Supporting Information

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SI Materials and Methods

Cell Lines and Reagents. The 4T1 and LLC cell lines were purchased from ATCC and maintained in high-glucose Dulbecco's modified Eagle medium (DMEM) plus L-GlutaMAX-1 [supplemented with 10% (vol/vol) FCS, 10 mM Hepes buffer, 10 mM sodium carbonate (4T1 cells)]. The 4T1 cell line was originally isolated from a spontaneously arising mammary tumor in a BALB/c mouse (1), whereas LLC cells originated from C57BL/6 mice. The 4T1 and LLC cells were injected in mice after less than 4 and 10 serial passages in vitro, respectively. All cell culture products were purchased from Life Technologies/Invitrogen.

Animals. All animal procedures were performed using 6- to 8-wk-old BALB/c female or C57BL/6 female mice (Jackson Laboratory). All experimental procedures involving mice were approved by the Animal Care and Use Committee of the Immune Disease Institute. All transplantation experiments were approved by the Institutional Animal Use and Care Committee of Massachusetts General Hospital.

Bone Marrow Transduction and Transplantation. Transduction/transplantation experiments were performed as described (2, 3). Briefly, ecotropic replication-defective retrovirus was generated by cotransfection of MSCV-IRES-GFP-p210-BCR-ABL or MSCV-IRES-GFP (vector control) and the *kat* packaging plasmids into 293 cells. The retrovirus was harvested and used to infect the bone marrow of 5-fluorouracil–pretreated C57BL/6 mice twice. Consequently, the bone marrow was transplanted into lethally irradiated (900 cGy) C57BL/6 mice in a dose of 3–5 $\times$ 10^5 cells/mouse by tail vein injection. Disease induction was monitored by weekly full blood count analyses by using a Vetscan 5 HM (Abaxis) and by flow cytometry of peripheral blood after red blood cell lysis (ACK lysing buffer; Lonza) and staining of leukocytes with a phycoerythrin (PE)-conjugated antibody to CD11b (clone M1/70; BioLegend). Disease burden was measured by percentage of GFP^+ $CD11b^+$ cells in peripheral blood. Necropsies were performed on euthanized mice, and spleen weights were recorded to confirm establishment of CML at the time the NET experiments were performed.

Induction of Solid Tumors. The 4T1 cells (4×10^5) were inoculated in the mammary fat pad of 6- to 8-wk-old BALB/c mice. For the LLC model, 5×10^5 cells were inoculated in the right flank of 6- to 8-wk-old C57BL/6 mice. Animals were monitored twice a week, at which time tumors were carefully measured using calipers. The tumor volume was calculated using the formula $V = lw^2 \times 0.4$, where l is the length and w the width (4). Mice were killed at the indicated time or when tumor volume reached 2,500 mm^3 or animals were moribund. For peripheral blood cell count, blood was collected using EDTA-coated capillaries and analyzed with a Hemavet hematology analyzer (Drew Scientific). At sacrifice, blood was collected through the retroorbital sinus into Tyrode's buffer containing 10 mM EDTA, centrifuged at 3,300 $\times g$ for plasma collection, and centrifuged again at 13,400 $\times g$ to remove any contaminating cells.

Injection of Low-Dose LPS. Fourteen-day tumor-bearing mice or rhG-CSF–treated mice were injected intraperitoneally with LPS (Sigma-Aldrich) at the indicated concentration. The mice were killed at different time points (as indicated) and bled into 3.2% (wt/vol) sodium citrate for plasma collection. A drop of blood was also collected in EDTA-coated capillaries for blood cell count.

DNase1 Treatment. Fourteen-day tumor-bearing or tumor-free mice were injected intraperitoneally with 50 μg of DNase1 (Pulmozyme; Genentech) or saline (APP Pharmaceuticals) 3 h before LPS injections for 1 h before tail-bleeding experiments.

Tail-Bleeding Time. Mice were anesthetized with 2.5% tribromoethanol, and tail-bleeding time was determined by removing 2 mm of the distal mouse tail and immediately immersing the tail in PBS at 37 °C. A complete cessation of bleeding for more than 120 s was defined as the bleeding time.

rhG-CSF Treatment. Mice received s.c. injections between the scapulae with 2.5 or 10 μg of rhG-CSF (Neupogen; Amgen) once daily for 4 d to stimulate durable granulopoiesis (5). Control mice received vehicle [5% (wt/vol) dextrose] following the same injection schedule. On day 5, plasma was collected, and peripheral blood neutrophils were isolated.

Anti–G-CSF Treatment. Mice were injected with 4×10^5 4T1 cells in the mammary fat pad. After 2 d, mice were injected intraperitoneally with 10 μg of mouse neutralizing monoclonal antibody to G-CSF (R&D Systems) or with a matching isotype control IgG (R&D Systems) daily for 12 d. On day 14, plasma was collected, and peripheral blood neutrophils were isolated.

Peripheral Blood Neutrophil Isolation. Mice were exsanguinated into PBS containing 1% (wt/vol) BSA and 15 mM EDTA. After centrifugation, blood cells were resuspended and layered onto a Percoll gradient of 78%, 69%, and 52% in PBS (vol/vol), centrifuged and cells at the 69%/78% interface were collected. Red blood cell contamination was eliminated by hypotonic lysis, and final cell concentration was determined by hemacytometer. Neutrophil purity was established to be routinely >90%, as assessed by Wright–Giemsa staining on cytospin.

Cell Sorting by Flow Cytometry. Flow cytometry–based cell sorting of GFP^+ or control GFP^- cells was performed after drawing of a leukocyte-specific and, consequently, a myeloid cell–specific gate by an SORP 7-laser LSR-II (BD Biosciences).

Induction of Extracellular DNA Traps in Vitro. Isolated peripheral blood neutrophils (1.5×10^4) were seeded into 96 wells and allowed to adhere at 37 °C and 5% CO_2 for 15 min before stimulation with PAF or LPS at the indicated concentrations for 1 or 2-1/2 h, respectively. DNA was stained with Hoechst-33342 (Invitrogen), and cells were fixed with 2% (vol/vol) paraformaldehyde before visualization using an epifluorescent Axiovert microscope (Zeiss). For quantification, NETs were counted from six different fields in triplicate wells for each condition and expressed as percentage of NET-forming cells per total number of cells in the field.

Quantification of Plasma VWF, Soluble P-Selectin, Fibrinogen, G-CSF, and DNA. The VWF ELISA was performed as described (6) using the level of VWF in pooled plasma of 20 C57BL/6J WT mice as a reference standard [normal mouse plasma (nmp)]. Plasma-soluble P-selectin, fibrinogen, G-CSF, TAT, and DNA levels were determined using the mouse sP-Selectin/CD62P Quantikine ELISA (R&D Systems), the mouse fibrinogen ELISA (Genway Biotech), the Quantikine mouse or human G-CSF ELISA (R&D Systems), human TAT ELISA (Affinity Biologicals), and Quant-iT Picogreen assay (Invitrogen) according to the instructions of the manufacturers.

Immunostaining of Neutrophils and NETs. Isolated neutrophils were plated on a cell culture slide and stimulated as described above. After stimulation, cells were fixed in 2% paraformaldehyde and permeabilized, and neutrophils were stained with a rat anti–Gr-1 antibody (BD Bioscience) and NETs with a rabbit anti–histone H3 antibody (Abcam). Alexa 555–conjugated goat anti-rat IgG or Alexa 488–conjugated goat anti-rabbit IgG were used as secondary antibodies (Invitrogen). For H3Cit staining, isolated neutrophil cytospins were stained with a rabbit anti–histone H3 (citrulline 2, 8, 17) antibody (Abcam) and Alexa 488–conjugated goat anti-rabbit IgG (Invitrogen) used as secondary antibody. All immunostainings were counterstained with Hoechst-33342 to visualize DNA, and slides were mounted with Fluoro-gel (Electron Microscopy Sciences) and observed under an epifluorescent Axiovert microscope (Zeiss).

Western Blot Analysis. Equal amounts of plasma were analyzed on a 4–20% SDS/PAGE gel and transferred onto Immobilon PVDF membranes using a Mini-Trans Blot Electrophoretic Transfer Cell System (Bio-Rad Laboratories). The membranes were blocked with 5% milk in TBS/0.05% Tween-20 overnight and blotted for 2 h with primary antibodies. Membranes were probed with a rabbit polyclonal anti-cathelicidin (CRAMP) or anti–histone H3 and anti–histone H3 (citrulline 2, 8, 17) antibodies (Abcam). Secondary antibodies consisted of horseradish peroxidase–conjugated anti-rabbit IgG (Bio-Rad Laboratories). Detection was carried out with a Pierce ECL Western Blotting Substrate (Thermo Scientific). After detection, membranes were stained with Coomassie blue to ensure equal loading of all wells.

Lung Histology and Immunostaining. Lungs were harvested from killed animals and fixed in zinc fixative (100 mM Tris-HCl containing 37 mM zinc chloride, 23 mM zinc acetate, and 3.2 mM calcium acetate). Paraffin-embedded sections were deparaffinized in xylenes and rehydrated through a graded alcohol series. Sections were stained with hematoxylin and eosin, mounted in DPX mountant (Fluka BioChemika), and observed by light microscopy. For fibrinogen/fibrin and VWF staining, the sections were stained with a sheep anti-fibrinogen antibody (ABD Biologicals) and rabbit anti–human VWF antibody (Dako) and incubated with anti-sheep Alexa-555 and anti-rabbit Alexa-488 (Invitrogen) as secondary antibodies. Sections were counterstained with Hoechst-33342 to visualize all nuclei, mounted with Fluoro-gel (Electron Microscopy Sciences), and observed under an epifluorescent Axiovert microscope (Zeiss).

Statistical Analysis. Data are represented as means ± SEM and were analyzed by a two sided Mann–Whitney test performed between groups. Regression analysis was performed with plasma DNA (μg/mL) as the dependent variable using IBM SPSS Statistics (IBM), version 19.0. For this model, the adjusted R^2 and the standardized regression coefficients (β) of the independent variables were calculated. All P values were considered significant at or below 0.05.

1. Aslakson CJ, Miller FR (1992) Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. *Cancer Res* 52:1399–1405.
2. Krause DS, Lazarides K, von Andrian UH, Van Etten RA (2006) Requirement for CD44 in homing and engraftment of BCR-ABL-expressing leukemic stem cells. *Nat Med* 12: 1175–1180.
3. Li S, Ilaria RL, Jr., Million RP, Daley GQ, Van Etten RA (1999) The P190, P210, and P230 forms of the BCR/ABL oncogene induce a similar chronic myeloid leukemia-like syndrome in mice but have different lymphoid leukemogenic activity. *J Exp Med* 189: 1399–1412.
4. Attia MA, Weiss DW (1966) Immunology of spontaneous mammary carcinomas in mice. V. Acquired tumor resistance and enhancement in strain A mice infected with mammary tumor virus. *Cancer Res* 26:1787–1800.
5. Scholz M, et al. (2009) A pharmacokinetic model of filgrastim and pegfilgrastim application in normal mice and those with cyclophosphamide-induced granulocytopaenia. *Cell Prolif* 42:813–822.
6. Brill A, et al. (2011) von Willebrand factor-mediated platelet adhesion is critical for deep vein thrombosis in mouse models. *Blood* 117:1400–1407.

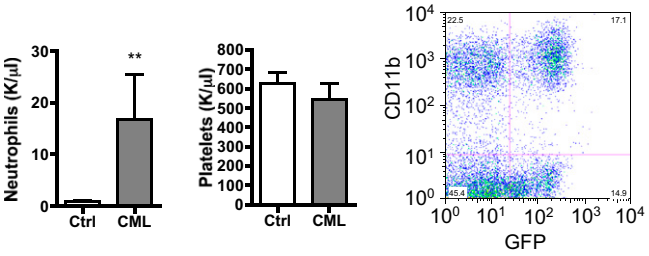

Fig. S1. Induction of CML-like disease increases neutrophils without affecting platelets. Bone marrow transduced with a retrovirus encoding BCR/ABL and GFP was transplanted into lethally irradiated C57BL/6 mice to induce CML. Control mice were transplanted with bone marrow transduced with a vector encoding only GFP (Ctrl). At days 18–20 posttransplantation, peripheral blood was collected, and numbers of neutrophils and platelets were evaluated in the blood of CML-like (gray) and control (white) mice ($n = 6$; $^{**}P < 0.01$). Representative flow cytometry dot plot showing the percentage of leukemic (BCR-ABL$^+$) cells in the peripheral blood assessed by the expression of CD11b and GFP. Data shown are means ± SEM.

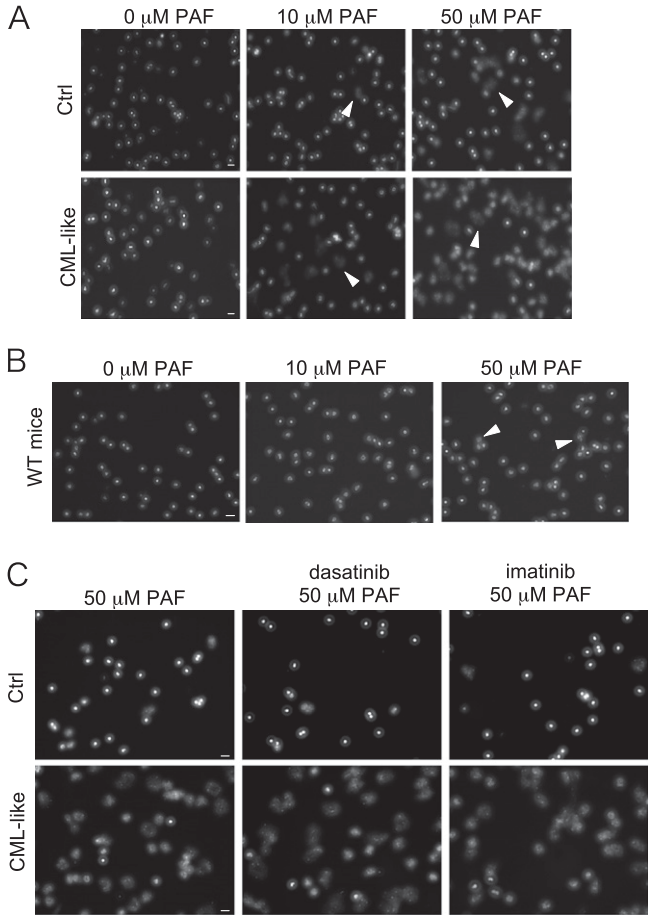

Fig. S2. Malignant and nonmalignant neutrophils from CML-like mice generate extracellular DNA traps. Representative fluorescent images of Hoechst staining after PAF stimulation (*A*) Nuclear decondensation of isolated neutrophils from CML-like mice shows a significant increase compared with control vector–transduced bone marrow recipients (Ctrl). Arrow, NET-forming cells. (Scale bar: 20 μm.) (*B*) NET formation of fluorescence-activated cell–sorted WT neutrophils (control for Fig. 1*D*). Arrow, NET-forming cells. (Scale bar: 20 μm.) (*C*) Neutrophils were isolated from CML-like or control mice and pretreated in vitro with dasatinib or imatinib, abl-specific tyrosine kinase inhibitors, at 10 μM for 4 h before PAF stimulation. This treatment induces apoptosis of leukemic cells but does not affect normal neutrophils. PAF stimulation showed that even after destruction of the malignant neutrophils, normal neutrophils from CML-like mice form NETs. (Scale bar: 20 μm.)

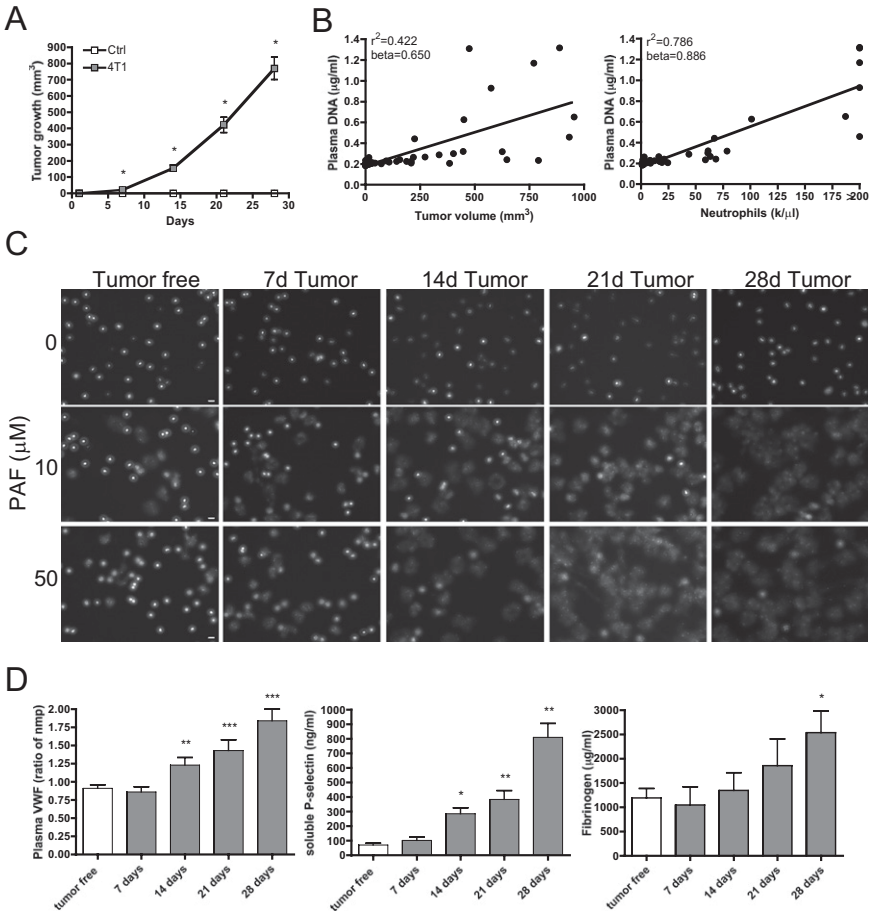

Fig. S3. The increase in plasma DNA in the 4T1 mammary carcinoma model is associated with NET formation and thrombosis markers. Tumor cells were injected in the mammary fat pad of BALB/c mice. (*A*) Tumor growth was measured every 7 d. (*B*) Plasma DNA was assessed as the dependent variable in a regression analysis. For this, the adjusted r^2 and the standardized regression coefficients (β) of the independent variables were calculated. The β indicates the change of the dependent variable, expressed in SD, when the independent variable increases 1 SD and all other variables in the model remain unchanged. The increase in plasma DNA is determined more by the number of neutrophils than by tumor size. (*C*) Representative fluorescent images showing chromatin decondensation after PAF stimulation and Hoechst staining of isolated neutrophils from tumor-bearing mice at different times after tumor cell injections show increased sensitivity to NET formation. (*D*) Plasma VWF, soluble P-selectin, and fibrinogen levels were determined by ELISA. Graphs represent means ± SEM (n = 6–10; *P < 0.05; **P < 0.01; ***P < 0.001).

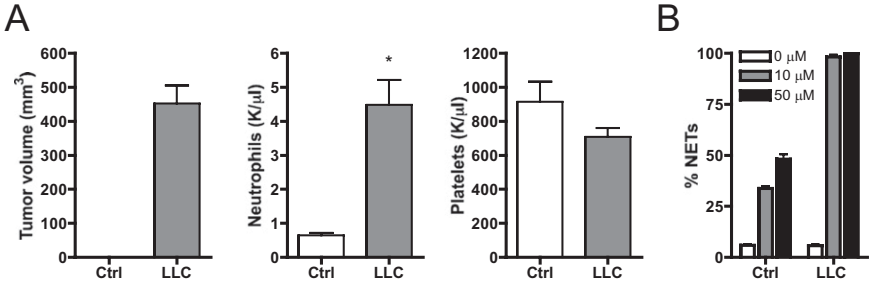

Fig. S4. Increased peripheral blood neutrophils in LLC tumor–bearing mice are more prone to NET formation. LLC tumor cells were injected in the right flank of C57BL/6 mice. (*A*) When tumor volume reached ~500 mm^3, peripheral blood neutrophil and platelet counts were determined (n = 4; *P < 0.05). (*B*) Quantification of NETs after PAF stimulation of isolated neutrophils from tumor-bearing mice showed a significant increase of NETosis compared with tumor-free mice. Data shown are means ± SEM.

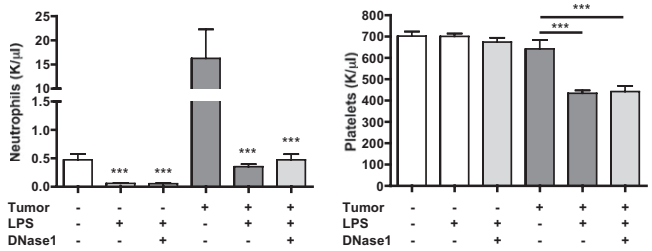

Fig. S5. DNase1 treatment of tumor-bearing mice does not prevent the reduction in neutrophil and platelet counts after LPS challenge. Fourteen-day tumor-bearing mice or tumor-free mice were injected with 50 μg of DNase1 or saline for 3 h before injection of LPS (1 mg/mL). The pretreatment with DNase1 did not prevent the reduction in the number of neutrophils and platelets observed 1 h after LPS injection (n = 5–9; ***P < 0.001). Data shown are means ± SEM.

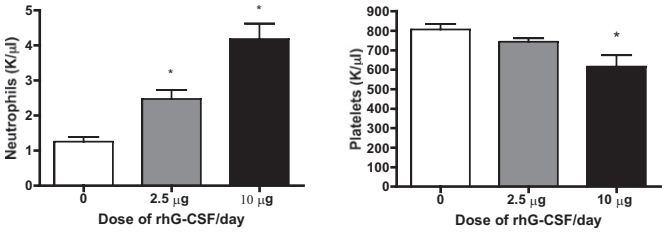

Fig. S6. rhG-CSF injection in WT mice results in an increase in peripheral neutrophils. rhG-CSF was injected s.c. daily at the indicated concentrations for 4 d. A dose-dependent increase in neutrophil counts and a reduction in platelet counts was observed (n = 4–5; *P < 0.05).