Supplemental Methods

Human platelet isolation

Human studies were performed according to principles of the Declaration of Helsinki. Informed consent was obtained from all participants. Blood was drawn from healthy volunteers and immediately mixed with 3.8% sodium citrate (10:1). Platelet-rich plasma (PRP) was obtained by centrifugation at 200 g for 20 min at room temperature. Platelets were washed twice at 1000 g for 10 min in the presence of 10 μg prostacyclin and resuspended in modified Tyrode's-HEPES Buffer (134 mM NaCl, 0.34 mM Na2HPO4, 2.9 mM KCl, 12 mM NaHCO3, 20 mM HEPES, 5 mM glucose, 1 mM MgCl2; pH 7.3).

Histone stimulation

Washed platelets were stimulated with ATP, nigericin, PAF (all from Sigma), mixture of all histones isolated from calf thymus (Worthington Biochem) or individual histones 1, 2a, 2b, 3 and 4 (50 μg/ml, New England BioLabs) and stained with FAM-YVAD-FMK FLICA™ (Bio-Rad) for 30 min at 37°C and anti-CD62P APC (eBioscience). Active Caspase-1 expression was analysed using an Accuri™ C6 flow cytometer and its software (BD Biosciences).

Light transmission aggregometry

Washed human platelets were warmed at 37°C in siliconized glass vials for 2 minutes before aggregation assays started. Aggregation was measured, under stirring conditions, in a ChronoLog model 700 aggregometer, using individual histones as agonists. Platelets were used at a concentration of $2x10^8$/mL.

Human neutrophil isolation

Blood was collected from healthy volunteers in EDTA-coated tubes (BD Vacutainer®) and allowed to cool at room temperature for 30 minutes. A gradient of Histopaque was achieved by carefully overlaying 2.5mL of Histopaque-1077 (Sigma Aldrich) onto the same volume of Histopaque-1119 (Sigma Aldrich) in a 15mL conical centrifuge tube and 5mL of blood layered onto this gradient. Following centrifugation at 700g for 45 minutes at room temperature, granulocytes formed an opaque layer at the lower Histopaque-1077/1119 interface. Cells were carefully collected and washed with phosphate buffered saline (PBS, Sigma Aldrich) at 200g for 8 minutes.

Platelet and neutrophil co-culture

Human neutrophils (2 x10^5 cells) were seeded on coverslips, previously coated with Poly-L-Lysine (Sigma Aldrich), for 1 h at 37°C. Neutrophils were then stimulated with PMA (10nM) for 2 h or left untreated. Autologous washed human platelets were seeded on neutrophils for 3 h at 37°C. Recovered platelets were stained with FAM-YVAD-FMK FLICA™ (Bio-Rad) and their expression of active caspase-1 assessed as described above.

Mice

C57BL/6 male mice were purchased from Charles River and kept at the Biomedical Services Unit at the University of Birmingham, UK, under standard conditions. Animal experiments were performed in accordance with the UK Animals in Scientific Procedures Act 1986 and were approved by the Local Animal Welfare Ethical Review

Body and the Home Office (United Kingdom; Project Licenses 40/3745 and PC427E5DD).

Stenosis of the inferior vena cava (IVC)

Briefly, mice of 8 to 10 weeks old were anesthetized with an isofluorane-oxygen mixture, a midabdominal incision was performed, the intestines were exteriorized and soaked in warm saline. All the IVC side branches were ligated. The IVC was gently isolated from aorta, a 7-0 polypropylene suture was placed over the IVC and ligated over a spacer (30G needle) and then, the spacer was removed, leading to a ~90% reduction in luminal area. Finally, the peritoneum was closed with silk suture and the skin was closed with staples. Thrombosis was evaluated at 2, 6 or 48 h post-surgery. Experiments examining caspase-1 inhibition *in vivo* were performed by intraperitoneal injection of either ac-yvad-cmk (10 mg/kg, InvivoGen) or vehicle control 24 h and 30 min before and 24 h after stenosis application, and thrombosis was evaluated at 48 h post stenosis.

Flow cytometry

For flow cytometric analysis, thrombi were dissected and digested in StemPro™ Accutase™ cell dissociation reagent (ThermoFisher Scientific) for 30 minutes on a shaking platform. Cell suspensions and any non-digested tissue were filtered through a 70 μm cell strainer and washed in RPMI-1640 (+2% FCS) (Sigma Aldrich). For peripheral blood staining, blood was collected by terminal cardiac puncture and stabilized by sodium citrate. Cells from both dissociated thrombi and blood were stained with FAM-YVAD-FMK FLICA™ (a fluorescent reagent, which labels activated caspase-1; Bio-Rad), anti-GPIIb/IIIa (JON/A, Emfret Analytics) and anti-CD41a APC

(eBioscience) and samples acquired with an Accuri™ C6 flow cytometer (BD Biosciences).

Immunofluorescence

Freshly dissected thrombi were embedded in OCT compound (Sakura Finetek), frozen in dry ice and stored at -80°C. Acetone-fixed frozen sections were blocked with 10% goat serum and stained with FAM-YVAD-FMK FLICA™ (for staining active caspase-1; Bio-Rad), SYTOX™ Orange (ThermoFisher Scientific), anti-histone 3 (citrulline R2 + R8 + R17) (Abcam), anti-CD41a (eBioscience), anti-myeloperoxidase (2D4, Abcam) and anti-ASC (Novus Biologicals), followed by Alexa-Fluor-labelled secondary antibodies (Invitrogen) where appropriate. Nuclei were stained by ToPro3 or Hoescht (ThermoFisher Scientific). Images were acquired using a Zeiss LSM780 confocal microscope.

Intravital microscopy

Mice underwent IVC stenosis and were allowed to recover. Two hours later, mice were anaesthetized using tribromoethanol and FAM-YVAD-FMK FLICA™ (100 μl of 15X solution, Bio-Rad) and SYTOX™ Orange (5 μM, ThermoFisher Scientific) and CD41 APC (1:50, eBioscience) were injected intravenously. Mice were placed into the supine position, the IVC was exposed and covered with a glass coverslip. The area adjacent to the ligation site was imaged by a 3i VIVO-SDC confocal system with Yokogawa CSU-10 and Photometrics Evolve EMCCD camera on an Olympus BX61WI upright microscope with an air objective x10. As the vessels being imaged were not flat, Z-projections were generated of the IVC wall to compensate for tilt.

Projections were generated and de-noised using FlatZ and channels were composited using ImageJ. FlatZ is freeware, available to download online (https://github.com/kavanagh21/FlatZ).

Western Blot

Thrombi were collected from the IVC either 6 or 48 h post stenosis, lysed and 150μg of total protein was loaded per lane and ran in NuPAGE Bis-Tris polyacrylamide gels (ThermoFisher Scientific). Proteins were transferred to PVC membranes using the Turbo Blot Transfer system, followed by blocking with 5% bovine serum albumin and blotted with anti-caspase-1 (p20) (Casper-1, AdipoGen®) or anti-α-Tubulin (B-5-1-2, Sigma Aldrich) antibodies.

The level of chemiluminescence was registered using the Odyssey® Fc imaging system (LI-COR). Densitometry of the bands was performed with Image Studio software version 3.1 (LI-COR) and normalized to the corresponding α-Tubulin band.

Statistical analysis

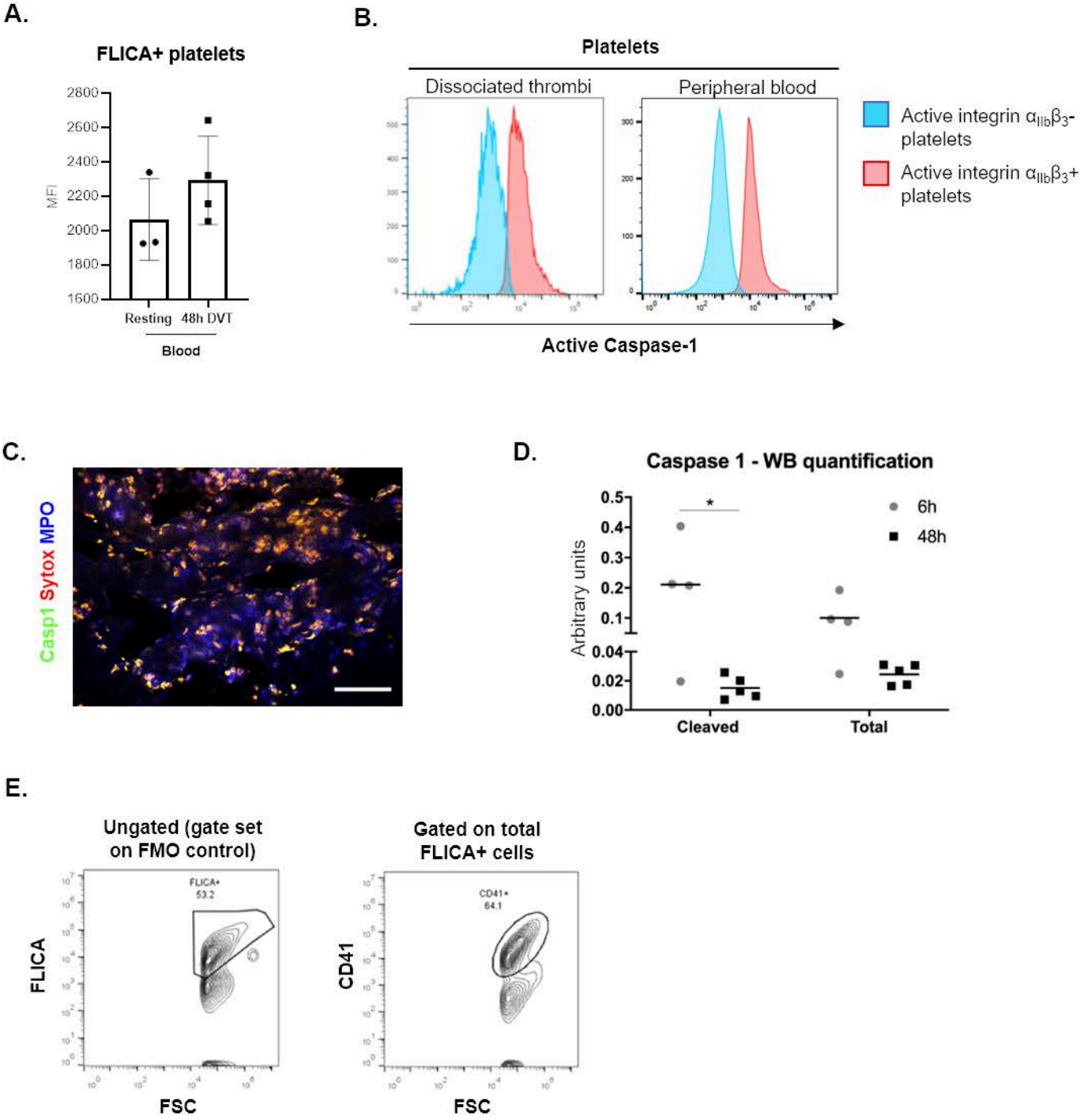
All data are presented as mean ± standard deviation (SD) with statistical significance taken at $P < 0.05$. Statistical analysis was performed as described in each section using GraphPad Prism 9.

Supplemental Figure 1. Activated platelets in DVT thrombi contain cleaved caspase-1. (A) Mean fluorescent intensity of FLICA incorporation by platelets in peripheral blood of both resting and 48 h DVT mice (n = 3-4, mean ± SD). (B) FLICA signal was assessed in integrin $\alpha_{IIb}\beta_3$+ and $\alpha_{IIb}\beta_3$- platelets, in both thrombi and peripheral blood in murine DVT. Representative plot of four independent experiments. (C) Representative immunofluorescence image of active caspase-1 (green), SYTOX™ Orange (red) and MPO (blue) in 48 h thrombus. Scale bar = 100 μm, n = 3. (D) Quantification of total and cleaved (activated) caspase-1 content in thrombus lysates at 48 and 6 h post IVC stenosis (n = 4-5). Two-way ANOVA, Sidak's multiple comparison test. *$p \leq 0.05$. (E) Representative flow cytometry plots of enzymatically digested 48 h murine thrombus (n = 3).

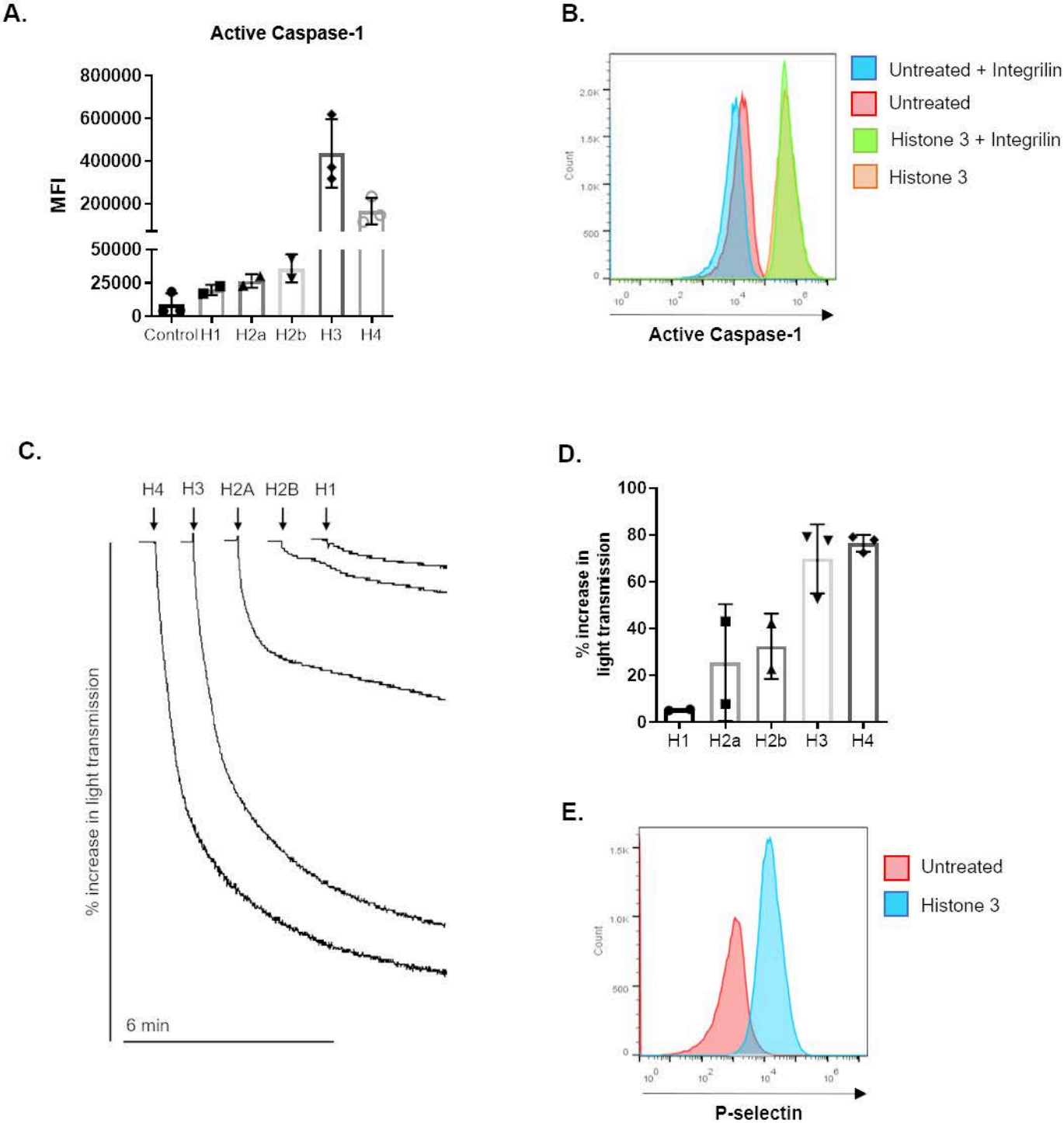
Supplemental Figure 2. Histones induce aggregation and expression of activation markers in washed human platelets. (A) Mean fluorescent intensity of FLICA incorporation by human platelets treated with individual human histones and evaluated by flow cytometry (n = 3, mean ± SD). (B) Levels of active caspase-1 were assessed in human washed platelets following stimulation with histone 3, both in the presence and absence of integrilin. Representative plot of three independent experiments. Representative curves (C) and quantification (D) of histone-induced aggregation of washed human platelets. (E) P-selectin expression by washed human platelets following stimulation with histone 3. Representative plot of three independent experiments.

Supplemental Figure 3. A schematic representation of the proposed interaction between NETs and inflammasomes in DVT initiation. Flow restriction induces

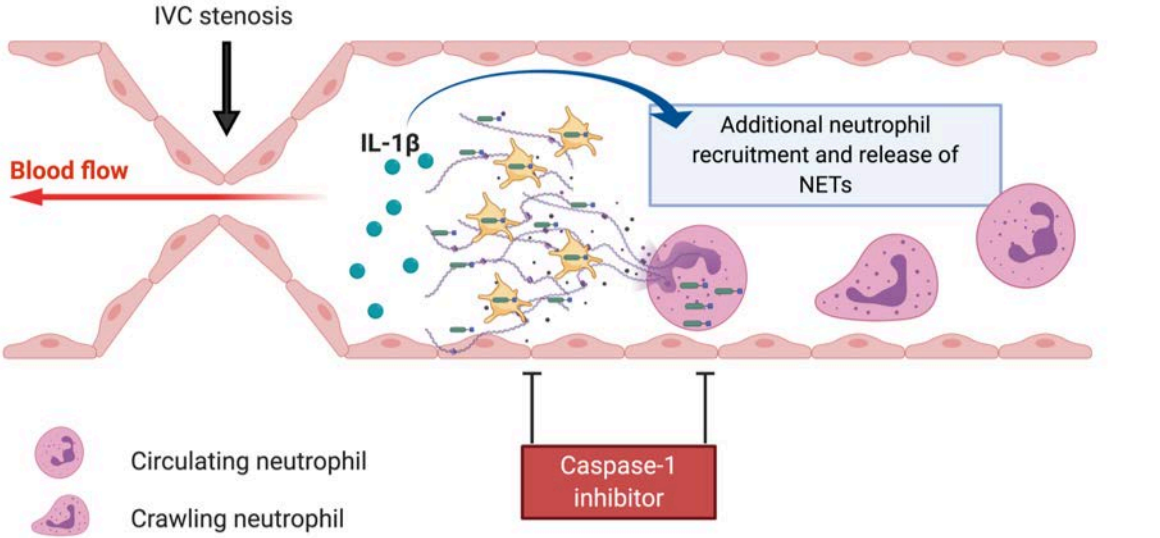
recruitment of both platelets and neutrophils to the vessel wall. Neutrophils release NETs, which recruit additional platelets, and platelets in turn stimulate NETosis. NETosis is accompanied by assembly of the NLRP3 inflammasomes in neutrophils. NETotic neutrophils and NETs (as a whole or their components histones) initiate formation of the inflammasome in platelets. This leads to activation of caspase-1 both inside the cells and on the NET surface. The resulting release of cleaved and activated IL-1β potentiates further recruitment of neutrophils, which accelerates thrombosis. Supplemental Figure 3 was created with BioRender.com and exported using a paid subscription.



Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3