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Neutrophil elastase-deficient mice form neutrophil extracellular traps in an experimental model of deep vein thrombosis

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Abstract

Background—Neutrophil serine proteases have been implicated in coagulation and neutrophil extracellular trap (NET) formation. In human neutrophils, neutrophil elastase (NE) translocates to the nucleus during NETosis and cleaves histones, thus aiding in chromatin decondensation. $NE^{-/-}$ mice were shown not to release NETs in response to microbes. However, mouse studies evaluating the role of NE in NET formation in sterile inflammation and thrombosis are lacking.

Objective—We wished to establish if neutrophils from $NE^{-/-}$ mice have a defect in NETosis, similar to peptidylarginine deiminase 4 ($PAD4^{-/-}$) mice, and how this might impact venous thrombosis, a model where NETs are produced and are crucial to thrombus development.

Methods—We performed in vitro NET assays using neutrophils from wild-type (WT), $NE^{-/-}$, SerpinB1 ($SB1^{-/-}$), and $NE^{-/-}SB1^{-/-}$ mice. We compared WT and $NE^{-/-}$ animals in the inferior vena cava stenosis model of deep vein thrombosis (DVT).

Results—NE-deficiency resulted in a small reduction in ionomycin-induced NET formation in vitro without affecting histone citrullination. However, NET production in response to PMA or PAF was normal in neutrophils from two independent NE-deficient mouse lines, or in $NE^{-/-}SB1^{-/-}$ as compared to $SB1^{-/-}$ neutrophils. NE-deficiency or inhibition did not prevent NETosis in vivo and DVT outcome.

Conclusions—NE is not required for NET formation in mice. $NE^{-/-}$ mice, which form pathological venous thrombi containing NETs, do not phenocopy $PAD4^{-/-}$ mice in in vitro

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Authorship contributions

K. Martinod designed and performed experiments, analyzed data, and wrote the paper. T. Witsch performed neutrophil elastase inhibition in vivo experiments and analyzed data. K. Farley performed experiments and analyzed data. M. Gallant provided expert technical assistance. E. Remold-O'Donnell provided mice, primers, helpful advice, analyzed data and contributed to discussions. D. D. Wagner supervised the study, designed experiments, analyzed data, and co-wrote the paper.

Disclosure

T. Witsch, M. Gallant, K. Martinod and E. Remold-O'Donnell report grants from NIH, during the conduct of the study.

NETosis assays or experimental venous thrombosis. Our study suggests that NET-targeted therapies need to be highly effective to have an impact on DVT.

Introduction

The serine protease function neutrophil elastase (NE) plays a crucial role in host defense [1] and is present on neutrophil extracellular traps that are released from activated neutrophils [2, 3]. In addition to being present on NETs, NE has also been implicated in chromatin decondensation in the process of forming NETs by cleaving histones. Indeed, NE translocates from azurophilic granules to the nucleus [4], which occurs before translocation of other granule contents such as myeloperoxidase (MPO). This occurs by a recently described mechanism independent of granule membrane fusion [4] but dependent on reactive oxygen species (ROS) and MPO [5]. In the Papayannopoulos et al. study, an elegant series of in vitro experiments was performed using isolated primary human neutrophils to show that NE, and not other serine proteases in the neutrophil such cathepsin G or proteinase 3, aids in chromatin decondensation during NETosis [4]. NE inhibitors greatly diminished NE entry into the nucleus, chromatin decondensation and NET formation in response to PMA. The effect of NE was also tested in mice, where intranasal instillation of *Klebsiella pneumoniae* in WT mice led to extensive fiber formation in the alveolar space, whereas $NE^{-/-}$ mouse lungs remained free of such NET-like fibers. While nuclear swelling was shown to be decreased by NE-deficiency using a cell-free assay, a quantitative in vitro NET assay remains to be performed using peripheral blood neutrophils which have not been activated in an inflammatory model. The role of NE in non-infectious disease models strongly affected by NET production was not studied. In addition, neutrophil serine proteases were shown to promote coagulation/thrombosis by cleaving tissue factor pathway inhibitor (TFPI) [6]. Interestingly, the serine protease inhibitor SerpinB1, a cytoplasmic protein abundant in neutrophils, was shown to have a regulatory role in NET formation [7]. SerpinB1 also translocates to the nucleus during NETosis in human neutrophils and SerpinB1-deficient mice ($SB1^{-/-}$) have exacerbated NET production in vitro and in vivo compared to WT mice [7]. We wished to examine whether the pro-NETosis phenotype would be reversed by NE deletion. Recently it was documented that NE on NETs retains its activity [8], and therefore we also wished to test the specific effect of NE on pathological venous thrombosis.

Materials and Methods

Mice

Experimental procedures in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the Immune Disease Institute and Boston Children's Hospital (Protocol No. 11-03-1919, 11-04-1848, 14-02-2609R, 14-03-2631R, 12-11-2317R). C57Bl/6J (WT) and $Elane^{tm1Sds}$/J ($NE^{-/-}$) mice [1] were purchased from The Jackson Laboratory. The independently generated neutrophil elastase null mice on the 129Sv background [9] in Figure 2 were made available by Jürgen Roes (University College London) and provided by Hongbo Luo (Boston Children's Hospital). Wild-type 129SvEv mice were from Taconic Labs. SerpinB1-null mice ($SB1^{-/-}$) backcrossed to C57BL/6 for 10

generations were previously described [7, 10]. The latter mice and the $NE^{-/-}$ mice on the C57BL/6 background [1] were crossed to generate $SB1^{-/-}NE^{-/-}$ mice. We also verified that $Elane^{tm1Sds}$/J mice did not express NE mRNA in isolated bone marrow cells using conventional PCR and the following primers: NE-forward, 5′-GTGGTGACTAACATGTGCCG-3′; NE-reverse, 5′-AATCCAGATCCACAGCCTCC-3′ (data not shown).

In vitro NET assays

Peripheral blood was collected via the retroorbital venous plexus and neutrophils were isolated from 6–18 week-old male or female WT or $NE^{-/-}$ mice as described [11]. Cells were assessed to be >90% pure by Wright-Giemsa stain. Neutrophils in RPMI/HEPES were incubated at 37°C in 5% CO_2 in glass-bottom plates for 20 min prior to stimulation with 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma) or 4 μM ionomycin (iono, Invitrogen). After 3.5 h, cells were fixed in 2% PFA and analyzed as described [12].

Bone marrow neutrophil isolation and NET assays were performed as previously described using Sytox Green staining [7].

Venous stenosis model

IVC stenosis experiments were performed as described under aseptic conditions [12, 13], with 90% ligation of the IVC and complete ligation of side branches to minimize variation among mice. Blood was collected 6 h or 48 h after stenosis via the retroorbital sinus, and thrombi harvested, measured and embedded in OCT for cryosectioning and histological analysis. All mice were given buprenorphine (0.1 mg/kg, s.c.) as an analgesic immediately before surgery and every 8–12 h subsequently. For neutrophil elastase inhibition experiments, mice received i.p. injections of sivelestat sodium tetrahydrate (Abcam, 30 mg/kg in 0.9% sterile saline) or vehicle 30 minutes prior to surgery and mice were euthanized after 6 h.

Blood cell and plasma analysis

Twenty-five μl of whole blood collected via the retroorbital sinus into EDTA-coated capillary tubes was analyzed by a Hemavet 950FS (Drew Scientific) for complete blood counts. At the time of sacrifice in DVT experiments, at least 250 μl of citrated whole blood was centrifuged to prepare platelet-poor plasma [12]. Plasma DNA concentrations were measured according to manufacturer's instructions using the Quant-iT™ PicoGreen® dsDNA Assay kit (Invitrogen).

Immunostaining and fluorescence microscopy

Fixed cells or tissue sections were immunostained as described [12]. Fluorescent images were acquired using an Axiovert 200 widefield fluorescence microscope (Zeiss). All channels were taken in greyscale with an Axiocam MRm monochromatic CCD camera (Zeiss), pseudocolored using Zeiss Axiovision software, and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistics

Data are presented as mean ± SEM unless otherwise noted and were analyzed using a one-way ANOVA with Bonferroni's posthoc analysis or Mann-Whitney U test. Thrombus frequencies were analyzed using chi-squared tests of contingency tables. All analyses were performed using GraphPad Prism software (Version 5.0). Results were considered significant at $p<0.05$. * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

Results/Discussion

Neutrophils from NE$^{-/-}$ mice present only a small reduction in NET production

We examined neutrophils from WT or neutrophil elastase-deficient mice for their ability to form NETs in response to non-microbial stimuli (Figure 1). For this we isolated WT and NE$^{-/-}$ neutrophils from mouse peripheral blood and stimulated them in parallel with PMA or ionomycin for 3.5 hours. The percentage of cells becoming hypercitrullinated at histone H3 was similar (Figure 1A), indicating the PAD4 is functional in these cells and that NE is not required for its nuclear translocation or activity. NET release is thought to be impaired in the NE$^{-/-}$ mice as shown by electron microscopy of *Klebsiella pneumonia* infected lungs [4]. Although we also did find that NE$^{-/-}$ neutrophils formed less NETs than WT, surprisingly this reduction was only about 40% compared to WT and a substantial portion of NE$^{-/-}$ neutrophils released NETs (Figure 1B). Representative images in Figure 1C show staining for H3Cit+ NETs in response to ionomycin as indicated by white arrows.

Of note, the reduction in NET formation in NE$^{-/-}$ neutrophils was only seen in response to stimulation with ionomycin. In response to PMA, NE−/− cells produced similar numbers of NETs compared to WT cells as quantified by nuclear morphology with cell-permeable Hoechst 33342 (Figure 1B) or cell-impermeable Sytox Green staining (Figures 2A and 2B). A lack of reduction of NET production with NE-deficiency was seen in both the C57BL/6 and the 129S6 backgrounds of mice (Figure 2) and was also seen with stimulation with platelet activating factor (PAF) (Figure 2). Interestingly, we saw that the increased NET formation seen in SB1$^{-/-}$ mice [7] was not abrogated by NE-deficiency, indicating that NE is not the only serine protease involved in this regulatory process (Figure 2B).

We also treated WT or NE$^{-/-}$ neutrophils with the NE inhibitor sivelestat (Figures 1A and 1B, dashed bars) to see if this would affect NETosis. Again, histone hypercitrullination was not impaired, while the percentage of cells releasing NETs was reduced in neutrophils from both WT and NE$^{-/-}$ mice as compared to vehicle-treated cells. This indicates that this particular, widely used NE inhibitor may have off target effects independent of neutrophil elastase activity. Indeed, while sivelestat was initially described as a selective inhibitor of NE in comparison with trypsin, thrombin, plasmin, kallekrein, chymotrypsin, and cathepsin G [14], it has been reported that sivelestat also efficiently inhibits proteinase 3 activity [15] and could possibly inhibit other enzymes.

NE$^{-/-}$ mice form NET-rich thrombi in the IVC stenosis model

Venous thrombosis in the IVC stenosis model of deep vein thrombosis is highly NET dependent [12, 13, 16]. In addition, NE was proposed to enhance coagulation [6], which

should enhance thrombosis. DVT is therefore an interesting setting in which to study the role of NE in NETosis and thrombosis. According to our in vitro results, NET formation should be slightly reduced in the $NE^{-/-}$ animals and any NETs being released would be devoid of neutrophil elastase. We hypothesized that thrombus size would be reduced in $NE^{-/-}$ mice since they form fewer NETs and inhibit less TFPI. Thrombus initiation frequency was unaffected in $NE^{-/-}$ compared to WT mice 6 h after partial IVC ligation (Figure 3A, B), but circulating plasma cell-free DNA levels were reduced by about 20% (Figure 3C, $P < 0.05$), indicating that fewer NETs are being released into circulation in the $NE^{-/-}$ mice. By 48 h, thrombus frequency was again similar between WT and $NE^{-/-}$ mice (Figure 3D, E), and $NE^{-/-}$ mice were capable of forming thrombi that were quite large in size (Figure 3D), indicating that the mutant mice don't have a major thrombotic defect and that the thrombi were stable. The approximate thrombus density of the mostly occlusive thrombi as assessed by a ratio of weight:length were similar between WT and $NE^{-/-}$ mice at both time points (6h: WT 2.20 ± 0.96 n=5, $NE^{-/-}$ 2.16 ± 0.73 n=5; 48h: WT 1.38 ± 0.97 n=5, $NE^{-/-}$ 2.23 ± 1.10 n=3). Thrombi from both the WT and $NE^{-/-}$ mice contained a characteristic NET meshwork [12, 17] at 48 h, visualized by extracellular H3Cit and DNA staining (Figure 3F). We also treated WT mice with sivelestat using a previously published dose of 30 mg/kg [18] and found that this treatment had no effect on thrombus formation or DNA levels in the plasma after 6 h (Figure 4). In mice which formed thrombi, there was a tendency toward reduced thrombus size with sivelestat treatment, but this was not statistically significant (WT 7.20 ± 0.644 mm, n=5; NE−/− 5.44 ± 0.601 mm, n=8; P = 0.06). Table 1 shows that neutrophil and platelet counts were similar after DVT between WT and $NE^{-/-}$ mice, as well as in WT mice that received NE inhibitor compared to vehicle.

The thrombotic potential of $NE^{-/-}$ mice greatly differs from that seen in $PAD4^{-/-}$ mice [12] or in mice treated with DNase [16, 17] in the same model. In IVC stenosis, $PAD4^{-/-}$ are highly protected from thrombosis and the few thrombi that do form don't appear to have NETs [12]. Also, recombinant DNase 1, which cleaves NETs [2] significantly reduces the incidence of thrombosis [16, 17]. The role of NE in thrombosis may not lie in its involvement in the generation of NETs, but rather NE may be active on released NETs. Therefore, studying the impact of NE on coagulation in vivo and on thrombus stability and resolution is of interest.

Taken together, these results show that slightly reduced NET formation has little beneficial impact in vivo in a non-infectious mouse model (DVT). This is in agreement with our observation that mice heterozygous for PAD4 showed no significant protection in DVT (unpublished result, Martinod and Wagner 2012). We had hypothesized that $NE^{-/-}$ mice would phenocopy $PAD4^{-/-}$ mice, which are completely unable to form NETs due to a lack of a histone-modifying enzyme required for chromatin decondensation [12, 19]. While we did see less NET release in the $NE^{-/-}$ blood neutrophils, this reduction was not observed after stimulation with PMA or PAF. Furthermore, NE was not required for the enhanced NETosis seen in $SB1^{-/-}$ mice. From our results, it would also appear that NE inhibitors used in mouse studies [20–22] may have additional non-specific activities besides inhibiting NE, as documented by sivelestat's inhibitory effect on NETosis in NE−/− neutrophils and lack of

NETosis reduction in vivo. For example, sivelestat was reported to also inhibit proteinase 3 (PR3) [15].

It appears that there are differences in the molecular mechanisms driving NETosis in murine versus human neutrophils. While human neutrophils may require neutrophil elastase to undergo NETosis, it is likely that several serine proteases such as cathepsin G (CatG), PR3, and NE may have overlapping pro-NETotic effects in the mouse. It was recently published that mice doubly deficient in NE and PR3 are protected from NET formation in atherosclerosis, a sterile setting dependent on cholesterol crystals for inducing NETs [23]. Clearly we now show that NE-deficiency is not sufficient to prevent NET formation in mouse neutrophils. In addition, the signaling pathways involved in NET formation during thrombosis, a sterile inflammatory process, may differ from the signaling pathways required for NETosis in response to infection.

Our results have important implications for designing therapeutics targeting prevention of NET formation. We have proposed that PAD4 would be an attractive candidate for this goal [24], but only recently have highly specific and effective PAD4 inhibitors been described [25] and their efficacy in vivo is still unknown. Targeting NE could have been beneficial in more aspects than just reducing NET formation, as protective effects have been seen using NE inhibitors in mouse models [20, 21, 26]. We now believe these were due to inhibition of NE secreted from neutrophils rather than an inhibition of NETosis. It appears that NE inhibition alone is unlikely to have major NET inhibition effects in the context of DVT. Even partial NET release seems sufficient to drive venous thrombus formation, as NETs avidly bind platelets, red blood cells [27], microparticles [28], and promote coagulation [6, 27]; this all promotes thrombus development [16, 27]. This does not mean that NE inhibitors would not have other beneficial effects in DVT, and the role of NE should be studied in the future in terms of its effect on thrombus stability, resolution, and post-thrombotic syndrome. In inflammation, when more neutrophils are recruited to inflammatory sites and NETs accumulate, the effect of a partial NET inhibition is also likely to be insignificant over time. We therefore do not recommend that $NE^{-/-}$ mice or NE inhibitors be used as a method in mouse models to assess the effects of a lack of NETosis. It will be important to develop inhibitors that stably and profoundly inhibit NETosis and/or degrade NETs with high efficacy to impact pathological thrombosis.

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Essentials

- Neutrophil elastase (NE) plays a role in extracellular trap formation (NETosis) triggered by microbes.
- The contribution of NE was evaluated in mouse NETosis models of sterile inflammation and thrombosis.
- We found little role for NE in mouse neutrophil NET production in vitro with non-infectious stimuli.
- NE-deficiency had no significant effect on thrombosis in the inferior vena cava stenosis model.

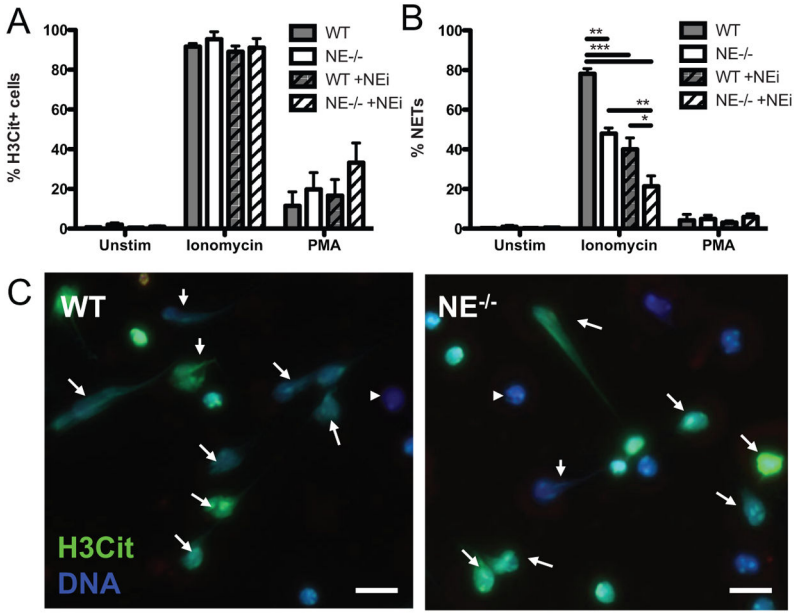

Figure 1. Neutrophil elastase-deficient neutrophils form fewer NETs in response to calcium ionophore but citrullination is unaffected

A. Neutrophils isolated from WT (gray bars) and $NE^{-/-}$ [1] blood (white bars) became hypercitrullinated at histone H3 to a similar degree in response to stimulation with ionomycin or PMA for 3.5 h. Neutrophil elastase inhibition (dashed bars) did not impact histone citrullination. n=5. Results are representative of 3 independent experiments. B. NET formation was similar between WT and $NE^{-/-}$ neutrophils in response to PMA, but was reduced by about 40% in $NE^{-/-}$ neutrophils in response to ionomycin. Neutrophil elastase inhibition similarly reduced NET formation in both WT and $NE^{-/-}$ neutrophils. n=5. Representative of 3 independent experiments. C. Representative micrographs of ionomycin-stimulated cells showing H3Cit-positive NETs forming both by WT and $NE^{-/-}$ neutrophils. White arrows indicate NETs. White arrowheads indicate H3Cit-negative cells. H3Cit, green; DNA, blue. Representative of n=10, scale bar, 20 μm. *P < 0.05, **P < 0.01, ***P < 0.001.

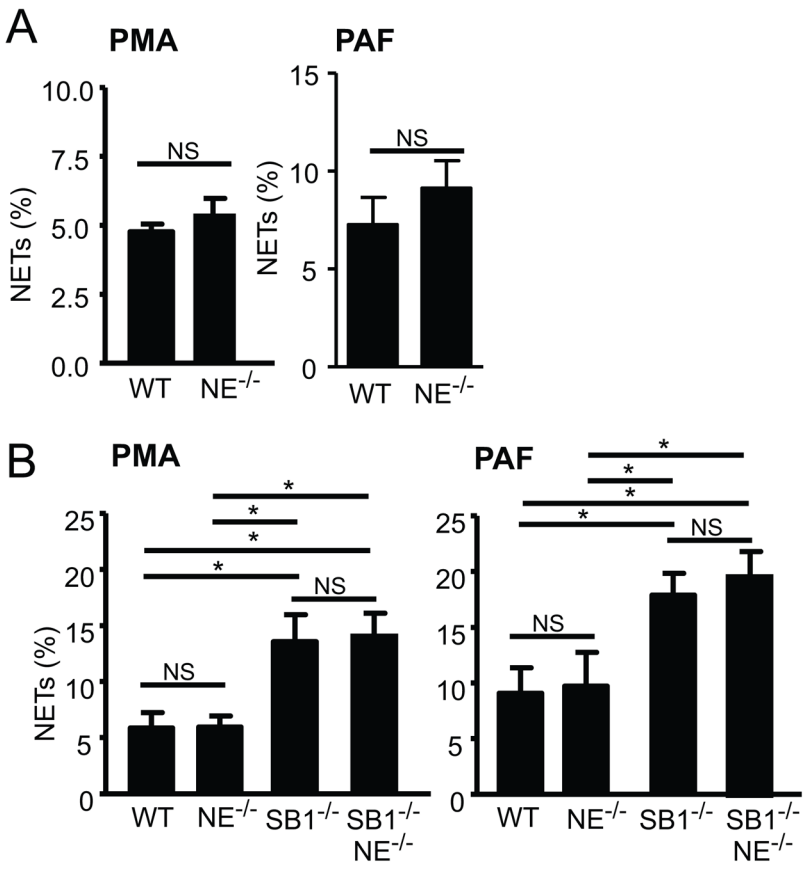

Figure 2. NET production by bone marrow neutrophils from two NE-null mouse lines do not differ from matched NE-expressing neutrophils

The NE-deficient mice in A and B are progeny of unrelated independent gene deletions [1, 9]. Bone marrow neutrophils were isolated and stimulated by PMA (left, 50 nM, 4 h) or PAF (right, 10 μm, 30 min) as previously described [7]. NETs were quantified in fluorescence images after Sytox staining. (*A*) $NE^{-/-}$ and WT neutrophils on a 129S6 background [9]. (*B*) $NE^{-/-}$, WT, $NE^{-/-}SB1^{-/-}$ and $SB1^{-/-}$ neutrophils on a C57Bl/6 background [1, 7, 10]. The data (% NETs) representing means ± SEM for 6 or more mice/group in 2 or more experiments, were analyzed by (*A*) unpaired *t* test or (*B*) ANOVA. *P < 0.05; NS, not significant.

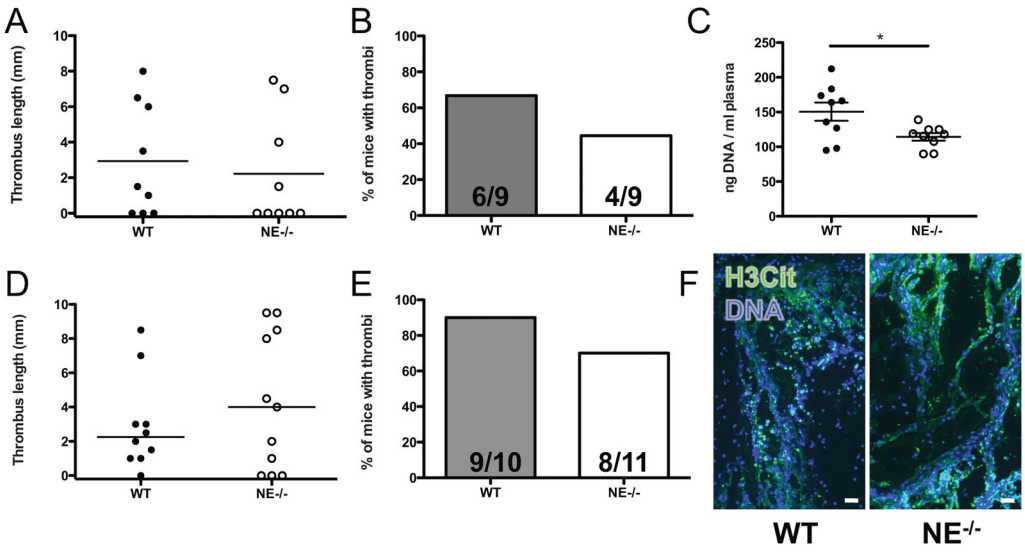

Figure 3. NE$^{-/-}$ mice form NET-containing thrombi in the venous stenosis model of DVT

A,B. NE$^{-/-}$ mice [1] formed thrombi to a similar extent to WT mice after 6 h of IVC stenosis. C. Plasma DNA was slightly decreased after 6 h in NE$^{-/-}$ mice compared to WT mice. D,E. At 48 h, NE$^{-/-}$ mice also presented with thrombi to a similar extent to WT mice. F. Both thrombi collected from WT or NE$^{-/-}$ mice 48 h after IVC stenosis showed an abundance of NETs as seen by H3Cit immunostaining (green) and extensive diffuse extracellular DNA staining (blue). Scale bar, 25 μm. *P < 0.05.

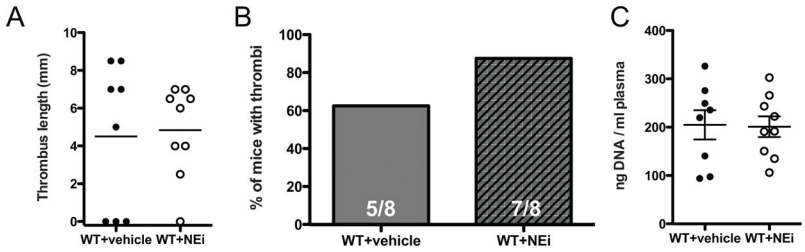

Figure 4. NE inhibition does not impair venous thrombus formation

Mice were injected with 30 mg/kg of sivelestat or vehicle 20 minutes prior to IVC ligation and then euthanized after 6 h to evaluate thrombus formation. A,B. NE inhibitor did not prevent thrombus formation in WT mice. C. Plasma DNA levels were similar between WT mice treated with vehicle or with sivelestat.

Table 1

Blood counts in mice at various time points in the venous stenosis model

A 25 μL blood sample was collected from WT or $NE^{-/-}$ mice via the retroorbital sinus either from non-operated mice, mice just prior to the time of sacrifice and thrombus harvest, or from WT mice treated with vehicle or neutrophil elastase inhibitor sivelestat (30 mg/kg) prior to thrombus harvest. Although platelet counts were slightly higher in the $NE^{-/-}$ group of non-operated mice compared to WT mice, there were no significant differences in platelet or neutrophil counts in mice after receiving IVC stenosis.

	WT	$NE^{-/-}$	*P*-value
Non-operated Neutrophils ($\times 10^3$/μl)	1.419 ± 0.112, n=8	2.250 ± 0.399, n=9	0.0539
Non-operated Platelets ($\times 10^3$/μl)	799.8 ± 24.97, n=8	900.6 ± 14.32, n=9	0.0045
6h IVC stenosis Neutrophils ($\times 10^3$/μl)	2.871 ± 0.280, n=9	3.573 ± 0.336, n=9	0.190
6h IVC stenosis Platelets ($\times 10^3$/μl)	623.9 ± 64.08, n=9	786.6 ± 53.79, n=9	0.0932
48h IVC stenosis Neutrophils ($\times 10^3$/μl)	1.941 ± 0.318, n=10	1.693 ± 0.173, n=11	0.751
48h IVC stenosis Platelets ($\times 10^3$/μl)	780.9 ± 55.13, n=10	708.9 ± 68.16, n=11	0.438

	WT+Vehicle	WT+NEi	*P*-value
6h IVC stenosis Neutrophils ($\times 10^3$/μl)	1.915 ± 0.270, n=8	2.108 ± 0.244, n=9	0.595
6h IVC stenosis Platelets ($\times 10^3$/μl)	630.1 ± 57.5, n=8	678.6 ± 34.6, n=9	0.470