BRIEF CONCLUSIVE REPORT

Treatment with DNases rescues hidden neutrophil elastase from aggregated NETs

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Abstract
The release of neutrophil extracellular traps (NETs) is one of the weapons neutrophils have in their armory. NETs consist of extracellular chromatin fibers decorated with a plethora of cytoplasmic and granular proteins, such as the antimicrobial serine protease neutrophil elastase (NE). Because the first description of NETs as beneficial to the host, reports on their double-faced role in health and disease have considerably increased recently. On one hand, NETs reportedly trap and kill bacteria and also participate in the resolution of the acute inflammation associated with infection and with tissue damage. On the other hand, numerous negative aspects of NETs contribute to the etiopathogenesis of autoimmune disorders. Employing soluble and solid fluorescent substrates, we demonstrate the interaction of NE with aggregated NETs (aggNETs), the limitation of its enzymatic activity and the containment of the enzyme from surrounding tissues. These events prevent the spread of inflammation and tissue damage. The detection of DNase 1-dependent elevation of NE activity attests the continuous presence of patrolling neutrophils forming NETs and aggNETs even under conditions physiologic conditions.

KEYWORDS
aggregated NETs, DNase 1, NETs, neutrophil elastase, neutrophils

INTRODUCTION
Neutrophils, the most abundant leukocytes, are the first cells attracted to the sites of inflammation and play an essential role in the initiation and modulation of inflammatory responses. Recruited from the blood vessels to the site of infection or sterile tissue damage, they phagocytose pathogens, generate reactive oxygen species, release microbicidal molecules (degranulation), and secrete proinflammatory cytokines. After fulfilling their mission, neutrophils undergo apoptosis and are ingested by tissue-resident macrophages.

Neutrophil extracellular trap (NET) formation at the site of bacterial infection has been initially described as a noncanonical function of neutrophils. NETs consist of decondensed chromatin fibers associated with cytoplasmic and granular proteins with antimicrobial activity, for example, histones, LL37, cathepsin G, neutrophil elastase (NE), and myeloperoxidase. Classical stimulators of NET formation are some bacterial endotoxins, crystals of monosodium urate (MSU), cytokines, and chemicals, such as PMA.

Autoimmune diseases are often characterized by an impaired clearance of cell remnants. DNA-containing cellular debris contributes
Much effort has been put forward to investigate the role of NETs in autoimmune diseases. A potentially pathogenic role of NETs has been shown for antineutrophil cytoplasmic antibodies-associated small-vessel vasculitides,\textsuperscript{12,13} rheumatoid arthritis,\textsuperscript{14} and systemic lupus erythematosus.\textsuperscript{15–17} Another intriguing deleterious effect of NET formation in the body is the accumulation of macroscopically visible aggregates of NETs that conditions pancreatitis initiated by ductal occlusion.\textsuperscript{18}

Contrastingly, aggregated NETs (aggNETs) have been shown to contribute to the resolution of sterile inflammation in gout, where they trap and degrade proinflammatory cytokines and chemokines. This stops subsequent chemotaxis and further infiltration and activation of neutrophils.\textsuperscript{6} A better understanding of the presence of NETs in various tissues and body fluids in health and disease is required to elucidate the contribution of NETs and aggNETs to the etiopathogenesis of several diseases.

The common method to detect NETs is the measurement of extracellular DNA\textsuperscript{19–21} and the visualization of DNA strains by immunocytochemistry.\textsuperscript{12,22–24} The sole detection of extracellular DNA with fibrillar pattern is not enough to prove the presence of NETs. It is widely accepted that the detection of specific NET-markers is necessary to confirm the presence of NETs. This refers to DNA, the main component of NETs, as well as to one of the proteins that are associated with decondensed chromatin, for example, NE, citrullinated histone H3, or myeloperoxidase. In some cases, detection by immunocytochemistry of protein markers is impeded by the intricate structure of aggNETs.

Endogenous macromolecular inhibitors, including \( \alpha \)-1-antitrypsin, \( \alpha \)-2-macroglobulin in the circulation, and the secretory leukocyte protease inhibitor in various epithelia, contain the lytic activity of NE released to extracellular space.\textsuperscript{1} The presence of NE in the interstitium has been demonstrated in severe conditions such as the emphysematous lung and the acute respiratory distress syndrome.\textsuperscript{25,26} Because NETs and aggNETs are heavily decorated with NE, we analyzed the interaction of the latter with DNA, the limitation of its enzymatic activity, and its containment from surrounding tissues. These events prevent the spread of inflammation and tissue damage. We argue for a regulatory role of aggNETs during neutrophil-driven inflammation, lowering the activity of NE and sequestering the enzyme from large molecular weight substrates. Furthermore, we show the presence of NETs and aggNETs in various body fluids attesting the continuous presence of patrolling neutrophils forming NETs even in health.

2 | MATERIALS AND METHODS

2.1 | Ethical issues

Human peripheral blood polymorphonuclear (PMN) cells were obtained from normal healthy donors and experiments were performed in full agreement with institutional guidelines and the approval of ethical committee of the University of Erlangen (permit #243_15 B). Written informed consent was given by each donor in accordance with the Declaration of Helsinki. All procedures involving animals were in accordance with institutional guidelines on animal welfare and were approved by the local Animal Care and Use Committees of the University Erlangen-Nürnberg (TS-12/2015 Medizin III Klin. Imm).

2.2 | Isolation of human PMN

Cells were isolated from venous blood of healthy donors. Briefly, anticoagulated blood was centrifuged for 30 min at 1100 rpm at room temperature (RT) employing Lymphoflot density gradients (Bio-Rad, Hercules, CA, USA). PMN layer on the top of red blood cells was collected. Next, PMN were subjected to 30 s hypotonic lysis. Cells were used immediately in experiments.

2.3 | Production of MSU crystals

A solution of 10 mM uric acid and 154 mM NaCl was adjusted to pH 7.2 and incubated for 72 h at RT. Created crystals were washed with 70% ethanol and dried under sterile conditions. Next, MSU crystals were sterilized at 180°C for 2 h and stored in PBS (pH 7.0).

2.4 | Generation of NETs and aggNETs for enzymatic assays

NETs and aggNETs were generated in V-bottom tubes with \( 5 \times 10^6 \) PMN/ml that had been settled to the bottom of the tube. These cells were stimulated with 10 ng/ml of PMA (Sigma Aldrich, Darmstadt, Germany) or 50 pg/cell of MSU crystals. NETs formed within 4 h in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C and 5% carbon dioxide. The settled aggNETs were washed 3 times with PBS before adding to the enzymatic assays.

2.5 | Analysis by microscopy of NETs/aggNETs

NETs and aggNETs were generated in chamber slides with \( 5 \times 10^6 \) PMN/ml that had been settled to the bottom of the chamber slide. These cells were stimulated with 10 ng/ml of PMA (Sigma Aldrich) or 50 pg/cell of MSU crystals. NETs formed within 4 h in RPMI medium (Thermo Fisher Scientific) at 37°C and 5% carbon dioxide and was stopped by addition of 4% paraformaldehyde (PFA; Sigma-Aldrich, St. Louis, MO, USA) for 15 min. AggNETs were blocked with 10% FBS for 1 h at RT. Next, primary antibody detecting NE (ab68672, Abcam, Cambridge, United Kingdom) and Cy5-conjugated secondary detection antibody AffiniPure Goat anti-rabbit IgG (H + L) (Jackson Immuno Research Labs, West Grove, PA, USA) were applied sequentially according to manufacturer’s instructions. We visualized DNA with Hoechst 33342 (Thermo Fisher Scientific). We analyzed the channel slides using a BX-7000 inverted fluorescence microscope (Keyence Corporation, Osaka, Japan). AggNETs for macrophotographs were stained with 1 µg/ml propidium iodide (PI). We took macrophotographs with the Greenough-stereomicroscope Stemi 305 trino...
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(Zeiss, Göttingen, Germany) equipped with an epifluorescence light source (530 nm) and red filter (650 nm).

2.6 | NE activity

All experiments were performed in reaction buffer consisting of PBS supplemented with 1 mM calcium and 6 mM magnesium. NE fluorogenic substrate MeOSuc-AAPV-AMC (Santa Cruz Biotechnology, Santa Cruz, California, USA, sc-201163) was used at 100 µM initial concentration. Measurements of the fluorescence were conducted on an Infinite 200 PRO plate reader (TECAN, Crailshaim, Germany) at 37°C (Ex.360 nm, Em. 465 nm). When indicated, NETs and aggNETs were treated with 20 µg/ml of human endonuclease DNase-1 (EC 3.1.21.1; Roche, Basel, Switzerland). Twenty µg/ml of micrococcal endo-exo-nuclease S7 (MNase; EC 3.1.31.1) was used in the assays with purified NE.

2.7 | Western blot analysis

Prepared PMA- and MSU-induced aggNETs were denatured in Laemmli buffer for 15 min at 95°C. Next, denaturing gradient SDS-PAGE (4-20% bis-acrylamide) was performed followed by Western Blot analysis (Immobilon-PSQ PVDF Membrane, 2 h, 80 V). Obtained membranes were blocked in 5% milk and incubated with primary rabbit anti-NE antibody (ab68672, Abcam) or primary rabbit anti-beta-actin antibody (8457S, Cell Signalling, Frankfurt am Main, Deutschland), at 1:1000 and with secondary goat anti-rabbit antibody conjugated with horseradish peroxidase at 1:10,000 (4030-05, SouthernBiotech, Birmingham, Alabama, USA). All incubations were conducted for 1 h at RT. Detection was performed with a Celvin S and the software SnapAndGo 1.6.1 (Biostep, Burkhardtsdorf, Germany).

2.8 | Digestion of gelatin-coated slides

Rounded cover slips were sterilized in 70% ethanol and placed in 24-well plates. Next, they were coated with 50 µg/ml of poly-L-lysine (P5899-5MG, Sigma Aldrich) and 0.5% glutaraldehyde (4995.1; Roth, Karlsruhe, Germany) for 20 min at RT. Fluorescein conjugated gelatin (G13187, Invitrogen by Thermo Fisher Scientific, Eugene, OR, USA) was used to coat the cover slips for 30 min at RT in the dark. Prepared PMA- and MSU-induced aggNETs were placed on the top of cover slips with or without addition of 20 µg/ml of DNase 1 for 30 minutes at 37°C. As a positive control 0.05 U/ml of purified NE was used. Plates were centrifuged (300 g, 5 min) and the fluorescence in the supernatants was measured on an Infinite 200 PRO plate reader (Ex.485 nm, Em. 535 nm).

2.9 | Murine samples

BALB/c mice were purchased from The Janvier Laboritories (Roubaix, France) and kept in the local animal facility with free access to food and water. We euthanized the mice with isoflurane before blood was drawn by cardiac puncture. We collected serum, gall fluid, and feces and stored them at –20°C until use.

2.10 | Human body fluid samples

Eye wash fluid was collected from normal healthy donors after at least 6 h and no more than 8 h of sleep by wash of the ocular surface with 5 ml of sterile 0.9% NaCl solution. Human gall fluid was obtained from hepatobiliary stents that were recovered during elective endoscopic retrograde cholangiopancreaticography and cryoconserved at –20°C for subsequent analyses.

2.11 | Enzymatic kinetics of NE

The kinetic model used to analyze the experimental results was the Michaelis-Menten equation:

\[ V = V_{\text{max}} \times \frac{S}{K_m + S} \]

where \( S \) is the concentration of the substrate; \( V_{\text{max}} \) represented the maximum reaction rate; and \( K_m \) was the substrate concentration at the half-maximal rate. The \( V_{\text{max}} \) and \( K_m \) values were obtained from a nonlinear regression between substrate concentrations and reaction velocities using the fit equation. Dataset used for analyses was obtained from at least 2 donors.

2.12 | Statistics

Statistics were calculated employing GraphPad Prism 5.03 software. We used 2-way ANOVA with Bonferroni posttest (Fig. 2A and B),
Active neutrophil elastase (NE) is released from neutrophil extracellular traps (NETs) and aggregated NETs (aggNETs) upon DNase 1 digestion. Quantification of NE enzymatic activity for (A, n = 2) PMA-induced or (B, n = 4) monosodium urate (MSU) crystals-induced aggNETs, with or w/o DNase 1 digestion. As a control of specific reaction NE inhibitor (sivelestat), was used. Depicted is NE mean fluorescence intensity (MFI) ± SEM of at least 3 technical replicates. Two-way ANOVA with Bonferroni posttest were used to evaluate differences among various conditions. Shown are, *P < 0.05, **P < 0.01, and ***P < 0.001. Michaelis-Menten analysis was used to characterize the enzymatic activity of NE in (C) PMA-induced or (D) MSU crystals-induced aggNETs. Western blots analysis of NE extracted from MSU and PMA-aggNETs generated from 3 neutrophil donors (E). Fluorescein release from a gelatin matrix in contact with aggNETs and its degradation products (F). Note, there was no intrinsic NE activity of our DNase 1 preparation (G).
Neutrophil elastase (NE) activity is inhibited by dsDNA. (A) The enzymatic activity of NE in the presence and absence of DNase 1 and MNase. (B) Residual enzymatic activity of NE after inhibition with sivelestat in the presence and absence of aforementioned nucleases. (C) The inhibition of NE activity by 10 µg/ml of dsDNA from herring sperm. All values depicted in the graphs were obtained after the reaction time of 120 min. Kruskal-Wallis statistic (A and B) and Student’s t-test (C) were employed to test the statistical significance; *P < 0.05, ***P < 0.001

Kruskal-Wallis test (Figs. 2F, 3A, B, and 4A-C) and Student’s t-test (Figs. 2E, G, 3C, and 4D). The P-values <0.05 were considered as statistically significant.

3 | RESULTS AND DISCUSSION

3.1 NETs form aggregates and tightly trap sterile particulate matter

We induced NETs by PMA (Fig. 1A) or MSU crystals (Fig. 1B) as reported before. Fluorescence staining revealed decondensed chromatin (PL, red) decorated by NE (green). Analysis of the colocalization of these markers is often employed for the detection of NETs. In high PMN densities, NETs reportedly aggregate and form so called aggNETs. In order to show differences between the aggregation in the presence and in the absence of particulate matter, we display the macroscopic appearance and PL epifluorescence of aggNETs induced by PMA (Fig. 1C) and MSU (Fig. 1D), both prone to DNase 1 digestion (Fig. 1E, F). PMA-induced aggNETs appear as a slack mass of extracellular DNA (PI positive, Fig. 1C). When compared to MSU-induced aggNETs the extracellular DNA appears more compacted (Fig. 1D). This suggests that, during aggNET formation, MSU crystals serve as a scaffold for the decondensed chromatin fibers. NE is a serine protease involved in the degradation of connective tissue components and the extracellular matrix. Its activity is controlled by endogenous macromolecular inhibitors, including α1-proteinase inhibitor, α2-macroglobulin, and secretory leukoproteinase inhibitor, thus warranting homeostasis. Under certain inflammatory conditions those inhibitors are inactivated by neutrophil oxidative products. This increases apparent NE activity and precipitates tissue damage. This imbalance is hypothesized to be involved in many disorders, such as pulmonary emphysema, adult respiratory distress syndrome, cystic fibrosis, and other acute and chronic inflammatory conditions. Furthermore, it was reported that aggNETs create a barrier between harmful endogenous or exogenous particles and surrounding tissues and thus contain inflammation. Moreover, aggNETs shield viable tissues from necrotic areas and may thus help to preserve the residual function of organ remains. Our data suggest a further function of aggNETs keeping away enzymatically active NE from extracellular matrix. This may reduce tissue damage and spread of inflammation.

3.2 Digestion with DNase 1 of NETs and aggNETs increased the apparent activity of NE

NE activity was measured in aggNETs (closed symbols) induced by PMA (Fig. 2A) or MSU crystals (Fig. 2B) in the absence and presence of DNase 1. The specificity of the reaction was confirmed by blocking substrate conversion with sivelestat, a specific NE inhibitor (open symbols). As shown in Fig. 2A and B, digestion with DNase 1 (squares) increased the NE activity in both kinds of aggNETs. PMA-induced aggNETs (Fig. 2A) show higher NE activity in comparison to MSU crystals-induced tophus-like aggNETs (Fig. 2B). This suggests that more NE is available to convert the low molecular weight substrate in the slack mesh of PMA-induced aggNETs (Fig. 1C) and supports the hypothesis that MSU crystals trigger more tight aggNETs (Fig. 1D). The accessibility of the enzymes contained in aggNETs to the external milieu is, therefore, regulated by the tightness of the chromatin mesh. In order to test whether the activity of the NE is affected by DNase 1, we calculated the Michaelis-Menten fit of the enzymatic activity of NE. As displayed in Fig. 2C and D the model fit predicts that more NE was available in the slack aggNETs, because \( V_{\text{max}} \) was never reached during the experiment, whereas MSU crystals-induced aggNETs had a calculated \( V_{\text{max}} \) of 14.4 without and 44.5 after digestion.
FIGURE 4 Traces of neutrophil extracellular traps (NETs) and aggregated NETs (aggNETs) are found in biologic samples. Quantitative NE enzymatic activity is shown for (A) gall fluid, (B) feces, (C) serum, and (D) eye wash. Depicted is the mean fluorescence intensity (MFI) of the converted substrate of NE (NE MFI). Plotted are values of individual subjects and means (red bar). Kruskal-Wallis or Student’s statistic were used to evaluate differences among various conditions, \* \( P < 0.05 \) and \** \( P < 0.001 \). Representative immunofluorescence pictures of aggNETs present in human gall fluid and eye wash (E). Scale bars, 20 \( \mu m \)

by DNase 1, respectively (Fig. 2D). These results demonstrate that the more relaxed structure of PMA-induced aggNETs allows easier access of the low molecular weight substrate to the NE contained inside the aggNETs. AggNETs preparations contained principally similar amounts of NE as estimated by Western blot (Fig. 2E) and DNase 1 did not possess intrinsic NE activity (Fig. 2G).

The neutrophil derived serine proteases have been identified as an inflammation-related pathogenic factor in the lung.28,30–34 Our observations support the notion that aggNETs limit tissue injury by sequestering enzymatically active NE from susceptible extracellular matrices and tissue surfaces, still allowing the degradation inside the aggNETs of small molecular weight inflammatory mediators such as cytokines and chemokines. Indeed, the release of enzymatically active NE from DNase 1 treated aggNETs leads to digestion of collagen under our experimental settings (Fig. 2F). We conclude that the sequestration of NE molecules inside the aggNETs allow only the digestion of soluble molecules able to enter the aggNETs. This partially protects the extracellular matrix from the proteolytic attack of NE as only molecules on the surface of aggNETs can reach the extracellular matrix. The disassembly of aggNETs in tissues can be therefore considered as an additional pathogenic factor in neutrophil mediated inflammation (Supplemental figure).

To show that digestion of the NET-DNA and not a putative interaction with NE of DNase 1 is responsible for the increased NE activity after treatment with the endonuclease DNase 1, we employed the endo-exo nuclease MNase to test its influence on the NE activity. Figure 3A shows the activity of purified NE after treatment with DNase 1 or MNase. The endonuclease DNase 1 did not influence the activity of NE, whereas the endo- and exonuclease MNase enhanced the conversion of the substrate and promoted the inhibition of NE by sivelestat (Fig. 3B). These observations argue for a close interaction of NE and an exonuclease substrate. The addition of herring sperm dsDNA also significantly inhibited NE activity (Fig. 3C). Previous reports on the interaction of NE and DNA demonstrate that positively charged NE interacts with negatively charged DNA in the bronchoalveolar fluid (BAF) of patients with cystic fibrosis.38,39 In the case of cystic fibrosis, the action of therapeutically administrated DNase causes an increase of the amount of active NE that contributes to the degradation of proteins that enhance the viscosity of BAF. Nevertheless, in the case of neutrophil-driven conditions affecting the pulmonary parenquima, an increased activity of NE results clearly disadvantageous.28–34 Based on these and our observations, we conclude that dsDNA in aggNETs physically interacts and interferes with the enzymatic activity of NE and thus plays a regulatory role at the sites of neutrophil-driven inflammation.

3.3 Digestion of DNA increased NET-associated NE activity in samples of body fluids

The formation of aggNETs reportedly causes pathologic ductal occlusion and pancreatitis due to patrolling neutrophils that enter the bicarbonate rich pancreatic ducts and execute NET formation and aggregation.18 Neutrophils patrol the ocular surface and form aggNETs to wipe out dust and microorganisms.41 NETs participate in the initiation and growth of gallstones.42 We assessed the NE activity in samples from murine gall fluid (Fig. 4A), feces (Fig. 4B), and serum (Fig. 4C), as well as eye wash (Fig. 4D and E) and gall fluid (Fig. 4E) samples from normal healthy donors. Inhibition by sivelestat of NE (open symbols) confirmed the specificity of the assay. We detected robust NE activity in murine gall fluid (Fig. 4A), feces (Fig. 4B), and human eye wash samples (Fig. 4D) only after digestion of the samples by DNase 1. For serum samples, no essential conversion of the substrate by NE was
to be detected (Fig. 4C). This can easily be explained by the presence in sera of anti-proteases like α1-antitrypsin, a natural agent blocking the activities of various serine proteases.43

Many reports have shown the presence of patrolling neutrophils in various body compartments under healthy conditions, such as the eye44 or sputum.45 Our results support aforementioned findings by the detection of aggNETs and their remnants in gall ducts, gut, and the ocular surface. Our results not only show the presence of NETs and aggNETs in biologic samples but also demonstrate the importance of NETs aggregation for the containment of tissue damage during neutrophil infiltration.

AUTHORSHIP
M.H., C.S., and, L.E.M. supervised the experiments and conducted data analysis. M.J.P. planned the experiments. M.J.P., A.M., J.H., J.K., C.M., L.P., and M.U. performed the experiments. M.L., C.S., and G.S. provided scientific input. M.J.P, M.H., L.E.M., and G.S. wrote and all the authors read and approved the manuscript. M.H., L.E.M., and C.S. equally contributed to senior authorship.

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DISCLOSURE
The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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