SUPPLEMENTAL DATA

Intravascular hemolysis triggers ADP-mediated generation of platelet-rich thrombi in pre-capillary pulmonary arterioles.

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SUPPLEMENTARY METHODS

Reagents

Violet 450 (V450) rat-anti mouse CD49b mAb (clone DX5) was purchased from BD Biosciences (San Jose, CA). FITC dextran (MW 70,000) was purchased from Molecular Probes Inc. (Eugene, OR). Adenosine 5′-diphosphate (ADP), thrombin from human plasma, eptifibatide acetate (eptifibatide) and prasugrel were purchased from Millipore-Sigma (St. Louis, MO). Heparin (1000 U/ml) was purchased from SAGENT Pharmaceuticals (Schaumburg, IL). Ketamine HCl (100 mg/ml) and Isothesia (isoflurane) were purchased from Henry Schein Animal Health (Dublin, OH). Xylazine (20 mg/ml) was purchased from AKORN, Inc. (Lake Forest, IL).

Mouse surgery

Mouse surgical procedure has been described elsewhere in detail (1-5). Mice were anesthetized with an intraperitoneal (i.p.) injection of 100 mg/kg ketamine HCl and 20 mg/kg xylazine. Following complete sedation, mice were given a 1 ml i.p. injection of warmed saline and placed on a heated stage in the supine position. A tracheotomy was performed and a short length of PE90 tubing was inserted into the incision site and fixed with the trachea using 3M Vetbond (3M Animal Care Products; St. Paul, MN). Next, the right carotid artery was cannulated with heparinized PE10 tubing. Mice were mechanically ventilated at 120 breaths/min with a tidal volume of 10 µl/g of body weight using a MiniVent Type 845 (Harvard Apparatus; Holliston, MA). The ventilator was also used to deliver maintenance anesthesia (1% isoflurane) with a FiO2 of 0.95. Mice were placed on the heated stage in the right lateral decubitus position. The left lobe of the lung was exposed through removal of the overlying skin, fat, and three to four anterior ribs. Bleeding was minimized by using a Thermal Cautery Unit (Geiger Medical Technologies; Council Bluffs, IA) during the

lung surgery. A small region of the lower half of the left lung was gently immobilized against a 12 mm round cover slip attached to a vacuum-enabled micro-machined thoracic lung window described elsewhere (1-6).

qFILM image processing

QFILM image processing has been already described elsewhere in detail (1, 2). Time series of qFILM 2D images were analyzed and processed in Nikon's NIS-Elements software. Image subtraction was used to remove autofluorescence. The signal-to-noise ratio was improved through application of a median filter and a noise-reduction algorithm. Signal contrast was further enhanced by adjusting the maxima and minima of the intensity histogram associated with each channel. Blue and green channels were pseudo-colored as green and purple, respectively to enhance contrast. As a result, platelets are shown in green and pulmonary microcirculation in purple in all figures and movies. All image processing operations were performed uniformly on every image frame and over the whole FOV in each frame. In some experiments, the peak inspiratory and/or expiratory frame in each breathing cycle was excluded to reduce z drift in the movies.

References

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4. Sparkenbaugh EM, Chen C, Brzoska T, Nguyen J, Wang S, Vercellotti GM, et al. Thrombin-mediated activation of PAR-1 contributes to microvascular stasis in mouse models of sickle cell disease. *Blood.* 2020.
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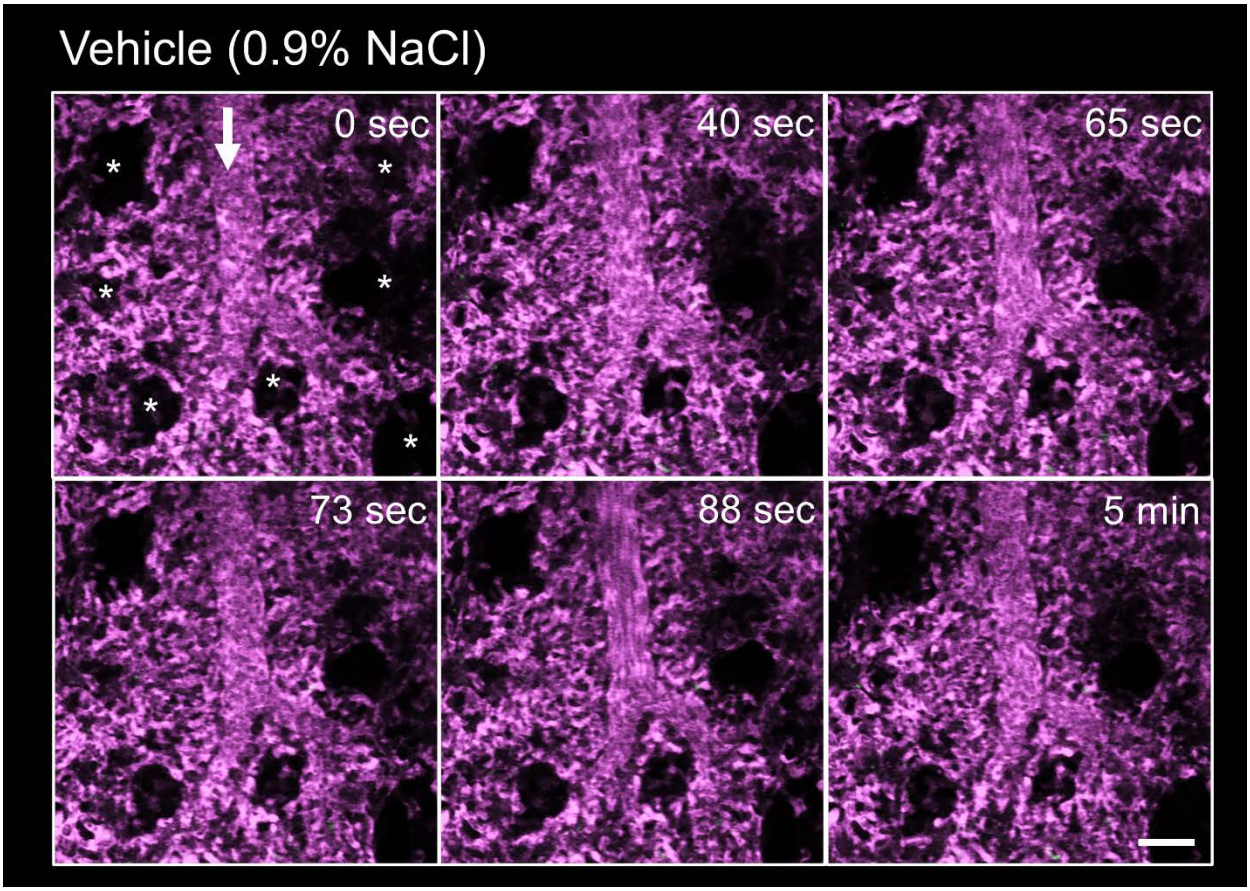
SUPPLEMENTAL FIGURE LEGENDS

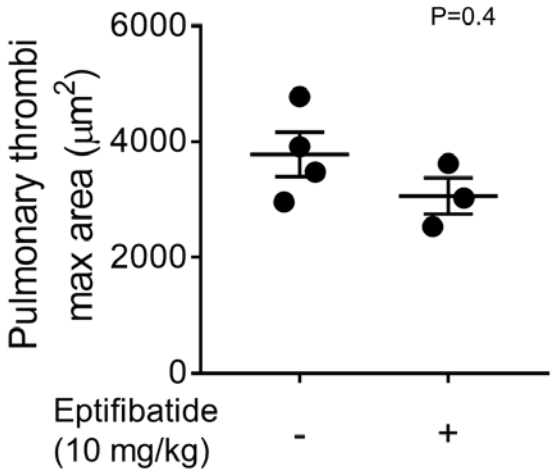
Supplemental Figure 1



Supplemental Figure 1. Pulmonary thrombosis is absent in mice administered IV saline. WT mouse was intravascularly (IV) administered with physiological saline (0.9% NaCl) and pulmonary circulation was imaged using quantitative fluorescence intravital lung microscopy (qFILM). qFILM images of the same field of view (FOV) at 6 different time points are shown to demonstrate absence of pulmonary thrombosis. t = 0 s corresponds to time point before and t > 0 s correspond to time points immediately following IV saline. Platelets (green) were stained by IV administration of V450-CD49b mAb and pulmonary microcirculation (purple) was visualized by IV administration of FITC-dextran. Pseudo-coloring was used for platelets and

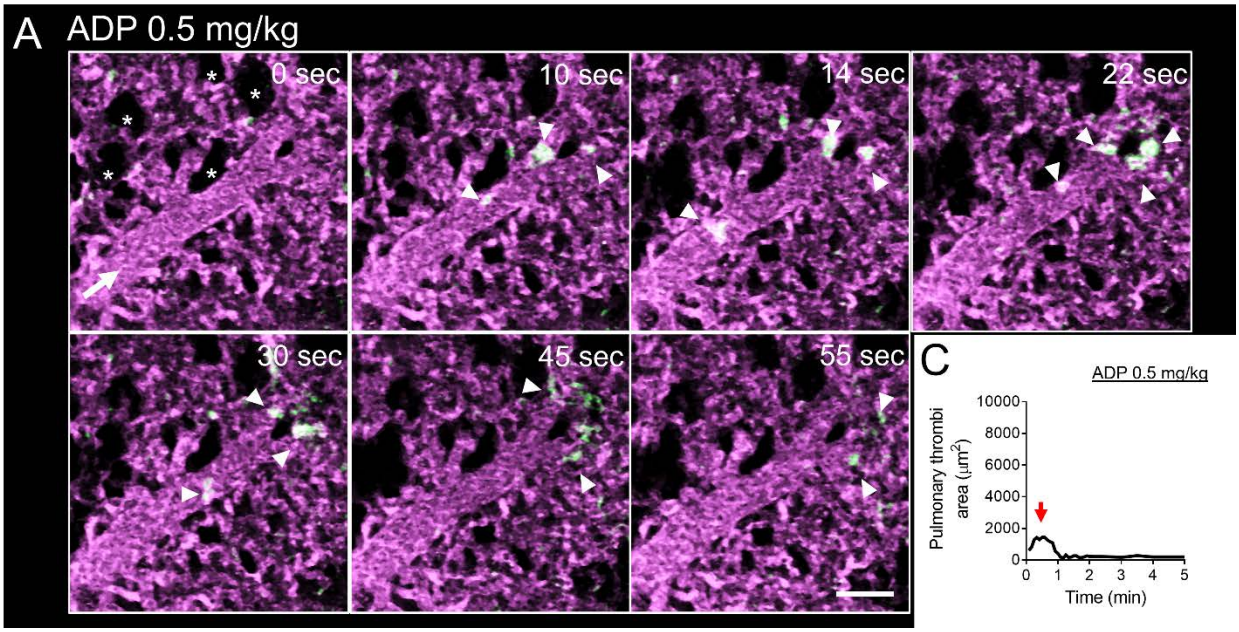
pulmonary vessels to enhance contrast. * denote the alveoli. White arrow marks the direction of blood flow within the feeding arteriole. The diameter of the arteriole is 42 μm. Scale bar 50 μm. Complete time series is shown in Supplemental Video 1.

Supplemental Figure 2



Supplemental Figure 2. Eptifibatide failed to prevent thrombin-triggered pulmonary arteriole thrombosis in mice. WT mice were intravascularly (IV) administered with 500 U/kg thrombin with (n = 3 mice) or without (n = 4 mice) IV administration of 10 mg/kg αIIbβ3-inhibitor (eptifibatide) 15 minutes before IV thrombin and pulmonary vasculature was visualized using quantitative fluorescence intravital lung microscopy (qFILM). QFILM images were analyzed to estimate pulmonary-thrombi-max-area as a quantitative measure of pulmonary thrombosis. Refer Methods for details. Pulmonary-thrombi-max-areas were compared using Wilcoxon-Mann-Whitney test. Mean pulmonary-thrombi-max-area was not significantly different (P = 0.4) between thrombin vs thrombin + eptifibatide. Data represent mean ± SE.

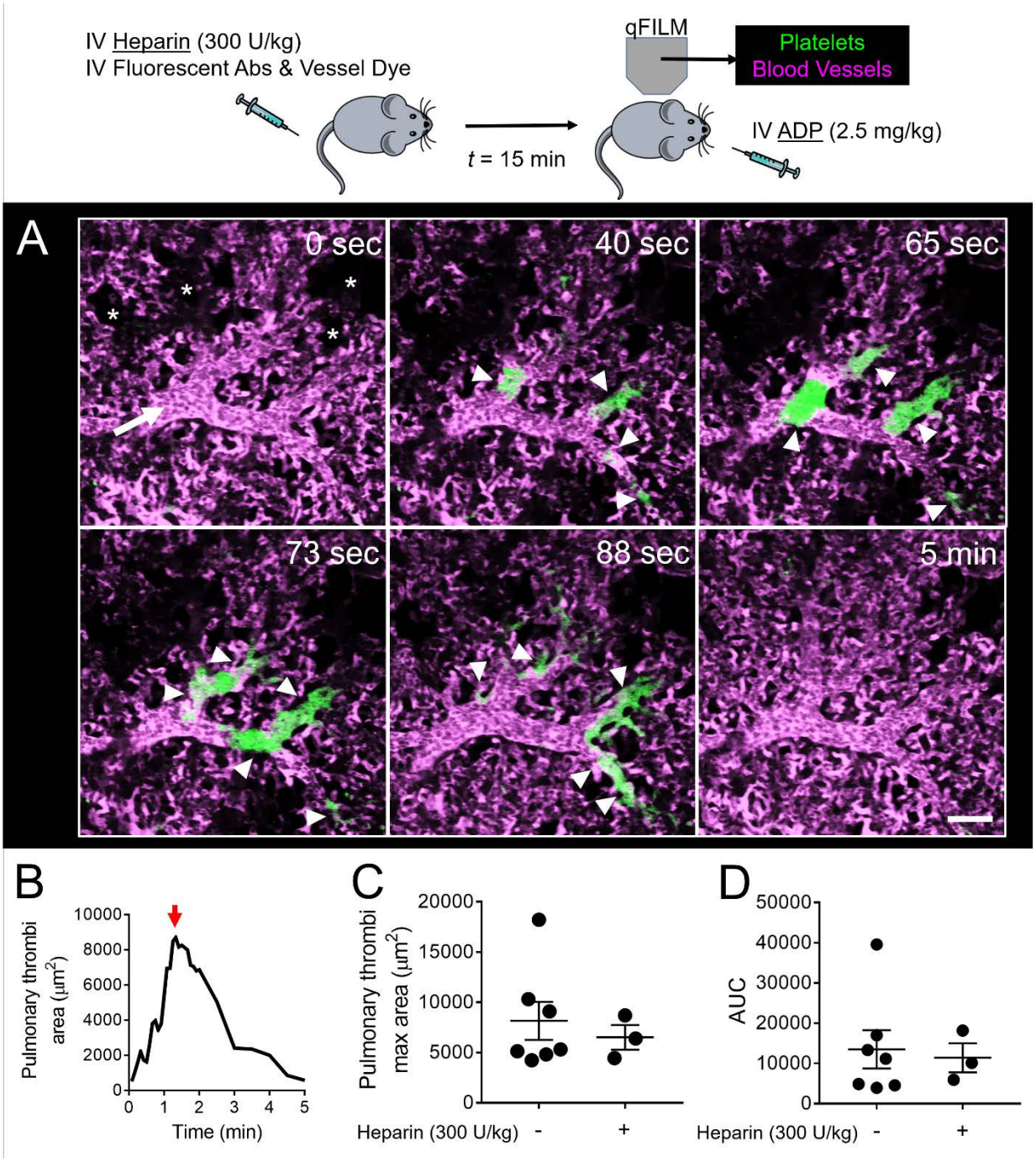
Supplemental Figure 3



Supplemental Figure 3. Low dose of ADP triggers mild pulmonary thrombosis. WT mice were intravascularly (IV) administered with 0.5 mg/kg ADP (n = 4 mice) and pulmonary circulation was imaged using quantitative fluorescence intravital lung microscopy (qFILM). **(A)** qFILM images of the same field of view (FOV) at 8 different time points are shown. t = 0 s corresponds to time point before IV ADP administration and other displayed time points are relative to IV ADP. Pulmonary thrombosis was absent at t = 0 s. Following 0.5 mg/kg IV ADP, small (size<500 μm^2) platelet-rich thrombi (white arrowheads) developed in the pulmonary arteriole (t=14 s). By t = 22 s, the thrombi were trapped in the arteriolar bottlenecks. Pulmonary thrombosis started to resolve by t = 30 s and completely resolved by t = 60 s. Platelets are shown in green and pulmonary microcirculation in purple. * denote alveoli. White arrow marks the direction of blood flow within the feeding arteriole. The diameter of the arteriole shown in A is 38 μm. Scale bar 50 μm. Complete qFILM time series corresponding to panels A is shown in Supplemental Videos 8. **(B)** Pulmonary thrombi area plotted as a function of time to show changes in the total area of platelet-rich thrombi

following 0.5 mg/kg IV ADP. Pulmonary thrombi maximum area (Pulmonary thrombi max area) value marked by red arrow.

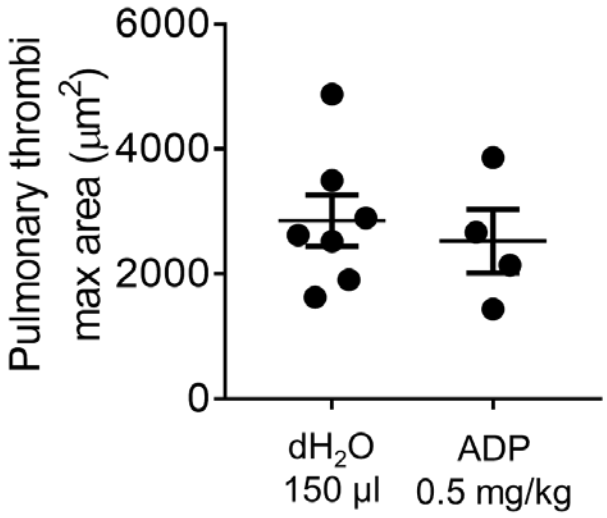
Supplemental Figure 4



Supplemental Figure 4. Heparin failed to prevent ADP-induced pulmonary thrombosis in mice. WT mice were intravascularly (IV) administrated with 2.5 mg/kg ADP with or without IV administration of 300 U/kg heparin 15 minutes before IV ADP. Pulmonary circulation was imaged

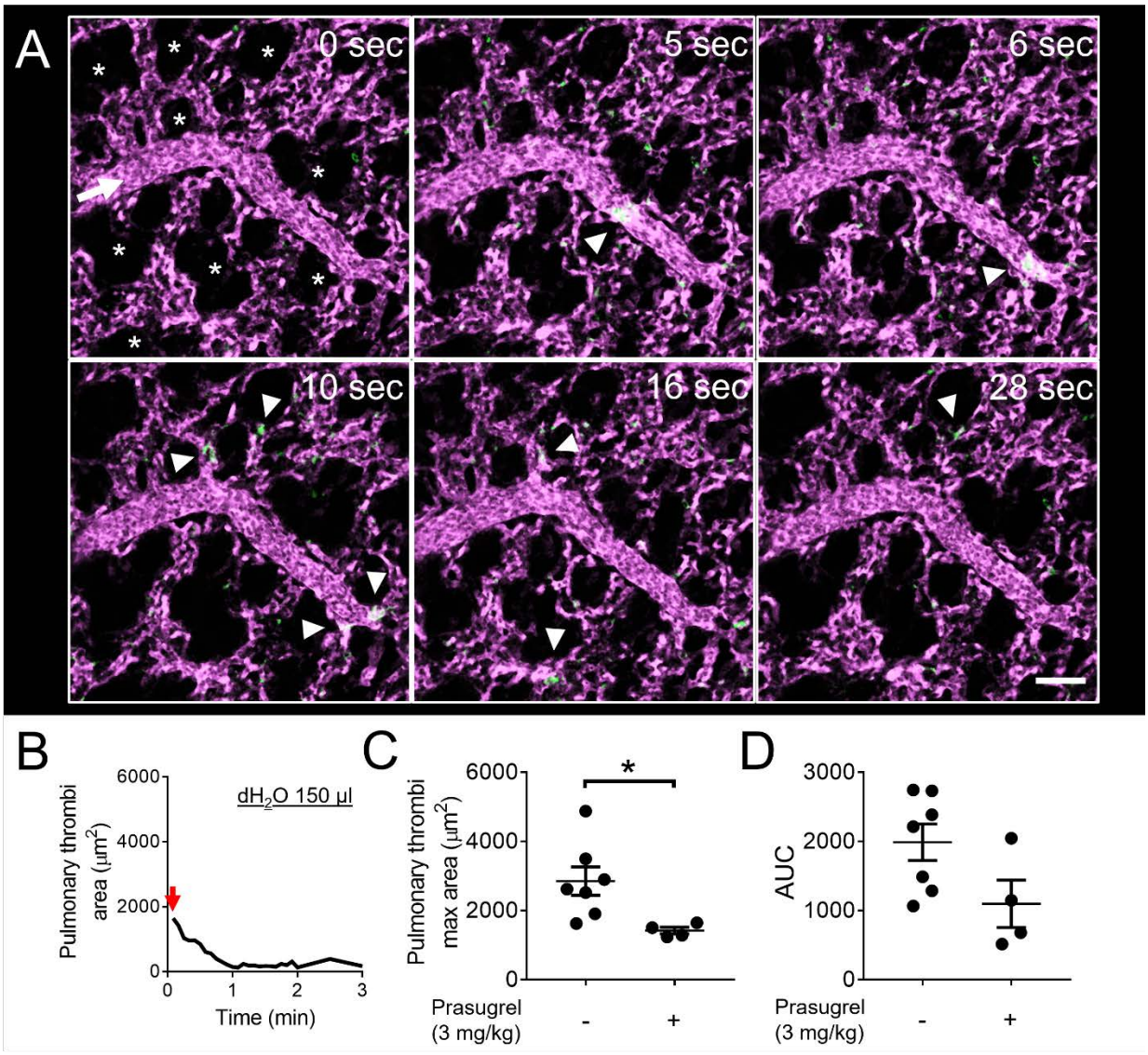
using quantitative fluorescence intravital lung microscopy (qFILM). Refer to experimental scheme shown on top. **(A)** qFILM images of the same field of view (FOV) at 6 different time points are shown. t = 0 s corresponds to time point before and t > 0 s correspond to time points immediately following IV ADP administration. Pulmonary thrombosis was absent at t = 0 s. Following 2.5 mg/kg IV ADP, medium (500-1000 μm^2) and large (>1000 μm^2) platelet-rich thrombi (white arrowheads) developed in the pulmonary arteriole by t = 40 s. The thrombi obstructed the pulmonary arteriolar bottlenecks by t = 73 s leading to loss of the pulmonary blood flow, which was evident by the absence of the vascular dye (purple fluorescence) in the capillaries. Pulmonary thrombosis resolved completely and the blood flow recovered by t = 5 min. Platelets (green) and pulmonary microcirculation (purple). * denote alveoli. White arrow marks the direction of blood flow within the feeding arteriole. The diameter of the arteriole is 37 μm. Scale bar 50 μm. Complete qFILM time series corresponding to panel A shown in Supplemental Video 10. **(B)** Pulmonary thrombi area plotted as a function of time for the FOV shown in panel A. Red arrow indicates pulmonary thrombi max area (Pulmonary thrombi max area). **(C)** Pulmonary thrombi max area and **(D)** area under the curve (AUC) in mice with (n = 3 mice) and without (n = 7 mice) pretreatment with 300 U/kg IV heparin prior to 2.5 mg/kg IV ADP. Pulmonary thrombi max area and AUC were estimated as described in Methods. Pulmonary thrombi max area and AUC were compared using Wilcoxon-Mann-Whitney test. Data represent mean ± SE.

Supplemental Figure 5



Supplemental Figure 5. Pulmonary thrombosis was identical in mice challenged with deionized water or ADP. WT mice were intravascularly (IV) administered with 150 µl dH_2O (n = 7 mice) or 0.5 mg/kg ADP (n = 4 mice) and pulmonary vasculature was visualized using quantitative fluorescence intravital lung microscopy (qFILM). QFILM images were analyzed to estimate pulmonary-thrombi-max-area as a quantitative measure of pulmonary thrombosis and compared using Wilcoxon-Mann-Whitney test. Refer Methods for details. Mean pulmonary-thrombi-max-area was not significantly different (P = 0.8) between dH_2O vs ADP. Data represent mean ± SE.

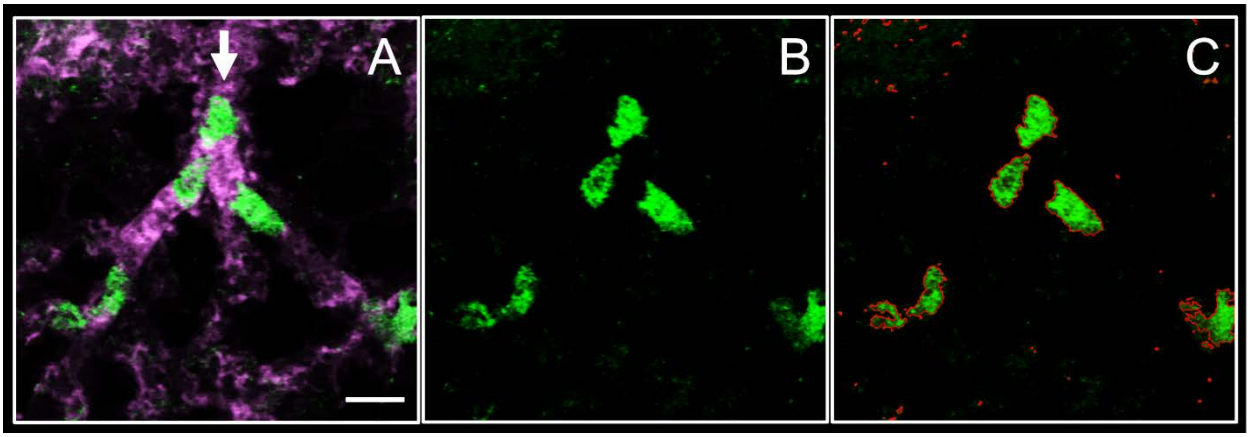
Supplemental Figure 6.



Supplemental Figure 6. Platelet-P2Y$_{12}$ inhibition attenuates hemolysis-induced pulmonary thrombosis in mice. WT mice were administrated by oral gavage (PO) with 3 mg/kg prasugrel (for more details refer to Methods) and intravascularly (IV) challenged with 150 µl dH_2O. Pulmonary circulation was imaged using quantitative fluorescence intravital lung microscopy (qFILM). **(A)** qFILM images of the same field of view (FOV) at 6 different time points are shown to assess the effect of prasugrel on the development of dH_2O-dependent pulmonary thrombosis. t

= 0 s corresponds to time point before and t > 0 s correspond to time points immediately following IV dH_2O administration. Pulmonary thrombosis was absent at t = 0 s. DH_2O-evoked pulmonary thrombosis was abrogated in mice pretreated with prasugrel. Platelets (green) and pulmonary microcirculation (purple). * denote alveoli. White arrow marks the direction of blood flow within the feeding arteriole. The diameter of the arteriole shown is 34 μm. Scale bar 50 μm. See also Supplemental Video 12 for the complete qFILM time series. **(B)** Pulmonary thrombi area plotted as a function of time for the FOV shown in panel A. Red arrow indicates pulmonary thrombi maximum area (Pulmonary thrombi max area). **(C)** Pulmonary thrombi max area and **(D)** area under the curve (AUC) in mice with (n = 4 mice) or without (n = 7 mice) pretreatment with 3 mg/kg PO prasugrel prior to IV dH_2O. Pulmonary thrombi max area and AUC were estimated as described in Methods. Pulmonary thrombi max area and AUC were compared using Wilcoxon-Mann-Whitney test. Data represent mean ± SE. *P < 0.05 when comparing with and without prasugrel pretreatment.

Supplemental Figure 7.



Supplemental Figure 7. Estimation of total pulmonary thrombi area from qFILM images. Platelets are shown in green and pulmonary microcirculation in purple. White arrow marks the direction of blood flow. Scale bar 50 μm. **(A)** Pulmonary arterioles were identified as blood vessels draining blood into smaller daughter arterioles followed by even smaller pulmonary capillaries. Platelet-rich pulmonary thrombi were defined as platelet aggregates (area > 10 μm^2) sequestered within the pre-capillary pulmonary arterioles and extending down into the pulmonary capillaries. Two-dimensional sizes (areas in μm^2) of platelet-rich thrombi were estimated in NIKON NIS-Elements software by converting **(B)** qFILM platelet-rich thrombi images into **(C)** binary images, and adjusting the image-intensity histograms uniformly over the entire field of view (FOV) to identify thrombi (green thrombi marked with red borders in C) in each image frame of the time-series. The sizes of all the platelet-rich thrombi (marked with red borders in C) in a single image frame were added to generate total pulmonary thrombi area, which was plotted as a function of time. Changes in total pulmonary thrombi area over time was used to calculate pulmonary-thrombi-maximum-area and area-under-the-curve (AUC). Refer to Methods for details on qFILM images analysis.

Supplemental Video Legends

Supplemental Video 1. Absence of pulmonary thrombosis in WT mouse following IV saline administration. Platelets (green) and pulmonary microcirculation (purple). White arrow-direction of blood flow within the feeding arteriole. 1.5x original acquisition rate.

Supplemental Video 2. Transient pulmonary thrombosis in WT mouse following 150 µl IV dH_2O. Platelets (green) and pulmonary microcirculation (purple). t = 0 s corresponds to time before and t > 0 s correspond to time following IV dH_2O administration, respectively. White arrow-direction of blood flow within the feeding arteriole.

Supplemental Video 3 Absence of pulmonary thrombosis in mouse pretreated with 10 mg/kg IV eptifibatide prior to 150 µl IV dH_2O. Platelets (green) and pulmonary microcirculation (purple). t = 0 s corresponds to time before and t > 0 s correspond to time following IV dH_2O administration, respectively. White arrow-direction of blood flow within the feeding arteriole. 1.5x original acquisition rate.

Supplemental Video 4. Pulmonary thrombosis in WT mouse following 250 U/kg IV thrombin administration. Platelets (green) and pulmonary microcirculation (purple). t = 0 s corresponds to time before and t > 0 s correspond to time following IV thrombin, respectively. White arrow-direction of blood flow within the feeding arteriole.1.5x original acquisition rate.

Supplemental Video 5. Lethal pulmonary thrombosis in WT mouse following 500 U/kg IV thrombin administration. Platelets (green) and pulmonary microcirculation (purple). t = 0 s

corresponds to time before and t > 0 s correspond to time following IV thrombin, respectively. White arrow-direction of blood flow within the feeding arteriole.1.5x original acquisition rate.

Supplemental Video 6. Three-Dimensional image of a massive pulmonary thrombi developed within a large pulmonary artery branch/arteriole in a mouse challenged with 500 U/kg IV thrombin. Platelets (green) and pulmonary microcirculation (purple).

Supplemental Video 7. Lethal pulmonary thrombosis in mouse pretreated with 10 mg/kg IV eptifibatide prior to 500 U/kg IV thrombin. Platelets (green) and pulmonary microcirculation (purple). t = 0 s corresponds to time before and t > 0 s correspond to time following IV thrombin, respectively. White arrow-direction of blood flow within the feeding arteriole. 1.5x original acquisition rate.

Supplemental Video 8. Transient pulmonary thrombosis in WT mouse following 0.5 mg/kg IV ADP. Platelets (green) and pulmonary microcirculation (purple). t = 0 s corresponds to time before and t > 0 s correspond to time following IV ADP administration, respectively. White arrow-direction of blood flow within the feeding arteriole. 1.5x original acquisition rate.

Supplemental Video 9. Transient pulmonary thrombosis in WT mouse following 2.5 mg/kg IV ADP. Platelets (green) and pulmonary microcirculation (purple). t = 0 s corresponds to time before and t > 0 s correspond to time following IV ADP administration, respectively. White arrow-direction of blood flow within the feeding arteriole. 1.5x original acquisition rate.

Supplemental Video 10. Transient pulmonary thrombosis in mouse pretreated with 300 U/kg IV heparin prior to 2.5 mg/kg IV ADP. Platelets (green) and pulmonary microcirculation (purple). t = 0 s corresponds to time before and t > 0 s correspond to time following IV ADP, respectively. White arrow-direction of blood flow within the feeding arteriole. 1.5x original acquisition rate.

Supplemental Video 11. Absence of pulmonary thrombosis in mouse pretreated with 10 mg/kg PO prasugrel prior to 150 µl IV dH_2O. Platelets (green) and pulmonary microcirculation (purple). t = 0 s corresponds to time before and t > 0 s correspond to time following IV dH_2O, respectively. White arrow-direction of blood flow in arteriole.

Supplemental Video 12. Restricted pulmonary thrombosis in mouse pretreated with 3 mg/kg PO prasugrel prior to 150 µl IV dH_2O. Platelets (green) and pulmonary microcirculation (purple). t = 0 s corresponds to time before and t > 0 s correspond to time following IV dH_2O, respectively. White arrow-direction of blood flow in arteriole.