Extracellular DNA, Neutrophil Extracellular Traps, and Inflammasome Activation in Severe Asthma

To cite this version:
Marrah Lachowicz-Scroggins, Eleanor Dunican, Annabelle Charbit, Wilfred Raymond, Mark Looney, et al.. Extracellular DNA, Neutrophil Extracellular Traps, and Inflammasome Activation in Severe Asthma. American Journal of Respiratory and Critical Care Medicine, 2019, 199 (9), pp.1076-1085. 10.1164/rccm.201810-1869OC . hal-02997946

HAL Id: hal-02997946
https://cnrs.hal.science/hal-02997946
Submitted on 10 Nov 2020

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Title: **EXTRACELLULAR DNA, NEUTROPHIL EXTRACELLULAR TRAPS, AND INFLAMMASOME ACTIVATION IN SEVERE ASThma**


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Author contributions: MLS, EMD and JVF designed the initial concept; MLS and EMD designed the experiments; MLS, EMD, ARC and WR carried out the experiments and/or analyzed the data; MRL and EL provided expertise and input on experimental design for NETs assays, MLS, EMD, MCP performed the statistical analysis; EMD, MCP, EDG, PGW, BRP, DTM, SAC, SCE, MWJ, NNJ, AMC, MC, ATH, ERB, MLF, SEW, EI, BDL and JVF aided in collection of and provided access to the longitudinal respiratory study samples for human analysis; MLS, EMD and JVF wrote the manuscript; MLS, EMD, MCP and JVF revised the manuscript; all authors reviewed the manuscript.
Abstract

Background: Extracellular DNA (eDNA) and neutrophil extracellular traps (NETs) are implicated in multiple inflammatory diseases. NETs mediate inflammasome activation and IL-1β secretion from monocytes and cause airway epithelial cell injury, but the role of eDNA, NETs, and IL-1β in asthma is uncertain.

Methods: We measured sputum eDNA in induced sputum from 399 asthmatics in the Severe Asthma Research Program (SARP)-3 and in 94 healthy controls. We subdivided asthma subjects into eDNA-low and -high subgroups to compare outcomes of asthma severity and of neutrophil and inflammasome activation. We also examined if NETs cause airway epithelial cell damage that can be prevented by deoxyribonuclease.

Results: We found that 13% of the SARP cohort is “eDNA-high”, as defined by sputum eDNA concentrations above the upper 95th centile value in health. Compared to eDNA-low asthma patients, eDNA-high patients had lower asthma control test scores, frequent history of chronic mucus hypersecretion, and frequent use of oral corticosteroids for maintenance of asthma control (all p values < 0.05). Sputum eDNA in asthma was associated with airway neutrophilic inflammation, increases in soluble NET components, and increases in caspase-1 activity and IL-1β (all p values < 0.001). In in vitro studies NETs caused cytotoxicity in airway epithelial cells that was prevented by disruption of NETs with deoxyribonuclease.

Conclusion: High extracellular DNA levels in sputum marks a subset of patients with more severe asthma who have neutrophil extracellular traps and markers of inflammasome activation in their airways.
Key words: asthma, neutrophils, extracellular DNA, caspase-1, myeloperoxidase, neutrophil extracellular traps, deoxyribonuclease, IL-1β.
Introduction

Although sputum neutrophils are associated with more severe subtypes of asthma [1], the role of neutrophils in asthma remains unclear. Recent studies have advanced understanding by drawing attention to the possibility of pro-inflammatory roles for extracellular DNA derived from neutrophils [2, 3] and the association between airway neutrophilia and airway inflammasome activation [4, 5]. Neutrophil-derived DNA is released in chromatin filaments that form web-like structures decorated with granular proteins called neutrophil extracellular traps (NETs), and NETs are established as mediators of a wide range of immune, inflammatory, and metabolic diseases [6]. The inflammasome is an intracellular molecular scaffold that supports autocatalytic cleavage of caspase-1 to drive processing and secretion of the pro-inflammatory cytokines IL-1β and IL18 [7]. IL1β is a prototypic proinflammatory cytokine [8, 9] and a plausible mediator of acute and chronic airway inflammation.

To explore the role of extracellular DNA (eDNA), NETs, and the inflammasome in severe forms of asthma, we measured multiple outcomes related to neutrophils, neutrophil activation, eDNA and the inflammasome in sputum from patients in the Severe Asthma Research Program (SARP)-3. We explored the relationship between sputum eDNA and asthma control outcomes, including outcomes related to mucus symptoms. In cell culture studies we also explored the effect of NETs on airway epithelial cells. Some of the results of the eDNA studies reported here have been previously been presented in abstract form [10].
Materials and Methods

Subjects

The Severe Asthma Research Program (SARP)-3 is a 3-year longitudinal cohort study in which 60% of subjects have severe asthma [11]. Severe asthma was defined according to the European Respiratory Society/American Thoracic Society consensus definition [12]. Data here is from 399 adult asthma participants who provided a sample of induced sputum that passed sputum quality assurance measures during two baseline visits. Healthy control subjects included 35 subjects recruited by SARP-3 and 59 healthy subjects recruited at UCSF. Patients with COPD were excluded from the study through exclusion of current tobacco smokers and those with significant past smoking history (>5 pack years if they were <30 years of age and >10 pack years if they were >30 years of age) and requirement for a positive methacholine challenge test or demonstration of bronchodilator reversibility to albuterol of at least 12%.

UCSF Healthy Subjects: Fifty-nine healthy control subjects had been recruited to research studies between 2005-2014. They had no history of pulmonary disease, atopic disease or allergic rhinitis, and had normal methacholine responses.

SARP-3 Healthy Subjects: Thirty-five healthy control subjects had no history of pulmonary disease, no history of atopic disease or allergic rhinitis, and had normal methacholine responses.

Chronic mucus hypersecretion (CMH): This was defined using the ATS/WHO definition of chronic bronchitis, which assesses chronic cough and sputum production in the preceding
2 years [13]. The specific question was: Have you had cough and sputum production on most days for at least 3 months a year for at least two consecutive years?

**Induced sputum:** Induced sputum (entire expectorate) was homogenized using methods previously described [14, 15] and centrifuged to yield a supernatant that was aliquoted for storage at -80°C. Each center shipped two aliquots to the UCSF SARP-3 center for measurement of analytes.

**Total and differential cell counts:** Cytocentrifuged sputum cells were stained with DiffQuik (ThermoFisher, Waltham, MA) and shipped from each SARP-3 center to the sputum cytology core (Wake Forest University) where the sputum cells were enumerated by light microscopy.

eDNA: eDNA was measured by first diluting the sputum supernatant sample 1:10 in 1X Tris-EDTA buffer. Next, double-stranded DNA (dsDNA) was quantified using Quant-iT™ PicoGreen™ dsDNA Assay Kit (ThermoFisher, Waltham, MA).

**Gene transcripts for IL-4, IL-5 and IL-13:** The combination of the gene expression levels of IL-4, IL-5, and IL-13 in a single quantitative metric ("Th2 gene mean") measures airway type 2 inflammation and we have recently reported the Th2 gene mean data in SARP-3 [16].

**Myeloperoxidase (MPO):** Induced sputum supernatant was diluted 1:2500 in 1X Tris-EDTA buffer and MPO was quantified using Human Myeloperoxidase DuoSet ELISA #DY3174 (R&D Systems, Minneapolis, MN).
**Soluble NETs components:** Induced sputum supernatant was diluted 1:10 in 1% BSA in PBS to measure neutrophil elastase (NE) and citrullinated histone (H3Cit) that are complexed to DNA using immunoassays previously described [17]. For the NE-DNA complexes, we used the C-17 sc-9520 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and for H3Cit-DNA complexes, we used the ab5103 antibody (Abcam, Cambridge, MA) as capture antibodies for sputum supernatant. An anti–DNA-HRP conjugate (Cell Death Detection ELISA Plus Kit, Roche, Indianapolis, IN) was used for the detection antibody. Luminescence at 450 nm was read using OptiBlaze ELISA femto solution (G-Biosciences, St. Louis, MO).

**Caspase-1 activity:** Caspase-1 was measured by diluting induced sputum supernatant 1:4 in PBS and incubating the sample 1:1 with Caspase-Glo® 1 buffer and Z-WEHD--aminoluciferin substrate using the Caspase-Glo® 1 Inflammasome assay #G9951 (Promega, Madison, WI)[18].

**IL-1β:** Sputum was diluted 1:5 in RD6-10 calibration diluent and IL-1β was quantified using the Human IL-1 beta/IL-1F2 QuantiGlo ELISA Kit #QLB00B (R&D Systems, Minneapolis, MN).

**IL-18:** Sputum was diluted 1:5 in RD1N calibration diluent and IL-18 was quantified using the Human Total IL-18/IL-1F4 Quantikine ELISA Kit #DL180 (R&D Systems, Minneapolis, MN).

**Western Blots:** 10 µL of sputum supernatant were denatured and run in 4-10% Bis-Tris gels in MES buffer on the NuPage system (Invitrogen). Proteins were transferred to PVDF membranes using the iBlot2 (Invitrogen) and blocked for 2h at room temperature in 5%
NFDM in TBS-T. Membranes were incubated overnight in primary antibody H3Cit (H3 ab5103, Abcam) and from Cell Signaling Inflammasome Antibody Sampler (#32961) caspase-1 D7F10, caspase-1 (asp297), IL-1β (D3UE3) and IL-1β(asp116) at a concentration of 1:1000 in TBS-T. Membranes were incubated the following day with goat anti-rabbit HRP 1:500 (Jackson Laboratories) for 2 hr at room temperature in TBS-T and developed using ECL Prime (GE Lifesciences). Membranes were viewed via chemiluminescence on a GE LAS 4010 Imager and sized using MagicMark (Invitrogen) on ImageQuant software.

**Neutrophil Extracellular Traps (NETs):** Neutrophils were isolated from 40 mL of blood from each of five healthy donors. Using Axis-Shield Polymorphprep (Cosmo Bio USA, Carlsbad, CA) by layering the blood over the density gradient solution and centrifuging. Neutrophils were re-suspended in RPMI media + 3% FCS at a density of 5 x10⁶ cells/mL. Neutrophils were stimulated with Phorbol 12-Myristate 13-Acetate (SigmaAldrich, St. Louis, MO) at a concentration of 250ng/ml until NETs were visualized [19].

**Stimulation of Airway Epithelial Cells with NETs:** Human bronchial epithelial cells were harvested from cadavers, as previously described [20]. Cells were plated on rat tail collagen coated flasks (Corning, Tewksbury, MA) and expanded in ROCK media [21]. Cells were plated on human placenta collagen-coated (SigmaAldrich, St. Louis, MO) 3460 or 3470 transwell inserts (ThermoFisher, Waltham, MA) submerged in Chu media [22], until cells formed confluence. After cells reached confluence on inserts (3-7 days), medium was changed to PnuemaCult ALI-media (StemCell Technologies, Cambridge, MA) and maintained at air-liquid interface for a minimum of three weeks before experimentation. The apical surface of the cells was exposed to either vehicle control (PBS) or NETs solution at a
DNA-NETs equivalent concentration of 6 µg/mL of DNA for 24 hours. In some experiments the NETs were disrupted by co-incubation for 24 hours with bovine pancreas DN25 (SigmaAldrich, St. Louis, MO) at a concentration of 10 Kunitz units per 6 µg of DNA-NETs.

Glucose-6-phosphate dehydrogenase (G6PD): G6PD is a cytosolic enzyme and leaks from cells when plasma membrane integrity is compromised [23]. We measured G6PD in the basolateral conditioned media using the Vybrant Cytotoxicity Assay #V23111 (ThermoFisher, Waltham, MA).

Gene Transcripts for IL-6 and IL-8 in Airway Epithelial cells: cDNA was generated from AECs RNA (20 ng) as previously described [24]. Measurement of genes associated with inflammation, remodeling and inflammasome were done via qPCR was performed using TaqMan Universal PCR Mix (ThermoFisher) on a ViiA7 and normalized to housekeeping genes as previously described [24, 25]. Primer sequences are provided in Supplemental Table 1.

IL-6 and IL-8 from Conditioned Media: Measurement of IL-6 and IL-8 was performed in 50 µL of basolateral condition media or cell culture media blank using the IL-6 and IL-8 Human ProcartaPlex™ Simplex Kits, Platinum (ThermoFisher) # EPXP010-10213-901 and EPXP010-10204-901, according to manufacture directions. Samples were read at the UCSF Parnassus Flow Cytometry Core using a Bio-Plex 200 and data generated in Bioplex 4.1 Software (Bio-Rad, Hercules, CA).

Statistical Methods

Analyses were performed using JMP 10 software package (SAS Institute, Cary, NC) and Stata 13.1 (StataCorp College Station TX). Two-group comparisons between eDNA-high
and eDNA-low asthma were made using Student’s t-test for continuous variables with roughly symmetric distributions, Wilcoxon’s rank-sum test for continuous variables with skewed distributions, and Pearson’s chi-square test for categorical variables. Spearman’s correlation was used to assess the relationships between continuous variables. Figures were generated using statistical software GraphPad Prism 7.0 (GraphPad Software, La Jolla CA). Box-and-whisker plots were prepared showing the median (marked by a horizontal line), 25% and 75% quartiles (box) and extreme values as far as 1.5 x interquartile range (IQR) beyond the limits of the box (whiskers). Data points further than 1.5IQR beyond the limits of the box are plotted as outliers. Tests were considered statistically significant with p-values represented as *p < 0.05, **p < 0.005 and ***p < 0.0001.
Results

Increased extracellular DNA (eDNA) in sputum from a subset of asthmatics reflects neutrophil activation.

The asthma patients were older and heavier than the healthy controls (Table 1). To calculate a reference interval for sputum supernatant eDNA in health, we followed the guidelines of the national committee for clinical laboratory standards [26]. First, we log transformed sputum eDNA values in healthy subjects to normalize the distribution and to determine the upper 95th centile value as the upper limit of normal (Fig 1A). This approach yielded 3.8 µg/mL as the cutoff value. We found that 13% of the asthma patients had sputum eDNA levels above 3.8 µg/mL, and we classified this asthma subgroup as “eDNA-high”. We classified the remaining asthma patients with sputum eDNA below 3.8 µg/mL as “eDNA-low”.

We considered the most likely cellular source of DNA in the sputum to be neutrophils, since the neutrophil is the most abundant granulocyte in sputum [27]. Indeed, among patients with asthma, we found no significant correlation between sputum eDNA and sputum eosinophils (Fig 1B) or between sputum eDNA and other measures of airway type 2 inflammation, such as the sputum Th2 gene mean [28] (Fig 1C). In contrast, sputum eDNA was significantly and positively correlated with sputum neutrophil % (Fig 1D), although the correlation was only moderate in strength. We reasoned that we would find a stronger relationship if we examined a marker of neutrophil activation. To explore the relationship between eDNA and neutrophil activation in sputum, we measured myeloperoxidase levels. Myeloperoxidase (MPO) is a peroxidase enzyme abundantly expressed by neutrophils and
secreted upon neutrophil activation by a wide variety of stimuli [29]. We found that sputum MPO levels strongly correlate with sputum eDNA levels (Fig 1E) and that sputum MPO levels were significantly higher in eDNA-high patients than in eDNA-low patients (Fig 1F). Taken together, these data indicate that activated neutrophils are the cellular source of eDNA in the asthma airway.

**eDNA-high asthma is characterized by multiple clinical indicators of asthma severity.**

We explored differences in the clinical features of the eDNA-high and -low asthma subgroups. Compared to eDNA-low asthma, we found that the demographic features of eDNA-high asthma were notable for older age and male predominance (Table 2). In comparing the clinical features of eDNA-high and -low patients, we noted that the eDNA-high subgroup had significantly lower asthma control test scores (Table 2, Fig 2A) and that the majority of eDNA-high patients had symptoms of chronic mucus hypersecretion (Table 2, Fig 2B). We also found that forced vital capacity was worse in eDNA-high asthma and the frequency of daily oral corticosteroid use in the eDNA-high subgroup was almost twice that in the eDNA-low subgroup (Table 2). Although the proportion of patients with 3 or more exacerbations in the previous year (exacerbation-prone) was higher in the eDNA-high subgroup than in the eDNA-low subgroup, the frequency of hospitalizations or emergency department visits for asthma in the prior year was not higher in the eDNA-high subgroup (Table 2). Regression analyses showed that age and gender did not significantly confound these relationships between sputum eDNA levels and clinical outcomes (Supplement Table 2).

**Soluble NETs components are increased in the eDNA-high asthma subgroup.**
We next returned to analyses of sputum and to measures of neutrophil extracellular traps (NETs). To quantify NETs, we measured complexes of neutrophil elastase (NE) and DNA (NE-DNA) and of citrullinated histone H3 (H3Cit) and DNA (H3Cit-DNA), using ELISAs recently described [17]. For these studies, we analyzed sputum from 44 eDNA-high patients, 42 randomly selected eDNA-low patients, and the 35 SARP healthy controls. We found that both NE-DNA and H3Cit-DNA complexes are increased in eDNA-high patients but not in eDNA-low patients (Fig 3A, 3B).

**Increased caspase-1 activity and IL-1β in sputum in eDNA-high asthma.**

To explore if caspase-1 activity is increased in sputum from eDNA-high patients, we assayed for caspase-1 using immunoblots and a recently described bioluminescent assay [30]. Caspases are inactive zymogens and caspase maturation into active caspase involves homodimerization and proteolytic processing to remove the pro-region and split the large subunit from the small subunit [9]. Using immunoblots, we found that increased full-length and activated caspase-1 protein (p20 and p10 bands, as indicated) and increased in eDNA-high patients (Fig 4A). Using the bioluminescent activity assay, we found very low caspase activity in sputum from healthy controls (n=35) and eDNA-low patients (n=42) but increased caspase activity in eDNA-high patients (n=44) (Fig 4B). To determine how much of the caspase activity detected in the bioluminescent assay reflects caspase 1, we measured caspase activity in four sputum samples in the presence of Ac-YVAD-CHO, a specific inhibitor of caspase-1. We found that Ac-YVAD-CHO decreased the caspase signal by more than 60% (Supplement Fig 1) indicating that caspase-1 is the dominant source of the bioluminescent signal but that some other caspases also contribute.
Increased IL-1β in sputum in eDNA-high asthma.

Since caspase-1 activity cleaves pro-forms of IL-1β to active mature forms, we looked for IL-1β in the sputum from eDNA-high patients. Using immunoblots to probe sputum supernatant from a limited number of subjects in each subgroup, we found increased IL-1β precursor and cleaved mature form of IL-1β in eDNA-high patients (Fig 4C). We also measured IL-1β using a high sensitivity ELISA. For these studies, we analyzed sputum from the 44 eDNA-high patients, 42 selected eDNA-low patients, and 35 SARP healthy controls. We found very low IL-1β levels in sputum from healthy controls and eDNA-low patients but easily detected and markedly increased IL-1β protein in eDNA-high patients (Fig 4D). We noted that the concentration of IL-1β in sputum varied widely in the asthma subgroup, and so we compared clinical variables among asthma patients with sputum IL-1β levels above and below the median split value. We found that the IL1β-high asthma subgroup had more severe asthma than the IL-1β-low subgroup. Specifically, compared to the IL-1β-low asthma subgroup, the IL-1β high asthma subgroup had a higher frequency of chronic bronchitis (29.6 vs. 61.4%, p=0.009), a higher frequency of being exacerbation prone (21.2 vs 46.9%, p=0.02), a lower FEV1 % predicted (69.0 v 85.0%, p<0.001), and a lower FVC percent predicted (78.0 vs. 94.0%, p<0.001).

Low levels of IL-18 in sputum in eDNA-high asthma.

IL-18 is a member of the IL-1 superfamily and, like IL-1β it is activated by caspase-1 in the inflammasome. In a subset of sputum samples from patients from the UCSF SARP-3 center, we assayed for IL-18 in sputum from healthy (n=14), eDNA-low (n=18) and eDNA-high (n=19) and subjects. We found that the levels of IL-18 were a log order lower than the
levels for sputum IL-1β and were not significantly different in the eDNA-high and low patients (Supplement Fig 2). Accordingly, we did not extend these IL-18 analyses to the full sputum samples set of eDNA-high and -low patients.

**NETs cause toxicity in airway epithelial cells**

In considering the pathogenic role of NETs in neutrophilic asthma, we hypothesized that NETs could produce airway injury by activating airway epithelial cells (AECs). To explore this possibility, we first generated NETs by activating neutrophils from freshly collected whole blood (five healthy donors) with phorbol 12-myristate 13-acetate (PMA). We then exposed airway epithelial cells in air liquid interface culture to NETs and found that they induced upregulation of IL-6 and IL-8 mRNA as well as secretion of these cytokines into the basolateral media (Fig. 5A and 5B). We also noted a large increase in G6PD release (Fig 5C). To control for the possible effect of PMA in the NETs preparations on AECs, in preliminary experiments, we included PMA as a control and found that it had no effect on G6PD release from epithelial cells (data not shown). We further found that the NET structure is required for this effect, because disrupting NETs with deoxyribonuclease (DNase1) largely prevented the NET-induced G6PD release (Fig 5C).
Discussion

We report that approximately 13% of the SARP-3 cohort has increased levels of extracellular DNA (eDNA) levels in their sputum. We find strong evidence that this extracellular DNA originates from neutrophils, including significant positive correlations between eDNA levels and sputum neutrophil percentages and eDNA levels and myeloperoxidase levels. Thus, extracellular DNA in sputum marks neutrophil activation in the airway and the relatively small but distinct subgroup of eDNA-high asthma patients are amongst the more severe patient in the SARP-3 cohort. Specifically, eDNA-high asthma is characterized by poor asthma control and frequent use of oral corticosteroids as maintenance therapy for asthma control. In addition, more than half of the eDNA-high asthma subgroup reported cough and sputum production in the questionnaire instrument used to capture symptoms of chronic mucus hypersecretion (CMH, also called chronic bronchitis).

Neutrophils can release their extracellular chromatin, nuclear protein, and serine proteases to form net-like structures called neutrophil extracellular traps (NETs)[6, 31, 32]. Soluble NET components such as neutrophil elastase-DNA (NE-DNA) complexes or citrullinated histone (H3Cit)-DNA complexes can be measured using immunoassays, and we find that NE-DNA and H3Cit-DNA complexes are increased in the sputum of the eDNA-high asthma subgroup. NETs are an important component of host defense against microbial infection, but they are known to cause collateral damage to the lung in pneumonia [17]. Indeed, NETs cause airway epithelial cell death and detachment and induce autoantigen production in airway epithelial cells [33-36]. We report here that NETs cause airway epithelial cell injury (G6PD release) and that NET structure is required for this effect, because disrupting
NETs with deoxyribonuclease blocks NET-induced G6PD release. This finding raises the intriguing possibility that inhaled deoxyribonuclease could prevent NET-driven epithelial cell damage in the airway and have therapeutic benefit in eDNA-high patients.

Consistent with previous studies [3], we found that IL-1β levels are increased in eDNA-high asthma, and other studies in severe asthma have also reported increased in sputum IL-1β [15, 37]. IL-1β is cleaved to its secreted cytokine isoform by caspase-1 that is activated in the inflammasome [7]. We show here that caspase-1 levels are increased in eDNA-high patients and thus provide direct evidence for inflammasome activation in at least a subset of asthma patients that is marked by high sputum levels of eDNA. Taken together, our data suggest that a subset of patients with severe asthma have neutrophil activation in their airways, including NETs, and that NETs may activate the inflammasome in resident cells such as monocytes or macrophages to cause secretion of IL-1β [38]. The reasons for neutrophil activation and inflammasome activation in eDNA-high asthma are not revealed by our study, but airway infection should be considered, especially since this inflammation signature (airway neutrophilia, inflammasome activation, and IL-1β responses) is present in a mouse model of infection-mediated steroid resistant asthma [4]. An alternative possibility is the caspase signal we detect in eDNA-high asthma is indicative of airway pyroptosis that could promote GSDMD-mediated NETosis [39, 40]. Although mitochondrial DNA (mtDNA) is a source of extracellular DNA in NETs, we did not investigate the role of mtDNA in our study. Viable eosinophils and neutrophils release mtDNA in a reactive oxygen species (ROS)-dependent manner, in the absence of nuclear proteins and independently of cell death [41, 42]. The presence of H3Cit suggests the source of eDNA is from the nucleus. Formation of H3Cit is part of chromatin decondensation in the nucleus during NETosis [43].
Given that we see nuclear proteins and a signature associated with IL-1β/caspase-1 activation in the sputum, our data suggests the source of eDNA is from the nucleus in activated cells undergoing cell death [44]. All participants in SARP-3 were enrolled at a time when they had been free of an airway infection or asthma exacerbation for at least 4 weeks, so it is unlikely that an acute airway infection explains neutrophil activation in their airways. But it is possible that these patients have chronic airway infection or microbial dysbiosis [45] - our study did not investigate this possibility. However, our findings do suggest novel treatment possibilities for eDNA-high asthma, such as disruption of NETs with inhaled deoxyribonuclease or treatments directed at the inflammasome, including inhibition of IL1β.
References


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Figure Legends

Figure 1: Increased extracellular DNA (eDNA) in sputum from a subset of asthmatics is associated with increased neutrophil numbers and neutrophil activation. (A) A subset of asthmatics has sputum eDNA levels above the 95th centile value in healthy control subjects. (B) Sputum eDNA levels are not significantly correlated with sputum eosinophils. (C) Sputum eDNA levels are not significantly correlated with the Th2 gene mean. (D) Sputum eDNA levels are significantly correlated with sputum neutrophils. (E) Sputum eDNA levels are significantly correlated with myeloperoxidase (MPO) levels. (F) Sputum MPO levels are significantly higher in eDNA-high asthma than in eDNA-low asthma. ***Indicates p < 0.001. Circles represent individual data points.

Figure 2: eDNA-high asthma is associated with poor asthma control and symptoms of chronic mucus hypersecretion but not with airway mucus plugging. (A) The asthma control test (ACT) score is significantly lower in eDNA-high asthma than in eDNA-low asthma. (B) Chronic mucus hypersecretion (CMH or chronic bronchitis) is more prevalent in eDNA-high asthma than in eDNA-low asthma. Chronic mucus hypersecretion data were available for 297 DNA-low patients and 40 DNA-high patients. *Indicates p < 0.05. ***Indicates p < 0.001. Circles represent individual data points.

Figure 3: Soluble NET complexes are higher in eDNA-high asthma than in eDNA-low asthma. (A) Neutrophil elastase (NE)-DNA complexes are significantly higher in eDNA-high asthma than in eDNA-low asthma. (B) Citrullinated histone H3 (H3Cit)-DNA complexes are significantly higher in eDNA-high asthma than in eDNA-low asthma. Healthy n=35, eDNA-low n=42 and eDNA-high n=44. RLU indicates relative luminometer units. *Indicates p < 0.05; ** Indicates p < 0.01; ***Indicates p < 0.001. Circles represent
individual data points. **Figure 4: Caspase-1 activity and IL-1β levels are higher in eDNA-high asthma than in eDNA-low asthma.** (A) Western blot data for caspase 1 in sputum from 3 healthy controls, 3 patients with eDNA-low asthma and 3 patients with eDNA-high asthma. The bands show caspase-1 zymogen and cleavage at Asp297 yielding the activated protease bands p20 and p10. (B) Bioluminescence data for caspase-1 in sputum showing significantly higher levels in eDNA-high asthma (n=44) than in eDNA-low asthma (n=42) or healthy controls (n=35). (C) Western blot data for IL1β in sputum from 3 healthy controls, 3 patients with eDNA-low asthma and 3 patients with eDNA-high asthma. The data show cleavage of IL-1β at Asp116 in eDNA asthma. (D) ELISA data for IL-1β in sputum showing are significantly higher levels in eDNA-high asthma (n=44) than in eDNA-low asthma (n=42) or healthy controls (n=35). ***Indicates p < 0.001. Circles represent individual data points. **Figure 5: NET-mediated injury to airway epithelial cells.** (A) Apical exposure of airway epithelial cells (AECs) grown at air liquid interface (ALI) to NETs (6 µg/mL) for 24h causes upregulation of gene expression for IL-6 and IL-8 (data are from epithelial cells from 7 donors). (B) Apical exposure of AECs) grown at ALI to NETs (6 µg/mL) for 24h causes release of IL-6 and IL-8 protein into the conditioned media (data are from epithelial cells from 7 donors). G6PD release into conditioned media of airway epithelial cells in air liquid interface culture following exposure to NETs (6 µg/mL) at the apical surface for 24 h in the presence or absence of DNase I co-treatment (data are from epithelial cells from 5 donors). Error bars represent S.E.M. *Indicates p<0.05 and ***indicates p < 0.0001. Circles represent individual data points.
Table 1. Characteristics of healthy subjects and subjects with asthma

<table>
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<th>Healthy SARP (n = 35)</th>
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<td>44 (28, 64)</td>
<td>62 (35, 78)</td>
<td>51 (34, 74)</td>
</tr>
<tr>
<td>Macrophages †</td>
<td>36 (28, 50)</td>
<td>25 (13, 50)</td>
<td>28 (13, 43)</td>
</tr>
<tr>
<td><strong>Blood counts (x10⁶/L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosinophils †</td>
<td>130 ± 106</td>
<td>143.7 ± 79.8</td>
<td>295 ± 279</td>
</tr>
<tr>
<td>Neutrophils †</td>
<td>3387 ± 1080</td>
<td>3269 ± 1044</td>
<td>4511 ± 2149</td>
</tr>
<tr>
<td>Serum IgE, IU/mL †</td>
<td>19 (10, 49)</td>
<td>42 (15.6,99.4)</td>
<td>153 (48, 363)</td>
</tr>
<tr>
<td>FeNO (ppb) †</td>
<td>16 (11, 21)</td>
<td>16 (11, 24)</td>
<td>22 (13, 38)</td>
</tr>
<tr>
<td>Pack year smoking history</td>
<td>-</td>
<td>-</td>
<td>0.88 ± 2.13</td>
</tr>
</tbody>
</table>

Data reported as mean ± standard deviation or median (interquartile range). UCSF=University of California San Francisco. SARP=Severe Asthma Research Program. FEV 1=forced expiratory volume in 1 s. FVC=forced vital capacity. FeNO= fractional exhaled nitric oxide. ppb=parts per billion.

* p<0.05 for comparison between Healthy UCSF and Asthma SARP
† p<0.01 for comparison between Healthy SARP and Asthma SARP
‡ p<0.001 for comparison between Healthy UCSF and Healthy SARP

Blood counts were not available for 2 healthy UCSF subjects, 5 healthy SARP subjects and 1 subject with asthma.

Serum IgE was not available for 2 healthy UCSF subjects, 5 healthy SARP subjects and 2 subjects with asthma.

FeNO was not measured in 11 healthy UCSF subjects, 3 healthy SARP subjects and 3 subjects with asthma.
| Table 2. Characteristics of subjects with asthma stratified by DNA concentration |
|---------------------------------------------------|-------------------------------|-------------------------------|-------------------------------|
|                                                   | All (n = 399)                 | eDNA-low (n = 346)            | eDNA-high (n = 53)            |
| Age (yr) ‡                                       | 47.6 ± 13.9                   | 46.6 ± 13.8                   | 54.0 ± 12.3                   |
| Female, no. (%) †                                 | 263 (65.9)                    | 238 (68.8)                    | 25 (47.2)                     |
| Body mass index (kg/m²)                           | 32.7 ± 8.6                    | 32.4 ± 8.3                    | 34.3 ± 10.2                   |
| Maintenance corticosteroid use, no. (%)           |                               |                               |                               |
| Inhaled, any dose                                 | 355 (89.0)                    | 358 (89.5)                    | 44 (95.7)                     |
| Inhaled, high dose                                | 246 (61.7)                    | 245 (61.3)                    | 32 (69.6)                     |
| Systemic †                                       | 66 (16.5)                     | 52 (15)                       | 14 (26.4)                     |
| Severe Asthma, no. (%)                            | 246 (61.7)                    | 211 (61.0)                    | 35 (66.0)                     |
| Exacerbations in last 12 months                   |                               |                               |                               |
| ER visits in last 12 months                       | 94 (23.6)                     | 84 (24.3)                     | 10 (18.9)                     |
| Hospitalizations in last 12 months                | 43 (10.8)                     | 38 (11.0)                     | 5 (9.4)                       |
| Exacerbation prone, no. (%) †                     | 94 (23.7)                     | 73 (21.1)                     | 21 (39.6)                     |
| Spirometry                                        |                               |                               |                               |
| FEV1% of predicted volume                         | 72.4 ± 19.2                   | 72.9 ± 19.2                   | 68.7 ± 19.3                   |
| FVC% of predicted volume ‡                        | 84.7 ± 16.5                   | 85.7 ± 16.6                   | 78.2 ± 14.4                   |
| FEV1/FVC                                          | 0.84 ± 0.12                   | 0.84 ± 0.12                   | 0.86 ± 0.14                   |
| FeNO (ppb)                                       | 22 (13, 38)                   | 22 (14, 38)                   | 20 (13, 38)                   |
| Blood                                            |                               |                               |                               |
| Neutrophil count (x10⁶/L) *                       | 4511 ± 2149                   | 4397 ± 1970                   | 5248 ± 2990                   |
| Eosinophil count (x10⁶/L)                         | 295 ± 279                     | 299 ± 292                     | 268 ± 180                     |
| IgE, IU/mL                                        | 153 (48, 363)                 | 154 (50, 368)                 | 129 (39, 320)                 |
| Sputum                                            |                               |                               |                               |
| Neutrophil count (x10⁶/L) ‡                       | 476                           | 407 (173, 921)                | 1553 (867, 5200)              |
| Neutrophil % ‡                                    | 52.1 (34, 74)                 | 49.5 (32, 68)                 | 79.4 (56, 90)                 |
| Eosinophil count (x10⁶/L) *                       | 7                             | 6 (0.3, 41)                   | 30 (2, 62)                    |
| Eosinophil %                                      | 0.8 (0.2, 3.0)                | 0.8 (0.2, 3.1)                | 0.7 (0.2, 2.6)                |
| Macrophage count (x10⁶/L) *                       | 244                           | 231 (109, 480)                | 384 (152, 873)                |
| Macrophage % ‡                                    | 27.6 (13, 43)                 | 29.2 (15, 45)                 | 14.5 (5, 30)                  |
| Pack year smoking history                         | 0.88 ± 2.13                   | 0.85 ± 2.02                   | 1.07 ± 2.75                   |

Data reported as mean ± standard deviation or median (interquartile range). ACT= Asthma Control Test. Exacerbations defined as taking a short course of oral corticosteroids for asthma (min. 3 days). Exacerbation prone defined as 3 or more exacerbations in the last 12 months.

* p<0.05 for comparison between DNA-low and DNA-high groups
† p<0.01 for comparison between DNA-low and DNA-high groups
‡ p<0.001 for comparison between DNA-low and DNA-high groups

Exacerbation data were missing in 3 DNA-low patients. FeNO measurements were missing in 2 DNA-low patients and 1 DNA-high patient. Blood counts were missing in 1 DNA-low patient. Serum IgE measurements were missing in 1 DNA-low patients and 1 DNA-high patient.
Figure 1

A. eDNA concentration, µg/mL

B. Sputum Eosinophils, %

C. Th2 Gene Mean

D. Sputum Neutrophils, %

E. MPO concentration, µg/mL

F. MPO concentration, µg/mL

Spearman's ρ = 0.64
p < 0.001

Spearman's ρ = -0.02
p = 0.8

Spearman's ρ = 0.34
p < 0.001

Spearman's ρ = 0.64
p < 0.001

Healthy Asthma
Figure 3

A. NE-DNA complexes (RLU x 10^3)

B. H3Cit-DNA complexes (RLU x 10^3)

Healthy  eDNA-low  eDNA-high

*  **  ***

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Figure 5

A. Log2 Normalized Gene Expression

B. pg/mL

C. G6PD Release (24 hours, RFU x 10^2)

IL-6  |  IL-8

Control | NETs

IL-6  |  IL-8

Control | NETs

Control | NETs + DNase I

Significance: * p < 0.05  *** p < 0.001

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Supplementary Appendix

Title: EXTRACELLULAR DNA, NEUTROPHIL EXTRACELLULAR TRAPS, AND INFLAMMASOME ACTIVATION IN SEVERE ASTHMA


Severe Asthma Research Program-3 Investigators

*These authors contributed equally.

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Email: john.fahy@ucsf.edu.
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer type</th>
<th>Sequence (5' to 3')</th>
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<td>PPIA</td>
<td>Forward RT</td>
<td>ATGAGAACTTCATCCTAAAGCATACG</td>
</tr>
<tr>
<td>PPIA</td>
<td>Reverse RT</td>
<td>TTGGCAGTGAGATGAAAAACT</td>
</tr>
<tr>
<td>PPIA</td>
<td>TaqMan Forward</td>
<td>ACGGGTCTCTGGCATCTTTGT</td>
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<tr>
<td>PPIA</td>
<td>TaqMan Probe</td>
<td>ATGGGAATAATGCTGGGACCAACACA-FAM(BHQ)</td>
</tr>
<tr>
<td>PPIA</td>
<td>TaqMan Reverse</td>
<td>GCAGATGAAAAACTGGGAACCA</td>
</tr>
<tr>
<td>EEF1A1</td>
<td>Forward RT</td>
<td>TGCTAACATGCCTTGGTTCAAG</td>
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<tr>
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<td>Reverse RT</td>
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<tr>
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<td>CCTTGCTTCAAGGGATGGAA</td>
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<td>TaqMan Probe</td>
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<tr>
<td>EEF1A1</td>
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<td>Forward RT</td>
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<tr>
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<td>TaqMan Probe</td>
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<td>TaqMan Reverse</td>
<td>GCCCTCTTCAAAAAACTTTCTCCACACCC</td>
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<td>Outcome variable</td>
<td>Predictor variable</td>
<td>Model 1</td>
</tr>
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<td>----------------------------------</td>
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</tr>
<tr>
<td>Asthma Control Test score</td>
<td>eDNA concentration</td>
<td>-0.21 (-0.36, -0.05) p=0.012</td>
</tr>
<tr>
<td>Chronic Mucus Hypersecretion</td>
<td>eDNA concentration</td>
<td>1.19 (1.10, 1.29) p&lt;0.001</td>
</tr>
<tr>
<td>FVC, % predicted</td>
<td>eDNA concentration</td>
<td>-1.12 (-1.66, -0.59) p&lt;0.001</td>
</tr>
<tr>
<td>Oral corticosteroid use</td>
<td>eDNA concentration</td>
<td>1.12 (1.04, 1.21) p=0.003</td>
</tr>
<tr>
<td>Exacerbation prone</td>
<td>eDNA concentration</td>
<td>1.11 (1.04, 1.19) p=0.003</td>
</tr>
</tbody>
</table>

Asthma Control Test and FVC outcome variables were analysed using linear regression models. Chronic mucus hypersecretion, oral corticosteroid use, and exacerbation prone outcome measures were analysed using logistic regression models. Data are presented as a &beta; coefficient or odds ratio, with 95% confidence interval and p value.

Model 1 – unadjusted model
Model 2 – model adjusted for age at enrolment
Model 3 – model adjusted for gender
Model 4 – model adjusted for age at enrolment and gender.
Supplement Figure 1: Caspase-1 activity in sputum is inhibited by the Ac-YVAD-CHO inhibitor. Ac-YVAD-CHO decreased the caspase bioluminescent signal (n=4).

Bars represent S.E.M. *Indicates p<0.05
Supplement Figure 2: IL-18 levels in sputum in asthma subgroups. IL-18 levels in sputum are low and not significant different among eDNA-high asthma (n=19), eDNA-low asthma (n=18) or healthy control (n=14) subgroups.