Supplemental Methods

Antibodies and fluorescent tracers. Anti-P-selectin (whole IgG; clone RB40.34) and anti-CD41 ($F(ab)_2$ fragments; clone MWReg30) were purchased from BD Biosciences (San Diego, CA). Anti-fibrin antibody hybridoma (clone 59D8) was a generous gift from Dr. Hartmut Weiler [1]. The hybridoma was injected into mice to generate antibodies by a commercial vendor (Covance, Princeton, NJ) and purified from mouse ascites by Dr. Rodney Camire (Children's Hospital of Philadelphia). Antibodies were labeled using Alexa-fluor monoclonal antibody labeling kits from Invitrogen (Carlsbad, CA). Alexa-fluor 488 and FITC-tagged dextrans with nominal molecular weights of 3 kD, 10 kD and 70 kD, as well as Alexa-fluor 488 BSA and fibrinogen, were purchased from Invitrogen.

Response to vascular injury in the mouse cremaster muscle microcirculation. Male mice 8-12 weeks of age were anesthetized via intraperitoneal injection of sodium pentobarbital (90 mg/kg). A cannula was introduced into the jugular vein for delivery of antibodies, dextran and additional anesthetic as needed. The cremaster muscle was exteriorized, cleaned of connective tissue, opened and spread flat on the glass coverslip of a custom built chamber for viewing by intravital microscopy. The cremaster preparation was continuously superfused with bicarbonate buffer warmed to 36.5˚C and bubbled with 95%N_2/5%CO_2.##The cremaster microcirculation was visualized using an Olympus BX61WI upright microscope with a 60X (0.9 NA) water immersion objective, coupled to a Yokogawa CSU-X1 spinning disk confocal scanner. Diode pumped solid state lasers (488 nm, 568 nm, 640 nm) with AOTF control were used as the fluorescence excitation light source (LaserStack, Intelligent Imaging Innovations, Denver, CO). Confocal fluorescence images were acquired using a CoolSnap HQ CCD digital camera (Photometrics, Tucson, AZ). The microscope, confocal scanner, lasers and camera were all controlled and synchronized using Slidebook 5.0 image acquisition and analysis software (Intelligent Imaging Innovations). 30-40 μm diameter arterioles with unperturbed blood flow were selected for study. Various combinations of anti-CD41 $F(ab)_2$ fragments (0.12 μg/g body weight; clone MWReg30, BD Bioscience), anti-P-selectin (0.2 μg/g body weight; clone RB40.34, BD Bioscience), anti-fibrin (0.2 μg/g

body weight; clone 59D8), Alexa-488 albumin and dextrans (3, 10 or 70 kD, Invitrogen) were infused intravenously via the jugular vein. Antibodies were labeled with Alexa fluor dye monoclonal antibody labeling kits (Alexa-488, Alexa-568 and Alexa-647) according to the manufacturers instructions (Invitrogen). The anti-CD41 antibody used (MWReg30) does not affect function, and we verified that MWReg30 $F(ab)_2$ fragments do not cause thrombocytopenia in mice (data not shown), as seen with whole IgG preparations of this antibody. The anti-P-selectin antibody used (clone RB40.34) is function blocking, but had no impact on platelet accumulation at the concentration used in these studies (data not shown).

Injury models. Vascular injury was induced with either a pulsed nitrogen dye laser (SRS NL100, 440 nm) focused on the vessel wall by the microscope objective, or via vessel wall puncture with a glass micropipette. In the case of the laser injury, the laser power was set to 55-65% and the laser fired at the vessel wall until a small number of red blood cells exited the lumen of the vessel (1-10 laser pulses). In some cases, the red cells entered the extravascular space, while in other cases they remained trapped within the layers of the vessel wall. For puncture injury, a glass micropipette (0.4 μm tip, World Precision Instruments (WPI), Inc.) controlled with a micromanipulator was positioned against an arteriole such that it slightly impinged on the vessel wall. Once in position, a piezotranslator (MPM20, WPI, Inc.) was used to rapidly advance the micropipette (10-20 μm distance at a rate of 20 mm/s) to breach the vessel wall. Puncture through the vessel wall was confirmed by visualization of blood cells moving around and adhering to the micropipette tip once it entered the lumen of the vessel. The micropipette was then rapidly retracted out of the vessel and field of view. In most cases, red blood cells did not exit the arteriole following retraction of the micropipette, but plasma was observed to leak from vessels following puncture (Supplemental video 1).

Embolization analysis. Embolization of platelets from the main platelet mass was assessed following laser and puncture injury by creating a region of interest encompassing the vessel lumen downstream from the platelet mass (Suppl Fig 2B). The area of CD41-positive and P-selective positive events

within this region over time was determined (Suppl Fig 2A). To compare results from multiple injuries, the sum area of each fluorescent channel (CD41 and P-selectin) over the entire 4 minute observation period was calculated for each thrombus and defined as the "integrated embolization area" reported in Fig 1D.

Studies with pharmacologic agents. Hirudin was a generous gift from Dr. Sriram Krishnaswamy (Children's Hospital of Philadelphia), and was infused intravenously via a jugular vein cannula (15 μg prior to each injury). Cangrelor (formerly AR-C69931MX) was a gift from The Medicines Company (Parsippany, NJ). Cangrelor is a fast acting, reversible $P2Y_{12}$ antagonist with a plasma half-life of 3-5 minutes[2]. It was administered via intravenous infusion through the jugular vein (0.75 μg prior to each injury). For both hirudin and cangrelor studies, multiple thrombi generated in the same mice prior to drug infusion served as controls.

Supplemental Literature Cited

1. Weiler-Guettler H, Christie PD, Beeler DL, et al. A targeted point mutation in thrombomodulin generates viable mice with a prethrombotic state. *J Clin Invest*. 1998;101(9):1983-1991.

2. Ferreiro JL, Ueno M, Angiolillo DJ. Cangrelor: a review on its mechanism of action and clinical development. *Expert Rev Cardiovasc Ther*. 2009;7(10):1195-1201.

Supplemental Figure Legends

Supplemental Figure 1: P-selectin expression is restricted to platelets following laser injury in mouse cremaster arterioles. A and B) Photomicrographs show representative arterioles following laser injury in a platelet-depleted mouse. Following surgical preparation for intravital microscopy, mice were infused with 50 μg anti-GPIb antibody (Emfret Analytics) to deplete platelets. After 90 minutes, mice were infused with anti-CD41 (red) and anti-P-selectin (green) antibodies prior to induction of several laser injuries as described in Methods. In each of the 3 images (A-C), the P-selectin channel alone is shown in the left panel, the CD41 channel in the middle panel and the composite of P-selectin, CD41 and brightfield in the right panel. In A, no platelets adhered at the site of injury, and no P-selectin expression is observed. Extensive red blood cell leakage into the extravascular space following injury is evident (due to platelet depletion). In B, a single platelet is adhered to the injury site and becomes P-selectin positive, but no P-selectin expression is observed on the vessel wall (endothelium). In contrast, extensive P-selectin expression is observed on the endothelium in a venule from the same mouse (C). The capture and display settings were identical for all of the images shown.

Supplemental Figure 2: P-selectin positive platelets are stably adherent during hemostatic plug formation in vivo. As described in the Supplemental Methods, values on the graph (A) report the area of CD41 (red line) or P-selectin (green line) positive events occurring in a region of the blood vessel under study downstream from the main platelet mass, as shown by the yellow box in the image at the right (B). The graph shows data from one thrombus that is representative of 10 thrombi analyzed in an identical fashion. Mean data are reported in Figure 1D.

Supplemental Figure 3: Limited access of anti-CD41 F(ab)2 fragments to the core region. Photomicrographs of a representative thrombus 20 minutes post-injury. Alexa-488 anti-CD41 was infused 20 minutes post-injury (panel i, green in the merge). Alexa-568 anti-CD41 (panel ii, red in the merge) and Alexa-647 anti-P-selectin (panel iii, blue in the merge) were infused prior to injury. The

brightfield image is shown (panel v) with binary fluorescence images overlaid to highlight the localization of the different antibodies within the thrombus (see color key at right for overlapping fluorophore colors). Note that while the red anti-CD41 antibody infused before the injury labeled all the platelets in the thrombus, the green anti-CD41 infused post-injury was unable to penetrate the thrombus core.

Supplemental Video legends

Supplemental Video 1: Laser vs. micropuncture injury in mouse cremaster muscle arterioles. Representative timelapse videos of platelet accumulation and activation following laser (left) and micropuncture injury (right) are shown side-by-side. Both videos are composites of 3 fluorescence channels overlaid on the brightfield image: platelets (CD41) are labeled red, P-selectin is green, and plasma (albumin) is blue. Overlay of CD41 and albumin appears magenta; overlay of albumin, CD41 and P-selectin is gray/white. Images were captured at 1.4 composite frames per second and playback is 10 frames per second. Timestamp is hh.mm:ss.000. Videos of 3D reconstructions of the same injuries are shown following the timelapse videos. The 3D images are isosurface views with platelets (CD41) in red and P-selectin in green. 3D images were created using Volocity image analysis software (Perkin Elmer).

Supplemental Video 2: Fibrinogen leakage into the extravascular space is rapidly converted to fibrin. Part 1 shows a timelapse video of fibrin formation as detected by an anti-fibrin antibody following laser-induced injury. Platelets (CD41) are red, anti-fibrin blue and the overlay of platelets and fibrin is magenta. Part 2 is a representative timelapse and 3D reconstruction video showing fibrinogen leakage following laser injury. Alexa-488 labeled fibrinogen was infused intravenously prior to injury. Timelapse video is a composite of 3 fluorescence channels overlaid on the brightfield image: platelets (CD41) are red, P-selectin blue and fibrin(ogen) green. Overlay of CD41 and P-selectin is magenta. Timestamp is hh.mm:ss.000. The gamma setting of the fibrinogen channel was set to 0.5 to increase the brightness of the fibrin fibers in the extravascular space. The 3D reconstruction was made from a series of z-plane images obtained following stable hemostatic plug formation. The CD41 (red) and P-selectin (blue) channels are shown in isosurface view and the fibrin(ogen) (green) in maximum intensity projection view. 3D images were constructed using Volocity image analysis software.

Supplemental Video 3: Platelet mass porosity measured using albumin to highlight the interplatelet plasma volume. Representative timelapse video showing fluorescently labeled albumin highlighting the plasma volume within a stable hemostatic plug. The video begins 20 minutes post-injury. Timing of albumin infusion is indicated. The left panel shows the albumin channel in gray scale. Note the appearance of albumin in the gaps between platelets within the thrombus. The right panel shows the albumin as a pseudocolor image indicating albumin intensity overlaid on the brightfield image. Timestamp is hh.mm:ss.000. Note the decreased albumin intensity (blue in the pseudocolor image) in the platelet mass core indicating less plasma volume in this region.

Supplemental Video 4: Entry of 10 kDa vs. 70 kDa dextran into the core region of a hemostatic plug. Representative timelapse videos of 10 kDa or 70 kDa dextran accessibility into hemostatic plugs 20 minutes post laser injury are shown. Both videos show the grayscale dextran channel fluorescence on the left, and a pseudocolor image of the dextran fluorescence within the platelet mass on the right. The area of the pseudocolor image was defined by anti-CD41 fluorescence. Timestamp is hh.mm:ss.000.

Supplemental Video 5: Laser-induced injury in mouse cremaster arterioles: effect of a $P2Y_{12}$ antagonist. Representative timelapse videos of platelet accumulation following laser injury before (left) and after (right) intravenous infusion of the $P2Y_{12}$ antagonist cangrelor are shown side-by-side. Both videos are composites of 2 fluorescence channels overlaid on the brightfield image: platelets (CD41) are labeled red and P-selectin is green. Overlay of CD41 and P-selectin appears yellow. Images were captured at 1.4 composite frames per second and playback is 10 frames per second. Timestamp is hh.mm:ss.000.

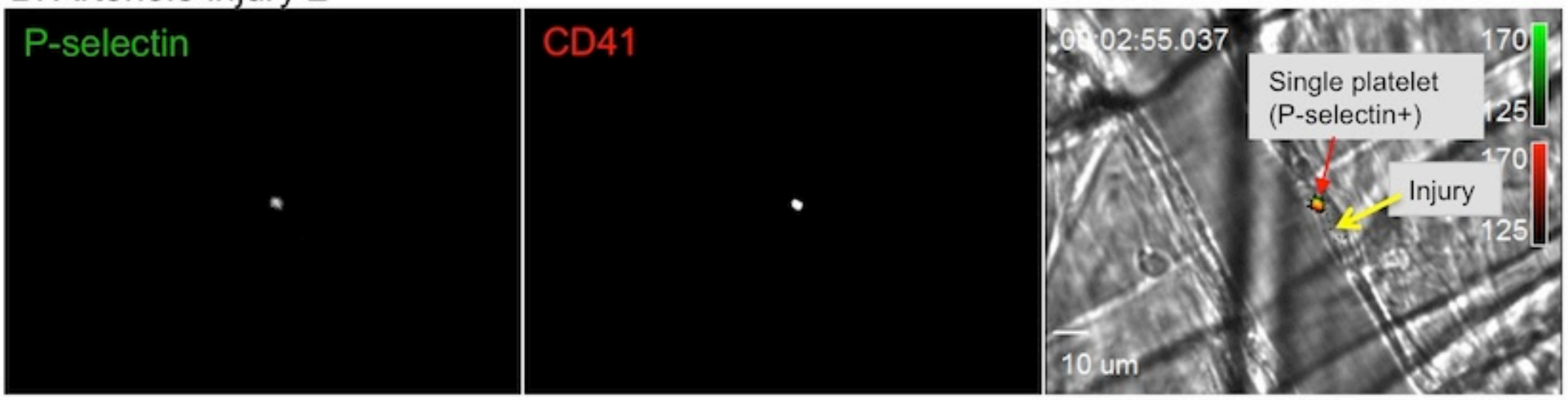
Supplemental Video 6: Laser-induced injury in mouse cremaster arterioles: effect of a thrombin inhibitor. Representative timelapse videos of platelet accumulation following laser injury before (left) and after (right) intravenous infusion of the direct thrombin inhibitor hirudin are shown side-by-side. Both videos are composites of 3 fluorescence channels overlaid on the brightfield image: platelets

(CD41) are labeled red, P-selectin is green and fibrin is blue. Overlay of CD41 and P-selectin appears yellow; overlay of CD41 and fibrin appears magenta; overlay of fibrin, CD41 and P-selectin is gray/white. Images were captured at 1.4 composite frames per second and playback is 10 frames per second. Timestamp is hh.mm:ss.000.

Supplemental Figure 1:

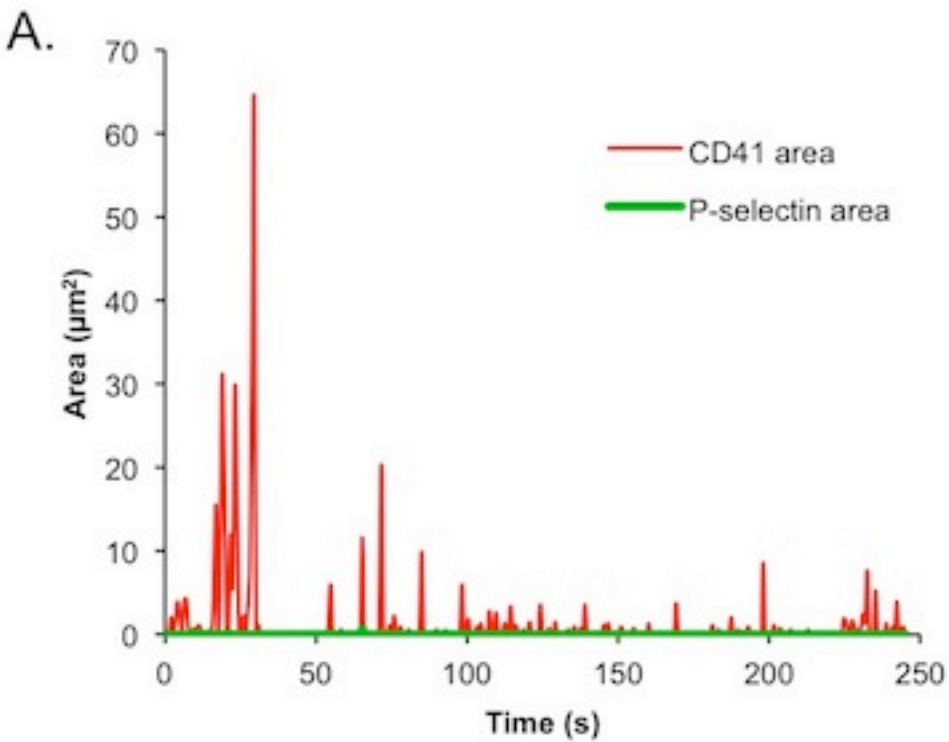
A. Arteriole injury 1

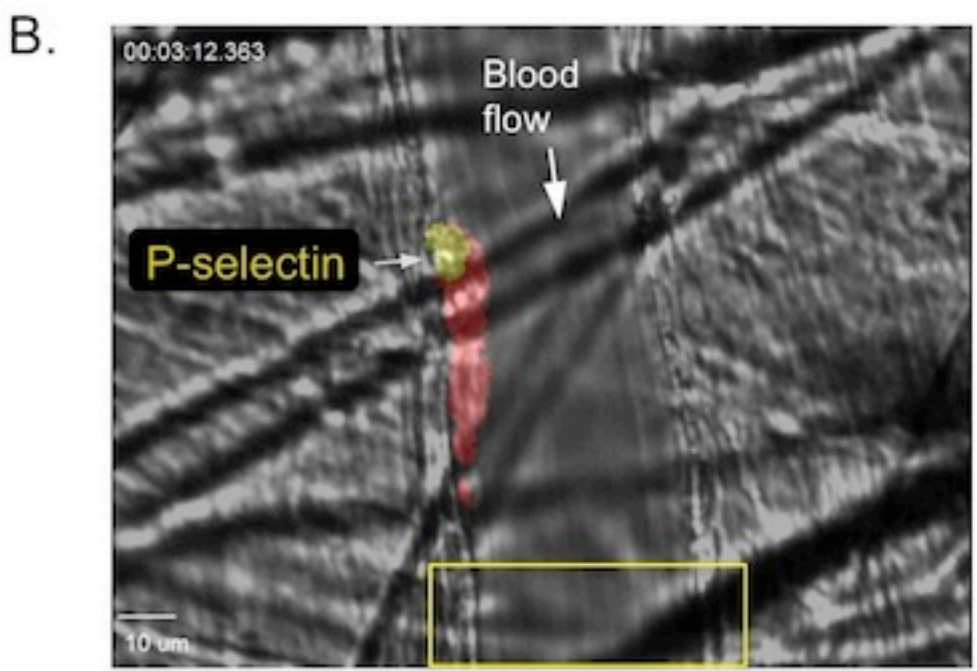
B. Arteriole injury 2



C. Uninjured venule

Supplemental Figure 2:





Supplemental Figure 3: