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Supplementary Information for

Interrelationships between structure and function during the hemostatic response to injury

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Supplemental Methods

Fluorescence image quantification Images were acquired as a series of z-planes using 2-photon microscopy. Hemostatic plugs were imaged from both sides for comparison of fluorescence in the intraluminal vs. extraluminal compartments. A region of interest away from the hemostatic plug was selected on the image taken from the intravascular side. A mask was created in this region and copied to all planes in the z-stack. The maximum pixel intensity of each channel within this mask was defined as the background fluorescence intensity. The total platelet volume was calculated as the volume of all CD41 positive pixels (i.e. pixels with a fluorescence intensity above background). The CD41 and P-selectin mean fluorescence intensities were calculated as the mean intensity of all CD41 positive pixels. Using the mean pixel intensity within the platelet volume was necessary to account for the large difference in platelet volume between in the intraluminal and extraluminal portions of hemostatic plugs (Figure 3C). As expected, the mean intensity of CD41 positive pixels was no different in the intraluminal vs. extraluminal images (Figure 3D), confirming the validity of this quantification approach. The sum fluorescence intensity was used to quantify total fibrin deposition on both the intraluminal and extraluminal sides of hemostatic plugs. The sum intensity reported is the total sum intensity of all fibrin positive pixels across all z-planes, minus the product of the fibrin background intensity times the total number of fibrin positive pixels. Image analysis was performed using Slidebook 6.0 imaging software (Intelligent Imaging Innovations, Denver, CO).

Supplemental Video Captions

Supplemental Video 1: 3D reconstruction of the intravascular portion of a jugular vein hemostatic plug. The first part of the video shows the z-series images obtained by 2-photon microscopy (5 μm z-step size), starting with the base of the platelet plug closest to the endothelial cell surface (z = 0 μm). Anti-CD41 (red) and anti-P-selectin (green) antibodies were infused prior to injury. Scale bar = 100 μm. The second part of the video shows the 3D isosurface view reconstructed from the z-series images. Grid size = 35 μm. The final frames in the video show the fluorescence maximum intensity projection image overlaid on the corresponding scanning electron micrograph.

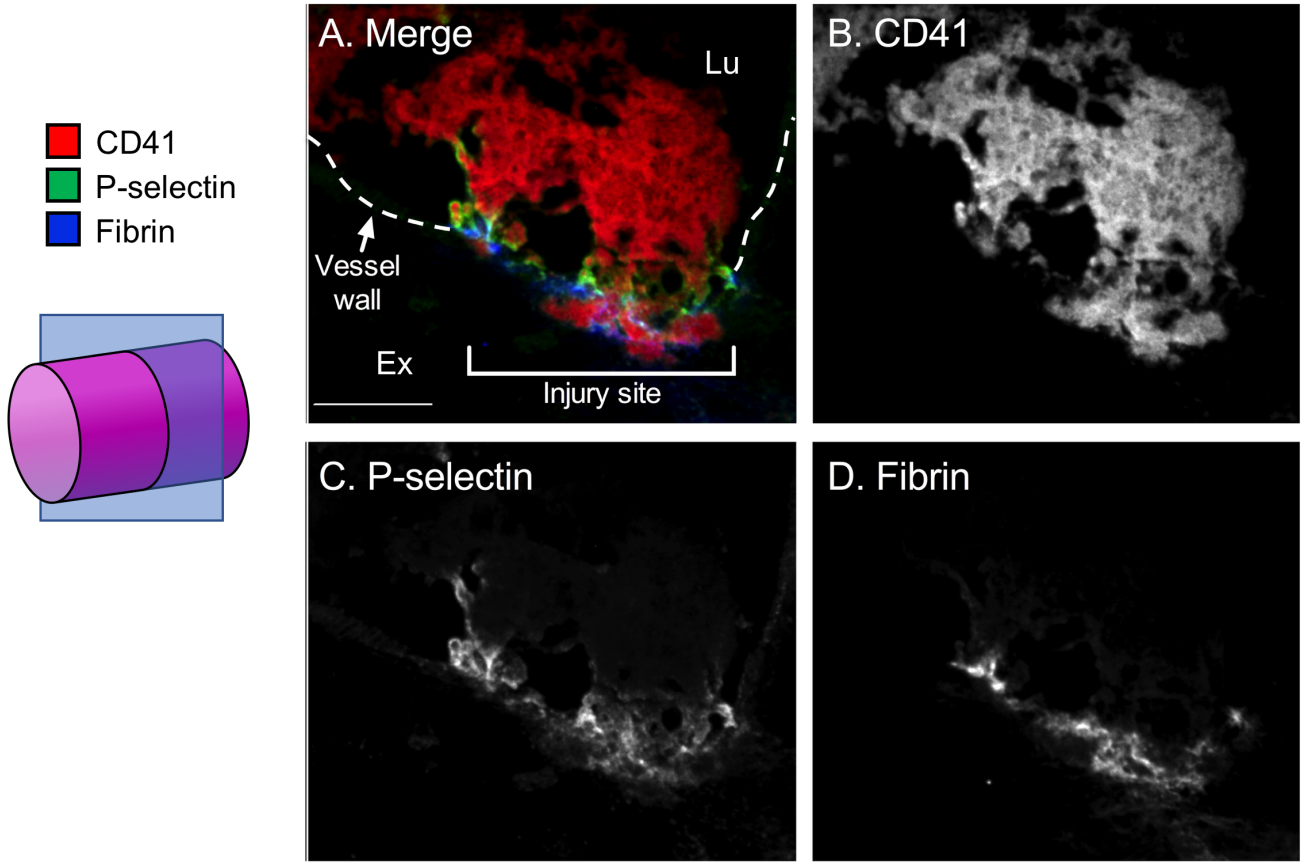
Supplemental Video 2: 3D reconstruction of the extravascular portion of a jugular vein hemostatic plug. The video shows the 3D isosurface view reconstructed from a series of z-plane images obtained by 2-photon microscopy (5 μm z-step size). Anti-CD41 (red) and Alexa-488 fibrinogen (green) were infused prior to injury. The hole created by the puncture injury may be seen filled with platelets (indicated as “injury site” in the video). Grid size = 68 μm. The final frames in the video show the fluorescence maximum intensity projection image overlaid on the corresponding scanning electron micrograph.

Supplemental Dataset Captions

Supplemental dataset S1: Scanning electron micrographs showing multiple perspectives of the intravascular portion of a jugular vein hemostatic plug. The interactive slideshow contains multiple SEM images of the hemostatic plug shown in Figure 1. Click on the blue hyperlink boxes to navigate to additional images. Scale bars are indicated on the micrographs. Note the difference in morphology between platelets on the luminal surface and those at the injury site boundary. The intact endothelium surrounding the injury site is also clearly seen.

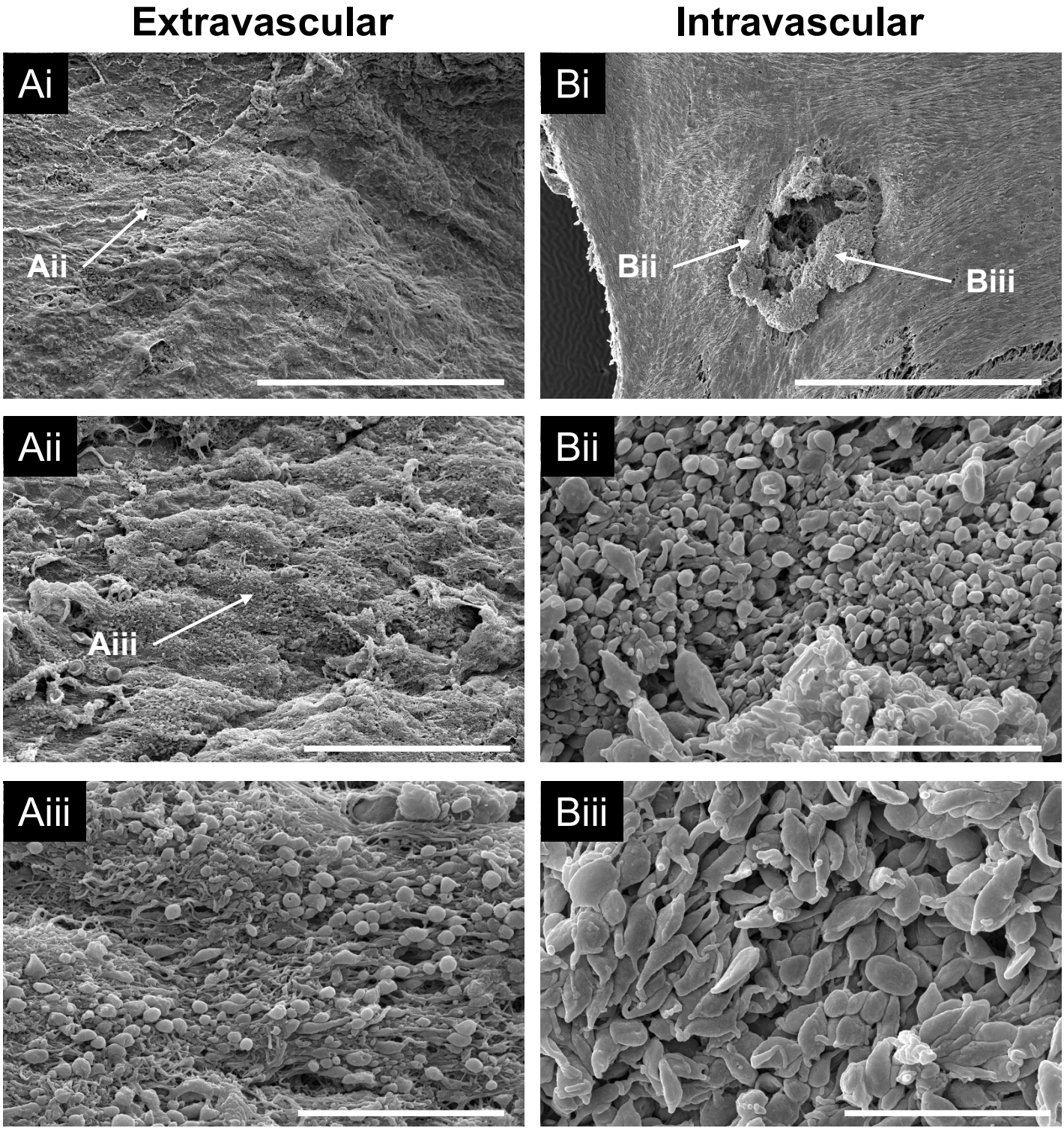
Supplemental dataset S2: Scanning electron micrographs showing multiple perspectives of the extravascular portion of a jugular vein hemostatic plug. The interactive slideshow contains multiple SEM images of the hemostatic plug shown in Figure 2. Click on the blue hyperlink boxes to navigate to additional images. Scale bars are indicated on the micrographs. The colorized image was artificially colored to highlight highly activated platelets (blue), minimally activated platelets (yellow), red blood cells (red) and fibrin (grayscale). Note the highly activated platelets and fibrin spread over a large area of the extravascular surface of the blood vessel. A few red blood cells may be observed trapped in the fibrin.

Supplemental Figure 1



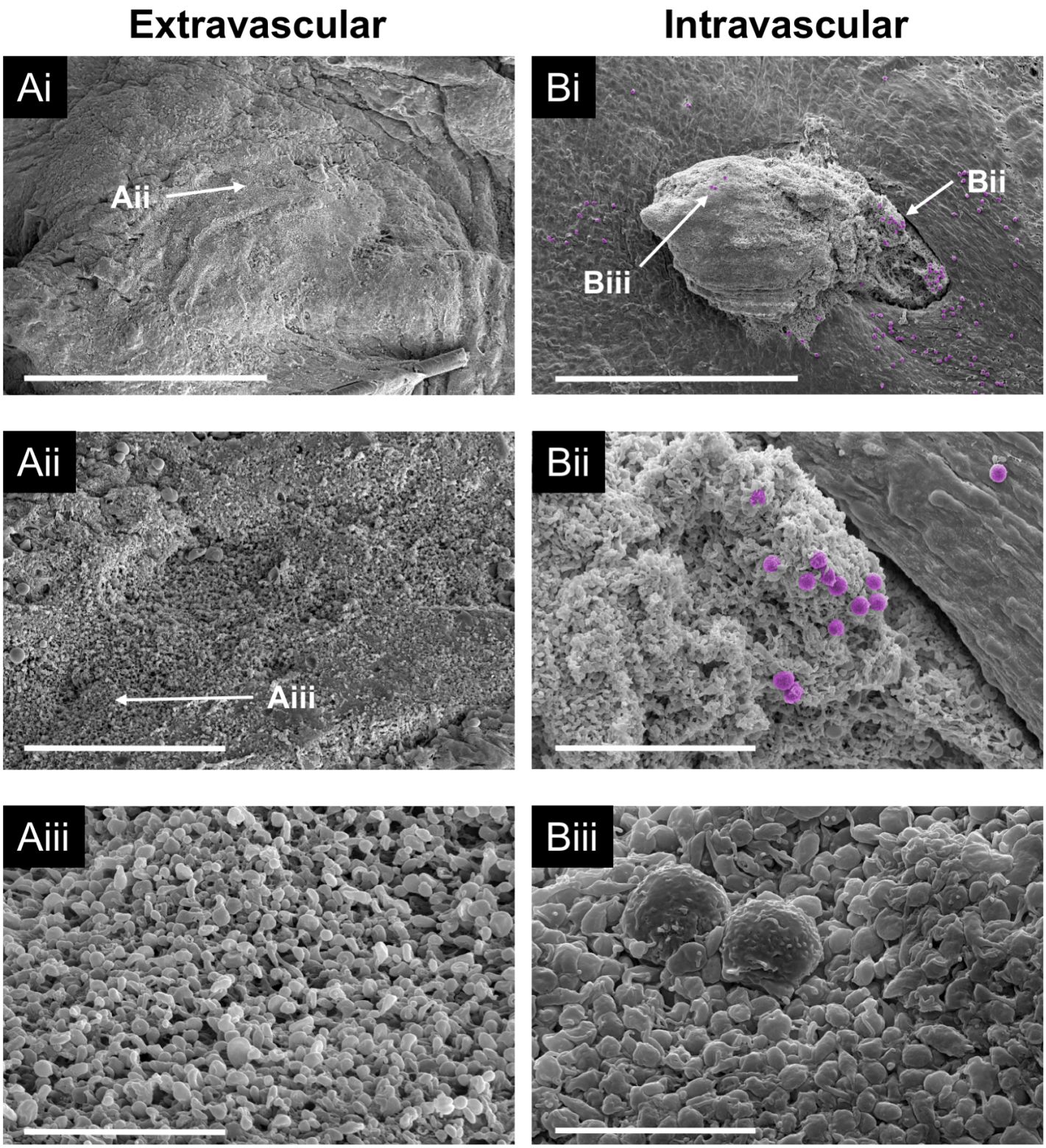
Supplemental Figure 1: Platelet activation gradient following large vein puncture injury. Micrographs show a representative hemostatic plug fixed 5 minutes following puncture injury. The sample was then frozen and sectioned to obtain cross sections (diagram at left shows perspective), followed by confocal fluorescence imaging. Panel A is the merged fluorescence image; CD41 is shown in red, P-selectin in green and fibrin in blue. The luminal side of the vessel wall is shown by the dotted line. The hole in the vessel wall is indicated as the injury site. "Lu" indicates the lumen and "Ex" indicates the extravascular side. Panels B-D show the individual fluorescence channels in grayscale, as indicated. Scale bar in A = 100 μm. Images shown are representative of 3 hemostatic plugs imaged following sectioning.

Supplemental Figure 2



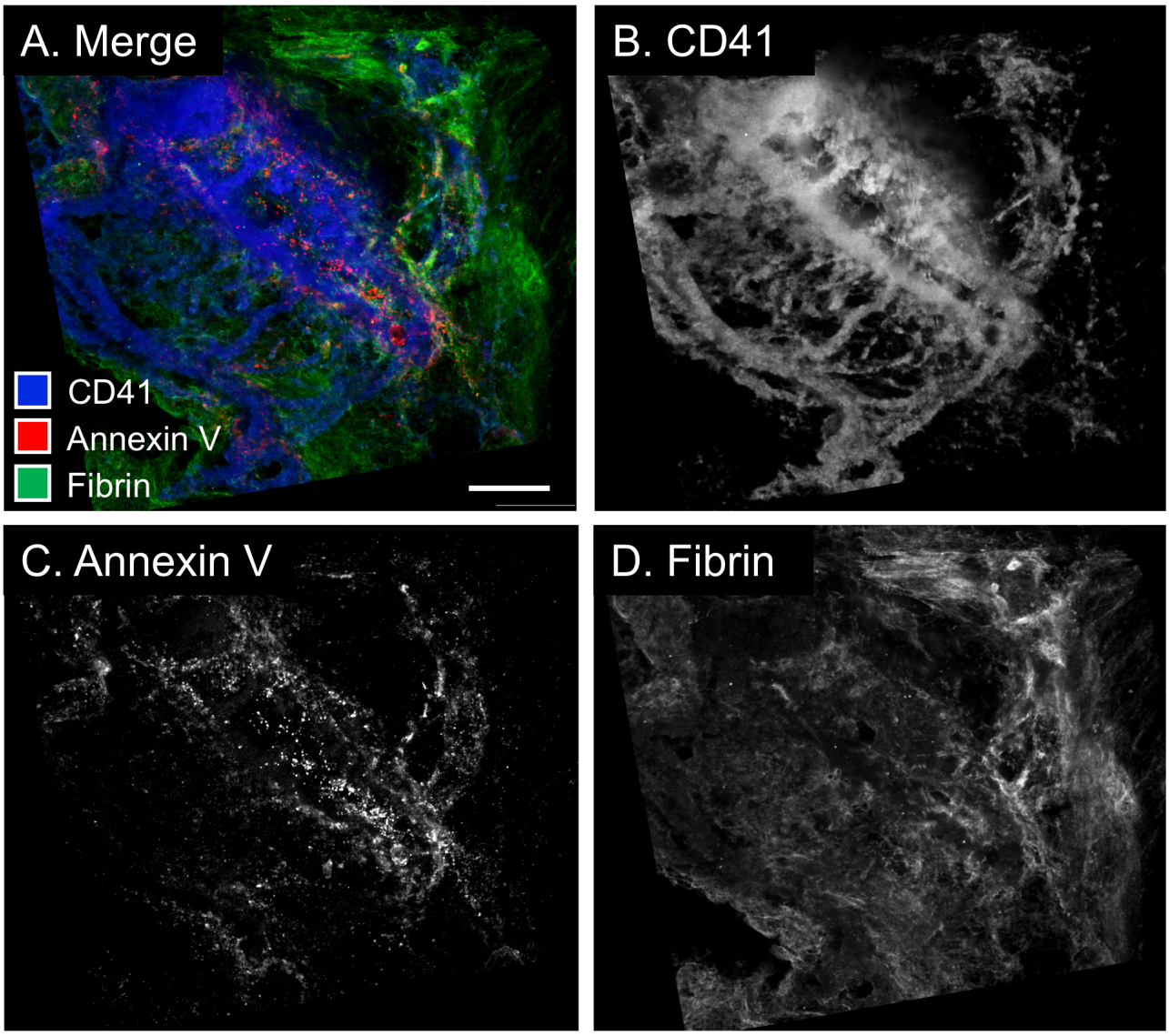
Supplemental Figure 2: Hemostatic plug formation 1 minute post-injury. Photomicrographs show representative hemostatic plugs 1 minute post-injury imaged from the extravascular (A, left panels) and intraluminal (B, right panels) side. Scale bars are 300 μm in Ai and Bi, 50 μm in Aii, and 10 μm in Bii, Aiii, and Biii. The white arrows indicate the regions shown in the higher magnification images. Note the contrasting platelet morphologies observed on the extravascular vs. intravascular sides of the hemostatic plugs, which is most apparent at the highest magnification (Aiii vs. Biii).

Supplemental Figure 3



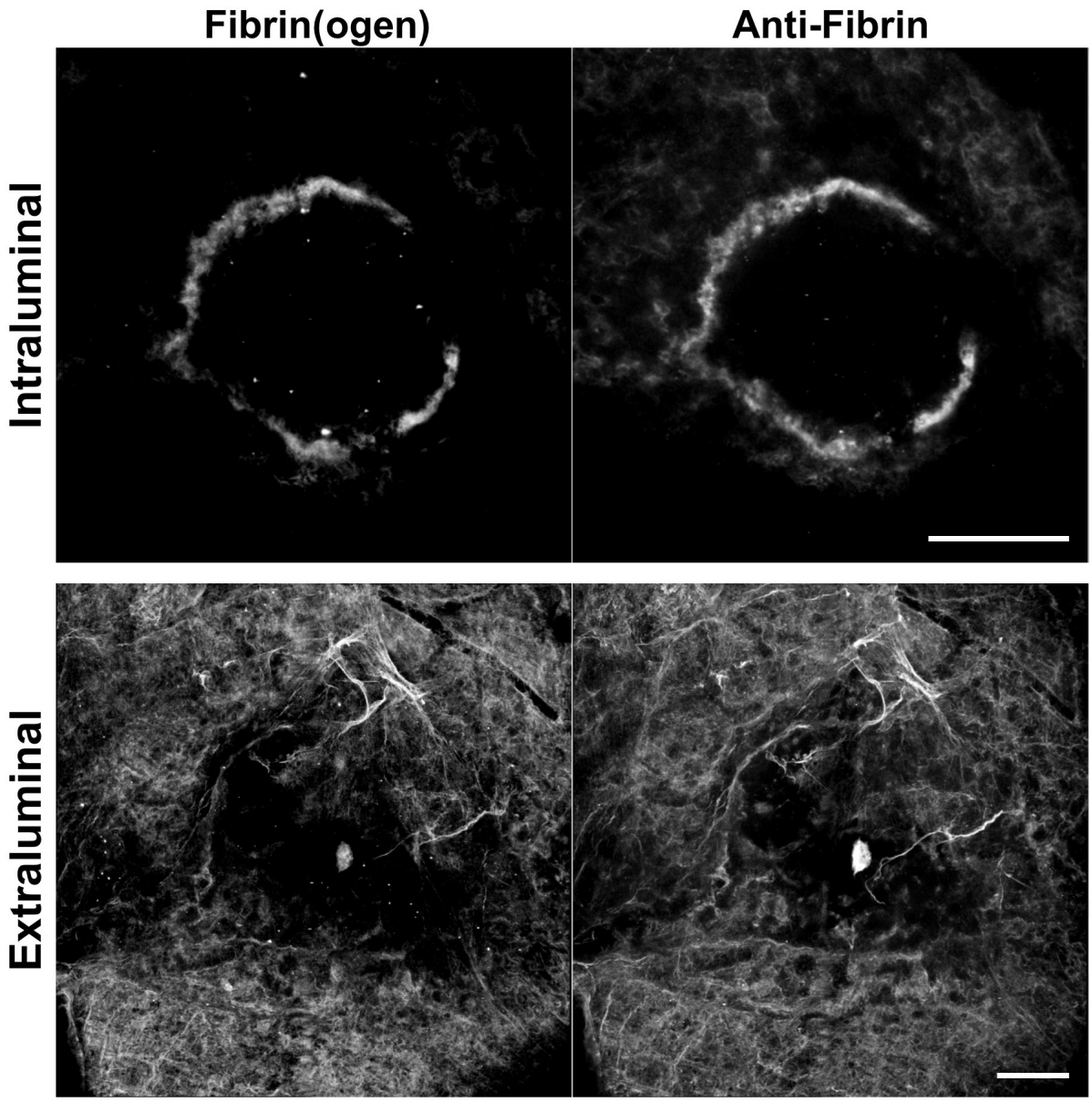
Supplemental Figure 3: Hemostatic plug formation 20 minutes post-injury. Photomicrographs show representative hemostatic plugs 20 minutes post-injury imaged from the extravascular (A, left panels) and intraluminal (B, right panels) side. Magnification increases from top to bottom. The white arrows indicate the regions shown in the higher magnification images. Leukocytes observed adhering to the intravascular portion of the hemostatic plug and surrounding vessel wall are pseudocolored purple for contrast (Bi and Bii). Scale bars are 300 μm in Ai and Bi, 50 μm in Aii and Bii, and 10 μm in Aiii and Biii.

Supplemental Figure 4



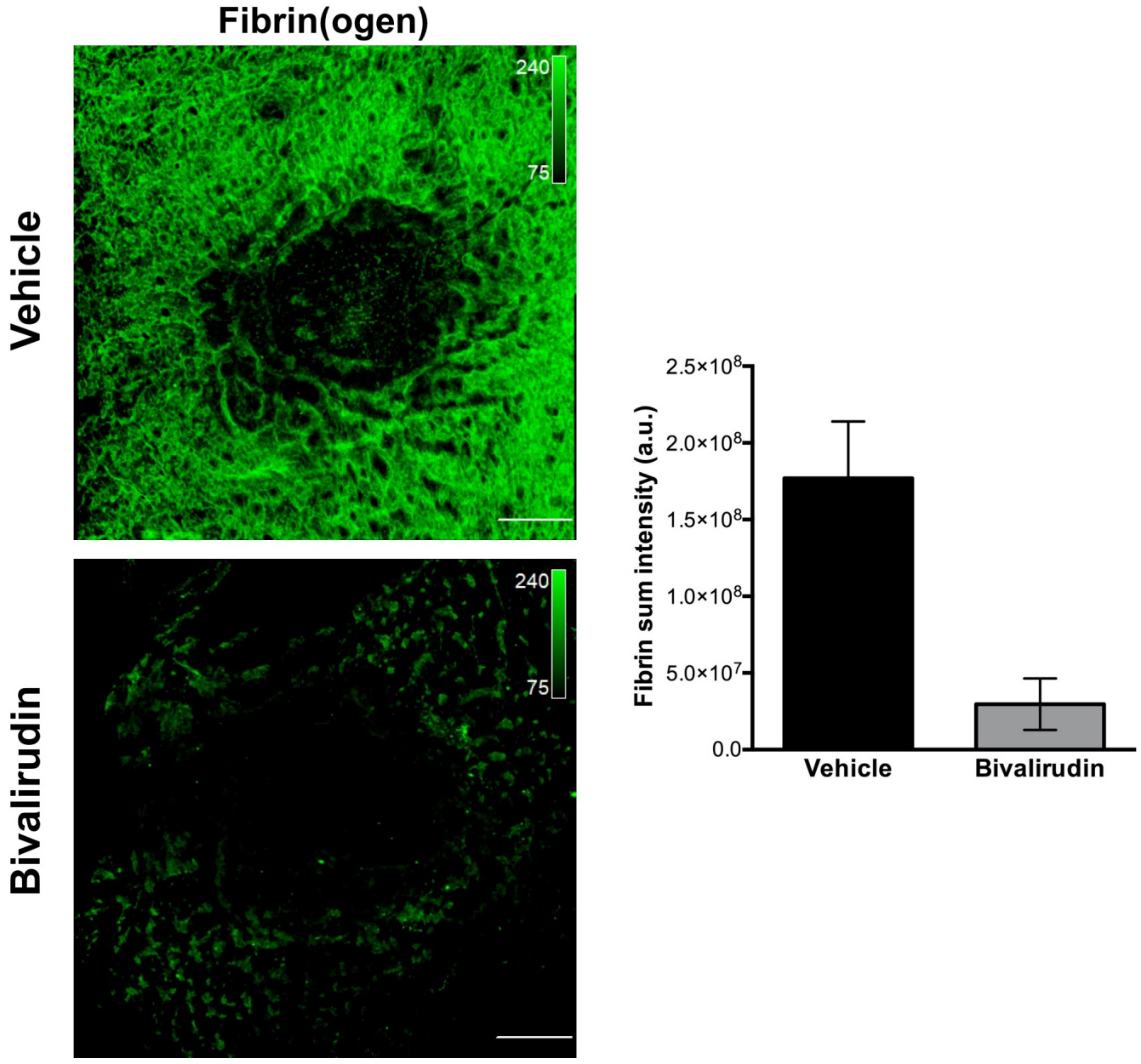
Supplemental Figure 4: Phosphatidylserine positive membranes in the extravascular side of a hemostatic plug. Micrographs show a representative hemostatic plug fixed 5 minutes post-injury. A-D) The merged fluorescence image (A) and individual fluorescence channels (B-D) are shown as indicated. The images are a maximum intensity projection of a series of z-plane images. Platelets are shown in blue (CD41), fibrin in green and Annexin V in red in the merge. Scale bar in A = 100 μm. Images shown are representative of >10 hemostatic plugs labeled with Annexin V.

Supplemental Figure 5



Supplemental Figure 5: Fluorescently labeled fibrinogen indicates fibrin formation. Micrographs show a representative hemostatic plug fixed 5 minutes post-injury imaged from the intraluminal (top panels) and extraluminal (bottom panels) sides. The images are a maximum intensity projection of a series of z-plane images. Left panels show Alexa-488 labeled fibrinogen; right panels show Alexa-568 labeled anti-fibrin antibody. Scale bars = 100 μm.

Supplemental Figure 6



Supplemental Figure 6: Fluorescently labeled fibrinogen indicates fibrin formation. Micrographs show representative hemostatic plugs fixed 5 minutes post-injury imaged from the extraluminal side. The images are a maximum intensity projection of a series of z-plane images. Hemostatic plugs were from mice treated with either vehicle (normal saline) or 1 μg/g body weight bivalirudin. Scale bars = 100 μm. Graph at the right shows fibrin sum intensity (mean±SEM) calculated from fluorescence images as described in the Supplemental Methods section.