Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps

Venizelos Papayannopoulos, Kathleen D. Metzler, Abdul Hakkim, and Arturo Zychlinsky

Department of Cellular Microbiology, Max Planck Institute for Infection Biology, Berlin 10117, Germany

---

Neutrophils release decondensed chromatin termed neutrophil extracellular traps (NETs) to trap and kill pathogens extracellularly. Reactive oxygen species are required to initiate NET formation but the downstream molecular mechanism is unknown. We show that upon activation, neutrophil elastase (NE) escapes from azurophilic granules and translocates to the nucleus, where it partially degrades specific histones, promoting chromatin decondensation. Subsequently, myeloperoxidase synergizes with NE in driving chromatin decondensation independent of its enzymatic activity. Accordingly, NE knockout mice do not form NETs in a pulmonary model of *Klebsiella pneumoniae* infection, which suggests that this defect may contribute to the immune deficiency of these mice. This mechanism provides for a novel function for serine proteases and highly charged granular proteins in the regulation of chromatin density, and reveals that the oxidative burst induces a selective release of granular proteins into the cytoplasm through an unknown mechanism.

---

**Introduction**

Neutrophils are the first line of immune defense (Lekstrom-Himes and Gallin, 2000; Nathan, 2006), and they combat pathogens by phagocytosis, degranulation, and the release of neutrophil extracellular traps (NETs; Brinkmann et al., 2004; Nauseef, 2007; Papayannopoulos and Zychlinsky, 2009). NETs are composed of decondensed chromatin and antimicrobial factors, including neutrophil elastase (NE) and myeloperoxidase (MPO; Brinkmann et al., 2004; Urban et al., 2009), and capture and kill bacteria, fungi, and parasites (Urban et al., 2006; Guimarães-Costa et al., 2009; Ramos-Kichik et al. 2009). NETs are implicated in immune defense, sepsis, and autoimmunity (Clark et al., 2007; Kessenbrock et al., 2009; Papayannopoulos and Zychlinsky, 2009; Hakkim et al., 2010). Mast cells, eosinophils, and plant cells also release DNA, which suggests that this may be a common strategy in immunity (von Köckritz-Blickwede et al., 2008; Yousefi et al., 2008; Wen et al., 2009).

NE and MPO are stored in azurophilic granules of naïve neutrophils (Borregaard and Cowland, 1997; Lominadze et al., 2005). NE is a neutrophil-specific serine protease that degrades virulence factors and kills bacteria (Lehrer and Ganz, 1990; Belaaouaj et al., 2000; Weinrauch et al., 2002). MPO catalyzes the oxidation of halides by hydrogen peroxide (Hazen et al., 1996; Eiserich et al., 1998; Nauseef, 2007). NE and MPO knockout mice are susceptible to bacterial and fungal infections (Belaaouaj et al., 1998; Aratani et al., 1999; Tkalec et al., 2000; Gaut et al., 2001; Belaaouaj, 2002). Interestingly, histones are the most abundant NET component and are potent antimicrobials (Hirsch, 1958; Kawasaki and Iwamuro, 2008; Urban et al., 2009).

Isolated human neutrophils release NETs 2–4 h after stimulation with microbes or activators of PKC such as PMA (Fuchs et al., 2007), but respond much faster when activated by platelet cells stimulated with LPS, a process thought to be relevant during sepsis (Clark et al., 2007).

NETs form via a novel form of cell death (Fuchs et al., 2007) that requires the production of reactive oxygen species (ROS). Neutrophils from chronic granulomatous disease patients with mutations in the NADPH oxidase that disrupt ROS production (Clark and Klebanoff, 1978) fail to form NETs (Fuchs et al., 2007; Bianchi et al., 2009). In neutrophils from healthy donors, ROS production is followed by the disassembly of the nuclear envelope. Chromatin decondenses in the cytoplasm and binds to granular and cytoplasmic antimicrobial proteins.

---

Correspondence to Arturo Zychlinsky: zychlinsky@mpiib-berlin.mpg.de

Abbreviations used in this paper: ABAH, 4-aminobenzoic acid hydrazide; CG, cathepsin G; CGi, CG inhibitor I; DPI, diphenyleneiodonium; G-CSF, granulocyte colony-stimulating factor; HSP, high-speed pellet; HSS, high-speed supernatant; LSS, low-speed supernatant; MPO, myeloperoxidase; NE, neutrophil elastase; NET, neutrophil extracellular trap; PAD4, peptidylarginine deiminase 4; PBMC, peripheral blood mononuclear cell; PR3, proteinase 3; PVDF, polyvinylidene fluoride; ROS, reactive oxygen species; SIPI, serum leukocyte protease inhibitor.

© 2010 Papayannopoulos et al. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike–No Mirror Sites license for the first six months after the publication date (see http://www.rupress.org/terms). After six months it is available under a Creative Commons License (Attribution–Noncommercial–Share Alike 3.0 Unported license, as described at http://creativecommons.org/licenses/by-nc-ss/3.0/).

Supplemental material can be found at: [http://doi.org/10.1083/jcb.201006052](http://doi.org/10.1083/jcb.201006052)
Figure 1. NE cleaves histones and promotes nuclear decondensation in vitro. (A) Nuclei isolated from neutrophils were incubated in buffer or in neutrophil-derived LSS lysates for 120 min at 37°C and labeled with Sytox green. Bar, 10 µm. (B) Neutrophil extracts are sufficient to decondense nuclei from other cell types. Nuclear decondensation of LSS extracts from HL-60 cells differentiated with RA, HeLa cells, PBMCs, and neutrophils were tested with nuclei.
Proteins before NET release. Chromatin decondensation and the association with antimicrobial proteins are two essential steps during NET formation. The molecular mechanism linking ROS production to chromatin decondensation and binding to antimicrobial proteins is unknown.

Here we show that NE is essential to initiate NET formation and that it synergizes with MPO to drive chromatin decondensation. Our findings reveal a novel mechanism to drive massive chromatin decondensation, and provide evidence for a novel pathway that allows granular proteins to leak into the cytoplasm.

Results

Neutrophil extracts promote chromatin decondensation

To identify factors involved in NET formation, we developed a cell-free nuclear decondensation assay using intact nuclei and cytoplasmic extracts from neutrophils and other control cells. Only the neutrophil-derived low-speed supernatant (LSS), containing cytoplasm and granules, decondensed nuclei from neutrophils, peripheral blood mononuclear cells (PBMCs), human leukemia-60 (HL-60), and HeLa cells (Fig. 1, A and B), which indicates that neutrophil LSS contains specific factors that decondense nuclei. Further separation of the LSS into cytoplasmic (high-speed supernatant [HSS]) and membrane/granule (high-speed pellet [HSP]) fractions showed that the decondensation activity partitioned with the HSP (Fig. 1 C). Neutrophils contain azurophilic, specific, and gelatinase granules (Borregaard and Cowland, 1997). The decondensation activity fractionated with the azurophilic granules (Fig. 1 C, fraction 3; Kjeldsen et al., 1994), which suggests that granular factors are not only components of NETs but are also involved in their formation.

NE is necessary and sufficient for chromatin decondensation

NE and MPO are two of the primary enzymes stored in azurophilic granules and are found in abundance in NETs. Unlike MPO, only NE sediments exclusively to the azurophilic fraction (Fig. 1 D, lane 3; Lominadze et al., 2005). We tested whether the activity of NE and MPO is required for nuclear decondensation. Chromatin decondensation was blocked by two NE inhibitors, GW311616A (NEi) and serum leukocyte protease inhibitor (SLPI; Macdonald et al., 2001), but not by the MPO inhibitor 4-aminobenzolic acid hydrazide (ABAH; Fig. 1 C; Kettle et al., 1997).

Azurophilic granules store two additional NE-related proteases, proteinase 3 (PR3) and cathepsin G (CG). Using protease-specific chromogenic peptides and histones as substrates, we show that NEi inhibits NE and PR3, but not CG (Fig. S1). SLPI is known to inhibit NE and CG but not PR3 (Rao et al., 1991). Considering the specificity of these inhibitors, these results indicate that PR3 is not sufficient to drive decondensation in vitro. In addition, CG inhibitor I (CGi), a CG-specific inhibitor, did not inhibit nuclear decondensation. Collectively, these data suggest that among these serine proteases, only NE is required for the decondensation of the nucleus.

NE degrades histones to promote nuclear decondensation

Because histones pack DNA, we examined whether histones H3 and H4 are degraded in nuclei treated with LSS. To distinguish between histone degradation and histone release, we separated the soluble unbound proteins (SF) from the nuclear fraction (NF) by filtration. Interestingly, H4, but not H3, was degraded in an NE-dependent manner (Fig. 1 E). We also monitored the kinetics of nuclear localization of NE and MPO in relation to histone degradation and nuclear decondensation. Both NE and MPO were detected in the nuclear fraction within the first 30 min. Notably, H1 was degraded early but decondensation coincided with H4 degradation at 150 min (Fig. 1 F), which suggests that nuclear decondensation is driven primarily by the degradation of core histones (Fig. 1 G). However, H1 may have to be degraded first to allow for the subsequent degradation of core histones.

Moreover, purified NE and PR3, but not CG, promoted nuclear decondensation in vitro (Fig. 1 H, Fig. S1 F, and not depicted). NE degraded H4 processively, whereas the other histones were only partially degraded (Fig. 1 I). Histone degradation was detectable by 30 min and coincided with nuclear decondensation (Fig. 1, H and I). As a control, we showed that NE completely degraded soluble histones purified from neutrophils (Fig. 1 J), which indicates that the pattern of histone degradation depends on chromatin structure and not on the intrinsic
susceptibility of histones to this protease. Similarly, purified PR3 cleaved nuclear histones in vitro (Fig. S1 E). In contrast, and consistent with our previous observations, nuclear and purified histones were poor substrates for CG (unpublished data).

To examine whether the degradation of histones is causing chromatin decondensation, we preincubated permeabilized nuclei with antihistone or a control antibody against CD63, a transmembrane granular protein. Nuclear decondensation was reduced by 50% in nuclei incubated with antibodies against H4, and to a lesser extent with antibodies against other core histones (Fig. S2 A), but not by the control antibody. Accordingly, H4 degradation was reduced in nuclei treated with antihistone antibodies (Fig. S2 B).

Interestingly, in a fractionation experiment, we purified H1 as an inhibitor of NET formation (unpublished data). H1 promotes the condensed, closed state of chromatin, which restricts its accessibility (Roche et al., 1985; Bustin et al., 2005; Woodcock et al., 2006). Nuclei pretreated or coincubated with H1 were resistant to decondensation and histone degradation by LSS and HSP (Fig. S2, C–E). Moreover, MPO failed to partition with the NF in nuclei pretreated with H1, which indicates that chromatin is the primary binding site of MPO in vitro (Fig. S2 D). Therefore, NE and MPO require access to the core histones to degrade them and induce decondensation.

**NE and MPO synergize to promote nuclear decondensation**

Because MPO localizes to NETs and binds to nuclei in vitro (Fig. 1 F), we tested whether MPO promotes nuclear decondensation (Brinkmann et al., 2004). At 1 µM, MPO alone had little effect, but it dramatically enhanced chromatin decondensation in the presence of NE (Fig. 1 H). Interestingly, MPO did not affect histone degradation, which suggests that it does not up-regulate NE activity (Fig. 1 I). MPO promoted nuclear decondensation in a dose-dependent manner that did not require its substrate H2O2 and was not inhibited by ABAH (Fig. 2, A and B). Furthermore, horseradish peroxidase, used as a control for enzymatic activity, did not promote chromatin decondensation (unpublished data). We concluded that MPO enhances NET formation independent of its enzymatic activity.

To better understand the synergy between NE and MPO, we measured its effect on nuclear decondensation driven by NE. Increasing amounts of MPO enhanced NE-mediated nuclear decondensation (Fig. 2 C). Surprisingly, high concentrations of NE inhibited nuclear decondensation (Fig. 2 D). This auto-inhibition could be caused by NE autodegradation. Alternatively, at high concentrations, NE could inhibit histone degradation. Accordingly, DNA has been shown to inhibit NE (Belorgey and Bieth, 1995). We confirmed the inhibitory effect of DNA on NE and CG, and found that it was reversed by the addition of NaCl (Fig. 3, A and B). Intact nuclei also inhibited NE activity, but this effect was not reversed by the addition of NaCl. Therefore, after histone cleavage, NE activity may be down-regulated by binding to DNA. Autoinhibition was not observed for PR3, which completely dissolved the nuclei at high concentrations (Fig. S1, E and F).

To investigate whether high concentrations of NE decreased histone degradation, we incubated nuclei with increasing NE concentrations and separated the nuclear (NF) and soluble (SF) fractions over filters (Fig. 2 E). H4 was degraded under optimal, but not autoinhibitory, concentrations of NE. Notably, NE activity is not required for NE binding to nuclei, as it partitioned with nuclei in the presence of NEi. The lower levels of NE in the NF fraction in the absence of NEi suggest that NE degrades itself in the nucleus (Fig. 2 E, left, asterisks).

We asked whether NE and MPO interact inside the nucleus by incubating nuclei with NE and low concentrations of MPO. MPO nuclear localization was independent of NE activity, as significant amounts of MPO were found in the nuclear fraction in the presence of NEi (Fig. 2 E, right). Moreover, we observed NE-dependent MPO degradation in the nucleus, but not in the soluble fraction, which indicates that these proteins come into close proximity in the nucleus.

**NE activity is required for NET formation in neutrophils**

To investigate whether NE activity is required for NET formation, we pretreated neutrophils isolated from healthy donors with NEi or CGI, and induced NET formation with PMA in the presence of Sytox, a cell-impermeable DNA dye. Untreated neutrophils and cells treated with CGI formed NETs efficiently 2–4 h after activation. Notably, NEi-treated neutrophils failed to make NETs, and appeared necrotic as determined by the Sytox-positive condensed nuclei (Fig. 3 A). To quantitate NET formation, we measured the DNA area of each Sytox-positive neutrophil and plotted the percentage of Sytox-positive cells against their corresponding nuclear area (Fig. 3 B). Our quantitation confirmed that neutrophil death is dramatically reduced in the presence of NEi (11.2% vs. 63.7%), whereas the low nuclear areas of the dead cells indicate that cells did not make NETs but were necrotic.

*Candida albicans* is a potent physiological activator of NET formation in isolated primary neutrophils. Neutrophils treated with NEi failed to make NETs efficiently when exposed to *C. albicans* (Fig. 3 C). The number of neutrophils that died without making NETs increased dramatically and was comparable to neutrophils treated with NEi and stimulated with PMA (Fig. 3 D). Interestingly, *C. albicans* induced NET formation with lower efficiency than PMA (Fig. 3 D), which indicates that when presented with microbes, neutrophils may respond in different ways, with some neutrophils consuming NE and MPO through phagocytosis or degranulation while others form NETs.

As controls, we show that NEi was not cytotoxic (Fig. 3 E) and did not inhibit ROS production, which was potently blocked by the NADPH oxidase inhibitor diphenylethiodonium (DPI; Fig. 3 F; Cross and Jones, 1986).

**NE cleaves histones during NET formation**

Because PMA is a more potent inducer of NETs than *C. albicans* and other microbes, we used it to examine whether histones are degraded in an NE-dependent manner during NET formation. Consistent with our in vitro data, H2B and H4 were
partially degraded during NET formation, whereas H3 shifted to a higher molecular weight species (Fig. 4 A). The presence of NEi inhibited the degradation of H4 and significantly reduced the degradation of H2B. NEi also prevented the formation of an H2A degradation product, which was observed in both naive and activated cells in the absence of the inhibitor. The higher...
Figure 3. NE is required for NET formation. (A) NEi but not CGI, a CG inhibitor, blocks NET formation. Purified human neutrophils, untreated or pretreated with NEi or CGI, were either activated with PMA for 4 h or left unactivated (naive) in the presence of 10% FCS. Shown are fluorescence images of cells in the presence of the cell-impermeable DNA dye Sytox green (left), and phase contrast images (right). Bar, 50 µm. (B) Quantitation of chromatin decondensation.
Histone degradation was detected before cell death and peaked during NET formation (Fig. 4 B). NET release and neutrophil death occurred simultaneously between 2 h and 3 h after stimulation, as indicated by the large DNA area of all Sytox-positive neutrophils, which was blocked in the presence of NEi (untreated) or in the absence of NEi (untreated). Histones were degraded 2–4 h after stimulation, coinciding with the peak of NET release (Fig. 4 B).

**Figure 4.** NE partially degrades core histones during NET formation. (A) Histone cleavage during NET formation is inhibited by NEi. Western immunoblotting against histones in lysates of naive (N) and PMA-activated neutrophils (PMA) in the presence (+NEi) or in the absence of NEi (untreated). (B and C) Quantitation of chromatin decondensation for the samples shown in A. (B) Untreated neutrophils. (C) Neutrophils treated with NEi. Shown are naive neutrophils at 0 h (gray) or 4 h (black), activated with PMA for 1 (yellow), 2 (orange), 3 (red), or 4 h (blue). (D) NE is not significantly externalized before NET formation. The time course of the release of NE into the medium by neutrophils activated with PMA measured by ELISA is shown. MNase was added to solubilize NE bound to DNA. Samples were normalized to NE levels from plated naive neutrophils lysed with 0.1% Triton X-100 (Total).

molecular weight H3 species in both activated and naive neutrophils were independent of NE activity and may represent other posttranslational modifications. Histones were degraded 2–4 h after stimulation, coinciding with the peak of NET release (Fig. 4 B).

Histone degradation was detected before cell death and peaked during NET formation (Fig. 4 B). NET release and neutrophil death occurred simultaneously between 2 h and 3 h after stimulation, as indicated by the large DNA area of all Sytox-positive neutrophils, which was blocked in the presence...
Figure 5. **NE, PR3, and MPO localization during NET formation.** (A–C) Naive and PMA-activated neutrophils in the presence or absence of NEi, fixed at the indicated time points and immunolabeled for NE (red) and PR3 (green; A), or NE (red) and MPO (green; B and C). DNA was stained with DRAQ5 (blue). (A) NE, and to a lesser extent PR3, translocate to the nucleus within 60 min after stimulation. (B) MPO associates with DNA before cell lysis but later than NE. (C) NEi prevents NE and MPO translocation to the nucleus, and chromatin decondensation. Bar, 5 µm. (D) NE is released from the granules.
of NEi (Fig. 4 C). By monitoring the release of NE during stimulation, we confirmed that significant levels of the NE remained intracellular during the first 2 h (Fig. 4 D).

**NE translocates to the nucleus during NET formation**

During NET formation, NE localized to the nucleus 60 min after stimulation, accompanied by reduced granular staining (Fig. 5 A, ii). In contrast, only low levels of PR3 were detected in the heterochromatin areas of the nucleus, arguing for a selective translocation of NE. After 120 min of stimulation, NE, but not PR3, was found predominantly in the decondensing nucleus in a diffuse gradient-like pattern (Fig. 5 A, iii). Interestingly, when neutrophils were treated with NEi, NE remained granular (Fig. 5 A, v), which suggests that NE activity is required for NE translocation. Because NE bound efficiently to isolated nuclei in the presence of NEi, NE activity may be required for granular release (Fig. 2 E).

In contrast to NE, MPO remained granular until the later stages of NET formation (Fig. 5 B, i), where it colocalized with NE and DNA in neutrophils undergoing NET release (Fig. 5 B, ii). Consistently, NE translocated faster than MPO in vitro (Fig. 1 F). Furthermore, the translocation of MPO was also blocked by NEi (Fig. 5 C).

We detected high levels of NE in the nuclei of neutrophils undergoing NET formation by immunohistological analysis, whereas NE activity was selectively decreased in the granules compared with MPO (Fig. 5 D). We did not detect NE activity in the nuclear fraction of either sample, as expected from the inhibitory effect of DNA (Fig. S3 A; Belorgey and Brieth, 1995). Moreover, CD63, a transmembrane protein found in azurophilic granule membranes, was not perinucleary at any point during NET formation. This indicates that the granules do not fuse with the nuclear membrane. Instead, NE is released from the granules and then translocates to the nucleus, a hypothesis that remains to be confirmed (Fig. S4).

Although it is conceivable that in isolated neutrophils, NE is first externalized and then binds to the nucleus of necrotic neutrophils, our observations argue against this possibility. The translocation of NE to the nucleus is selective and occurs within 1 h after activation (Fig. 5 A, iii), whereas neutrophil death occurs only after 3 h of stimulation with PMA (Fig. 4 B). Moreover, NE is not significantly externalized during that time (Fig. 4 D). Finally, cell death is primarily caused by NET formation, as neutrophil death is strikingly reduced when NE activity is inhibited (Fig. 3 B). However, such a mechanism may represent an alternative route to NET formation during an infection where externalized NE may process nuclei derived from necrotic bystander cells.

**NE is required for NET formation in vivo**

We examined NET formation in the lungs of wild type (WT) and NE knockout mice infected intranasally with a sublethal dose of *Klebsiella pneumoniae*, a Gram-negative bacterium that causes pneumonia. Neutrophils were massively recruited to the lungs 48 h after infection in both groups of animals, confirming that the loss of NE does not interfere with neutrophil recruitment (Hirche et al., 2004). The lungs of WT mice contained decondensed web-like structures that stain with antibodies against MPO and an H2A-H2B-DNA complex (Fig. 6 A, i; Brinkmann et al., 2004). NETs have also been observed in a mouse pneumococcal pneumonia model (Beiter et al., 2006). In contrast, in the lungs of infected NE knockout animals, neutrophils exhibited condensed nuclei, and MPO did not colocalize with the DNA marker but remained granular (Fig. 6 A, ii).

We also analyzed these sections by scanning electron microscopy. NETs trapping bacteria were present in abundance in the lungs of WT animals (Fig. 6 B, i and ii). In contrast, the lungs of NE knockout mice were devoid of structures resembling NETs on lung tissues and bacterial surfaces (Fig. 6 B, iii and iv). The absence of NETs in NE single knockout animals indicates that the extracellular DNA detected in the lungs of WT mice does not originate from bacterial biofilm, as such structures would be present in both groups of mice. Furthermore, these results suggest that PR3 cannot complement the loss of NE in NET formation.

In addition, we examined nuclear decondensation by extracts derived from mouse peritoneal neutrophils. LSS extracts from WT, but not from NE knockout mouse peritoneal neutrophils, decondensed nuclei in vitro (Fig. 6 C). Despite our efforts, peritoneal mouse neutrophils responded poorly to stimulation ex vivo, reflecting our current inability to potently stimulate NET formation in these isolated cells (Fig. S5). Collectively, these results indicate that NE is the major protease driving nuclear decondensation during NET formation in vivo.

**Discussion**

According to our model, ROS production leads to the release of NE and subsequently MPO from azurophilic granules. NE translocates first to the nucleus, where it digests nucleosomal chromatin state, but the details of the molecular mechanism remain to be addressed.

In addition to histone degradation, posttranslational modifications may also play a role during NET formation. We observed...
Figure 6. **NE knockout mice fail to form NETs.** (A) Representative fluorescence images of the lungs of WT (i) and NE knockout (ii) mice infected with *K. pneumoniae*, and stained with antibodies against MPO (green) and against a DNA/histone complex (red). The lungs of WT mice (i) contain decondensed web-like chromatin structures that stain for MPO (arrow). In contrast, in the lungs of NE knockout mice, all neutrophils appear naive, with condensed nuclei and granular MPO staining (asterisks). Bar, 20 µm. (B) NETs (arrows) trapping bacteria are detected in scanning electron micrographs of WT mouse lungs (i and ii) infected with *K. pneumoniae*. The lungs of NE knockout animals are devoid of NETs (iii and iv). Bars: (i and iii) 100 nm; (ii and iv) 1 µm. (C) Lysates from NE knockout mouse peritoneal neutrophils lack nuclear decondensation activity in vitro. Cell-free nuclear decondensation assays of mouse peritoneal nuclei treated for 2 h with LSS extracts from peritoneal neutrophils derived from WT and NE knockout mice are shown. ***, P < 0.0001.
Figure 7. Model of NET formation. In resting neutrophils, NE and MPO are stored in the azurophilic granules. Upon activation and ROS production, NE escapes the granules and translocates to the nucleus. In the nucleus, NE cleaves histones and promotes chromatin decondensation. MPO binds to chromatin in the late stages of the process. MPO binding promotes further decondensation. NE and MPO cooperatively enhance chromatin decondensation, leading to cell rupture and NET release.

An important role of NETs may be the sequestration of the neutrophil’s toxic antimicrobials to minimize tissue damage (Henson and Johnston, 1987; Weiss, 1989; Xu et al., 2009). Using antimicrobials as key factors in NET formation ensures that these toxic proteins are sequestered by chromatin. A two-step mechanism provides the necessary time for these proteins to bind to chromatin before neutrophil rupture. Allowing NE to operate early protects other NET proteins from being degraded by NE while it is still active inside the nucleus. Moreover, the inhibitory effect of DNA on NE activity may slow down the degradation of DNA-bound proteins, preserving their antimicrobial capacity. Interestingly, although histone degradation fragments are more potent antimicrobials than the intact proteins (Kawasaki and Iwamuro, 2008), the degradation of histones reduces cytotoxicity against the host during sepsis (Xu et al., 2009).

The requirement of neutrophil-specific factors explains why thus far extracellular trap formation is restricted to granulocytes, which also produce the highest levels of ROS (Segal, 2005). Mast cells express mast cell protease II, and eosinophil elastase is found in eosinophils (Woodbury et al., 1978; Lungarella et al., 1992). However, eosinophils may not require...
proteases, as they release their mitochondrial DNA, which lacks histones (Yousefi et al., 2008). Moreover, the contribution of other cell-specific peroxidases (Henderson and Kaliner, 1979; Ten et al., 1989) in ET formation may be closely associated with their ability to interact with DNA.

The immunodeficiency of NE and MPO knockout mice may be due in part to a defect in NET formation. Our findings may help explain the nonredundancy of NE with other related serine proteases. Mutations in NE are associated with neutropenias that are treatable with granulocyte-colony-stimulating factor (G-CSF; Ancillif et al., 2001; Aprikyan and Dale, 2001). Patients treated with G-CSF display neutrophils devoid of granular proteins (Horwitz et al., 2007). In spite of having near normal neutrophil counts, death from sepsis still poses a high risk in patients treated with G-CSF. With G-CSF treatment, mortality caused by sepsis has decreased from 6% to 0.9% per year (Rosenberg et al., 2006). The remaining risk in neutropenic patients with mutations in NE may be due in part to a defect in NET formation. Because the molecules involved in NET formation play important roles in other neutrophil antimicrobial mechanisms, it is difficult to dissect the contribution of NETs in immune defense. Notably, microbes have evolved virulence factors to degrade NETs (Beiter et al., 2006; Buchanan et al., 2006; Walker et al., 2007; Lauth et al., 2009). Therefore, it could be informative to document the types of microbes that infect patients with different immunodeficiencies, and associate them to their susceptibility to NETs.

The advances presented here uncover a novel mechanism for NET formation that may help to better understand and treat human immunodeficiency, sepsis, and autoimmune disease.

Materials and methods

Cell isolation and NET formation
Blood was drawn from healthy volunteers, and neutrophils were isolated over a Histopaque 1119 bed and a discontinuous Percoll gradient as described previously (Fuchs et al., 2007). Cells were stored in HBSS (without calcium or magnesium) before experiments. PBMCs were isolated from the residual Percoll. Granule fractions were resuspended into the original 1.050, 1.090, and 1.120 g/ml Percoll gradient as described previously (Kjeldsen et al., 1994; Lominadze et al., 2005). The data were corrected to a percentage of Sytox-positive cells by dividing the Sytox-positive counts by the total number of cells as determined from corresponding phase-contrast images, and plotted as the percentage of all cells that were positive for Sytox for each DNA area range.

Cell-free nuclear decondensation assay over filters
We seeded 5 × 10⁴ neutrophils per well in 24-well plates, in HBSS (+) medium supplemented with 10% human serum at an MOI of 10. C. albicans were grown overnight at 30°C in YPD media and subcultured to reach an exponential phase. Sytox images were taken 3 h after infection.

Histone degradation during NET formation
At each time point, the medium was removed and 5 × 10⁶ neutrophils were resuspended in 300 µl of 1× SDS loading buffer. Samples were resolved by SDS-PAGE electrophoresis, transferred to PVDF, blocked in 5% BSA, and labeled with primary antibodies and secondary horseradish peroxidase–conjugated antibodies (see Antibodies for Western immunoblotting).

Extracellular NE was measured by removing half the volume of media. NE levels were quantitated by ELISA. MNase (2 U/ml) was present throughout the time course to solubilize any extracellular NE bound to DNA.

Antibodies for Western immunoblotting

1:1,000 rabbit anti-H2A (2578, recognizes the C terminus; Cell Signaling Technology), 1:5,000 anti-H2B (07-371, recognizes aa 118–126; Millipore), 1:10,000 anti-H3 (07-690, recognizes the C terminus; Millipore), 1:5,000 anti-H4 (04-858, epitope mapped to aa 25–28; Millipore), and the pan-histone antibody MAB052 (1:500; Millipore) for analysis of H1. Rabbit anti-NE (ab21595; Abcam) was used at 1:200, or rabbit anti-NE (EMD) was used at 1:500. 1:10,000 anti-MPO (A0398; Dako), 1:500 anti-gelatinase (Dako), and 1:500 anti-lactoferrin (Sigma-Aldrich) were also used. Secondary antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc.) were used at a 1:20,000 dilution.

Immunostaining and microscopy
Cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, blocked with 3% BSA, and stained with the following primary antibodies: 1:500 mouse anti-NE [in house], 1:200 rabbit anti-NE (EMD), 1:200 rabbit anti-MPO (Dako), 1:200 EPC rabbit anti-PR3, 1:200 mouse anti-CD63 (CBL 553; Millipore), and DRAQ5 (Biostatus Limited). Secondary antibodies conjugated with Cy2 and Cy3 (Invitrogen) were used. Confocal images were obtained using a confocal fluorescence microscope (TCS-SP; Leica) and a Fluorostar objective lens; images were captured using ImageJ image processing software. The area of Sytox signal for 300–500 cells per sample was individually measured. For quantitation of nuclear decondensation, we plotted the mean DNA area derived from each nucleus (circles) and the standard deviation of the values (bars) denoting the range of areas for each condition. For NET formation, the distribution of the number of cells across the range of nuclear area was obtained using the frequency function in Excel (Microsoft). The data were corrected to a percentage of Sytox-positive cells by dividing the Sytox-positive counts by the total number of cells as determined from corresponding phase-contrast images, and plotted as the percentage of all cells that were positive for Sytox for each DNA area range.

Cell-free nuclear decondensation assay
Reactions of 10 µl of LSS extract at ~10 mg/ml total protein (derived from 3–5 × 10⁵ neutrophils/ml), the equivalent amount of HSP, or isolated granules, were mixed with 10⁶ nuclei and Sytox. Before incubation, 3-µl aliquots were transferred onto 12-well, 5-mm diagnostic slides (Menzel-Glaser) and covered with 20 × 50 mm coverslips. Reactions were performed in a humidified chamber at 37°C for 120 min (unless otherwise specified). Sytox-labeled nuclei were analyzed by fluorescence microscopy.
using Leica software. Epifluorescence images were obtained using an epifluorescence microscope (Axioplan; Carl Zeiss, Inc.) and 10×, 20×, or 40× objective lenses. Images were captured using a ProRes camera and software (Jenoptik).

Luminal assay
For ROS production, 10^5 neutrophils, either untreated or treated with 5 µM NEI, 5 µM CGP, or 10 µM of the NADPH oxidase inhibitor DPI, were activated with 100 nM PMA. ROS was measured by monitoring luminal fluorescence (50 µM) in the presence of 1.2 U/ml horseradish peroxidase.

Cytotoxicity assay
Cells were either untreated or treated with NEi at the indicated concentrations. After 5 h of incubation, the medium was collected, and LDH release was measured using the LDH cytotoxicity detection kit (Tokara Bio, Inc.) and normalized to the total LDH of an equivalent number of lysed neutrophils.

Mouse lung infections
Five WT 129S2 mice and five NEi [129S2 NE KO] knockout mice were infected with 2 × 10^5 Klebsiella pneumonia [KPS2145; Benghezal et al., 2007]. The bacteria were picked from an overnight plate and diluted into PBS. Mice were anesthetized and infected intranasally with 50 µl of PBS solution containing bacteria. 48 h later, mice were sacrificed, and lungs were removed and fixed with 2% PFA overnight. Lungs were embedded in paraffin and sectioned into 5-µm sections. Paraffin was removed with Neo Clear (Merck) and samples were rehydrated in ethanol. Samples were incubated in PBS containing 0.075% Tween 20 for 30 min at 50°C to expose the antibodies, blocked with 5% FCS/5% donkey serum, and stained with rabbit anti-MPO (1:50; Dako) and a mouse Fabs against a H2A–H2B–DNA complex (a gift from Marc Monestier, Department of Microbiology and Immunology, School of Medicine, Temple University, Philadelphia, Pennsylvania; Lomman et al., 1992) fused to ATTO 550. Lung inflammation and neutrophil recruitment were evaluated by hematoxylin and eosin stains. The experiment was repeated twice with five and seven mice per group, respectively.

For scanning electron microscopy, paraffin-embedded samples were rehydrated, postfixed with glutaraldehyde, contrasted using repeated changes of 0.5% OsO4 and 0.05% tannic acid, dehydrated in a graded ethanol series, critical-point dried, and coated with 5 nm platinum/carbon. Samples were obtained with a field emission scanning electron microscope (Leo 1550; Carl Zeiss, Inc.), and images were analyzed with SmartSEM software (Carl Zeiss, Inc.).

Animal experiments are in compliance with the German animal protection law and have been officially approved by the Landesamt fur Gesundheit und Soziales, Berlin.

Mouse peritoneal cells
Mouse peritoneal cells were collected 5 h after injection of 1 ml of thioglycollate into the peritoneal cavity of 10-wk-old mice. The peritoneum was lavaged with 10 ml of PBS to collect the neutrophils. Neutrophils were stained with rabbit anti-MPO (1:50; Dako) and a mouse Fabs against the histone-DNA complex. V. Papayannopoulos designed the project and all the experiments. V. Papayannopoulos performed all experiments with the exception of experiments performed by K.D. Metzler and where assisted by A. Hakkim. V. Papayannopoulos wrote the manuscript. K.D. Metzler performed all mouse neutrophil immunofluorescence stains and imaging (Fig. 4), the nuclear digestion with purified NE [Fig. 1, H and I], the blots of granule fractions (Fig. 1 D), and the NE ELISA (Fig. 4 B). A. Hakkim developed the mouse infection model and performed the mouse lung infections and lung isolation. A. Hakkim and V. Papayannopoulos adjusted and optimized the sublethal dose of K. pneumoniae for knockout animals. V. Papayannopoulos performed lung processing, staining, and image analysis of lung sections. A. Zychlinsky advised and coordinated the project. A. Zychlinsky supervised the writing of the manuscript.

This work was funded by the Max Planck Society. V. Papayannopoulos was supported by an EMBO Long term fellowship. None of the authors have any commercial or other conflict of interest.

Submitted: 9 June 2010
Accepted: 29 September 2010

References
Ancliff, P.J., R.E. Gale, R. Liesner, I.M. Hann, and D.C. Linch. 2001. Mutations in the ELA2 gene encoding neutrophil elastase are present in most patients with sporadic severe congenital neutropenia but only in some patients with the familial form of the disease. Blood. 98:2643–2650. doi:10.1182/blood.V98.9.2643


Benghezal, M., E. Adam, A. Lucas, C. Burn, M.G. Orchard, C. Deuschel, E. Valentino, S. Braillard, J.P. Paccaud, and P. Cossin. 2007. Inhibitors of


Figure S1. Specificity of the NE inhibitor. (A) NEi, but not CGi, inhibits the proteolytic activity of NE. Initial proteolytic rates (plotted as µmols of consumed elastase substrate I [MeOSuc-Ala-Ala-Pro-Val-pNA]/min) of commercially available purified NE (50 nM) alone, or in the presence of 10 µM NEi or 10 µM CGi. Although 0.5 µM NEi was also tested and is sufficient to inhibit NE activity (not depicted), we used higher concentrations to test the specificity of the inhibitor in the concentrations used in our experiments. The chromogenic elastase substrate I (300 µM) was used in all reactions. (B) NEi does not inhibit the proteolytic activity of CG. Commercially available purified CG (50 nM) and chromogenic CG substrate I (300 µM) were used. (C) NEi inhibits the proteolytic activity of purified PR3 against elastase substrate I. (D) NEi and SLPI inhibit the proteolytic activity against an NE-specific peptide substrate in blood neutrophil–derived LSS extracts (0.5 mg/ml). Initial proteolytic rates are plotted as µmols of consumed elastase substrate I/min. NEi was tested at 1 µM and 10 µM. SLPI, which inhibits both CG and NE, was used at 0.5 µM and 5 µM for comparison. (E) NEi does not inhibit the proteolytic activity against a CG-specific peptide substrate in blood neutrophil–derived LSS extracts (0.5 mg/ml). In contrast, CG-specific proteolytic activity was inhibited by SLPI. Mean values and the standard deviation from triplicate samples for each condition are presented (error bars). (F) PR3 cleaves histones in vitro. Because the available chromogenic PR3 substrates perform poorly, we tested PR3 at the indicated concentrations against isolated intact nuclei for 2 h at 37°C. Interestingly, PR3 displays a preference for H4 over other core histones. The proteolytic activity against H4 is blocked by 10 µM NEi. The PR3-dependent degradation of histones was also confirmed against soluble purified histones (not depicted). (G) NEi inhibits PR3-mediated nuclear decondensation in vitro. The indicated concentrations of PR3 were incubated with nuclei for 2 h at 37°C in the absence (left) or presence (right) of 10 µM NEi. These are the same samples as in E. When nuclei were treated with 3 µM PR3, decondensation was so extensive that only diffuse continuous DNA clouds were observed, and these could not be measured microscopically.
Figure S2. **Histone degradation mediates NE-mediated nuclear decondensation.** (A) Anti-histone antibodies reduce NE-mediated nuclear decondensation. Nuclei were preincubated with antibodies against H4, H2B, H3, and CD63 (control) in the presence of 0.01% Nonidet-P40. Subsequently, the samples were incubated in buffer (B) or in buffer containing 1 µM purified NE. Permeabilization enhanced NE-mediated nuclear decondensation, as compared with Fig. 2 C, but no decondensation was observed in nuclei treated with Nonidet-P40 in the absence of NE. (B) H4 degradation is reduced in the presence of anti-histone blocking antibodies. After incubation, the samples from A were analyzed by SDS-PAGE and immunoblotted to determine H4 degradation. H4 was nearly completely degraded by NE in nuclei treated with anti-CD63 antibody (control). Degradation was reduced in nuclei treated with anti-H4, anti-H2, and anti-H3 antibodies. Different molecular weight H4 species appear when the reaction was performed in the presence of different anti-histone antibodies. (C) The addition of histone H1 reduces nuclear decondensation. Nuclei were treated with buffer (B), buffer (B) + 12 µM H1, LSS, or LSS + 12 µM H1. Quantitation of nuclear decondensation by Sytox (DNA) was performed by fluorescence imaging after 2 h of incubation at 37°C. (D and E) Pretreatment with H1 prevents nucleosomal histone degradation and nuclear decondensation. (D) Nuclei were pretreated with buffer (untreated) or with 12 µM H1 (H1) for 1 h at 25°C. Buffer or crude neutrophil membrane fraction (HSP) was added to nuclei for 2 h. HSP in the absence of nuclei was also included to indicate the total MPO levels in the extract. An aliquot of the total unfiltered reaction (T) was removed and the rest was separated into the nuclear fraction (NF) and the soluble fraction (SF) over filters. The fractions were analyzed by SDS-PAGE immunoblotting, and labeled with a pan-histone and an anti-MPO antibody. Pretreatment with H1 prevented the binding of MPO to nuclei. (E) Nuclear decondensation of samples in D.
Figure S3. **DNA and nuclear binding inhibit NE activity.** Azurophilic granule (AZ) extracts (0.1 mg/ml total protein) were incubated for 30 min at 4°C, either alone (open bars) or with 0.3 mg/ml plasmid DNA (gray bars), or $2 \times 10^4$/ml nuclei derived from RA-differentiated HL-60 cells (shaded bars). Before enzymatic reactions, the indicated samples were treated with 600 mM NaCl for 5 min at 4°C to dissociate potential ionic interactions. All samples were then diluted 20-fold, and proteolytic activity against elastase substrate I (A) and CG substrate I (B) was measured at 25°C. Mean values and the standard deviation from triplicate samples for each condition are presented (error bars).
Figure S4. **Azurophilic granules do not fuse with the nuclear membrane during NET formation.** Naive or PMA-stimulated human neutrophils were fixed and immunolabeled for the transmembrane azurophilic granule marker CD63 (red) and for NE (green), and stained with the DNA dye DRAQ5 (blue). No CD63 staining was associated with the nucleus in activated neutrophils undergoing NET formation, as would be expected if the granular membranes were fusing with the perinuclear membrane or the ER. Arrows indicate perinuclear NE staining in the absence of CD63 staining during the early stages of NET formation. Bar, 3 µm.
Figure S5. Mouse peritoneal neutrophils do not form NETs in response to PMA. Peritoneal neutrophils were thioglycolate-elicited from either WT (129S2; black lines) or NE knockout (129S2 NE KO; gray lines) mice. Cells were stimulated with PMA. Sytox was added and cells were examined by microscopy at the indicated time points. The distribution of the fraction of Sytox-positive nuclei versus their respective chromatin area is shown. Red numbers on the x axis denote decondensation levels that are attributable to NETs produced by human blood-derived neutrophils. Although the neutrophils were stimulated with PMA, their nuclei fail to decondense effectively, and no differences were observed between WT and mutant strains.