Clinical & Experimental Immunology
The Journal of Translational Immunology

doi: 10.1111/cei.13392
Clinical and Experimental Immunology ORIGINAL ARTICLE

EDITORS' CHOICE

Neutrophil proteases degrade autoepitopes of NET-associated proteins

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Accepted for publication 27 October 2019
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Summary

Neutrophils can form neutrophil extracellular traps (NETs) to capture microbes and facilitate their clearance. NETs consist of decondensed chromatin decorated with anti-microbial proteins. Here, we describe the effect of neutrophil proteases on the protein content of NETs. We show that the neutrophil serine proteases degrade several neutrophil proteins associated with NETs. Interestingly, the anti-bacterial proteins associated with NETs, such as myeloperoxidase, calgranulin B and neutrophil elastase (NE), seem to be less susceptible to proteolytic degradation than other NET proteins, such as actin and MNDA. NETs have been proposed to play a role in autoimmune reactions. Our data demonstrate that a large number of the autoepitopes of NET proteins that are recognized by autoantibodies produced by systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) patients are also removed by the proteases. In conclusion, neutrophil serine proteases have a major impact on the NET proteome and the proteolytic changes of NET-associated proteins may counteract autoimmune reactions to NET components.

Keywords: autoepitopes, NETosis, neutrophil proteases, rheumatoid arthritis, systemic lupus erythematosus

Introduction

Neutrophil extracellular traps (NETs) are anti-microbial structures, composed of sticky chromatin fibers expelled by neutrophils, in which bacteria and other microbes are captured and thus prevented from spreading [1,2]. The NETs contain several anti-microbial proteins such as histones, neutrophil elastase (NE), calgranulin B (also known as S100A9) and myeloperoxidase (MPO), but the bacterial killing capacity of NETs is still controversial [3,4]. NETs are often characterized as a double-edged sword, as they defend against pathogens, but may have detrimental effects when they are not properly cleared by circulating DNases [5,6].

Autoimmune diseases were among the first diseases that were reported to be associated with (aberrant) NET formation [7]. Neutrophils from patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and other autoimmune diseases were shown to have an increased propensity to form NETs *in vitro* [8–10]. Furthermore, antibodies of patients with these diseases were shown to bind NETs, and their plasma was also able to induce NET formation in healthy control neutrophils, due probably to an inflammatory feedforward loop [11,12].

Other types of disorders that have been associated with defective clearing of NETs are renal disorders, severe sepsis, diabetes, deep vein thrombosis and cancers [5,13–15]. Defective clearance of NETs can lead to clogging of veins and ducts, and the anti-microbial proteins within the NETs are able to damage healthy tissues [6,11,16]. The neutrophil proteases such as neutrophil elastase (NE), cathepsin G (CG) and matrix metalloproteinase 9 (MMP9) are particularly notorious in this light [6,10,11,17]. NE and CG on NETs were shown to be able to awaken dormant cancer cells via the remodeling of the extracellular matrix [18]. Finally, NET proteases are capable to activate the complement and coagulation cascades, which might stimulate an immune response by attracting other immune cells [19]. Conversely, NET-associated proteases are also able to dampen the immune response, as shown by their ability to cleave Toll-like receptors and cytokines [20–23].

The activity of NE also plays an important role in the formation of NETs and, accordingly, inhibitors of NE have

been shown to block phorbol myristate acetate (PMA)-induced NET formation *in vitro* [24]. After the induction of NET formation, NE degrades the actin skeleton and migrates to the nucleus to cleave histones, especially H3 and H4, which induces DNA decondensation [24,25]. DNA decondensation leads to swelling of the cell and eventually to rupture of the cell membrane. After DNA decondensation has initiated there is a so-called 'point of no return', after which there is no way back and the chromatin is released [26,27]. During the process, peptidylarginine deiminase 4 (PAD4) may become activated due to the rise in intracellular calcium levels and citrullinate histones to facilitate chromatin decondensation [27,28]. We and others have previously shown that the citrullinated N-terminal tail of H3 is cleaved off during NET formation [29,30].

We hypothesized that the degradation of NET-associated proteins by neutrophil proteases is not limited to the cleavage of histones. Other proteins on the NETs might also be cleaved to different extents, and this may also affect the recognition sites of autoantibodies directed to NET-associated autoantigens. Here, we used the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) to study the impact of neutrophil proteases on the integrity of proteins and the presence of autoepitopes on NETs. To avoid PMSF inhibiting NET formation, PMSF was added after the 'point of no return'.

Materials and methods

Chemicals, antibodies and sera

Reagents were purchased from the following suppliers: alpha-1-antitrypsin (Sigma, St Louis, MO, USA; cat.no A6150), serum-free Dulbecco's modified Eagle's medium (DMEM)/F12 medium without phenol red (Gibco, Carlsbad, CA, USA; cat. no 11039-021), lymphoprep (Stemcell Technologies, Vancouver, Canada; cat. no. #07851), micrococcal nuclease (Boehringer Mannheim, Mannheim, Germany; MNase, cat. no 85446620), pefabloc SC (VWR cat. no 1.24839.0100), PMA (Sigma Aldrich, St Louis, MO, USA; cat. no P1585), PMSF (Sigma Aldrich; cat.no P7626), Sytox Green (Thermo Fisher Scientific, Fremont, CA, USA; cat. no S7020) and 3,3′,5,5′-tetramethylbenzidine (TMB) (Invitrogen, cat. no 00-4201-56). All antibodies used in this study are listed in Table 1.

Serum samples of SLE and RA patients were collected at the Radboud University Medical Center in Nijmegen and healthy control sera were collected at the Sanquin Blood Bank in Nijmegen. Patient sera were used in accordance with the code of conduct of research with human material in the Netherlands. The collection of serum samples was approved by the local Medical Ethical Committee.

Table 1. List of antibodies used in this study

Antibody against	Supplier	Catalog number
α-enolase	Santa Cruz	sc-100812
Actin	Sigma Aldrich	A2066
calgranulin B	Neomarkers	MAC387
GAPDH	Cell Signalling	2118
H2B	Abcam	ab52599
H3	Abcam	ab195277
H3 (citrullinated)	Abcam	ab5103
H4	Abcam	ab177840
MNDA	Abcam	ab188566
MPO	R&D Systems	3174
NE	Abcam	ab68672
PAD4	Abcam	ab128086
IRDye 800CW-conjugated goat-anti human IgG	Licor	925-32232
IRDye 800CW-conjugated goat-anti rabbit IgG	Licor	926-32211
IRDye 680RD-conjugated goat anti-mouse IgG	Licor	925-68070
HRP-conjugated rabbit anti-human IgA, IgG, IgM	Dako	P0212

GAPDH = glyceraldehyde 3-phosphate dehydrogenase; H2B = histone 2B; H3 = histone 3; H4 = histone 4; MNDA = myeloid nuclear differentiation antigen; MPO = myeloperoxidase; NE = neutrophil elastase; PAD4 = peptidylarginine deiminase 4; HRP = horseradish peroxidase.

Neutrophil isolation and stimulation in the absence and presence of PMSF

Blood of healthy volunteers was collected in ethylenediamine tetraacetic acid (EDTA) anti-coagulation tubes. Blood was diluted 1 : 1 in phosphate-buffered saline (PBS) and layered on top of lymphoprep, according to the manufacturer's protocol. The polymorphonuclear cell (PMN) layer was collected and red blood cells were lysed by treatment with milliQ for 20 s and re-establishing physiological conditions with ×10 PBS. The PMNs were then resuspended in serum-free DMEM/F12 medium without phenol red. For each experiment, 30 million PMNs in 15-ml medium were incubated in a 10-cm Petri dish. The PMNs were left to adhere to the plate for 30 min at 37°C, after which 5 nM PMA was added. In samples that were treated with PMSF, 0·5 mM PMSF was added 2 h after PMA addition and this was repeated every 15 min until NET harvesting (Fig. 1). These studies were approved by the local Medical Ethical Committee.

NET harvesting and preparation for protein gel analysis

Three hours after PMA incubation, the neutrophil cultures were washed once with PBS to eliminate cellular proteins (Supporting information, Fig. S1). After washing, the NETs were harvested in 4-ml medium supplemented with

5 U/ml MNase. In the PMSF-treated samples, 0·5 mM PMSF was added to the MNase solution. After incubation for 10 min at 37°C, the supernatant was collected and EDTA was added (5 mM final concentration) to inhibit MNase activity. Subsequently, the samples were spun for 5 min at 1500 ***g*** to remove cell debris, after which a small sample (50 µl) was taken to measure the DNA content with Sytox Green (50 µl, 2·5 µM). The NET harvests were precipitated by adding 1 : 3 ice-cold acetone and incubating overnight at –20°C. Precipitated material was collected by centrifugation for 15 min at 21 000 ***g*** and the pellet was dissolved in 300 µl sample buffer [1% sodium dodecyl sulfide (SDS)/2·5% beta-mercapto ethanol/5% glycerol/0·0025% bromophenol blue/32 mM Tris/HCl, pH 6.8], sonicated (10 cycles, 30 s on, 30 s off) and heated for 5 min at 95°C.

SDS-polyacrylamide gel electrophoresis (PAGE) and protein detection

The NET samples were separated by electrophoresis using 15% SDS-polyacrylamide gels, after which the proteins were transferred to nitrocellulose membranes. The nitrocellulose blots were cut into strips to allow for multiple antibody or patient serum incubations. The strips were first blocked for 1 h at room temperature in 5% (v/v) non-fat dry milk in PBS supplemented with 0·5% Tween-20. Subsequently, strips were incubated for 1·5 h at 37°C with autoimmune patient sera [diluted 1 : 100 in 5% (v/v) non-fat dry milk in PBS/0·5% Tween-20] or with specific antibodies [see Table 1, diluted 1 : 1000 in 5% (v/v) non-fat dry milk in PBS/0·5% Tween-20]. Next, the strips were washed three times for 5 min in 5% (v/v) non-fat dry milk in PBS/0.5% Tween-20, before incubation with IRDye-conjugated secondary antibodies [diluted 1 : 5000 in 5% (v/v) non-fat dry milk in PBS/0·5% Tween-20] for 1 h. Depending on the primary incubation, IRDye 800CW-conjugated goat anti-human, IRDye 800CW-conjugated goat anti-rabbit or IRDye 680RD-conjugated goat anti-mouse immunoglobulins were used. After washing in 5% (v/v) non-fat dry milk in PBS/0·5% Tween-20, PBS/0·5% Tween-20 and PBS, the antibody binding to the strips was visualized using a Li-cor Odyssey imaging system. The intensity of the bands was quantified using Image Studio Lite software (version 5.2.5; Li-cor, Lincoln, NE, USA).

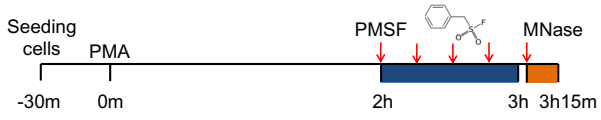

Fig. 1. Schematic representation of the experimental set-up. Starting 2 h after stimulation of the neutrophils, phenylmethylsulfonyl fluoride (PMSF) was added every 15 min (red arrows; the chemical structure represents PMSF) to inhibit neutrophil proteases. Also, the MNase solution used for harvesting of the neutrophil extracellular traps (NETs) contained PMSF.

Enzyme-linked immunosorbent assay (ELISA)

Fresh NET samples (100 µl undiluted per well) were coated on MaxiSorp plates overnight at 4°C. After coating, the wells were blocked with 5% (v/v) non-fat dry milk in PBS supplemented with 0·5% Tween-20 (200 µl per well) for 1 h at room temperature. The immobilized NETs were incubated with SLE or RA sera for 1·5 h at 37°C [diluted 1 : 100 in 5% (v/v) non-fat dry milk in PBS/0·5% Tween-20, 100 µl per well]. The wells were washed three times with PBS, 0·5% Tween-20, before incubation with horseradish peroxidase (HRP)-conjugated rabbit anti-human immunoglobulin [diluted 1 : 2000 in 5% (v/v) non-fat dry milk in PBS/0·5% Tween-20, 100 µl per well] for 1 h at room temperature. After washing with PBS/0·5% Tween-20 and PBS, TMB substrate solution (100 µl per well) was added and the reaction was stopped with H_2SO_4 (2 M, 100 µl per well). The absorbance at 450 nm was measured using a microplate reader (Tecan Sunrise, Männedorf, Switzerland).

Statistical analyses

Quantitative data are presented as mean plus standard deviation, and the significance of differences between the data was determined by Student's *t*-test using GraphPad Prism software (version 5.03, GraphPad Software, Inc., San Diego, CA, USA).

Results

Neutrophil proteases degrade NET proteins

To investigate the effect of serine proteases on the NET proteome, neutrophils were stimulated with PMA and NET formation and harvesting were allowed to occur either in the presence or absence of PMSF. PMSF was added to the neutrophil cultures after 2 h of PMA stimulation; i.e. after most of the neutrophils have passed the point of no return in the NET formation process [26]. When PMSF was added before this time-point, NET formation was strongly inhibited (Supporting information, Fig. S2). After 3 h, NET material was collected by incubation with MNase solution supplemented with PMSF and subsequently acetone precipitated for analysis by SDS-PAGE (Fig. 2a). In the absence of PMSF, the intensity of many protein bands was strongly reduced compared to the bands obtained in the presence of PMSF, indicating that the activity of neutrophil serine proteases affects a broad spectrum of NET proteins. To exclude that this was caused by a general reduction of NET formation, we measured the DNA content of both samples with Sytox green. We found that NETs produced

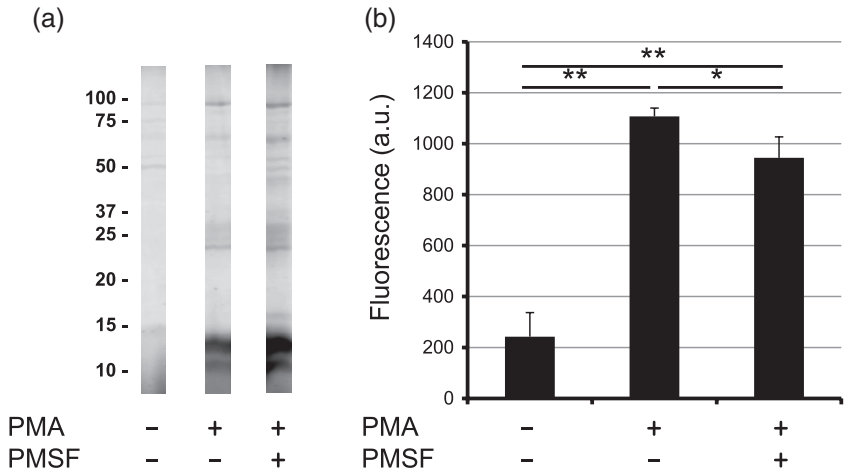

Fig. 2. Neutrophil extracellular trap (NET) formation in the absence and presence of phenylmethylsulfonyl fluoride (PMSF). (a) NET proteins harvested from NETs produced by neutrophils stimulated with phorbol myristate acetate (PMA) and by non-stimulated neutrophils as a control, and produced with (+) or without (–) PMSF were separated by sodium dodecyl sulphate-polymerase gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue. The position of molecular weight markers (kDa) is indicated on the left. (b) NET quantification by Sytox green (DNA) of non-stimulated cells and neutrophils stimulated with PMA in the presence or absence of PMSF. a.u. = arbitrary units. Mean plus standard error of the mean of at least 10 biological replicates. *$P < 0{\cdot}05$, **$P < 0{\cdot}001$, paired Student's t-test.

and harvested without PMSF appeared to contain slightly more DNA than the NETs obtained with PMSF (Fig. 2b). This difference is due partly to reduced NET formation (Supporting information, Fig. S2), and also to less efficient release of NET material, probably caused by inhibition of MNase by PMSF (Supporting information, Fig. S3). Thus, even though fewer NETs were harvested in the presence of PMSF, many proteins appeared to be more abundant in this material than in the NETs obtained in the absence of PMSF, substantiating the reduction of their levels when serine proteases are active.

To study these effects for individual proteins, Western blotting was performed using antibodies against proteins that are known to be associated with NETs [31–35]. The results showed that not all proteins on NETs are equally susceptible to serine protease modification (Fig. 3a). In particular, the proteins that have been reported to contain antibacterial activity, such as MPO, calgranulin B and NE, were found to be less strongly affected by the proteases than other NET proteins, such as actin and myeloid nuclear differentiation antigen (MNDA). However, this is not a general phenomenon, because α-enolase, which is not an established antimicrobial protein, is relatively resistant against proteolysis. Furthermore, the NET-associated histones are also cleaved by the proteases, albeit at moderate levels, which results in relatively stable cleavage products (Fig. 3b). As histones have also been suggested to display anti-microbial activity, we hypothesize that these cleavage products also have this activity [36]. Similar results were obtained when pefabloc (a water-stable variant of PMSF) or alpha-1-antitrypsin (most abundant protease inhibitor in human serum) were used as protease inhibitor (Supporting information, Fig. S4). We conclude that the serine proteases modify a broad range of NET-associated proteins, although the anti-bacterial proteins may be less susceptible to such modifications.

Neutrophil proteases affect autoepitopes on NETs

Patients with autoimmune diseases such as SLE and RA often have autoantibodies against NET components in their sera [8,9]. The immunogenicity of the NETs might be due in part be to generation of neoepitopes by neutrophil protease activities. Alternatively, the NET proteases might lead to the disruption of autoepitopes and thereby reduce the immunogenicity of the NETs. To investigate the effect of neutrophil serine protease activity on the recognition of autoantigenic proteins associated with NETs, their recognition by SLE and RA patient sera was analysed by immunoblotting and by ELISA. Immunoblotting analyses with 25 SLE and 25 RA sera showed that 14 SLE and seven RA sera were reactive with NET proteins (Fig. 4a). A striking difference was observed between the reactivities with proteins from NETs produced in the presence and absence of PMSF. Without protease inhibition approximately 90% of the autoreactive bands were not detected, strongly suggesting that many autoepitopes were degraded. These results were corroborated by the analysis of 44 SLE and 44 RA sera by ELISA, which also showed a substantial difference in reactivity between NETs produced with and without PMSF. The number of anti-NET protein-positive sera was reduced, for SLE from 45 to 39% and for RA from 30 to 25%, when PMSF was omitted (Fig. 4b). The most strongly reactive sera for PMSF-treated NETs

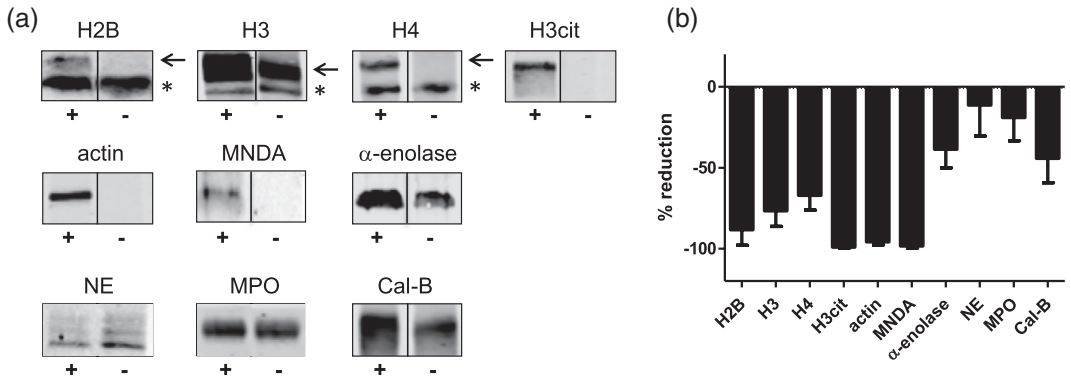

Fig. 3. Effect of proteases on neutrophil extracellular trap (NET) proteome. (a) Western blots containing NET proteins were incubated with antibodies to the indicated proteins. Cal-B = calgranulin-B; H2B = histone 2B; H3 = histone 3; H3cit = citrullinated histone 3; H4 = histone 4; MNDA = myeloid nuclear differentiation antigen; MPO = myeloperoxidase; NE = neutrophil elastase. The presence (+) or absence (–) of phenylmethylsulfonyl fluoride (PMSF) during NET production is indicated. Arrows mark the positions of the full-length histones; asterisks indicate stable degradation products. (b) Relative reduction of the full-length NET-associated proteins in the absence of PMSF compared to NETs treated with PMSF. Signal intensity was quantified using Image Studio Lite software (version 5.2.5, Licor). Mean plus standard deviation of at least three biological replicates.

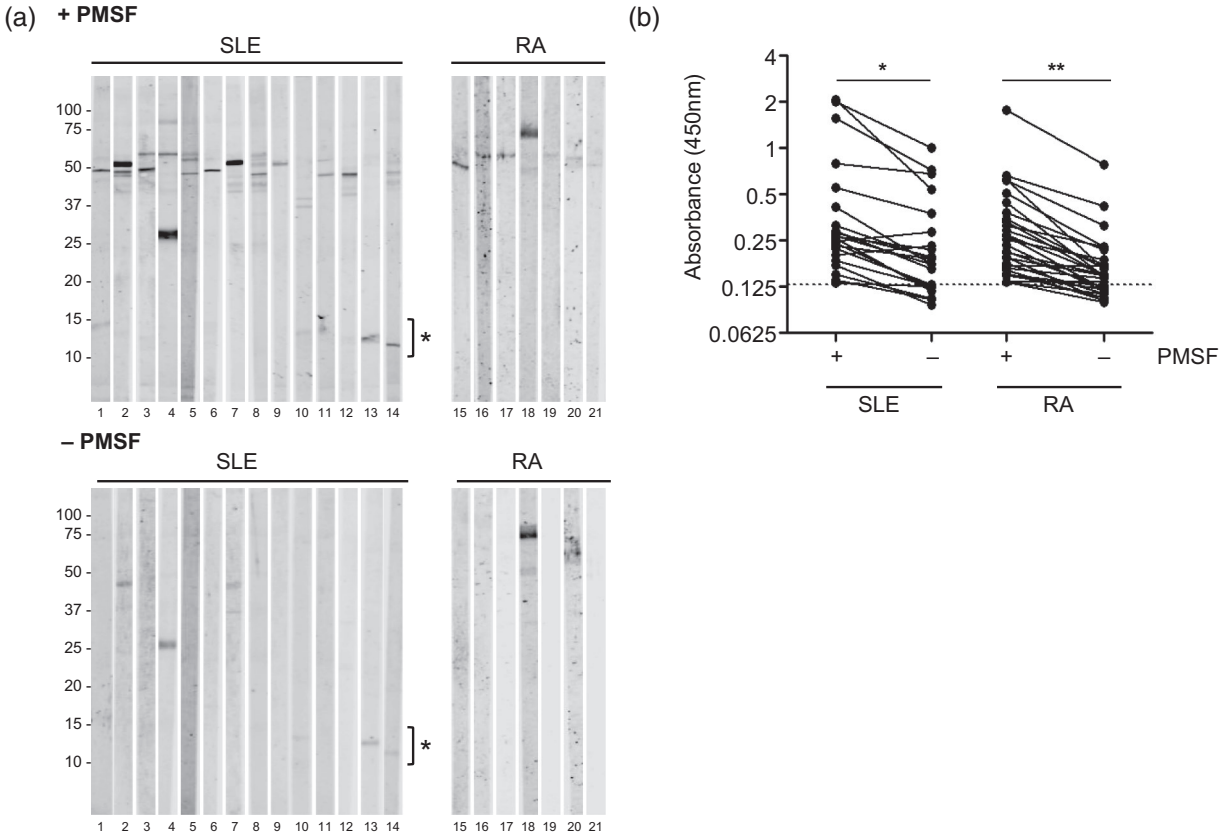

Fig. 4. Neutrophil proteases degrade autoepitopes that are recognized by systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) sera. (a) Reactivity of SLE and RA sera with proteins from neutrophil extracellular traps (NETs) produced with (upper panel) or without phenylmethylsulfonyl fluoride (PMSF) (lower panel) and analysed by immunoblotting. *Reactivity with polypeptides of approximately 10–15 kDa. Representative results of three independent analyses are shown. (b) Reactivity of SLE and RA sera with NET proteins, analysed by enzyme-linked immunosorbent assay (ELISA). The dotted line represents the cut-off value based on the mean plus two times the standard deviation of healthy control sera in the same assay with NETs produced in the presence of PMSF. *$P < 0{\cdot}05$, **$P < 0{\cdot}001$, Student's *t*-test.

are the same as those for non-treated NETs. Notably, the sera that are reactive with polypeptides migrating between 10 and 15 kDa (Fig. 4a, lanes 10, 13 and 14) were also reactive with NETs formed without PMSF. These polypeptides may represent histones, which would be consistent with the observation that histones associated with NETs are less prone to degradation (Fig. 3a).

Discussion

This study shows that many proteins associated with NETs, including several autoantigenic proteins, are efficiently degraded by neutrophil proteases in cell cultures. PMSF is able to rescue these NET proteins from degradation by inhibiting the neutrophil serine proteases, such as CG, proteinase 3 and NE. As the reactivity of SLE and RA autoantibodies with NET proteins was strongly reduced when NETs were generated in the absence of PMSF, it is tempting to speculate that the modification of NET proteins by neutrophil proteases contributes to the prevention of autoimmune reactions.

The experiments shown here were performed *in vitro*, which very probably does not completely mimic the *in-vivo* situation. Human blood contains endogenous protease inhibitors such as alpha-1-antitrypsin and other serpins that are able to inhibit neutrophil proteases [20,37]. These endogenous inhibitors may inhibit the activity of proteases on NETs. However, recent studies have shown that the neutrophil proteases on NETs cannot be completely inhibited, because they are able to damage the endothelium [18,38]. Possibly, their local concentration on NETs is high enough to exert their proteolytic function, even in the presence of these inhibitors [20]. The activity of the proteases of locally activated neutrophils may be highly dependent on the microenvironment and, therefore, the level by which NET proteins are cleaved may differ among different tissues. In our study, we used the general serine protease inhibitor PMSF as an easy-to-use compound to inhibit all the serine proteases that might be active during NET formation and NET harvesting in cell cultures. It is important to note that the water-stable variant pefabloc and purified alpha-1-antitrypsin gave similar results, although the efficiency of protease inhibition was less that that observed with PMSF, which might be due to the fact that these inhibitors were added only once instead of four times (Supporting information, Fig. S4). Further studies are required to reveal which neutrophil protease(s) is/are responsible for alteration of the NET proteome.

The association of NETs with the autoimmune response in SLE and RA [8,9] might involve (i) the ineffective clearance of NETs contributing to the autoimmune response, (ii) the formation of neoepitopes due to modification of NET-associated proteins by neutrophil proteases and/or (iii) the formation of immune complexes when antibodies to NET components are already present, propagating the inflammatory process. Our data confirm the presence of anti-NET autoantibodies in SLE and RA sera, with a higher frequency in SLE. SLE autoantibodies are generally targeting intracellular proteins, particularly nuclear proteins, whereas the main class of RA autoantibodies is directed towards citrullinated epitopes. Citrullination is mediated by PAD4, which is also expressed in neutrophils and has been reported to become activated during NET formation [25]. However, NET formation induced by PMA may be associated with relatively low levels of PAD4 activity, and as a result the NET samples will not contain many citrullinated proteins [29]. As SLE sera are known to contain a much greater diversity of autoantibodies, it is not surprising to observe more anti-NET reactivity in SLE than in RA sera.

The identity of the autoantigenic proteins and their epitopes on the NETs remains unclear, and will be a topic of further research. The 10–15-kDa bands recognized by the SLE sera used in lanes 10, 13 and 14 in Fig. 4 might represent histones, although these sera did not react with purified recombinant H2A, H2B, H3 or H4 in Western blotting or in ELISA (data not shown). This might be due to the absence of post-translational modifications.

Hakkim and coworkers showed that low DNase levels in the sera of SLE patients correlate with disease severity [5]. They hypothesized that NETs play a crucial role in the pathogenesis of SLE, which explains why low DNase levels (and thus low NET clearance) correlates so well with disease severity. Our results indicate that besides circulating DNases, also neutrophil proteases might contribute to the reduction of autoimmune reactivity by eliminating autoepitopes on NETs. As our *in-vitro* experiments did not take into account the presence of circulating protease inhibitors such as alpha-1-antitrypsin or other serpins, this hypothesis needs to be tested *in vivo*.

In conclusion, our results demonstrate that neutrophil serine proteases degrade NET-associated proteins to different extents. Anti-bacterial proteins such as NE, MPO and calgranulin B seem less prone to modification by the proteases. The degradation of NET proteins also reduces their recognition by autoantibodies, which indicates that NET-associated proteases might also have protective functions against autoimmunity.

Acknowledgements

The authors would like to acknowledge Dr Helen Wright (University of Liverpool) for kindly supplying some of the antibodies used in this study. Furthermore, the authors would like to thank Annemarie van der Heijden (Radboud University Nijmegen) for technical assistance.

Disclosures

The authors declare no conflicting interests.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1. Cellular proteins PAD4 and GAPDH are not detected in NETs. Total neutrophil lysate and two independently prepared NET preparations with PMSF were analysed by immunoblotting using antibodies to MPO, PAD4 and GAPDH. NETs were harvested after washing once with PBS.

Fig. S2. Effect of PMSF on NET formation. The upper panels show the different experimental setups of the experiments shown in the lower panel. The lower panel shows the time lapse of NET formation as monitored by Sytox Green of neutrophils stimulated with and without PMA and treated with or without PMSF at different time points. The black line shows regular NET formation after stimulation with PMA. The red line represents the conditions used in the experiments shown in the main text, with PMSF added every 15 minutes after 2 hours of PMA stimulation. When PMSF was added earlier, either from the start of the experiment (green line), or after 60 minutes of PMA stimulation (orange line), NET formation was markedly inhibited. In the absence of PMA, no NET formation was detected, irrespective of the addition of PMSF 2 hours after the start of the experiment (blue and yellow lines respectively). Data of two samples in two independent experiments ($n = 4$). Mean plus standard deviation are shown. a.u. = arbitrary units.

Fig. S3. Effect of PMSF on MNase. NET formation was induced by PMA and neutrophils were treated with or without PMSF, added 2 hours after PMA stimulation. Cultures were washed once with PBS before harvesting the NETs with MNase in the presence or absence of PMSF. Average plus standard deviation of 3 independent experiments ($n = 3$). A.u. = arbitrary units.

Fig. S4. Alpha-1-antitrypsin (α1AT) and pefabloc inhibit degradation of NET proteins. NET formation was induced by PMA and neutrophils were treated with or without inhibitors at the indicated concentrations. In contrast to PMSF, which was added every 15 minutes after 2 hours of neutrophil stimulation, pefabloc (a water stable variant of PMSF) and α1AT were added only once after 2 hours of PMA stimulation. NET harvests were analysed by immunoblotting using an antibody against citrullinated H3.

