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# Neutrophils and neutrophil extracellular traps enhance venous thrombosis in mice bearing human pancreatic tumors

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## ABSTRACT

Pancreatic cancer is associated with a high incidence of venous thromboembolism. Neutrophils have been shown to contribute to thrombosis in part by releasing neutrophil extracellular traps (NET). A recent study showed that increased plasma levels of the NET biomarker, citrullinated histone H3 (H3Cit), are associated with venous thromboembolism in patients with pancreatic and lung cancer but not in those with other types of cancer, including breast cancer. In this study, we examined the contribution of neutrophils and NET to venous thrombosis in nude mice bearing human pancreatic tumors. We found that tumor-bearing mice had increased circulating neutrophil counts and levels of granulocyte-colony stimulating factor, neutrophil elastase, H3Cit and cell-free DNA compared with controls. In addition, thrombi from tumor-bearing mice contained increased levels of the neutrophil marker Ly6G, as well as higher levels of H3Cit and cell-free DNA. Thrombi from tumor-bearing mice also had denser fibrin with thinner fibers consistent with increased thrombin generation. Importantly, either neutrophil depletion or administration of DNase I reduced the thrombus size in tumor-bearing but not in control mice. Our results, together with clinical data, suggest that neutrophils and NET contribute to venous thrombosis in patients with pancreatic cancer.

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## Introduction

Cancer patients have a 4- to 7-fold increased risk of venous thromboembolism (VTE) compared with the general population.[1] However, the rates of VTE vary in different cancer types. For instance, breast cancer has a low rate whereas pancreatic cancer has a high rate of VTE.[2] This variability suggests that there may be cancer type-specific mechanisms of VTE.[3] For instance, we found an association between levels of circulating extracellular vesicle tissue factor activity and VTE in pancreatic cancer in two studies and a borderline significance in a third study.[4-6] Circulating tumor-derived, tissue factor-positive extracellular vesicles are also observed in mice bearing human pancreatic tumors[7-10] Importantly, we have shown that these tumor-derived, human tissue factor-positive extracellular vesicles enhance venous thrombosis in mice.[10]

Leukocytosis is often observed in cancer patients, particularly patients with lung and colorectal cancer.[3] Leukocytosis is also associated with VTE in cancer patients, and is a component of the Khorana Risk Score for predicting chemotherapy-asso-

ciated thrombosis in ambulatory cancer patients.[11-13] In addition, some patients have increased circulating levels of hematopoietic cytokines, such as granulocyte-colony stimulating factor (G-CSF).[14] The coagulation cascade is activated by pathogens as part of the innate immune system to limit dissemination of infection.[15] Recently, the term "immunothrombosis" was introduced to describe the contribution of immune cells to thrombus.[16] Activated monocytes can trigger thrombosis by expressing tissue factor.[17] Activated neutrophils release proteases, such as neutrophil elastase (NE), which enhance thrombosis by degrading the anticoagulant protein tissue factor pathway inhibitor.[18] In addition, neutrophils release neutrophil extracellular traps (NET). NET are composed of extracellular chromatin components and neutrophil granule proteins that enhance thrombosis by capturing platelets and procoagulant extracellular vesicles.[19-22] NET are present in both arterial and venous thrombi.[19,23,24] NET can also obstruct smaller blood vessels in a coagulation-independent manner.[25] Interestingly, two studies showed that neutrophils contribute to thrombosis in the mouse inferior vena cava (IVC) stenosis model, although this was not observed in a third study.[20,26,27] In contrast, neutrophil depletion did not affect thrombosis in the IVC stasis model.[28]

There is a wide range of agonists that can induce NET formation.[29] In neutrophils histone citrullination by peptidylarginine deiminases (PAD), including PAD4, is considered a driver of chromatin decondensation and subsequent NET formation.[30] PAD4 is also expressed by the human breast cancer cell line MCF7.[31] Citrullinated histones, such as citrullinated histone H3 (H3Cit), are therefore widely used as a biomarker of NET formation. In mice, it has been proposed that PAD4 is required for NET formation.[32] Indeed, PAD4$^{-/-}$ mice have smaller thrombi in the IVC stenosis model.[33] However, a recent study found that inhibition of PAD did not affect human neutrophil NET formation induced by a variety of pathogens,[29] suggesting that certain forms of NET formation can occur without PAD. Interestingly, a recent study found an association between plasma levels of H3Cit and VTE in patients with pancreatic and lung cancer but not in those with other types of cancer, such as breast cancer.[34] In another study plasma levels of nucleosomes and cell-free DNA (cfDNA) were higher in cancer patients than in healthy controls, but these are not NET-specific biomarkers.[35]

Neutrophilia was observed in mice bearing murine breast 4T1 tumors and human pancreatic BxPc-3 tumors.[10,36-38] In addition, mice bearing 4T1 breast tumors had increased levels of circulating markers of neutrophil activation and NET, such as H3Cit and myeloperoxidase.[37,38] Furthermore, tumor-bearing mice had more rapid thrombotic occlusion in a jugular vein Rose Bengal/laser-induced injury model.[38] Interestingly, administration of DNase I to degrade cfDNA and NET did not affect thrombotic occlusion in control mice but provided protection from the enhanced venous thrombosis observed in tumor-bearing mice.[38] These studies suggest that neutrophils and NET contribute to venous thrombosis in a murine breast cancer model.

In the light of recent clinical data suggesting a role of NET in VTE in patients with pancreatic cancer,[34] we investigated the contribution of neutrophils and NET to venous thrombosis in mice bearing human pancreatic BxPc-3 tumors.

## Methods

### Cells and the mouse tumor model

We used a human pancreatic cancer cell line BxPc-3 expressing the firefly luciferase reporter.[10] BxPc-3 tumors were grown in the pancreas of Crl:NU-*Foxn1*$^{nu}$ male mice (nude mice) and monitored by measuring luciferase expression.[10] We used mice with tumors weighing from 1.5 to 3.9 grams. All animal studies were approved by the University of North Carolina at Chapel Hill Animal Care and Use Committee, and complied with National Institutes of Health guidelines.

### Measurement of blood cells

A Hemavet HV950FS (Drew Scientific, Miami Lakes, FL, USA) was used to count neutrophils.

### Preparation of plasma and measurement of plasma biomarkers

Blood was collected[39] and plasma was prepared by centrifuging the blood at 4500 x *g* for 15 min. CfDNA was quantified as described elsewhere.[40] Mouse G-CSF and NE were measured using commercially available enzyme-linked immunosorbent assays (R&D systems, Minneapolis, MN, USA). H3Cit was measured using an in-house enzyme-linked immunosorbent assay.[41]

### Thrombosis model

We used the IVC stasis model of thrombosis.[10]

### Western blot analysis of thrombus samples

Processing of thrombi and detection of primary antibodies is described in the *Online Supplementary Methods*. Membranes were probed with 2 μg/mL anti-Ly6G (BioXCell, West Lebanon, NH, USA), a 1,000-fold dilution of anti-β-actin (Abgent, San Diego, CA, USA), a 2,000-fold dilution of anti-PAD4 (Abcam), 1 μg/mL anti-H3Cit (Abcam) or 0.5 μg/mL anti-histone H3 (Abcam) primary antibody.

### Immunofluorescence

Analysis of thrombi by immunofluorescence is described in the *Online Supplementary Methods*. Areas of different fluorescent signals were quantified using Image J software.

### Scanning electron microscopy

Analysis of thrombi by scanning electron microscopy is described in the *Online Supplementary Methods*.

### Neutrophil depletion

Neutrophils were depleted by intravenous administration of 5 mg/kg anti-Ly6G antibody (BioXCell) 24 h and 1 h before IVC stasis. A rat IgG (Sigma-Aldrich) was used as a control.

### DNase I treatment

DNase I (50 U/mouse (Genentech, South San Francisco, CA, USA) or phosphate-buffered saline was intravenously administered to mice 1 h before and 24 h after IVC stasis.

### Statistical analysis

Data are shown as mean ± standard error of the means for normally distributed data or median ± interquartile range for non-normally distributed data. The Shapiro-Wilk test was used to determine normality. For the majority of the studies two-group comparisons, the unpaired two-tailed Student t-test or the Mann-Whitney U-test was used depending on the data distribution. For the ultrasound data, two-way analysis of variance with the Sidak multiple comparison test was used. These statistical analyses were

performed with GraphPad PRISM version 7.03 (GraphPad Software, La Jolla, CA, USA). For immunofluorescence data and scanning electron microscopy data, bootstrap simulations of the chosen quantiles of the distribution of the measured values were performed[42] followed by a Bootstrap Kuiper test for identity of the quantile distributions.[43] These statistical analyses are described in detail in the *Online Supplementary Material*. *P* values <0.05 were considered statistically significant for all experiments.

## Results

### Measurement of circulating neutrophil counts and different biomarkers in mice bearing human pancreatic tumors

We measured the numbers of neutrophils in whole blood and various circulating biomarkers in the plasma of mice bearing human BxPc-3 pancreatic tumors (1.5-3.9 grams) and in control mice. We observed significant increases in neutrophil numbers and mouse G-CSF levels in tumor-bearing mice compared with those in controls (Figure 1A, B). We did not observe an increase in human G-CSF (*data not shown*). We found a significant correlation between circulating neutrophil count and mouse G-CSF level (r = 0.83, *P*=0.02, Spearman test). In addition, we found significant increases in the neutrophil biomarker NE and the NET biomarker H3Cit in tumor-bearing mice compared with controls (Figure 1C, D). Finally, the amount of cfDNA was significantly greater in tumor-bearing mice than in controls (Figure 1E).

The human breast cancer cell line MCF7 expresses PAD4.[31] We therefore determined whether BxPc-3 cells also express PAD4. Western blotting showed that the BxPc-3 cells did express PAD4 protein (*data not shown*). Tumor cells may, therefore, also contribute to plasma H3Cit.

### Measurement of neutrophils, histone H3 and citrullinated histone H3 in thrombi from mice bearing human pancreatic tumors

We generated thrombi in control and tumor-bearing mice using the IVC stasis model. Using western blotting, we measured levels of Ly6G (a neutrophil marker), β-actin (a housekeeping gene), histone H3 (a marker of cellular content), and H3Cit (a NET biomarker) in thrombi from mice bearing human pancreatic tumors and control mice (Figure 2A). Thrombi from tumor-bearing mice had higher levels of Ly6G, β-actin, histone H3, and H3Cit compared with the levels in thrombi from control mice (Figure 2 B-E). The H3Cit/H3 ratio for thrombi from control mice was ~1 (Figure 2F). Interestingly, there was significant variation in the H3Cit/H3 ratio in thrombi from tumor-bearing mice (Figure 2F).

Next, we measured the cfDNA and H3Cit content in thrombi from mice bearing human pancreatic tumors and in thrombi from controls using immunofluorescence. Consistent with the data from the western blot analysis, thrombi from tumor-bearing mice had increased levels of cfDNA and H3Cit compared with levels of these biomarkers in thrombi from control mice (Figure 3).

Finally, we analyzed the composition of thrombi from control and tumor-bearing mice by scanning electron microscopy. Red blood cells were decreased in thrombi from tumor-bearing mice compared with thrombi from control mice (Figure 4A). In addition, we observed an increase in fibrin density and a decrease in fibrin fiber thickness in thrombi from tumor-bearing mice compared with thrombi from controls (Figure 4B). Previous studies

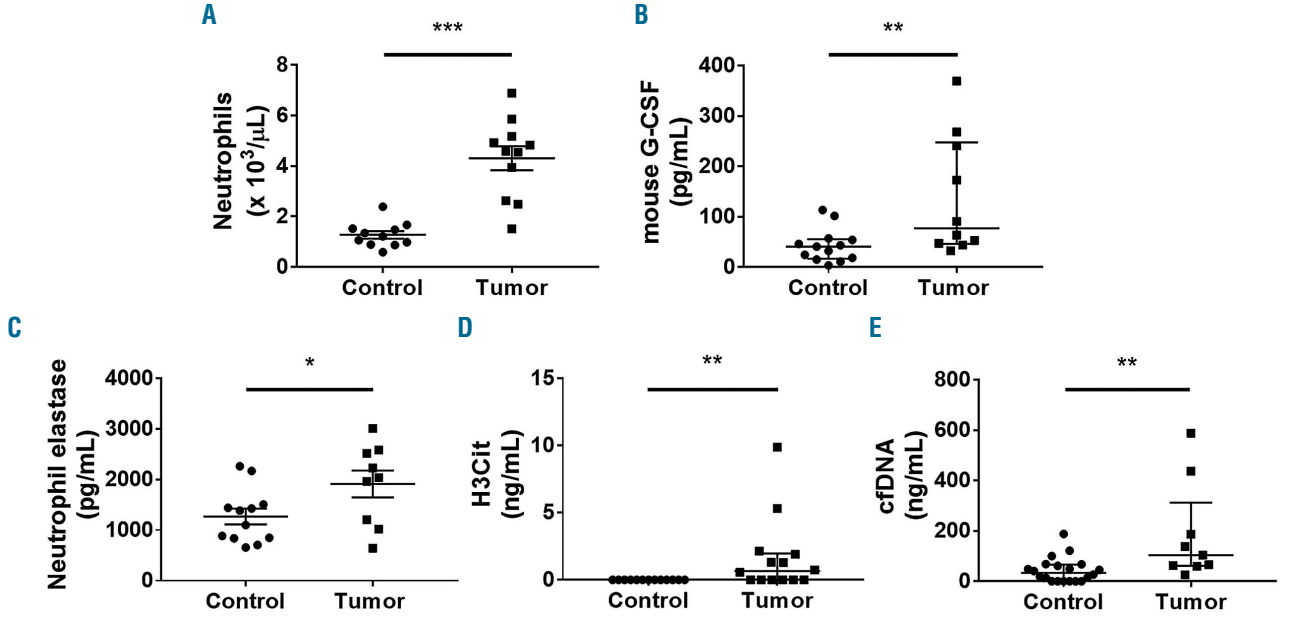


**Figure 1. Levels of neutrophils and circulating biomarkers in blood samples.** (A-E) Neutrophil counts in whole blood (A) and circulating mouse granulocyte-colony stimulating factor (G-CSF) (B), neutrophil elastase (C), citrullinated histone H3 (H3Cit) (D), and cell-free (cf) DNA (E) in plasma of control and tumor-bearing mice. Nine to 20 mice were used for each group. Data were analyzed with either the unpaired *t*-test or the Mann-Whitney U-test, depending on the type of data distribution. **P*<0.05, ***P*<0.01, ****P*<0.001.

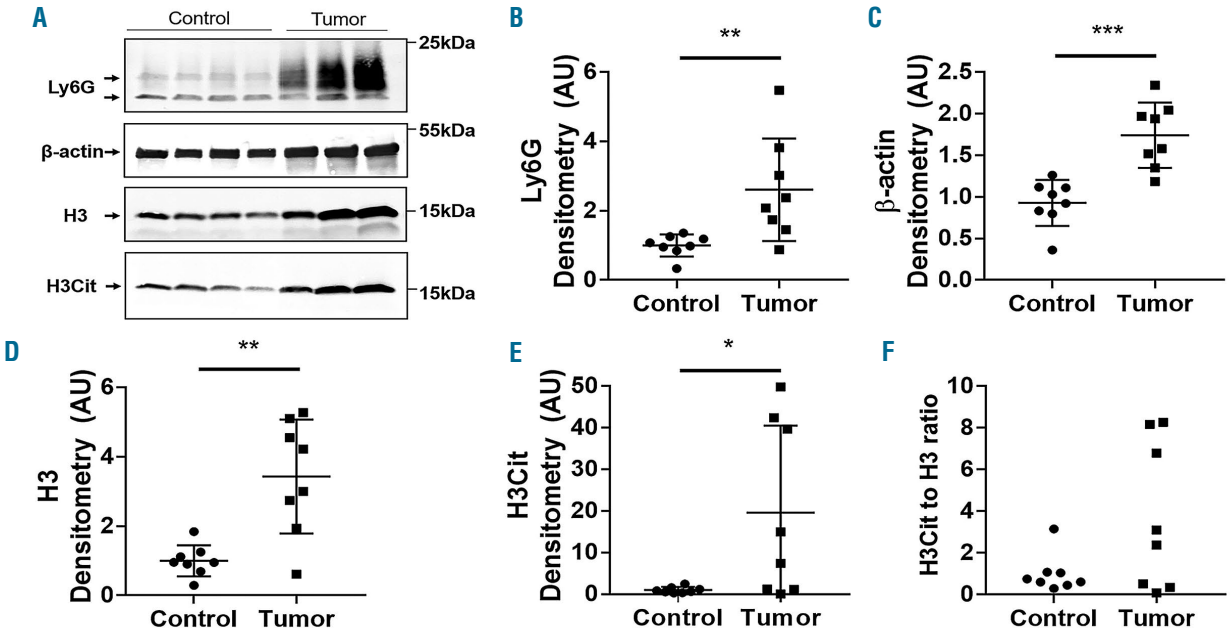


**Figure 2. Analysis of thrombus constituents.** (A) Representative western blot of Ly6G, β-actin, histone H3, and citrullinated histone H3 (H3Cit) in thrombi from control and tumor-bearing mice. Arrows on the left of the panel indicate the target proteins and the size of the molecular weight markers are shown on the right. (B-E) Levels of the different markers were quantified by densitometric analysis. (F) The H3Cit/H3 ratio for thrombi from control and tumor-bearing mice are shown. AU: arbitrary unit. Eight mice were used for each group. Data were analyzed with the unpaired *t*-test or the Mann-Whitney U-test depending on the type of data distribution. **P*<0.05, ***P*<0.01, ****P*<0.001.

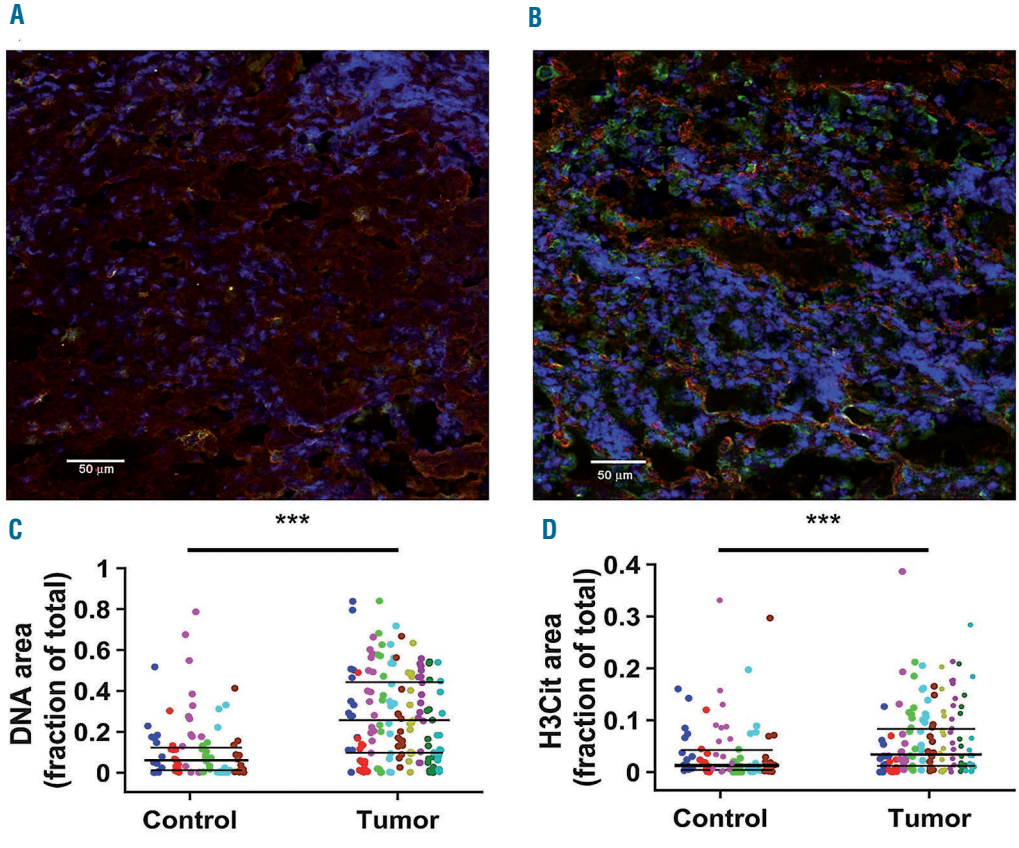


**Figure 3. Analysis of thrombi by immunofluorescence.** (A, B) Thrombus sections from control (A) and tumor-bearing (B) mice were stained for cell-free DNA (TOTO-3, blue), citrullinated histone H3 (H3Cit; immunostained, green) and fibrin (immunostained, red) and examined with confocal laser scanning microscopy (Zeiss LSM 710, Carl Zeiss, Jena, Germany). (C, D) The area occupied by the DNA (C) and H3Cit (D) signal was quantified in 15 randomly selected images from each thrombus shown with the same color. Lines indicate the median values of the bottom, median and top quartiles calculated from the data of 90 images from six animals of the control group (shown in different colors) and 150 images from ten animals of the tumor group (shown in different colors). The statistical analysis was performed using 90 or 150 input data according to the two-step procedure described in the *Online Supplement* (Bootstrap Kuiper test *P*<0.001 for all three quartiles of the datasets in panels C and D).

have shown that increased levels of thrombin lead to the generation of denser fibrin with thinner fibers.[44]

### Effect of neutrophil depletion on thrombus size in mice bearing human pancreatic tumors

Thrombi in the group of tumor-bearing mice treated with IgG weighed significantly more than those in controls [controls *vs.* tumor-bearing mice (median [range]): 16.4 [13.8-22.4] g *vs.* 31.9 [29.1-36.5] g; $P<0.001$, n=4-5]. Next, we investigated the role of neutrophils in thrombus formation in tumor-bearing mice and control mice by depleting neutrophils using the anti-Ly6G antibody 1A8. Administration of 1A8 significantly decreased levels of circulating neutrophils in both control mice [IgG *vs.* 1A8 (median [range]): 2.9 [1.9-4.4] x $10^3$/μL *vs.* 0.3 [0.2-1.3] x $10^3$/μL; $P<0.05$, n=4-5] and mice bearing human pancreatic tumors [IgG *vs.* 1A8 (median [range]): 6.7 [3.8-8.5] x $10^3$/μL *vs.* 1.1 [1.0-3.7] x $10^3$/μL; $P<0.05$, n=4-5]. Depletion of neutrophils decreased thrombus weight in tumor-bearing mice but not in control mice (Figure 5).

### Effect of DNase I administration on thrombus size in mice bearing human pancreatic tumors

Thrombus weight was significantly increased in the vehicle treatment group of tumor-bearing mice compared with that of control mice [controls *vs.* tumor-bearing mice (median [range]): 15.7 [12.1-25.2] g *vs.* 27.25 [22.8-47.8] g; $P<0.05$, n=5-6]. We determined the effect of DNase I administration on the size of thrombi in mice bearing human pancreatic tumors. DNase I degrades both cfDNA and NET. We found that administration of DNase I significantly reduced thrombus weight in tumor-bearing mice but did not affect thrombus weight in control mice (Figure 6).

## Discussion

We observed a striking increase in neutrophils in the circulation of nude mice bearing human pancreatic tumors compared with controls. In addition, we found increased levels of plasma biomarkers that either increase neutrophil

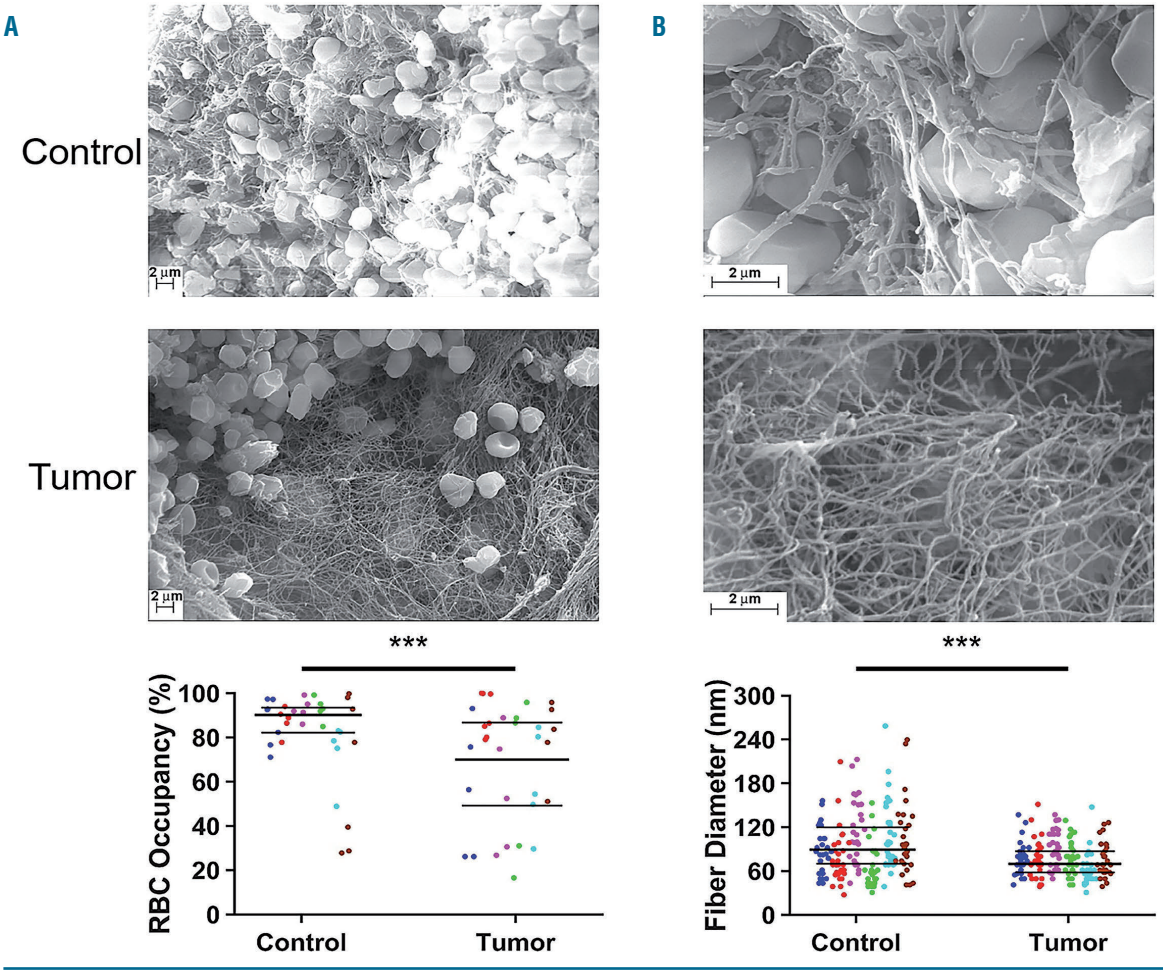


**Figure 4. Analysis of thrombi by scanning electron microscopy.** (A, B) The red blood cell (RBC) and fibrin content in thrombi from control and tumor-bearing mice were assessed by scanning electron microscopy using a SEM EVO40 (Carl Zeiss GmbH, Oberkochen, Germany). Upper panels are representative images of thrombi from control and tumor-bearing mice. RBC occupancy was quantified in five to seven randomly selected images from each animal (A, data from each thrombus are shown with the same color in the lower panel). The diameter of 300 fibrin fibers from separate parts of each thrombus was measured (B, every 10$^{th}$ measured value is plotted in the same color for each thrombus in the lower panel). Lines indicate the median values of the bottom, median and top quartiles calculated from the data of six animals from each group (shown in different colors) based on 35 images in the control group and 32 images in the tumor group. The statistical analysis was performed using 35 or 32 input data for RBC occupancy and 1,800 data for fiber diameter according to the two-step procedure described in the *Online Supplement* (Bootstrap Kuiper test $P<0.001$ for all three quartiles of the datasets in the lower panels).

counts, such as G-CSF, or are biomarkers of activated neutrophils and NET, such as NE and H3Cit, in tumor-bearing mice compared with controls. We also observed increased levels of the neutrophil biomarker Ly6G and the NET biomarker H3Cit in thrombi from tumor-bearing mice. Finally, depletion of neutrophils reduced thrombus size in tumor-bearing mice but not in control mice. The lack of effect on thrombus size in neutrophil-depleted control mice is consistent with a previous study showing that neutrophil depletion did not reduce thrombus size in mice in an IVC stasis model.[28] Taken together, these data suggest that neutrophils contribute to increased venous thrombosis observed in tumor-bearing mice.

We found that tumor-bearing mice have increased levels of mouse G-CSF and there was a significant positive correlation between levels of mouse G-CSF and neutrophil numbers. This suggests that increased levels of G-CSF drive the increase in neutrophil numbers. At present, we do not know the cellular source of G-CSF in tumor-bearing mice but this cytokine is normally expressed by endothelial cells, macrophages and immune cells.[45] In support of this, mice with murine breast 4T1 tumors exhibit increased plasma levels of G-CSF and administration of an anti-G-CSF antibody reduces neutrophil counts.[37] G-CSF level and neutrophil count may, therefore, represent novel biomarkers of VTE risk in cancer patients.

It has been hypothesized that G-CSF primes neutrophils to undergo NET formation in tumor-bearing mice.[37,46] In one study it was found that the percentage of H3Cit-positive neutrophils was increased in tumor-bearing mice compared with controls.[37] Furthermore, neutrophils isolated from mice bearing murine 4T1 tumors treated with an anti-G-CSF antibody had significantly less NET formation compared with those from tumor-bearing mice treated with an isotype control.[37] In addition, administration of recombinant G-CSF to mice bearing murine melanoma

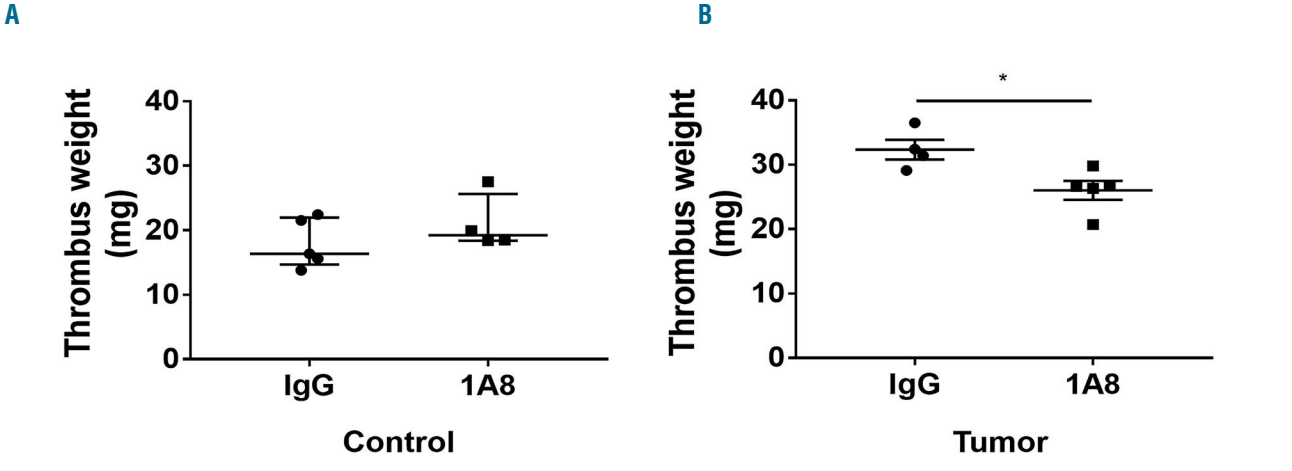


**Figure 5. Effect of neutrophil depletion on venous thrombosis in control and tumor-bearing mice.** (A, B) Control and tumor-bearing mice received an anti-mouse Ly6G antibody (clone: 1A8) or a control IgG (5 mg/kg) 24 h and 1 h before inferior vena cava stasis. Thrombi from control (A) and tumor-bearing mice (B) were collected at 48 h and weighed. Four to five mice were used for each group. Data were analyzed with either the unpaired *t*-test or the Mann-Whitney U-test depending on the type of data distribution. *$P$<0.05.

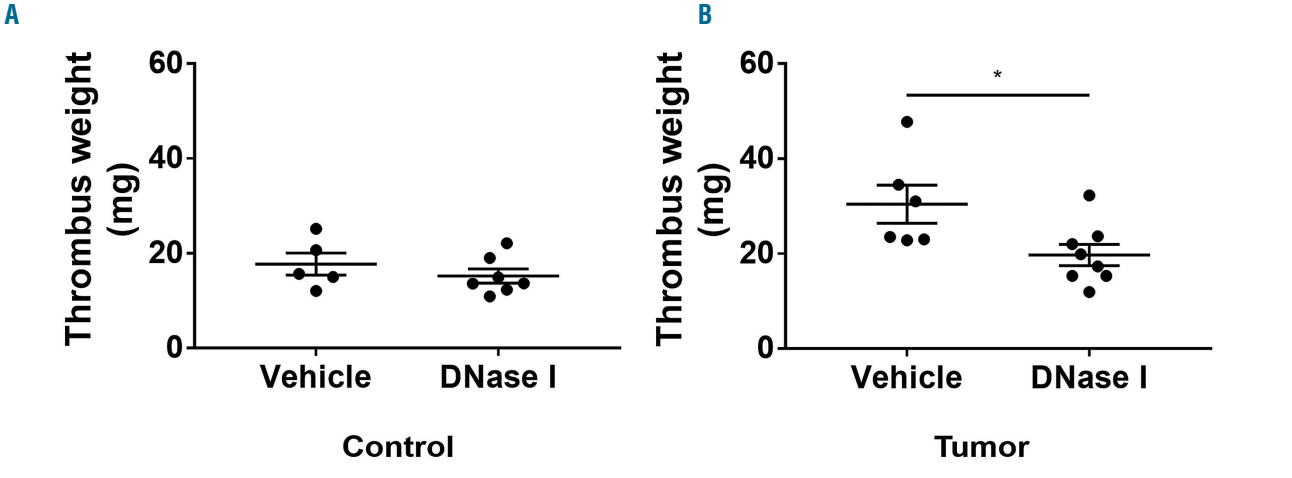


**Figure 6. Effect of DNase I treatment on venous thrombosis in control and tumor-bearing mice.** Mice received vehicle or DNase I (50 U/mouse) 1 h before and 24 h after inferior vena cava stasis. Thrombi from control (A) and tumor-bearing mice (B) were collected at 48 h and weighed. Five to seven mice were used for each group. Data were analyzed with the unpaired *t*-test. *$P$<0.05.

B16 tumors, which do not produce G-CSF, increased H3Cit in tumors in a PAD4-dependent manner.[46,47] We observed a large variation in the H3Cit/H3 ratio in thrombi from tumor-bearing mice which appears to be due to different levels of H3Cit in the thrombi. At present, we do not know the reason for this range of H3Cit in thrombi from tumor-bearing mice but it may reflect differences in G-CSF levels and the degree of neutrophil priming in the different tumor-bearing mice.

Plasma cfDNA and the NET biomarker, H3Cit, were increased in tumor-bearing mice compared with controls. Tumors are known to release cfDNA into the blood.[48] In addition, we observed that BxPc-3 cells express PAD4 and therefore tumors may also contribute to the plasma levels of H3Cit. Thrombi from tumor-bearing mice also had increased levels cfDNA and H3Cit compared with thrombi from controls. Importantly, administration of DNase I reduced thrombus size in tumor-bearing mice but not in control mice. Similarly, a previous study showed that DNase I did not affect jugular vein occlusion times in control mice but prolonged the time to occlusion in 4T1 tumor-bearing mice.[38] We observed that DNase I reduced thrombus size more effectively than depletion of neutrophils. It is possible that DNase I is more effective than neutrophil depletion because it digests not only NET but also cfDNA which may also be released by cancer cells and may enhance thrombosis by activating factor XII.[40,49]

In the present study, we observed decreased numbers of red blood cells in thrombi from tumor-bearing mice compared with controls. This is consistent with our recent study that showed a decrease in red blood cell-rich areas and an increase in inflammatory cell-rich areas in thrombi from tumor-bearing mice compared with controls.[10] In the current study, we did not observe an increase in neutrophils in thrombi of tumor-bearing mice because these cells were not preserved during the preparation of the samples for scanning electron microscopy. In addition, thrombi from tumor-bearing mice had a denser fibrin network with thinner fibrin fibers compared with thrombi from control mice. *In vitro* experiments showed that higher thrombin produces thrombi with a denser fibrin network with thinner fibrin fibers.[44] In our model of cancer-associated thrombosis, it is likely that tumor-derived, tissue factor-positive extracellular vesicles increase the thrombin concentration in thrombi and this results in the formation of a denser fibrin network with thinner fibers. Indeed, in our previous study,[10] we observed that thrombi from BxPc-3 tumor-bearing mice had increased levels of human tissue factor activity derived from extracellular vesicles released from BxPc-3 tumors. In addition, a previous study showed that extracellular vesicles bind to NET via a phosphatidylserine-histone interaction.[50]

In summary, we have demonstrated a contribution of neutrophils to venous thrombosis in a mouse model of pancreatic cancer-associated venous thrombosis. Our data, taken together with a clinical study showing an association between circulating H3Cit and VTE in pancreatic cancer, support the notion that neutrophils and NET formation enhance venous thrombosis in pancreatic cancer.

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