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Emerging Evidence Supports the Hypothesis that Neutrophil Extracellular Traps are a Major Factor in Genesis and Progression of Chronic Obstructive Pulmonary Disease

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ABSTRACT

Since their discovery about fifteen years ago, neutrophil extracellular traps (NETs) have been recognized as an intrinsic part of vertebrate innate immunity and inflammatory response. Consisting of entangled strands of extracellular DNA decorated with histones, elastase, myeloperoxidase and other proteins, NETs entrap and kill pathogens, but are increasingly also found to contribute to acute and chronic inflammatory disease due to their toxicity to host cell and autoimmunity induction. Chronic obstructive pulmonary disease (COPD) turned out to be among the major disorders involving overshooting formation of NETs and associated adverse effect. In the present review, we summarize the progress in knowledge on the role of NETs in COPD pathology made since our first reports on this subject. We highlight recent substantial advances and discuss possible cause-and-effect relationships, connections with common comorbidities and interactions with drugs, also to illustrate the importance of NETs as a future diagnostic tool and target for new medication strategies.

COPD is a progressive inflammatory airway disease, usually following long-term exposure to environmental insults. The main causal factor for developing COPD is inhaled tobacco smoke[1]. COPD affects around 10% of the adult population in industrialized countries[2] and has substantial impact on the quality of life and life expectancy[3]. It is the third leading cause of death on the global scale[4] and the sixth leading cause of death in countries with high socio-demographic index[5]. The disease varies in clinical presentation, often involving recurrent bacterial infection, massive neutrophil infiltration, and emphysematous alveolar wall destruction[6]. COPD is frequently still characterised into distinct 'phenotypes' based on varying criteria[7–9] (caveats defined by Agusti[10]). In many cases, periods of stable condition alternate with episodes of worsening (exacerbations), leading to increasing small airway obstruction and lung function impairment. Lung function decline is also a key basis of disease assessment according to international guidelines[11].

Extracellular traps (ETs) consist of entangled threads of DNA with dimensions down to 2 nm, associated with histones, elastase, myeloperoxidase (MPO) and other proteins that are antimicrobially effective, but also potentially cytotoxic[12,13]. ET formation (ETosis) by phagocytes is an intrinsic part of vertebrate innate pathogen defense and inflammatory response[12]. In humans, ETs are most frequently formed by neutrophils, then being abbreviated as NETs. Evidence on the existence of NETs is quite recent, first traces (although not explicitly designated) dating back to 1996[14], followed

by a comprehensive description not until 2004[15]. Since then, NET formation (NETosis) has turned out to be a versatile and multifaceted process with diverging signaling pathways and morphological execution[12,16,17]. NETosis is induced by a variety of host- or pathogen-derived molecular signals including chemokines such as inlerleukin-8 (IL-8), tumour necrosis factor alpha (TNFα), lipopolysaccharids, N-formylmethionyl-leucyl-phenylalanine (fMLP) and antineutrophil cytoplasmic antibodies (ANCAs), but also by calcium ionophores[18,19] and pharmacological agents such as phorbol-12-myristate-13-acetate (PMA)[12,15] and nicotine[20,21]. Some variants of NETosis including those assumed to be modifications of the autophagy pathway depend on reactive oxygen species (ROS) which may act on the process from both inside and outside the cell[22–24]. The main source of ROS involved in NETosis pathways are most probably subtypes of NADPH oxidase (Nox), but involvement of ROS from other sources (e.g. mitochondrial oxphos complexes, catalytic activity of myeloperoxidase, and nitric oxide synthase) is also proven[25–29]. ROS-independent NETosis is mediated by calcium ionophores[19] and uric acid[30]. Transcriptome analyses have shown that DNA transcription at multiple chromosome loci during chromatin decondensation is a general feature of NETosis and occurs faster in the ROS-independent form than in the ROS/Nox-dependent form[31]. However, LPS- and PMA-induced NETs have been shown not to require protein translation for their formation, all necessary factors being already contained in the differentiated neutrophils emerging from bone marrow[32].

Upon mitogenic stimulation and independent of ROS, NET-forming neutrophils were shown to employ the cell cycle kinases CDK4/6 for an incomplete activation of cell cycle reactions advancing until nuclear envelope breakdown[33].

Before this background, nuclear chromatin is increasingly placed in a dual assignment, with a fully-fledged second function in immunity that combines direct anti-pathogen action with a prominent alerting role via activation of DNA receptors such as Toll-like receptor(TLR)-9[34].

The best described form of NETosis releases DNA from nuclear chromatin and leads to cell death[12,35]. Chromatin decondensation depending on histone citrullination by peptidyl arginine deiminase 4 (PAD4)[36,37] is commonly regarded as a key feature of this type and used as an immunodiagnostic tool. Together with nuclear swelling, loss of intracellular organization, membrane rupture and extrusion of cell content, this constitutes a sequential morphological pattern also referred to as 'NETotic cascade'[12,38]. Caveats resulting from recent re-evaluation of PAD-mediated citrullination[24] require further consideration. Other mechanisms have been shown to leave NET-forming cells viable, and may also utilise mitochondrial DNA[16,39].

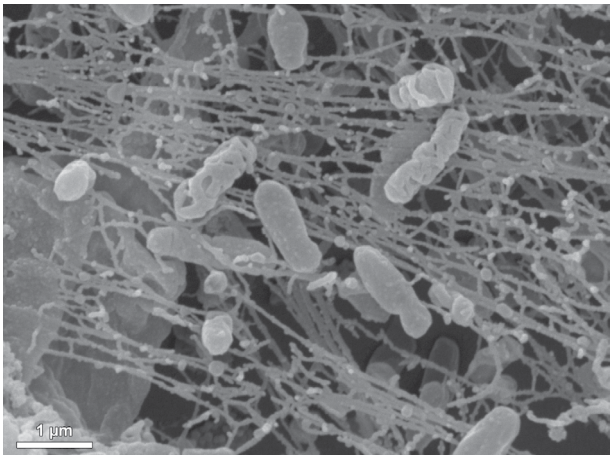

Figure 1: Scanning electron micrograph of in-vitro induced NETs entrapping *Pseudomonas* bacteria (Image: Astrid Obermayer, Salzburg).

Since their discovery[15], NETs have been found to act in a fragile balance between beneficial and harmful. On the one hand, they aid the entrapment and removal of prokaryotic and eukaryotic pathogens[40] (Figure 1) and are also formed during viral infections[41]. On the other hand, NETs have been identified as toxic to host cells[42], contributors to organ failure (e.g by interaction with platelets[43]), and potent inductors of autoimmunity[44]. Their formation in excess or in the wrong place, or their insufficient clearance, was found to be highly associated with severe acute illness and chronic inflammation[45,46]. This applies particularly to airway diseases such as allergic asthma[47], cystic fibrosis (CF)[48] and COPD.

Just like with CF, COPD had been a long-standing candidate for NETosis-mediated negative effects due to association with bacterial and viral infection[49,50] and neutrophil infiltration[6]. Previous work from our lab using a combination of electron microscopy, fluorescence light microscopy and CLSM image-based morphometry has for the first time confirmed the presence of large amounts of NETs in the sputa of patients with exacerbated COPD. NET abundance was found to correlate with the degree of airflow limitation as measured by forced expiratory volume in one second (FEV1). We also found elevated NETosis in stable COPD and in smokers with still normal lung function[13,51]. These findings have since been substantially confirmed and expanded by the work of other groups: Extracellular DNA levels and content of NET associated protein in sputum has been shown to be significantly higher in COPD patients than in controls[52,53], and spontaneous NET formation of neutrophils isolated from COPD sputum was found to be increased[52]. Interestingly, peripheral blood neutrophils of exacerbating COPD patients were found to diverge from

those of stable COPD patients and controls in that they showed decreased *in vitro* NET formation in response to inflammatory stimuli, even though the plasma of the exacerbators contained elevated levels of extracellular DNA[54]. The culminating work to date is perhaps the comprehensive study of Dicker et al[55]. Applying a variety of methods to measure NET content in COPD sputa, these authors show a significant association between sputum NET concentrations and various parameters of disease severity. By presenting new assays for facilitated non-microscopy based quantification of NETs, they made an important step further toward making sputum NET content usable as a diagnostic tool applicable in routine laboratory analysis. By showing that sputum NET concentrations correlate with a decreased microbiome diversity, and that the presence of NETs reduces airway neutrophil phagocytosis, scientific progress was also achieved in testing the potential role of microbiota in this context. Other recent work using *in vitro* and *ex vivo* test systems has further corroborated the promotive role of cigarette smoke on ET formation by neutrophils and macrophages (METs), accompanied by the release of NE and matrix metalloproteinases (MMPs), and initiation of T-cell-mediated immune response[56,57].

All this provides substantial support to the hypothesis that excess NET formation together with an unfavourable change in the phagocytosis/NETosis-balance[58] impairs full clearance of bacterial infections in COPD airways, thus driving a self-perpetuating cycle of inflammation and NET formation, as already shown for cystic fibrosis[59].

The increasing amount of data on the subject (evidence also comprehensively reviewed by Liu et al.[60]) encourages to further intensify research effort on the role of NETs in COPD, specifically to establish a more complete picture of the cause-and-effect relationships between the various aspects of the disease. Specific topics deserving to be further pursued concern molecular and biochemical interactions of intrinsic NETosis factors with the tissue environment (topics i – iv), and interrelations with individual circumstances of the disease, therapy and life cycle (topics v – viii).

(i) Histones. Core histones are known to account for 70% of all NET-associated proteins[61], and nucleosome histone complexes are major structural constituents of fine NET threads[13]. Together with MPO, extracellular histones have been identified as main factors in NET-mediated cytotoxicity[62]. Signalling pathways involve Toll-like receptors, complement molecules, membrane-bound phospholipids, and proinflammatory chemokine release mediated by MyD88, NFκB, and inflammasomes[63,64]. Citrullinated histone H3 was found to evoke endothelial barrier dysfunction via opening of adherens junctions and promotion of stress fibre formation in the cytoskeleton[65]. The relevance of all this is still unproven for COPD, as is the extent to which histone co-aggregation with pentraxin 3 (PTX3), a further protein component of NETs, is able to protect COPD airway epithelia from histone-mediated cytotoxicity[66].

(ii) Neutrophil elastase (NE). The serine protease NE (elastase 2) is another protein that is usually abundantly present in NETs. It has been attributed with a crucial role in emphysema development in animal model studies and human COPD already before NETs were first identified[67]. Over the years, a variety of animal models of emphysema induction by elastase instillation to the airways have been developed and tested, alone or in conjunction with other agents such as cigarette smoke, for suitability to mimic emphysema development at temporal, histological and molecular levels[68–70]. Regulatory pathways through which NE contributes to emphysema development are multifactorial and interdigitated, involving matrix metalloproteinases (MMPs), AMP-activated protein kinase (AMPK), α1,6-fucosyltransferase (Fut8), Wnt3a/b-catenin, and nuclear factor erythroid-2 related factor-2 (Nrf2), among others[71,72]. A relation to NADPH oxidase 2 (Nox)-dependent ROS production[72,73] and ROS-mediated regulation of neutrophil interleukin-1 beta (IL-1β) secretion is obvious, differential coupling to pathways regulating autophagy and apoptosis seems probable[74,75]. However, it is unclear how all this contributes to the interdependency between NETosis and emphysema in COPD, – all the more so because the origin of extracellular NE may be diverse (see topic iii). An insufficiently explained side aspect in this context is also whether COPD emphysema and idiopathic pulmonary fibrosis (IPF) are in fact final conditions resulting from divergent derangement of a similar set of signalling pathways under similar noxious influence[76].

(iii) Origin of neutrophil serine proteases (NSPs). Even when found associated with NETs, neutrophil proteases (in addition to NE also cathepsin G and proteinase 3[77]) may derive from NETosis-independent exocytosis (degranulation) induced by various factors[78,79], including macrolid antibiotics[80]. The contribution of NETosis-independent NSPs in COPD-related tissue damage may be substantial, not least due to secondary effects such as NSP-induced increased activation of proinflammatory cytokines (e.g. IL-1a, IL-36)[81,82].

(iv) ETs formed by eosinophils (EETs) and macrophages (METs). Eosinophilic inflammation is a common phenotype in COPD[83,84]. Eosinophils have been shown to undergo at least two types of EET formation: cell death ETosis that cytolytically releases eosinophil granules[85], and a specialised type of ETosis during which mitochondrial DNA is extruded in a catapult-like manner[86]. Recent research further strengthens a possible role of eosinophils in the pathophysiology of COPD phenotypes, notably already in the initial phase, and irrespective of whether patients quit

smoking, with EETosis cell debris acting as a promoter of uncontrolled NETosis[8]. Recent work additionally suggests a relevant contribution of METs in COPD emphysema, especially under the influence of cigarette smoke[56].

(v) Longitudinal relations. The information that NETosis intensity in COPD correlates with exacerbation derives mainly from cross sectional studies[51,55], and provide no evidence on the course of NET levels through the sequence of exacerbations, their regressions, and intermittent stable phases in the individual patient. Accordingly, it is presently still impossible to discern whether increased NETosis is a result of, or a reason for exacerbations or whether NETs levels have an influence on the general progression of the disease.

(vi) Comorbidities. COPD is in many cases associated with comorbidities including lung cancer and other cancers, cardiovascular disease and diabetes. The crucial connecting factor appears to be systemic inflammation which is inherent to COPD and provides a promoting environment for various comorbidities[87]. In this context, ample evidence suggests that particularly the role of NETs and NET-components is worthy further examination. Deregulated inflammation with aberrant release of cytokines and ROS is known to generate a carcinogenic milieu in the lungs[88], and NETs have been shown to be promotors of a tumorigenic microenvironment[89]. A similar association with NETosis has been established for cardiovascular disease including acute coronary syndrome[46,90] and for diabetes[91,92].

(vii) Drugs and stimulants. Influence on COPD NETosis by adjuvant medication and stimulants is as yet unexplored. Candidate substances among medical drugs include N-acetylcysteine (applied for routine mucolytic treatment) and phosphodiesterase (PDE_4) inhibitors. *In vitro* data show that N-acetylcysteine is able to reduce the ability of human neutrophils to form NETs under low ROS levels[93]. Implications under the high and heterogeneous ROS levels in COPD airways[94] are presently unexplored. PDE_4 inhibitors are increasingly established in COPD medication to improve lung function and reduce the likelihood of exacerbations. Although there is as yet no direct evidence that PDE_4 inhibitors affect NETosis, work on prostaglandin (PGE_2) has shown that NETosis may be downregulated by cyclic adenosine monophosphate (cAMP). There is still no enquiry as to how PDE_4 inhibitor-mediated elevated intracellular cAMP exerts influence on airway neutrophil NETosis. A further open issue in this context is NETosis promotion by nicotine[20,21] in persistently smoking COPD subjects.

(viii) Influence of age. COPD-associated NETosis is linked to age-related phenomena in at least two ways: On the one hand, NETosis-driven chronic low-grade inflammation and high ROS levels accelerate tissue senescence via various molecular mechanisms also entailing depletion of precursor cells[7,95,96]. This designates NETosis as a major factor in lung 'inflammaging'[97,98], and supports the classification of COPD as a condition of accelerated/abnormal lung aging[7,99]. On the other hand, advancing age of both, the individual cells and the organism, modulates the neutrophils' capacity to release NETs. This likely alters the balance of beneficial and detrimental effects of NETosis throughout the course of life in a complex and therapeutically relevant manner[100].

In conclusion, the research in the wake of our first publications on the subject [13,51] has to a large extent confirmed the hypothesis that NETosis levels in COPD correlate with disease severity, thus further highlighting the relevance of COPD-related NETosis as a promising target of diagnose and new medication strategies. Data available to date provide sound encouragement for several promising lines of research to be followed in the immediate future in order to improve the overall clinical outcome of the disease. The restricted scope of clinical trials on human patients highlights the need to improve and standardise preclinical *in vitro* and animal models for study of the NETosis-COPD relationship. This encourages effort to better adapt *ex vivo-in vitro* smoke models of NETosis induction[57,101], and to standardise the diversity of animal COPD models (e.g.[102,103]) to facilitate representative validation of biomarkers and testing of therapy targets and new therapeutic agents. Refined *in vitro* and animal models may help to assess the effectiveness and side effects of NETosis inhibitors[104–106]. Prominently, knowledge about cooperative signalling in neutrophil recruitment and the as yet limited efficacy of cytokine blocking therapies in COPD[107] may be expanded. Directions to be further followed are pointed by work comparing dual CXC-motiv-chemokine receptor 1/2 (CXCR1/2) inhibition vs. selective blocking of CXCR2[108], or cooperative P-selectin glycoprotein ligand-1 (PSGL-1) and CXCR2 signaling in deep vein thrombosis[109].

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