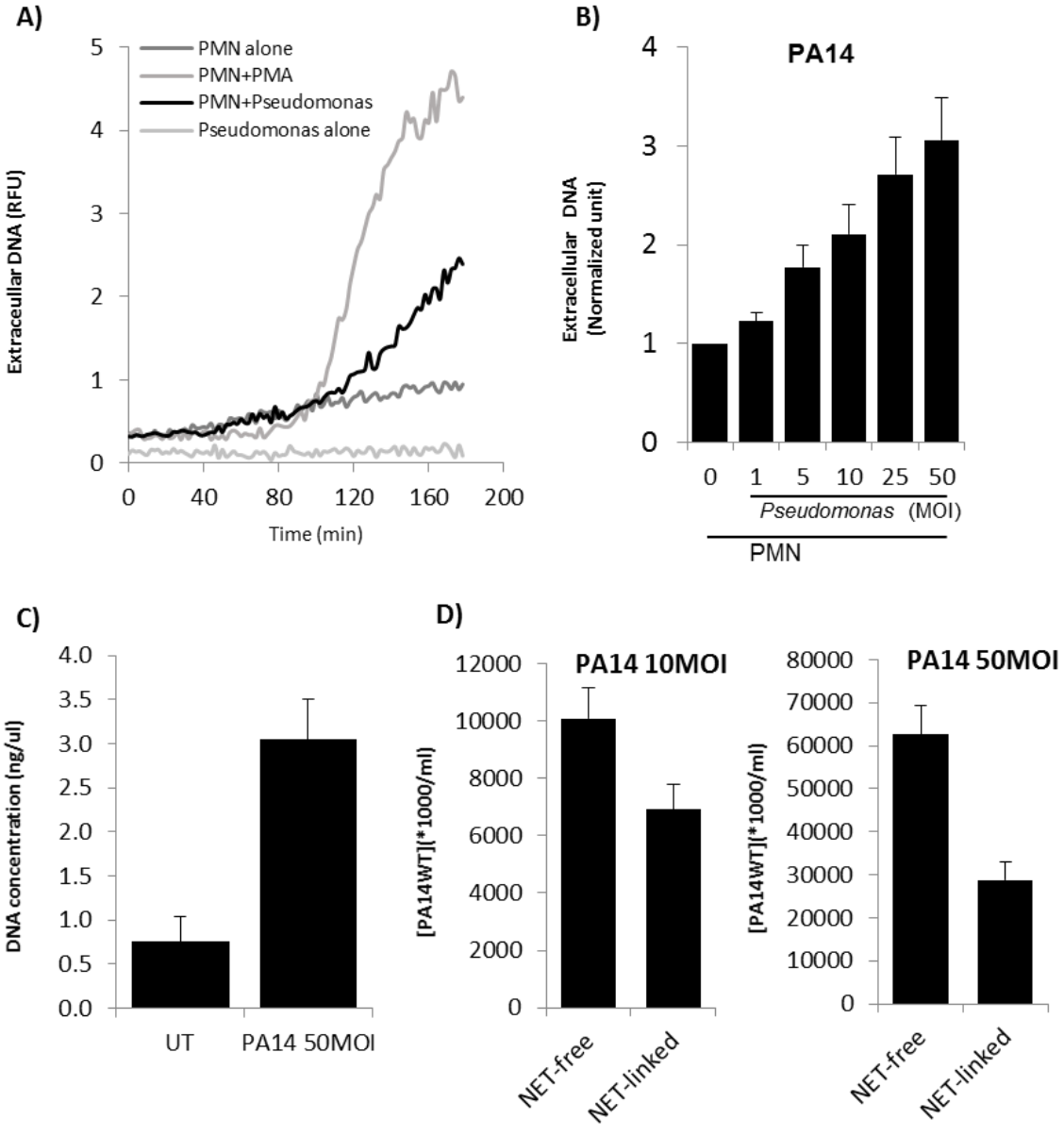

Figure 1-1. *P. aeruginosa*-induced extracellular DNA release from human neutrophils.

Extracellular DNA release from neutrophils in response to bacterial stimulation was detected via a Sytox orange based-microplate assay. (A) Time kinetics illustrated a response stimulated by *P. aeruginosa* PA14 50 MOI beginning around 90 min. (B) PA14-induced extracellular DNA release was measured and analyzed. A pattern of increasing bacterial MOI-dependent stimulation was seen (Mean±S.E.M, *n*=5). (C) To verify that neutrophils release their DNA extracellularly, DNA concentration after neutrophil incubation with PA14 50 MOI was quantitated via Sytox orange assay. Calf thymus DNA was used as the standard for calibration (Mean±S.E.M, *n*=3). (D) NET-free and NET-linked bacteria were collected and cultured for 16 hrs. Bacterial association was compared between the two as described in the Materials and Methods section (Mean±S.E.M, *n*=5).

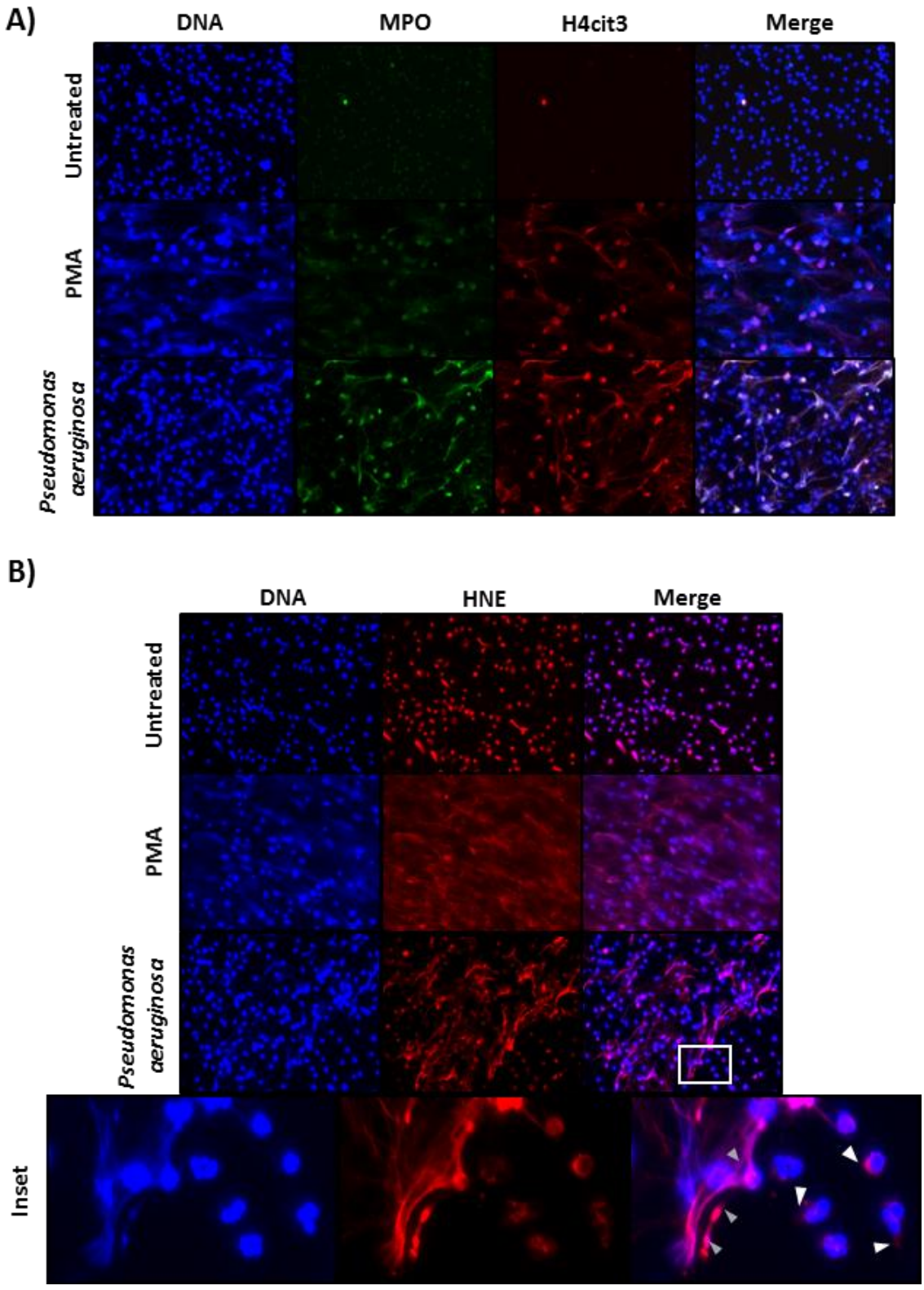
A)
DNA
MPO
H4cit3
Merge
Untreated
PMA
Pseudomonas aeruginosa
B)
DNA
HNE
Merge
Untreated
PMA
Pseudomonas aeruginosa
Inset

Figure 1-2.MPO, HNE and citrullinated histones co-localize with extracellular DNA in *P. aeruginosa*-triggered NETs.

PA14-triggered NET formation contains H4cit3, MPO, and HNE. (A) MPO (green) and H4cit3 (red) co-localize with extracellular DNA (blue) comprising NETs. Adherent human neutrophils were stimulated by opsonized *P. aeruginosa* PA14 (50:1 MOI), 100 nM PMA, or were left unstimulated (3 h). Neutrophils were fixed and stained with anti-MPO-FITC Ab or rabbit anti-H4cit3 primary and donkey anti-rabbit Alexa Fluor® 594 secondary Abs. DNA was counterstained by DAPI. One representative experiment, n = 5. Original magnification x400. (B) Localization of HNE in PA14- or PMA-stimulated human neutrophils was detected by immunofluorescence (rabbit anti-HNE primary Ab used, followed by anti-rabbit 594 secondary Ab). Both intracellular and extracellular DNA were stained with DAPI. One representative measurement, *n*=4. Original magnification ×400. (C) Inset of the HNE immunostaining from (B) illustrates the fine details of HNE localization in NETs after PA14 exposure (gray arrowheads). As expected, resting neutrophils fail to demonstrate any overlap of HNE with intact nuclear DNA (white arrowheads). Representative images, *n*=5. Original magnification ×2000.

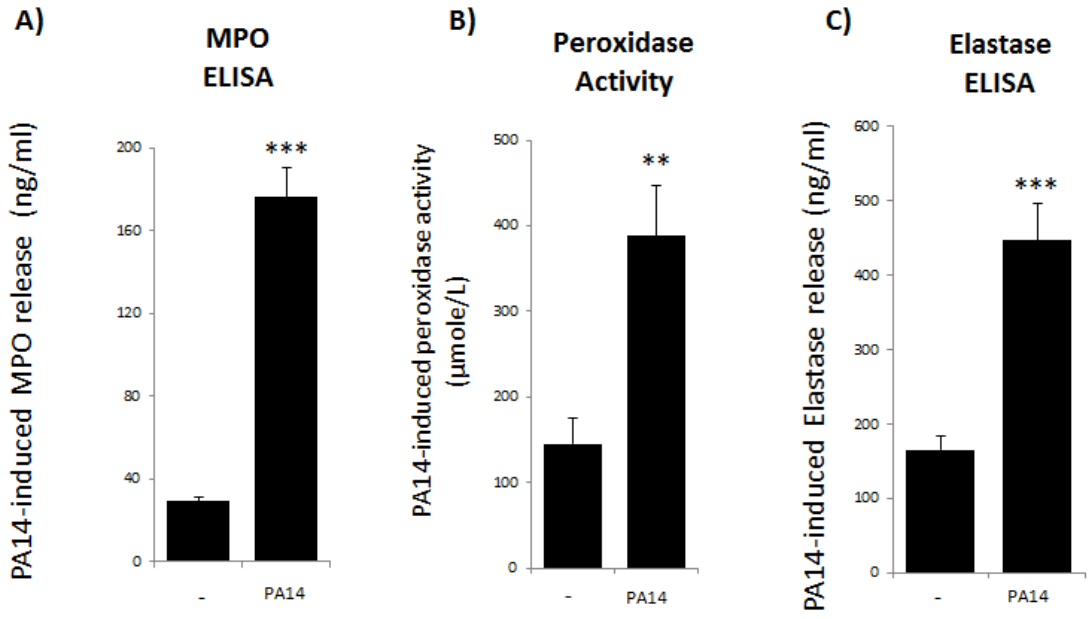

Figure 1-3. Neutrophils release active MPO and elastase upon *P. aeruginosa* challenge.

Adherent human neutrophils were exposed to *P. aeruginosa* PA14 (50 MOI) for 2.5 to 3 hrs. MPO and HNE concentration were determined by ELISA. Cell supernatants were diluted 1:100 (A and C). (B) *P. aeruginosa* PA14 increases extracellular peroxidase activity in neutrophils. Peroxidase activity was assayed by hydrogen peroxide-dependent Amplex Red oxidation in neutrophil supernatants. Mean±S.E.M, *n*=8. **P<0.01, ***P<0.001.

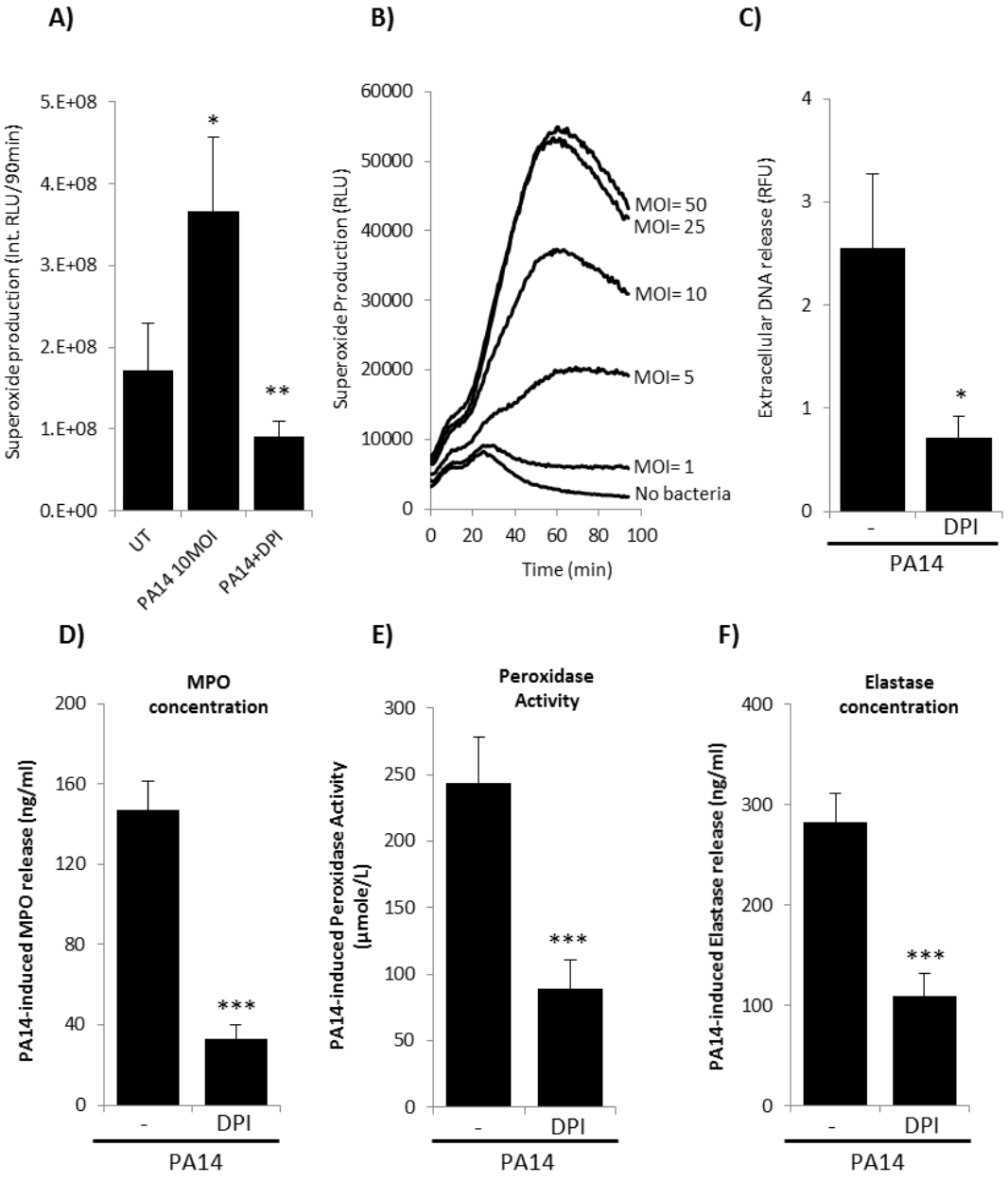

Figure 1-4. NADPH oxidase plays a crucial role in activating neutrophils for NETosis in response to stimulation by *P. aeruginosa*.

(A) Superoxide generation by neutrophils was measured by Diogenes superoxide measurement. NADPH oxidase of adherent neutrophils was selectively inhibited by DPI

(10 μM). Inhibitor was added 15 min prior to stimulation. PA14 (10 MOI) was added to the neutrophils, and this mixture was centrifuged at 500 X g for 2 min. Chemiluminescence was measured for 90 min. Mean±S.E.M, *n*=4. (B) Neutrophils were allowed to adhere for 15 min prior to being stimulated by *P. aeruginosa* PA14 in a dose dependent manner (1 to 50 MOI, *n*=4). After stimulation by PA14, neutrophils and bacteria were immediately centrifuged for 2 min at 500 X g to force close contact. Chemiluminescence was measured for 90 min. (C) Extracellular DNA release was measured by fluorescence generated by *P. aeruginosa* PA14 (10 MOI)-stimulated neutrophils pretreated with 10 μM DPI, as compared to PA14 (10 MOI)-stimulated neutrophils with no pretreatment. Sytox Orange (0.2 %) was used as a membrane impermeable DNA-binding dye to detect extracellular DNA release. Mean±SEM, *n*=5. (D and F) PA14-induced MPO and HNE release were measured by ELISA and quantified as ng/ml. Neutrophils were treated with or without DPI (10 μM) and immediately stimulated by PA14 (50 MOI). Stimulated neutrophils were incubated for 3 hrs at 37℃ prior to supernatant collection. Supernatants were diluted 1:100 for MPO ELISA and 1:30 for HNE ELISA, respectively. Mean±S.E.M, *n*=8. (E) PA14-induced peroxidase activity was determined by Amplex Red assay with undiluted supernatant samples. 100 μM Amplex red and 100 μM hydrogen peroxide mixed solution was used for analysis. Mean±S.E.M, *n*=8. *P<0.05, ***P<0.001.

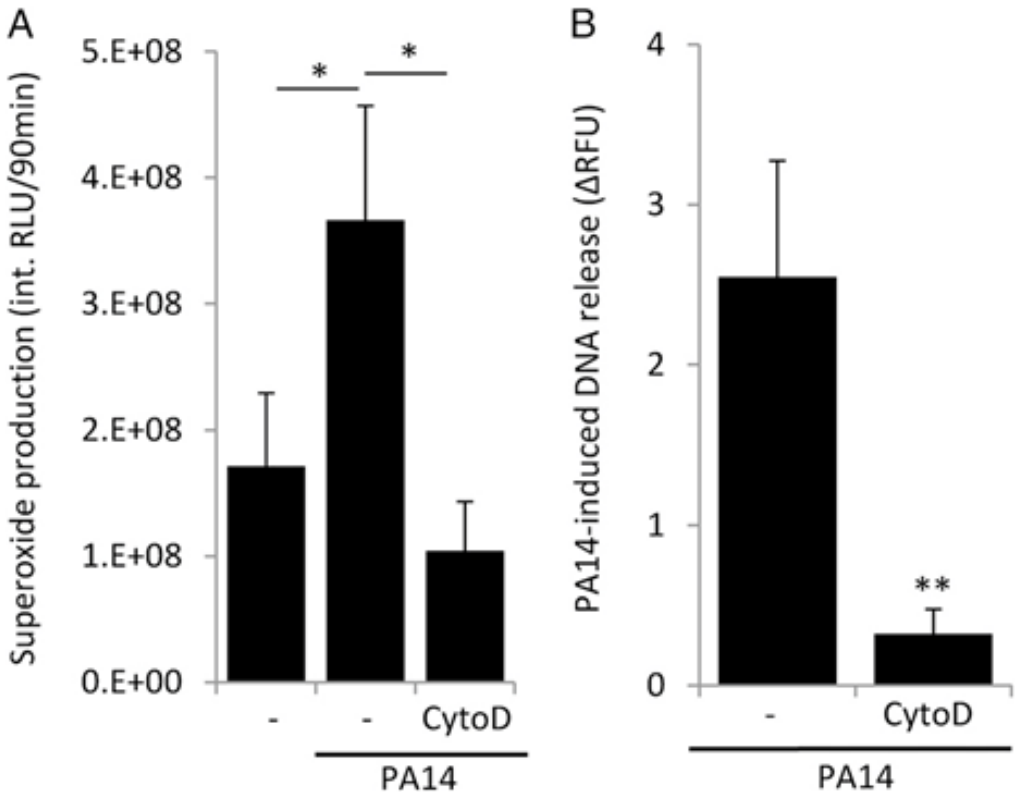

Figure 1-5. Cytoskeletal movement is required for NET release in response to *P. aeruginosa* stimulation.

Cytochalasin D, an actin polymerization inhibitor, suppressed *P. aeruginosa*-triggered respiratory burst and DNA release. Human neutrophils were stimulated with PA14 (10 MOI, 3 hr) in the presence or absence of 10 μM cytochalasin D. Superoxide production was measured with Diogenes superoxide-specific chemiluminescence kit (integrated relative luminescence units, int. RLU). Mean±S.E.M, n = 3. (B) Extracellular DNA release was measured by Sytox Orange fluorescence in neutrophils exposed to PA14 (10 MOI, 3hr) in the presence of 10 μM cytochalasin D. PA14-stimulated value (ΔRFU) is shown after subtraction of background activity. Mean± S.E.M, n=3. *p<0.05, **p<0.01.

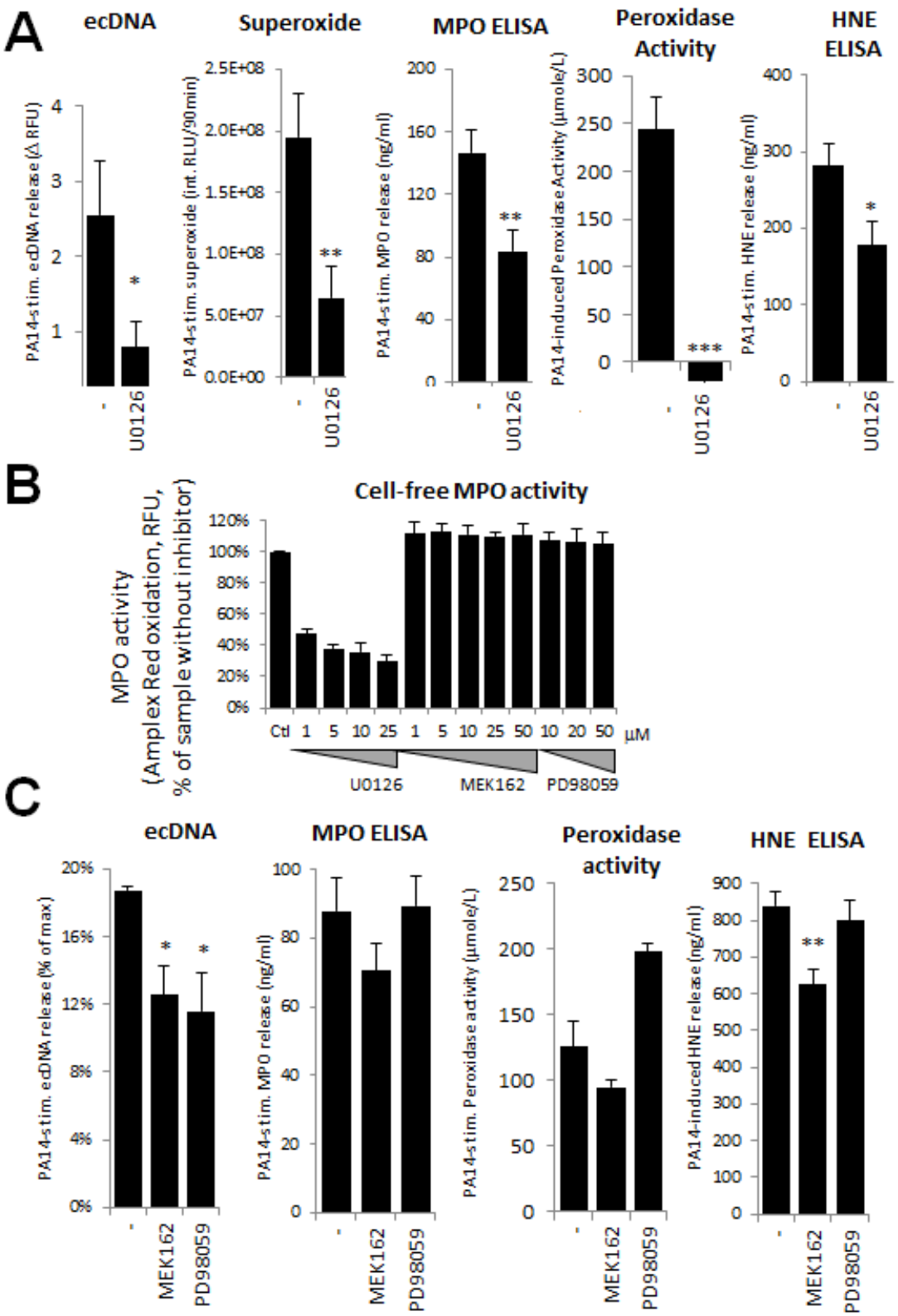
A
ecDNA
PA14-stim. ecDNA release (Δ RFU)
Superoxide
PA14-stim. superoxide (int. RLU/90min)
MPO ELISA
PA14-stim. MPO release (ng/ml)
Peroxidase Activity
PA14-induced Peroxidase Activity (µmole/L)
HNE ELISA
PA14-stim. HNE release (ng/ml)
-
U0126
B
Cell-free MPO activity
MPO activity
(Amplex Red oxidation, RFU, % of sample without inhibitor)
Ctl 1 5 10 25 1 5 10 25 50 10 20 50 µM
U0126
MEK162
PD98059
C
ecDNA
PA14-stim. ecDNA release (% of max)
MPO ELISA
PA14-stim. MPO release (ng/ml)
Peroxidase activity
PA14-stim. Peroxidase activity (µmole/L)
HNE ELISA
PA14-induced HNE release (ng/ml)
-
MEK162
PD98059

Figure 1-6. MEK1/2-Erk1/2 signaling partially contributes to NET release in response to *P. aeruginosa* stimulation.

(A) *P. aeruginosa* PA14-stimulated NET formation, superoxide generation, MPO release, HNE release and peroxidase activity were measured by Sytox Orange fluorescence, Diogenes, ELISA and Amplex red based peroxidase activity assay, respectively. U0126 (25 μM), a highly selective MEK1/2 kinase inhibitor, was used for inhibition of MEK1/2 activity. PA14 50 MOI was incubated with adherent human neutrophils for 3 hrs, followed by measurement of each aforementioned parameter. (B) Enzymatically active MPO (500 μM) was tested in a cell-free system via the Amplex red oxidation protocol. Three MEK kinase inhibitors, U0126, MEK162 and PD98059, were used at the indicated doses to test off-target effects. Experimental groups were normalized to the inhibitor-free group. Mean± S.E.M, n=2. (C) In the presence or absence of MEK162 (50 μM) or PD98059 (20 μM), neutrophils were co-incubated with PA14 50 MOI for 3 hrs. Extracellular DNA, MPO, HNE and peroxidase activity were tested. Mean± S.E.M, n=4. ANOVA, Tukey's post hoc analysis was done. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

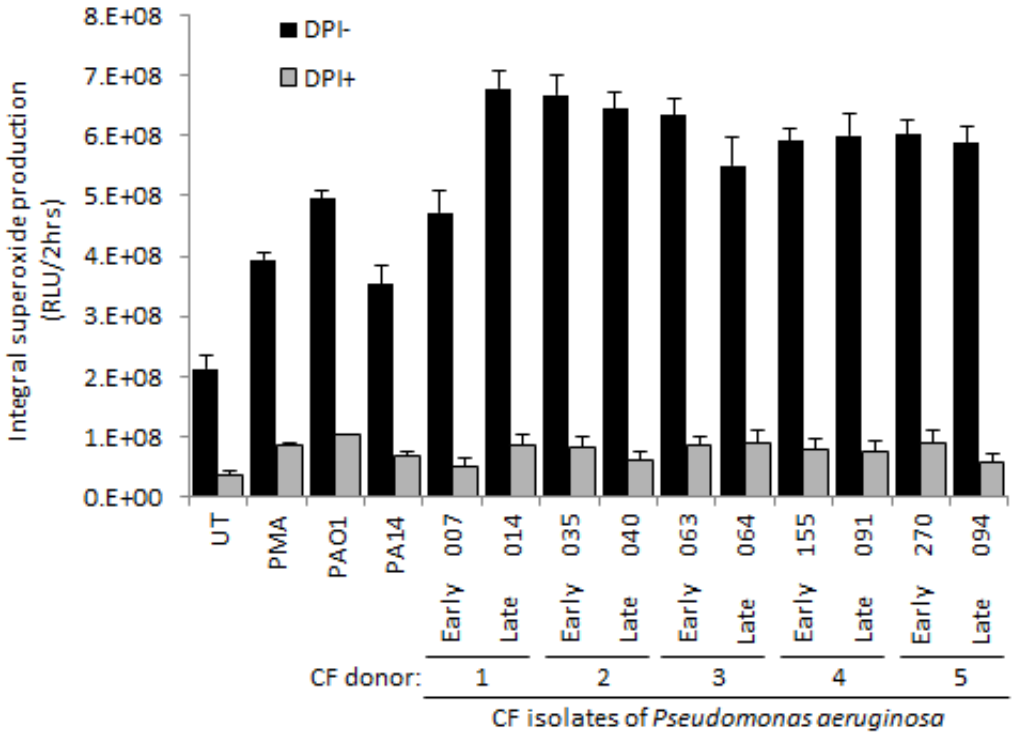

Figure 2-1. Detection of superoxide production by neutrophils stimulated by *P. aeruginosa* clinical isolates.

Adherent human neutrophils were exposed to CF clinical isolates of *P. aeruginosa* (10 MOI) for 2 hrs in the presence of the superoxide-specific Diogenes chemiluminescence detection reagent. Integrated luminescence values were detected for the duration of the measurement and reported as "RLU/2 hrs". Positive control cells were stimulated with 100 nM PMA, whereas negative control cells were left unstimulated (UT). The NADPH oxidase inhibitor diphenylene iodonium (DPI, 10 μM) blocked *P. aeruginosa*-triggered superoxide production. Neutrophils were incubated in HBSS containing 5 mM glucose, 10 mM HEPES and 1% autologous serum. Mean±S.E.M, *n*=3.

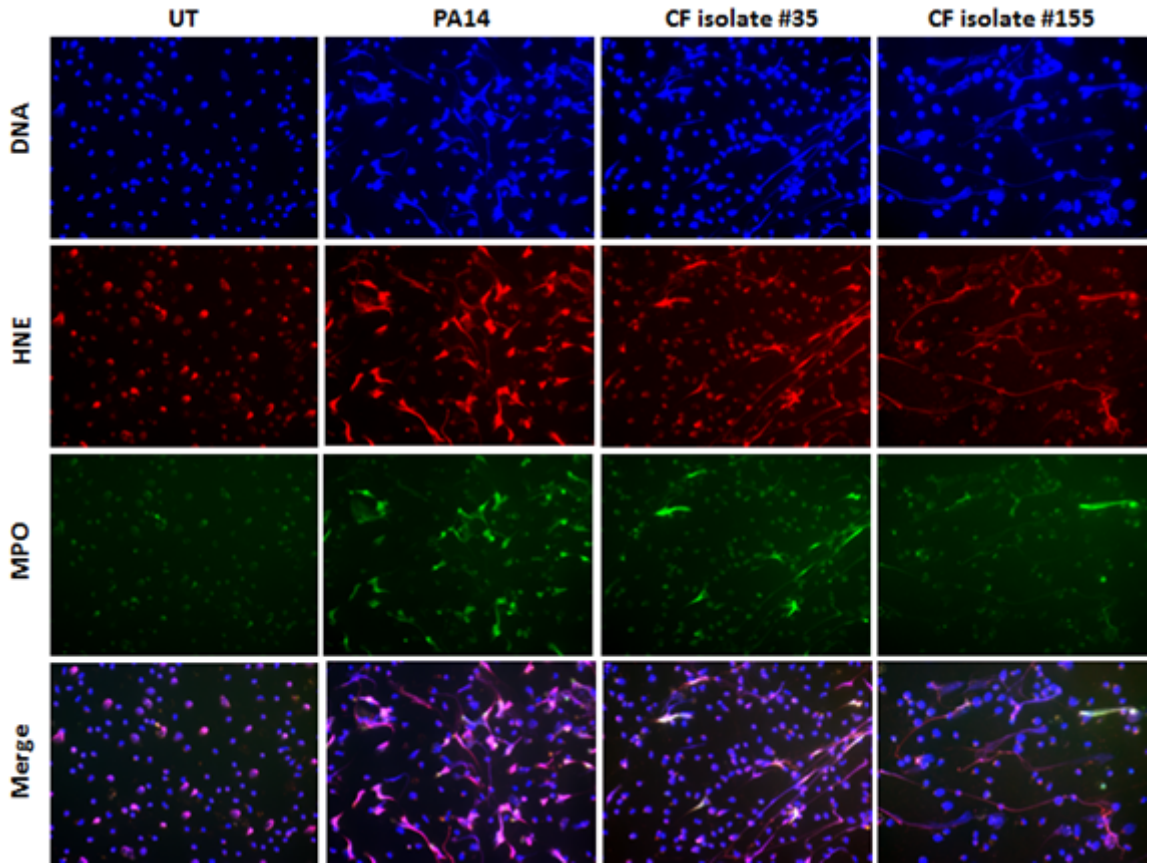

Figure 2-2. Immunostaining of NET formation induced by *P. aeruginosa* clinical isolates.

Adherent neutrophils, prepared in medium containing autologous serum (0.2%), were incubated with the indicated stimuli for 3 hrs at 37˚C. Samples were fixed with 4% paraformaldehyde and subjected to immunofluorescence staining. Extracellular DNA (blue) was stained with DAPI. MPO (green) was stained with FITC-labeled anti-MPO antibody. HNE (red) was detected by a combination of staining with primary anti-HNE antibody and secondary donkey Alexa 594-labeled anti-rabbit antibody. Images obtained at individual wavelengths. Merged versions are shown. One representative experiment, *n* = 3. Original magnification ×400.

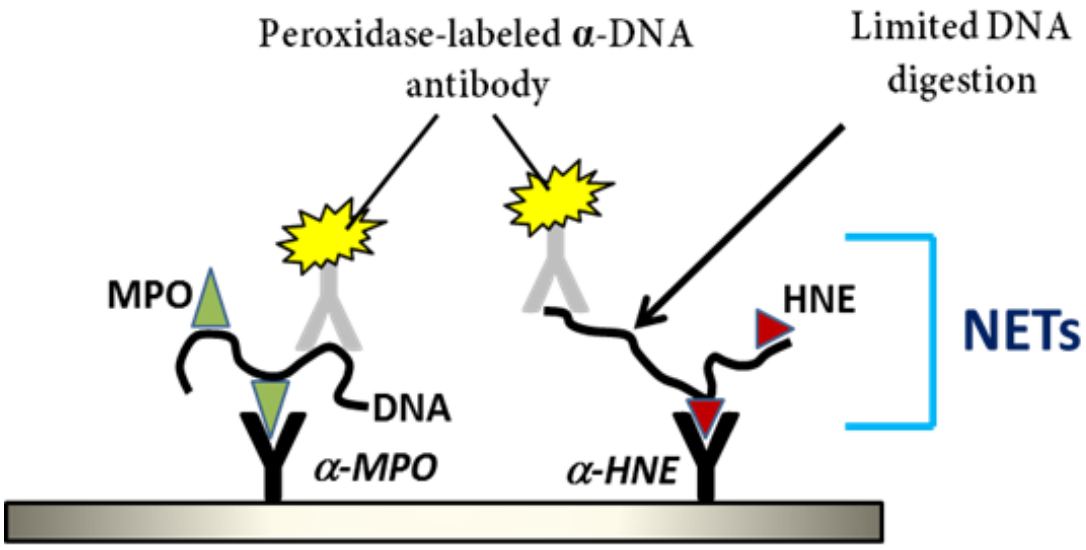

Figure 2-3. Schematic diagram explaining the MPO-DNA and HNE-DNA ELISA methods.

To quantitate measures of NET formation, MPO-DNA and HNE-DNA complexes, supernatants of activated neutrophils were digested in a limited fashion by DNAseI for 15 min at RT. A reaction was stopped with 20 mM EGTA in PBS, then added to ELISA microplates coated with either anti-MPO or anti-HNE capture Abs. After three washes, anti-DNA Ab conjugated with horseradish peroxidase was added. The colorimetric signal was obtained by adding TMB, the peroxidase substrate. The reaction was stopped by adding acid (HCl). Results were collected by measuring absorbance in a microplate spectrophotometer.

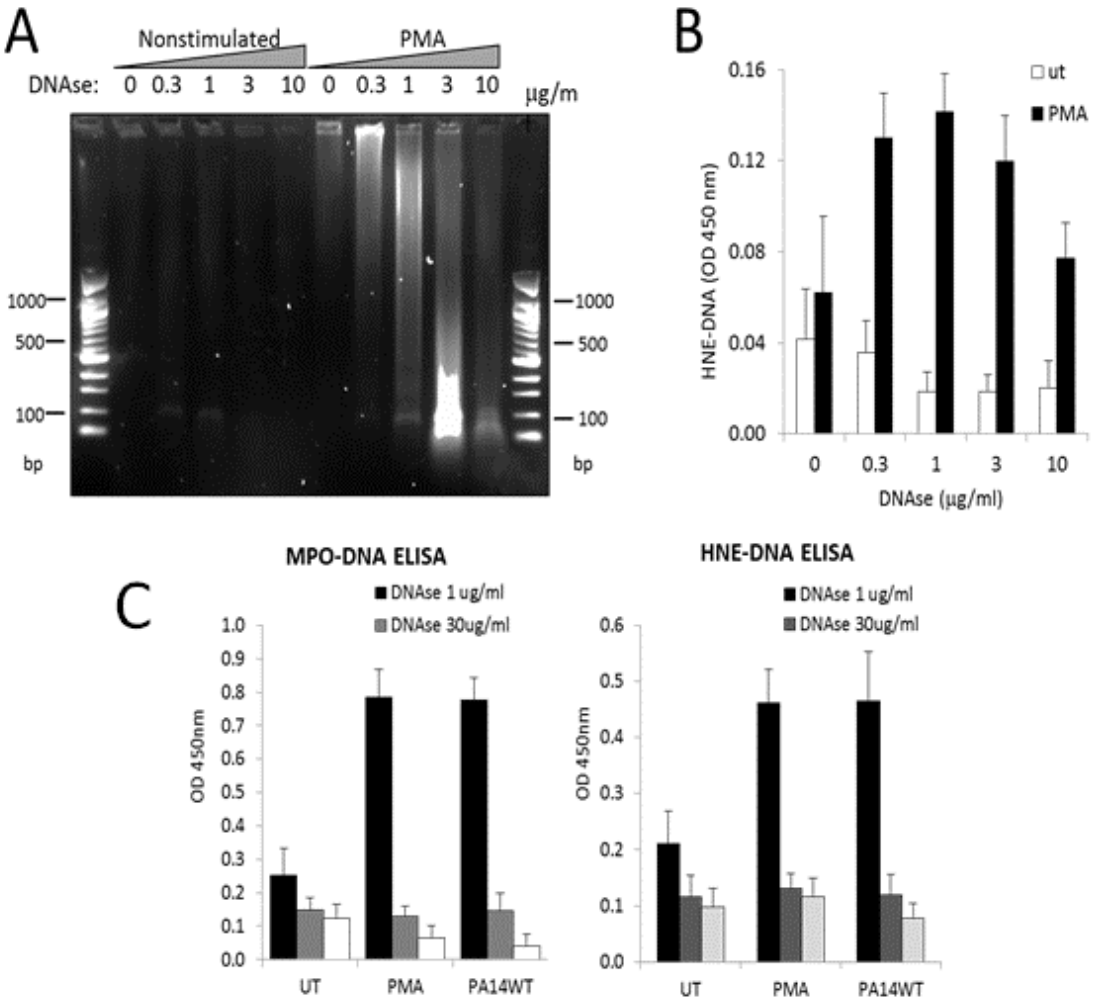

Figure 2-4. Optimization of the DNAseI treatment.

(A) Dose-dependent effect of DNAseI on NET-DNA digestion. DNAseI (0-10 µg/ml) was added to supernatants of human neutrophils left unstimulated or treated with 100 nM PMA (4 hrs). Digested supernatants were analyzed on a 0.8% agarose gel. (B) Neutrophil supernatants produced according to (A) were diluted 100-fold and subjected to HNE-DNA ELISA. Absorbance values indicative of NET release are expressed as a function of the DNAseI concentration used (0-10 µg/ml). (C) Optimization of DNAseI to measure MPO-DNA and HNE-DNA. Mean±S.E.M, *n*=6.

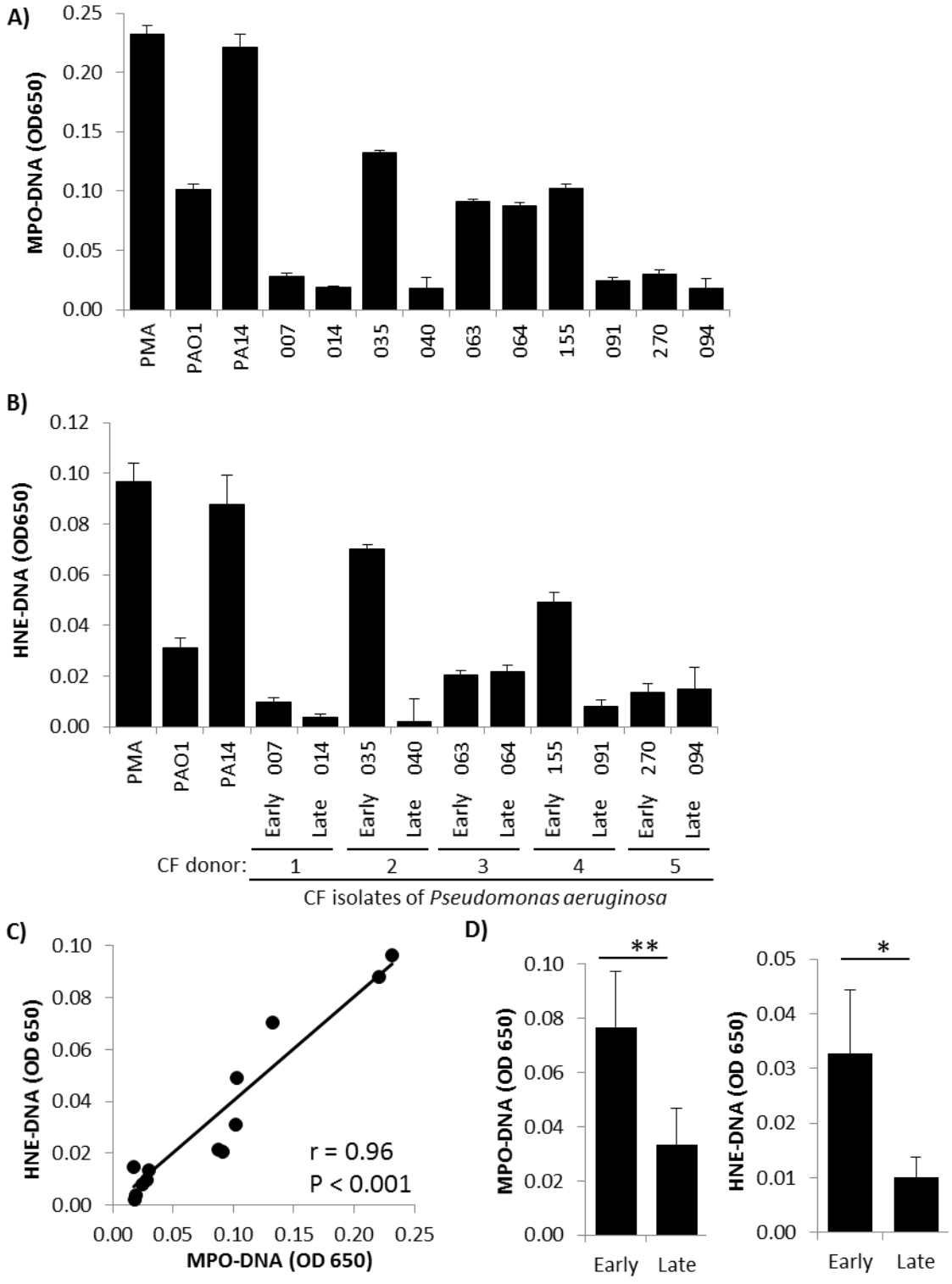
A)
MPO-DNA (OD650)
0.25
0.20
0.15
0.10
0.05
0.00
PMA
PAO1
PA14
007
014
035
040
063
064
155
091
270
094
B)
HNE-DNA (OD650)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
PMA
PAO1
PA14
007
014
035
040
063
064
155
091
270
094
Early
Late
CF donor:
1
2
3
4
5
CF isolates of Pseudomonas aeruginosa
C)
HNE-DNA (OD 650)
MPO-DNA (OD 650)
r = 0.96
P < 0.001
D)
MPO-DNA (OD 650)
**
Early
Late
HNE-DNA (OD 650)
*
Early
Late

Figure 2-5. MPO-DNA and HNE-DNA complex formation induced through neutrophil stimulation by *P. aeruginosa* clinical isolates.

Clinical isolates of *P. aeruginosa* and laboratory strains PA14 and PAO1 were cultured overnight and incubated with human neutrophils at PA:PMN MOI=10:1 for 3 hrs (A and B). Ten CF clinical isolates obtained from 5 different CF patients suffering from *P. aeruginosa* infection and severe lung disease were used in this study. For each patient, we studied an "early" and "late" clinical isolate. DNAse-digested supernatants were subjected to MPO-DNA (A) and HNE-DNA (B) ELISA assays. 100 nM PMA was used as a positive control. Signal of unstimulated cells was subtracted. Mean±S.E.M, n=3. (C) MPO-DNA and HNE-DNA were directly correlated. (D) MPO-DNA and HNE-DNA were analyzed according to the "early" versus "late" nature of the clinical isolates. Mean±S.E.M, n=5. *, $p<0.05$, **, $p<0.01$.

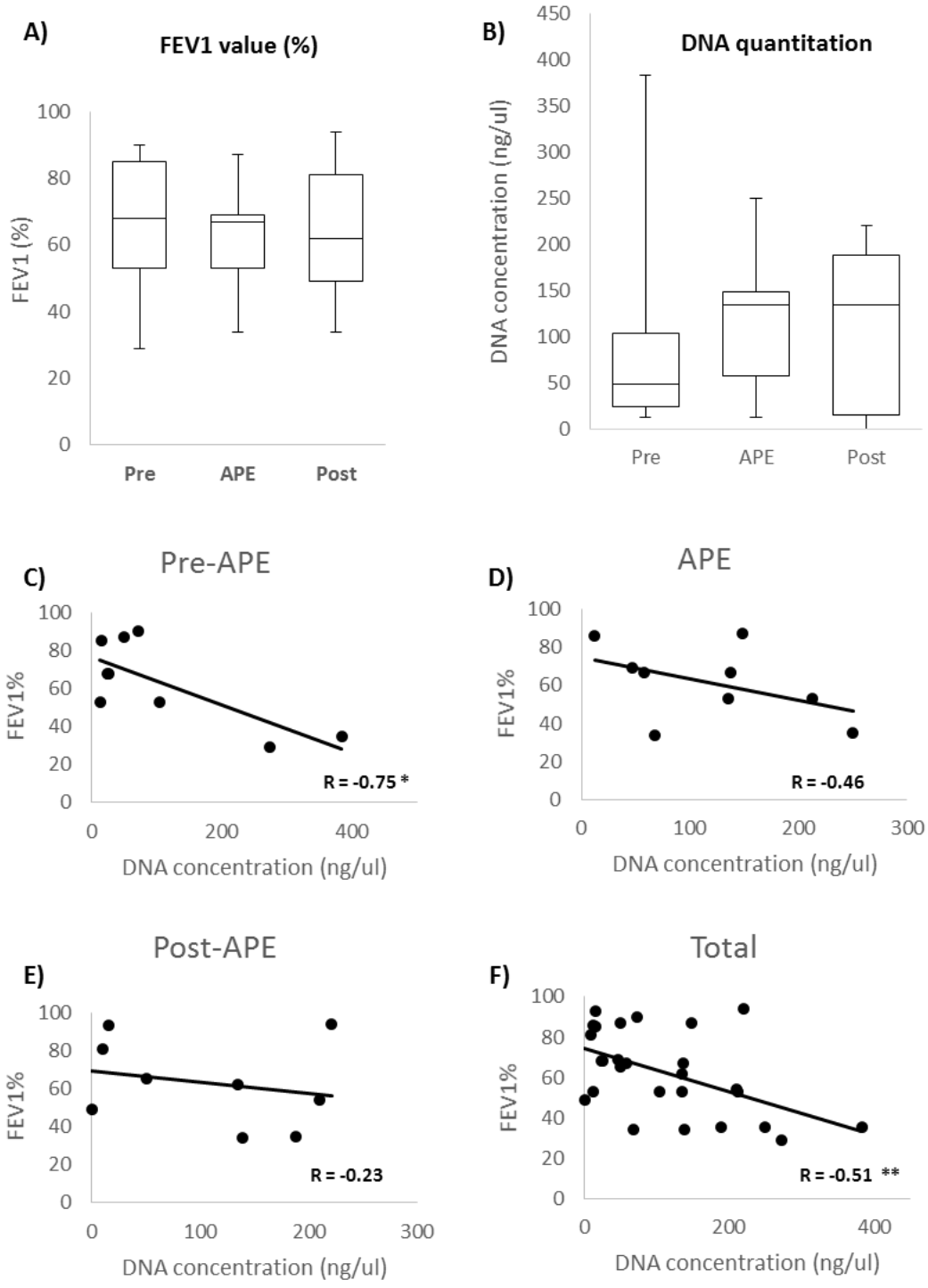
A)
FEV1 value (%)
FEV1 (%)
100
80
60
40
20
0
Pre
APE
Post
B)
DNA quantitation
DNA concentration (ng/ul)
450
400
350
300
250
200
150
100
50
0
Pre
APE
Post
C)
Pre-APE
FEV1%
R = -0.75 *
DNA concentration (ng/ul)
D)
APE
FEV1%
R = -0.46
DNA concentration (ng/ul)
E)
Post-APE
FEV1%
R = -0.23
DNA concentration (ng/ul)
F)
Total
FEV1%
R = -0.51 **
DNA concentration (ng/ul)

Figure 3-1. Association of extracellular DNA and FEV_1 value with acute pulmonary exacerbation (APE) stage.

FEV_1 values were analyzed in a box and whisker plot to determine its difference among three disease stages (A). DNA concentration was assayed via a Sytox Orange based-measurement of DNA in CF sputum samples. Calf thymus DNA was used for standard calibration (B). The three stages or total CF sputa were correlated by FEV_1 value and DNA concentration (C-F). Pearson correlation coefficient, r, was utilized, and its significance is shown as * P value<0.05, ** P value<0.01, n=5.

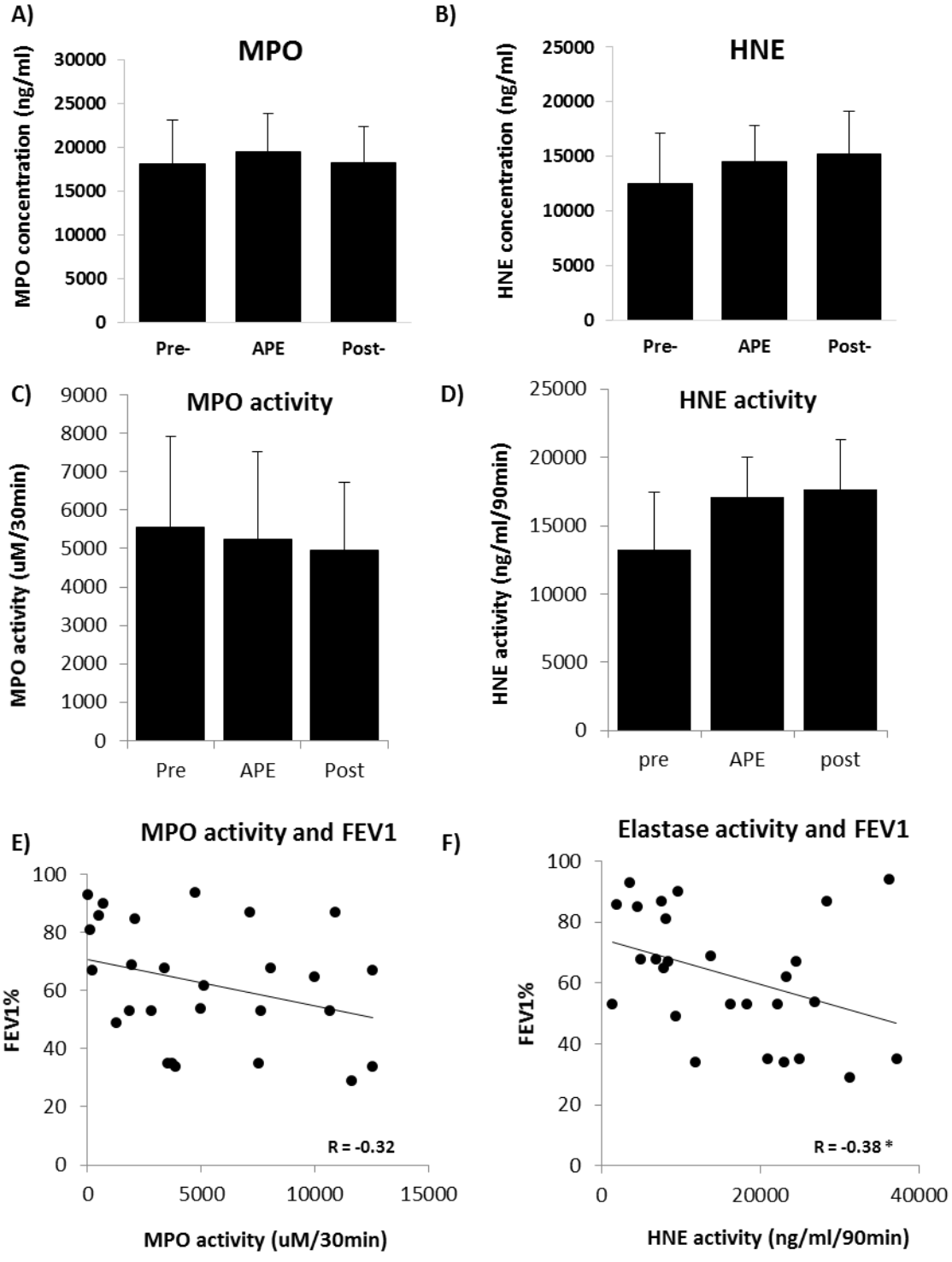
A)
MPO
MPO concentration (ng/ml)
30000
25000
20000
15000
10000
5000
0
Pre-
APE
Post-
B)
HNE
HNE concentration (ng/ml)
25000
20000
15000
10000
5000
0
Pre-
APE
Post-
C)
MPO activity
MPO activity (uM/30min)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Pre
APE
Post
D)
HNE activity
HNE activity (ng/ml/90min)
25000
20000
15000
10000
5000
0
pre
APE
post
E)
MPO activity and FEV1
FEV1%
100
80
60
40
20
0
R = -0.32
0
5000
10000
15000
MPO activity (uM/30min)
F)
Elastase activity and FEV1
FEV1%
100
80
60
40
20
0
R = -0.38 *
0
20000
40000
HNE activity (ng/ml/90min)

Figure 3-2. MPO and HNE inversely correlate with FEV_1 value.

MPO and HNE protein levels were analyzed by ELISA (A and B). Peroxidase activity was measured with a 100 μM Amplex Red and 100 μM H_2O_2 mixed solution (C). HNE activity was determined using an elastase activity kit (D). MPO and HNE activities of whole CF sputum were correlated with FEV_1 value (E and F). Pearson correlation coefficient, r, was utilized for this correlation, and its significance is shown as * P value<0.05, Mean±S.E.M, *n*=3.

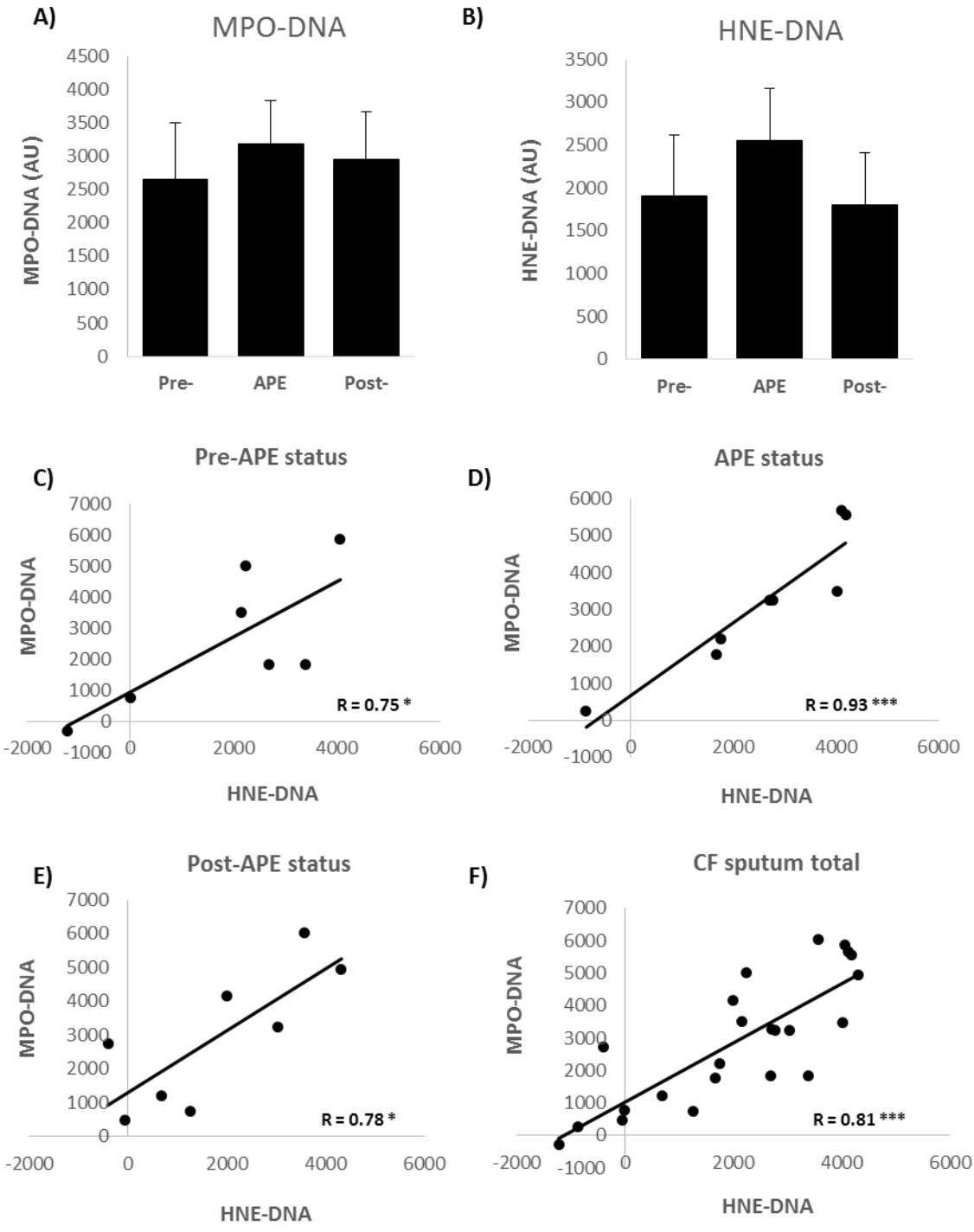
A)
MPO-DNA
MPO-DNA (AU)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Pre-
APE
Post-
B)
HNE-DNA
HNE-DNA (AU)
3500
3000
2500
2000
1500
1000
500
0
Pre-
APE
Post-
C)
Pre-APE status
MPO-DNA
HNE-DNA
R = 0.75 *
D)
APE status
MPO-DNA
HNE-DNA
R = 0.93 ***
E)
Post-APE status
MPO-DNA
HNE-DNA
R = 0.78 *
F)
CF sputum total
MPO-DNA
HNE-DNA
R = 0.81 ***

Figure 3-3. Analysis of NET-associated proteins, MPO-DNA and HNE-DNA, in CF patient sputum samples.

MPO-DNA and HNE-DNA complexes were assayed by our previously established NET marker detection protocol (A and B). Samples diluted 2000-fold in PBS were used for this analysis. (AU) indicates arbitrary units based on comparison to PMA, the positive control. Positive control and all samples were digested by *Alu*I restriction enzyme, cutting 5′-AG|CT-3′, for 30 min at RT. The three categorized or whole CF sputum were correlated in terms of MPO-DNA and HNE-DNA (C-F). Pearson correlation coefficient, r, was utilized for this correlation, and its significance is shown as * P value<0.05, *** P value<0.001, Mean±S.E.M, *n*=6.

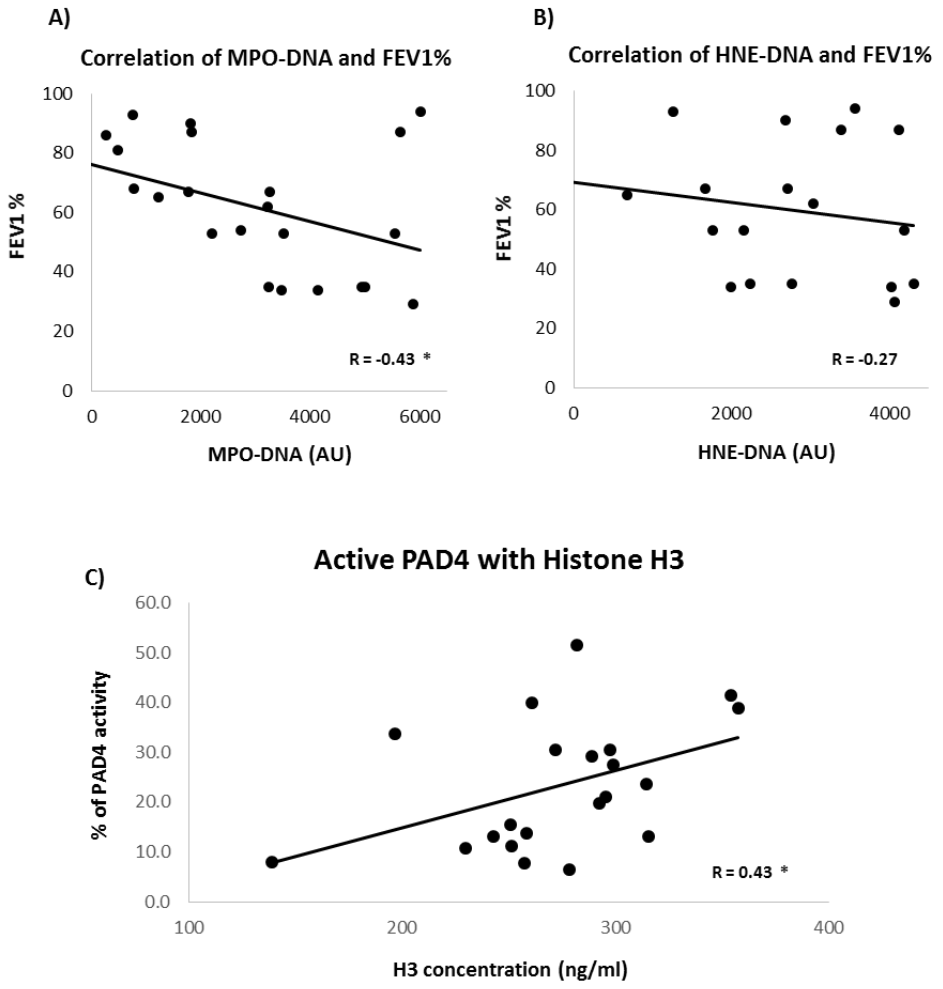

Figure 3-4. NET-associated proteins inversely correlates with FEV_1 value, but active PAD4 directly correlates with histone level in CF sputum samples.

MPO-DNA and HNE-DNA were measured in sputum samples from 23 patients. These data were correlated with FEV_1 value to test whether CF lung function decline relates to NET release. MPO-DNA displayed a significant inverse correlation with FEV_1 value (R= -0.43). On the other hand, HNE-DNA demonstrated only a slight inverse correlation (R= -0.27) with FEV_1 value (A and B). Active PAD4 directly correlated (R= 0.43) with histone H3 level. Significance $p<0.05$. Repeated experiments were done three times for (A and B) and two times for C.

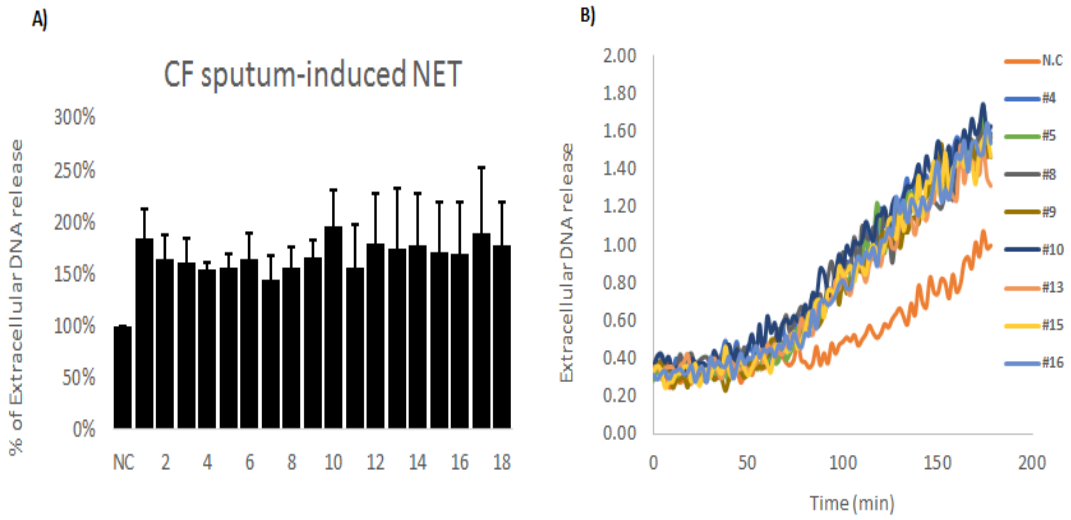

Figure 3-5. CF sputum alone induced extracellular DNA release from activated neutrophils.

100,000 neutrophils/well were stimulated by 500-fold diluted CF sputum samples, or left untreated, for 3 hrs (A). Sytox Orange-based measurement of extracellular DNA release was monitored for 3 hrs. (B) RFU, kinetics of extracellular DNA release induced by a selected 9 CF sputum samples versus that induced by the assay buffer treated control. Corresponds to level of NET formation. Mean±S.E.M, Samples *n*=18, repeated experiments were done six times. One representative kinetics for B out of 6 experiments.

CHAPTER 6

DISCUSSION

Cystic fibrosis is one of the most common human genetic disorders, affecting mostly Caucasians [60,86,95]. The presence of the main CF pathogen, *P. aeruginosa* promotes neutrophil recruitment to the airways [23]. Due to arduous elimination of the pathogen in CF, neutrophils are believed to undergo unnecessary overactivation, causing tissue damage and contributing to lung failure [28,35,86]. Levels of HNE are strikingly increased in CF newborns and adults [35]. Another major granule protein, myeloperoxidase and its activity are dramatically increased in CF patients [29,30]. Additionally, anti-neutrophil cytoplasmic autoantibodies (ANCA), elevated levels of IL-8, and robust neutrophil recruitment are typically found in CF patient airways [22,23,34,36]. NETs are one of the crucial innate immune responses of neutrophil granulocytes [14-19]. However, uncontrolled NET release offers a potential mechanism for neutrophil activation in CF airways [64,113,119]. NETs and their relevance to CF disease has not yet been fully defined. Thus, understanding the contribution of NET formation to CF airway pathophysiology is of clinical importance. NET formation requires superoxide production, extrusion of nuclear and/or mitochondrial DNA associated with citrullinated histones and several neutrophil antimicrobial proteins such as MPO and HNE [33,37,66,106,126]. The aforementioned proteins, oxidative stress and extracellular DNA are all markers of CF inflammation and are correlated with the poor lung function of CF patients [23,29,36,86]. The main airway pathogen of CF patients is *P. aeruginosa*. Although accumulation of

neutrophil markers and a presence of *P. aeruginosa* bacilli in chronic CF airways have been well-documented, NET formation induced by the bacteria and its contribution to CF lung pathology are poorly understood. Thus, in chapters 2, 3 and 4, we described *P. aeruginosa* triggered-NET formation and investigated its clinical relevance in CF airway disease.

***Pseudomonas aeruginosa*-stimulated NET formation.**

First, we examined whether *P. aeruginosa* can trigger NET formation in our *in vitro* experimental model. We found that the main effector response of neutrophils upon exposure to PA14 was NET formation (Figure 1-1 to 1-2). Human neutrophils exposed to PA14 showed clearly increased levels of extracellular DNA release (Figure 1-1 A and C). PA14-activated neutrophils induced DNA release in a dose-dependent manner. (Figure 1-1 B). PA14 was ensnared by 41.3% and 29.7% when human neutrophils were co-incubated with 10 MOI and 50 MOI PA14, respectively (Figure 1-1 D). To visualize this extracellular DNA release, we examined the presence of NET-specific markers in the released DNA by immunostaining. Our results clearly demonstrate co-localization of MPO, HNE, citrullinated histone H4 and extracellular DNA (Figure 1-2). This supports our hypothesis that NET formation induced by PA14 is the main effector response of neutrophils to *P. aeruginosa*. Highly elevated levels of MPO and HNE are found in CF airways, and they have been correlated with CF lung disease severity [29,30,34,36]. Thus, we investigated the levels of released MPO and HNE from PA14-stimulated neutrophils. We found that MPO and HNE were released from PA14-exposed neutrophils and peroxidase activity was also elevated in the supernatants (Figure 1-3). This suggests that *P. aeruginosa* bacilli are able to promote MPO and HNE release from human neutrophils by NET formation. The

NADPH oxidase is involved in NET formation by producing superoxide anions in PMA-stimulated neutrophils [42,68]. However, in response to high doses of uric acid (8 mg/dl), NET formation is triggered by an NADPH oxidase-independent mechanism [88]. Therefore, we investigated whether *P. aeruginosa* requires the host NADPH oxidase activity to promote NET formation. PA14 clearly stimulated superoxide production in a dose-dependent manner, and it was significantly inhibited by DPI pretreatment (Figure 1-4 A and B). This demonstrates that PA14 mainly promotes superoxide generation from activated neutrophils via the NADPH oxidase. DPI also inhibited NET formation, MPO, HNE release and peroxidase activity (Figure 1-4). This phenomenon suggested that the NADPH oxidase-dependent NET formation activated by *P. aeruginosa* bacilli enables maximal release of MPO and HNE. In a previous report, we showed that NET formation triggered by calcium pyrophosphate dihydrate crystals requires an intact cytoskeleton [43]. Similarly, this intact cytoskeleton was also required for the PA14-stimulated NET formation (Figure 1-5). Previously, the MEK/ERK signaling pathway was shown to be involved in the formation of NETs [42,45]. By investigating the involvement of MEK/ERK in PA14-induced NET formation, we found the dramatic inhibitory effects of U0126 (Figure 1-6). These data show that the U0126 compound affected MPO activity even in a cell-free *in vitro* system, whereas MEK162 and PD98059 did not (Figure 1-6 B). One possible explanation for these results is the chemical structure of U0126 has four primary amines and two disulfide bonds, suggesting it could be an excellent scavenger of hypochlorous ions [49,89]. MEK162 and PD98059 significantly inhibited DNA release but not MPO release and its activity. Only MEK162 significantly decreased HNE release (Figure 1-6 C). It implies that the MEK/ERK pathway is involved in PA14-induced NET

formation, however, this minor effect does not significantly alter MPO and HNE release, since they can be also released by degranulation (NET-independent pathway). *P. aeruginosa* can build up resistance to NET-mediated killing in CF airways [16]. Failing to remove *P. aeruginosa* bacilli efficiently in CF airways causes neutrophils to release their dangerous granule components, MPO and HNE into the airway lumen, instead. Although the CF airway environment is very complex, and various mechanisms are associated with dysfunction of neutrophils, our observation suggested that *P. aeruginosa*-induced NET formation is one of the critical mechanisms responsible for the release of excessive inflammatory mediators from neutrophils in CF.

Release of MPO-DNA and HNE-DNA complexes triggered by 'early' and 'late' phase CF clinical isolates

In chapter 3, we studied whether clinical isolates of *P. aeruginosa* can promote the release of NETs from neutrophils. Moreover, we quantitatively determined the release of NETs induced by these isolates via detecting MPO-DNA and HNE-DNA complexes. As seen in Figure 2-1, clinical isolates activated neutrophils more than laboratory strain PAO1 and PA14WT in integral superoxide production. The presented clinical isolates #35 and #155 showed a clear formation of NET linked with HNE and MPO (Figure 2-2). These data suggest that CF clinical isolates of *P. aeruginosa* promote NET formation by activating neutrophils predominantly through NADPH oxidase activity (Figure 2-1 and 2-2), which supports the hypothesis that chronic infection by *P. aeruginosa* activates neutrophils and promotes NET formation in the CF airway environment. To quantitatively evaluate NETs, we modified a previously established MPO-DNA detection method [59] and developed a new HNE-DNA detection method (both referred to as NET-ELISAs)

(Figure 2-3). NETs formed specific protein-DNA complexes released only by cells undergoing NET formation, not apoptotic neutrophils [90]. Therefore, these ELISA methods can be used for detection of extended NET markers, protein-DNA complexes (see detail methodology in Materials and Methods). *P. aeruginosa* infection is the critical issue in CF airways. Planktonic growth transitions to non-planktonic growth, which builds up a mucoidic polysaccharide-rich formation known as a biofilm [1,7-9,75,76]. This biofilm protects against certain antibiotics, such as ciprofloxacin, polymyxin B, tobramycin and more [91]. Our observation in Figure 2-5 indicates that 'early' phase *P. aeruginosa* induces NET formation significantly more than corresponding 'late' phase *P. aeruginosa*. Dr. Young and colleagues found that the 'late' phase clinical isolates showed enhanced-resistance to NET-mediated killing compared to 'early' phase isolates [16]. Similar to the results we have seen, Dr. Gadjeva *et al.* found reduced formation of NETs in response to mucoidic strains of *P. aeruginosa* [124]. In addition, Dr. Gadjeva proposed a pathoadaptation model of *P. aeruginosa* [125]. This model suggests that 'early' phase *P. aeruginosa* vigorously stimulates neutrophils to release NETs by secreting virulence factors. Although NETs sequester bacteria, they fail to successfully eliminate bacteria and instead provide a scaffold for bacterial colonization. This continuous bacterial survival finally leads to the acquisition of mutations, turning to mucoidic, 'late' phase strains that are less effectively captured by NETs [125]. Our results and others support that reduced stimulation of NET formation is a part of the adaptation route of *P. aeruginosa*, and the chronic infectious stage that occurs by evading the neutrophil immune response may influence increased biofilm formation in CF airways.

Correlation of clinical parameters and levels of NETs

CF patients typically demonstrate declining FEV_1% values [30,36] and high amounts of extracellular DNA present in their airways [92,93]. Several reports showed elevated DNA levels in CF sputum that correlated with CF lung function decline [28,86,94]. We investigated whether CF patients undergoing acute pulmonary exacerbation (APE) may have worse FEV_1% values and elevated extracellular DNA present in their sputum. After separating CF sputum samples obtained from Emory+Children's Pediatric Research Center CF Discovery Core at the Emory University (Atlanta, GA) into pre-APE, APE, and post-APE stages, larger amounts of extracellular DNA were seen in APE and post-APE stages than in pre-APE stage samples (with the exception of one outlier, Figure 3-1 B). As expected, FEV_1% values of the APE stage corresponded to a lower FEV_1 percentile, consistently within the narrow range in the Q3 quartile (Figure 3-1 A). This implies that CF patients during the APE stage may have more NET-mediated inflammatory markers expressed. To confirm this, we measured DNA concentration in CF sputum samples and correlated the results with FEV_1% values among the three different categories. As seen in Figure 3-1 C-F, FEV_1% values were significantly inversely correlated with extracellular DNA release in pre-APE and total CF sputum. APE and post-APE stages displayed a similar tendency but were not statistically significant. Based on our survey, it appears that DNA concentration related more to the numeric FEV_1% value than to symptom-based APE stage categorization. MPO and HNE release were not significantly different among the three categories. Their activities also showed no significance, but HNE activity did demonstrate a tendency in which the pre-APE stage had a lower level of activity than the other two stages (Figure 3-2 D). However, once we analyzed those activities in

relation to FEV_1%, active HNE showed a significant inverse correlation with FEV_1% values (Figure 3-2 F). Active MPO also demonstrated this tendency, suggesting active MPO hypersecretion may influence CF lung function (Figure 3-2 E). Other groups investigated and confirmed that MPO, HNE, IL-8 and GM-CSF hypersecretion was found in CF sputum samples and inversely correlated with FEV_1% value [28,30,35,36,95]. Dr. Kim *et al*. found that increased levels of MPO, IL-8 and DNA are significantly inversely correlated with cough transportability (CTR) and FEV_1% in CF patients but not in chronic bronchitis patients [28]. Dr. Van der Vliet *et al*. showed that the increase levels of MPO oxidation and reactive nitrogen species which may originate from MPO-derived oxidation of nitrite in CF sputum, insisting these increased activities may contribute to bronchial injury and respiratory failure in CF [29]. Other articles demonstrated that increased MPO activity and HNE concentration are inversely correlated with FEV_1% value [30,34]. Dr. Mayer-Hamblett *et al*. showed that increased levels of neutrophils, IL-8, HNE and *P. aeruginosa* bacilli are all found in CF sputum samples, and those increased levels are inversely correlated with FEV_1% value [36]. Our results also support the findings that MPO and HNE concentrations and activities were highly detectable in CF sputum and were inversely correlated with FEV_1% (Figure 3-2 E-F). High DNA concentration is also inversely correlated with FEV_1% (Figure 3-1 C-F). Along with others, our data suggest that neutrophil hyper-activation is significantly associated with lung function decline. These findings may provide insight into the creation of a diagnostic tool for measuring CF pathophysiology more distinctly. However, the relationship between NET-associated inflammatory mediators, such as MPO-DNA and HNE-DNA, and CF sputum has not yet been studied. Currently, little is known about NETs in CF. Dr. Manzenreiter *et al*. showed

the existence of NETs, underlying accumulation of a gel-like structure in CF sputum [63]. Dr. Papayannopoulos *et al*. showed the paradoxical beneficial effects of HNE that cleave histones and enhances the access of exogenous nuclease to DNA, improving solubilization of CF sputum [64]. Dr. Dubois and colleagues displayed clear NET-forming neutrophils in CF sputum by transmission electron microscopy [128]. However, all of the above findings failed to quantification of NETs. We believe that quantification of NETs is of great importance to investigate if NET formation is significantly related to CF disease progression. Thus, we next investigated the presence of NET-associated mediators in CF sputum by utilizing the NET-ELISA protocol used in Chapter 3. The results of this experiment are significant because it is the first assay to quantify NETs in the CF field. As expected, MPO-DNA and HNE-DNA complexes were found in CF sputum samples (Figure 3-3 A-B). MPO-DNA and HNE-DNA levels were higher during the APE stage than the other two stages, but this tendency was not significant (Figure 3-3 A-B). Based on the correlation between MPO-DNA and HNE-DNA, the two main biomarkers for NET-associated mediators were directly correlated (Figure 3-3 C-F). NET markers existed abundantly in all APE stages, suggesting that neutrophil activation and NET release occurred promptly at the early stage of CF disease progression. We next questioned whether NET-associated markers are critically related to CF disease severity. Excitingly, MPO-DNA and HNE-DNA demonstrated an inverse correlation with FEV_1% value (Figure 3-4 A-B). This result indicates that both degranulation and NET formation affect pulmonary function in CF. This data supports our hypothesis that the release of NETs in CF airways significantly influences lung function decline. In NET formation, decondensation of the DNA precedes histone citrullination [37,39]. This process requires

PAD4 activity [37,39]. Dr. Manzenreiter *et al*. found citrullinated histone H3 in CF sputum [63]. It is not currently known whether PAD4 activity influences CF airway inflammation. To test this, we assessed active PAD4 and histone H3 concentrations (Figure 3-4 C). A total of 23 CF sputum samples demonstrated a significant direct correlation between active PAD4 and histone H3 levels. This data indicate that elevated histone levels require active PAD4, and this occurs in the CF airway environment. Additionally, continuous hyperactivation of neutrophils in CF patients is thought to exacerbate CF [28,30,35,36,86]. Therefore, we investigated whether supernatants of CF sputum samples induce release of the extracellular DNA in human neutrophils. A total of 18 blind-tested CF sputum samples displayed an increased level of extracellular DNA release compared to spontaneous activation of neutrophils (Figure 3-5). In our results, CF sputum has a large number of MPO, HNE, DNA, HNE-DNA and MPO-DNA complexes (Figure 3-1, 3-2 and 3-3). As seen in Figure 3-5, our observation suggests that CF sputum influences neutrophils to release DNA, by which the critical components of neutrophils in CF sputum stimulate other neutrophils, which may contribute to CF disease pathogenesis. Certain types of autoantibodies, such as BPI-ANCA, have been found in CF [22]. Dr. Zychlinsky and colleagues suggested that major neutrophil products MPO and PR3 are the main targets of ANCA, autoantibodies directed against antigen present in the cytoplasm of neutrophils [21]. The ANCA presence is also found in Wegners' granulomatosis [21]. Additional autoantibodies of neutrophils were found in CF and RA such as BPI-ANCA [22] and ACPA [41], respectively. Dr. Kessenbrock *et al*. provided evidence of NET release induced by ANCA, containing targeted autoantigens MPO and PR3 [81]. This suggests that NET formation is highly associated with antigen presentation and production of those

autoantibodies. In SLE, NETs have a crucial role in disease progression. Dr. Leffler *et al*. suggested that abnormal clearance of NETs in SLE flare (severe) stage increases C1q complement deposition, potentially exacerbating SLE [129]. Moreover, they found that SLE flare stage sera have elevated anti-dsDNA and anti-histone autoantibodies levels which are all crucial components of NETs [129]. Dr. Lande and colleagues found that the neutrophil antimicrobial peptide LL-37 was tightly bound to NETs and existed in circulating DNA-containing immune complexes in SLE patient serum samples [130]. The NET complex, DNA-LL-37, efficiently triggered the activation of plasmacytoid dendritic cells (pDCs) via TLR9, suggesting a mechanism for the chronic release of immunogenic complexes in SLE [130]. Drs. Radic and Marion proposed a model for the chronic activation of neutrophils releasing NETs in glomerulonephritis progress in SLE [131]. In brief, the cross-reactive anti-DNA autoantibody binds to glomerular basement membrane (GBM) or mesangial matrix (MM) of the kidney. Neutrophils are recruited by activated complement. Complement-activated neutrophils upregulate FcγR. FcγR-activated neutrophils are able to release NETs. NETs can also bind to GBM/MM-bound anti-DNA autoantibody [131]. Non-GBM/MM cross-reactive autoantibodies can bind to GBM/MM-bound NETs to build up large-lattice immune complexes to recruit more neutrophils and/or monocytes and macrophages [131]. As seen from the above literature, NET-related autoantibodies have been frequently found in autoimmune diseases, and NET-linked autoantibodies play a pivotal role in autoimmune diseases.

In summary, from chapter two to four, we evaluated which mechanisms mediate *P. aeruginosa*-activated release of NETs. The NADPH oxidase and an intact cytoskeleton are necessary to release NETs. However, the MEK/ERK signaling pathway contribution to

NET formation is minimal. These data are novel findings on how *P. aeruginosa* stimulates neutrophils to release NETs. Our newly developed NET-ELISA methods were used for detection of NET-associated proteins as NET markers. MPO-DNA and HNE-DNA complexes were significantly associated with clinical isolates of *P. aeruginosa*-induced NET formation. 'Early' phase clinical isolates of *P. aeruginosa* increased the release of NETs significantly more than 'late' phase isolates. This may be caused by acquired resistance of *P. aeruginosa* against NET-mediated killing over the course of infection to prolong bacterial survival. Furthermore, NETs were found in CF sputum. Our results suggest that our NET ELISAs could be used to quantitate NETs in clinical samples. In biological circumstances, we propose that *P. aeruginosa*-induced NET formation provides the main mechanism of tissue-damaging neutrophil release of DNA and granule markers in CF airways.

CHAPTER 7

MATERIALS AND METHODS

Human subject statement

Healthy human subjects were recruited under the guidelines of an IRB-approved protocol (University of Georgia, IRB 2012-10769-9) and provided written informed consent. Human subjects included cystic fibrosis patients participating in an observational study of CF lung disease severity, "Genetics of CF Lung Disease" (Seattle Children's Hospital institutional review board approved protocol 10855 and Partners Healthcare Systems/Massachusetts General Hospital institutional review board approved protocol 2011P000544). Protocols and informed consent procedures were approved by the Institutional Review Board of the Seattle Children's Hospital, and Partners Healthcare Systems / Massachusetts General Hospital. Studies were conducted in accordance with the ethical guidelines of the Declaration of Helsinki. Human subjects recruited under the guidelines of the Seattle Children's Hospital IRB-approved protocol 10855 provided written informed consent for storage of specimens and data for use in future cystic fibrosis research (i.e., specimen and data banking). Written informed consent for storage of specimens and data for future research use was received from parents or legal guardians of minors. The Partners Healthcare Systems / Massachusetts General Hospital IRB assumed regulatory responsibility for a portion of the "Genetics of CF Lung Disease" study after the principal investigator relocated from Seattle Children's Hospital to Massachusetts General Hospital, then reviewed and approved the use of previously stored specimens and data for

the specific studies described below, under 45 CFR46.110 and 21 CFR56.110 (expedited review of minimal risk human subjects research). All 5 CF patients in this study were homozygous for the F508del allele of the CFTR gene. They were categorized as having "severe" lung disease if they were in the highest or lowest quintile for age of airway obstruction, as assessed by their median forced expiratory volume during the initial second of exhalation.

Neutrophil purification

Neutrophil granulocytes were obtained from fresh human peripheral blood of healthy volunteers. Red blood cells were removed by dextran sedimentation of the anticoagulant-treated blood, and neutrophils were separated using multistep Percoll gradient centrifugation. Leukocytes were layered on top of a 5-phase Percoll gradient (0.66 g/ml, 0.71 g/ml, 0.76 g/ml, 0.81 g/ml and 0.86 g/ml, Sigma), and the 71-76-81 (w/v) % Percoll layers containing neutrophils were collected. Cells were stored in a 1:1 mix of autologous serum and RPMI-1640 medium (Life Technologies) until use at room temperature. Serum was prepared from coagulated blood by centrifugation and sterile filtration. Calcium- and magnesium-containing HBSS (Mediatech) mixed with 1% autologous serum was used as assay buffer for Chapter 2. Chapter 3 and 4 assays did not include autologous serum unless otherwise mentioned. In Chapter 3, the assay buffer contained 5 mM glucose and 10 mM HEPES in HBSS. For Chapter 4, HBSS alone was used for all experiments.

Human CF sputum samples

APE stage categorized CF sputum samples were obtained from Emory+Children's Pediatric Research Center CF Discovery Core at the Emory University (Atlanta, GA).

Blind tested CF sputum samples for neutrophil activation assay were kindly provided by Dr. Rabindra Tirouvanziam, Emory University, Atlanta, GA.

Pseudomonas aeruginosa strains

Clinical isolates of *P. aeruginosa* bacilli were cultured from sputum or oropharyngeal swabs obtained from CF patients participating in the "Genetics of CF Lung Disease" observational study. These clinical isolates were kindly provided from Dr. Samuel Moskowitz in Massachusetts General Hospital. Isolates were categorized as "early" or "late" relative to the course of each patient's onset of infection. Early isolates were obtained from 3 months to 11 years of age; late isolates were obtained 5 to 20 years after collection of an early isolate from each patient. All clinical isolates were stored in Luria-Bertani broth with 16% glycerol at -80℃. PAO1 and PA14 were stored at -80˚C. All bacteria were cultured in LB broth overnight, and measured for bacterial concentration set at 10^9/ml in 200 µl (OD 0.6) by measuring absorbance at 600 nm.

DNA gel electrophoresis

Human neutrophils were plated at 250,000/well in 96-well plates, and stimulated with PMA or bacteria. Incubation was followed by DNAseI (Roche) treatment for 15 min at room temperature, allowing for limited DNA digestion of NETs. This treatment was then halted with final 20 mM EGTA in PBS. Supernatants were harvested, centrifuged and mixed with 10x loading buffer. Aliquots were analyzed by agarose DNA gel electrophoresis (0.8% agarose, Gel Start DNA-detecting dye (Lonza), 140 V). Images were taken and analyzed with Gel-Doc EZ Imager (BioRad).

Superoxide production

Superoxide production was measured by Diogenes superoxide-specific chemiluminescence assay kit (National Diagnosics). Adherent neutrophils in HBSS containing 5 mM glucose, 10 mM HEPES and with or without 1% autologous serum were exposed to 100 nM PMA or different strains of *P. aeruginosa* (PAO1, PA14, CF isolates; 10 or 50 MOI). Superoxide production was recorded for 90 to 120 min by measuring chemiluminescence in 96-well white plates using a microplate luminometer (Varioskan Flash, Thermo Scientific). Integrated luminescence values were calculated and shown as "RLU or int. RLU/90 min or int. RLU/2 hrs".

MPO-DNA and HNE-DNA ELISA

After stimulation, neutrophils were treated with DNAseI (0-10 μg/ml, Roche) for 15 min at room temperature. DNAse digestion was stopped by adding final 2.5 mM EGTA (Sigma). Supernatants were collected, centrifuged and diluted 10- to 100-fold in PBS + final 2.5 mM EGTA for Chapter 3 experiments. For the Chapter 4 experiments, *Alu*I restriction enzyme was used for limited digestion at 5′-AG|CT-3′. These samples were further diluted 2000-fold in HBSS. Diluted supernatants were added to high-binding 96-well ELISA miroplates (Greiner Bio-One) pretreated with either capture anti-MPO (1:2000, Upstate, rabbit) or anti-HNE (1:2000, Calbiochem, rabbit) antibodies overnight and subsequently blocked with 5% BSA (containing 0.1% human albumin) in PBS for at least 1-2 hrs. After overnight incubation of samples and three washes with 0.05% Tween-20 in PBS, secondary anti-DNA-POD (horse radish peroxidase conjugated anti-DNA antibody, mouse, 1:500, Roche) was added and allowed to incubate 30-60 min at room temperature. After three washes, TMB substrate (Thermo Scientific) was added, and the

reaction was subsequently stopped with 1 N HCl solution. Absorbance values were read at 450 nm, or 650 nm without stopping, by Eon microplate spectrophotometer (BioTek). Background absorbance values of the medium and untreated neutrophils were subtracted.

Sytox Orange-based extracellular DNA measurement

The neutrophils (100,000 cells/well) were seeded on the poly D-lysine coated 96well black transparent-bottom plates (Thermo Scientific) and allowed additional 10 min incubation to adhere the cells at 37˚C. All buffer contained 1 % autologous serum and 10 μM Sytox Orange (Life Technologies) as a membrane impermeable DNA-binding dye in HBSS. For test with inhibitors, cells were pretreated with selective inhibitors for 15 min prior to bacterial infection. Neutrophils were infected with 1 to 50 MOI *P. aeruginosa* PA14 or 10 MOI was used for inhibitor assay. PAO1 and clinical isolates 10 MOI were used for Sytox Orange assay. Fluorescence (ex 530 nm, em 590 nm) was measured in a fluorescence plate reader, Varioskan Ascent (Thermo Scientific) for 3 hrs at 37˚C. Fluorescence signals by 10 mg/ml saponin (Sigma) with neutrophils was referred as maximal signal (100%). Unknown samples were normalized to saponin maximal signal and expressed as '% of extracellular DNA release'. Relative Fluorescence Units (RFU) are also used for kinetic assay. Diphenylene iodonium (DPI, NADPH oxidase inhibitor, 10 μM), U0126 (MEK1/2 inhibitor, 25 μM), MEK162 (MEK1/2 inhibitor, 50 μM), PD98059 (MEK1 inhibitor, 20 μM) and Cytochalasin D (cytoskeleton inhibitor, 10 μM) were used for inhibitor tests. All chemicals were purchased from Sigma-Aldrich.

Measurement of "NET-free" and "NET-linked" bacterial numbers

Human neutrophils (100,000/wells) were adhered on the poly-D-lysine-coated transparent 96-well plates (Thermo Scientific) in HBSS. Bacteria were inoculated at two

different doses: PA14/neutrophil MOI=10:1 or 50:1. After 3 hrs incubation at 37˚C, supernatants were carefully collected and referred to "NET-free" fraction. Absence of DNA in this fraction was confirmed by DNA gel electrophoresis. Equal volume of assay medium containing 30 μg/ml DNAseI (Roche) was added back to the neutrophils and incubated for 15 min at RT. This DNAseI treated fraction was referred to "NET-linked". After centrifugation, concentration of PA14 bacteria in each fraction sample was determined by a microplate-based bacterial growth assay protocol described in detail [132]. In brief, the samples were diluted 10-fold in HBSS, and mixed with Luria-Bertani (LB) broth for a final 50-fold dilution. Samples were incubated in a microplate reader for 16 hours at 37˚C. This protocol allows the bacteria to grow in a timely manner in the plate. This assay adopted the principle of real-time polymerase chain reaction. The OD 0.2 (600 nm) was fixed as threshold for each growth of bacteria. The PA14 alone was used for standard control to compare with each group's growth curve. The number of surviving bacteria was calculated with the formula as $f \times e^{-g \times t_{inc,}}$ where *t*inc states incubation time required to attain the threshold OD value, *f* is a numeric number, and *g* is the slope of the line which indicate the rate of bacterial reproduction.

Measurement of DNA concentration

Collected supernatant from either CF sputum or PA14-stimulated neutrophil was diluted in HBSS 50-fold and mixed with Sytox Orange 10 μM to determine the concentration of extracellular DNA. Calf Thymus DNA (Boston bioproducts) was used as the standard and for calibration.

Immunostaining and fluorescent microscopy

Neutrophils were seeded on 12-mm glass coverslips (VWR International) in 24-well plates (Thermo Scientific). PA14, clinical isolates #035 and #155 were pre-opsonized with 10 % autologous serum for 30 min at 37˚C. After washing with HBSS, pre-opsonized bacteria were added to neutrophils and co-incubated in HBSS containing 0.2 % autologous serum for 3 hrs at 37˚C. Samples were fixed with 4 % paraformaldehyde (Affymetrix) and blocked with 5 % normal donkey serum (Jackson Immunoresearch Laboratories) with 0.1 % saponin (Sigma) in PBS for 30 min at RT. The following primary antibodies were used: clone MPO-7 monoclonal mouse anti-human MPO/FITC Ab (1:500, Dako), polyclonal rabbit anti-histone H4 (citrulline 3, 1:1000, Millipore) and rabbit anti-HNE (1:1000, Millipore) overnight at 4℃. For HNE and H4cit3 staining, Alexa Fluor® 594–labeled donkey anti-rabbit secondary Ab was used for 1 h (1:2000; Molecular Probes). Samples were stained with DAPI (1:20,000; Molecular Probes) for 2 min at RT and washed with PBS three times. Specimens were mounted with ProLong Antifade (Molecular Probes) and analyzed by Zeiss AxioCam HRM fluorescence microscopy (Axioplan2 imaging software).

MPO and HNE ELISA

MPO concentration in human neutrophil supernatants were measured by commercial ELISA kit (R&D Systems). Standard serial dilution was prepared from 125 ng/ml to use quantification of unknown samples. HNE was evaluated by ELISA. Supernatants diluted with coating buffer (25 mM carbonate, 25 mM bicarbonate, pH 9.6) were incubated overnight at 4˚C in 96-well high-binding microloan ELISA plates (Greiner bio-one). After blocking with 1 percent BSA for 1 hour at RT, rabbit anti-HNE polyclonal antibody (1:500 in PBS, Calbiochem) was added and allowed to incubate 2 hrs at RT. After three times washing with

0.05 % Tween 20, samples were incubated with horseradish peroxidase (HRP)-linked donkey anti-rabbit antibody (1:2000, GE Healthcare) for 1 hour at RT. 3,3',5,5'-tetramethybenzidine (TMB, Thermo Scientific) peroxidase substrate solution was used for blue coloration development. Reaction was stopped by adding 1 N hydrochloric acid (HCl, Sigma), and absorption was read at 450 nm wavelength by Eon microplate photometer (BioTek). Purified HNE standard (stock 1 mg/ml, CellSciences) was used to evaluate HNE concentrations in unknown samples.

Measurement of peroxidase activity

Peroxidase activity was assessed by hydrogen peroxide-dependent oxidation of Amplex Red (Molecular Probes). Undiluted neutrophil supernatants (50 μl) were added to 96-well nontransparent black microplates (Costar) and mixed with assay solution including 100 μM hydrogen peroxide (Sigma) and 100 μM Amplex Red. Fluorescent product was measured by a fluorescence plate reader (Varioskan Ascent, Thermo Scientific) for 30 min at 37˚C with excitation 560 nm and emission 590 nm wavelengths. Active MPO was used as a standard and for calculation of enzymatic activity of peroxidase in supernatant (stock 20 mmole/L).

Cell-free MPO activity test

Enzymatic activity of 500 μM MPO was measured with the Amplex Red oxidation method in a cell-free system in the presence of MEK1/2 inhibitors with indicated micromole concentration (U0126, MEK162 and PD98059). Results were normalized to inhibitor-free sample.

Measurement of PAD4 activity

PAD4 activity assay were performed by following manufacturer's protocol (PAD4 inhibitor screening assay kit, Cayman Chemical Company). In brief, 10 μl of CF sputum/each

sample were used for this assay. One hundred fifty microliter assay buffer was used for active PAD4 standard and sample dilution. After incubation at 37˚C for 30 min, 10 µl of PAD4 ammonia detector was added, followed PAD4 stop solution. After 15 min incubation at 37˚C, samples were read on a microplate reader, Varioskan (Thermo Scientific) with 405 nm excitation and 470 nm emission settings.

Total histone H3 evaluation

The Total Histone H3 ELISA kit was used by following manufacturer's protocol (Active Motif). Briefly, 50 µl of CF sputum/each sample were diluted into PBS (120 µl) after limited digestion of extracellular DNA by *Alu*I 100 U/ml (NEB) with CutSmart Buffer (NEB) for 30 min at RT. The diluted samples, total volume 50 µl/each well, were added onto the H3 histone pre-coated wells in triplicates. At this time, pre-diluted histone H3 standard (2000 ng/ml highest concentration) was added to the indicated wells for the calibration. After 1 hr agitating incubation, the anti-H3 antibody (1:1000) was added and incubated for 1 hr at RT with agitation. After three washes, HRP-conjugated anti-rabbit IgG antibody (1:2000) was added and incubated for 1 hr without agitation at RT. Following washes, TMB solution was added and the plate was read at 450 nm absorbance followed stop solution.

Statistical analysis

Data are shown as mean +/- SEM. Significance of independent variables was calculated with Student's t-test or one-way ANOVA with Tukey posttest for multiple comparisons. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Pearson correlation coefficient, r, was used for consideration of degree of correlation, and *p* value was used for significance.

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