Effects of ibrutinib treatment on murine platelet function during inflammation and in primary hemostasis

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Supplemental Information

Supplemental Materials and Methods

Materials

The following materials/reagents were used: low–molecular weight enoxaparin sodium (Lovenox) (Sanofi-Aventis), heparin-coated capillaries (VWR), ketamine and xylazine (Med-Vet International), ibrutinib (PCI-32765) (Selleck Chemicals), convulxin (provided by K.J. Clemetson, Theodor Kocher Institute, Bern, Switzerland), PAR4p (Advanced Chemtech), collagen (Chronolog), recombinant mouse podoplanin/Fc chimera protein (R&D Systems), BSA (US Biological), PGI_2 (Cayman Chemical), Surflash 18G I.V. catheters (Terumo Medical), *P. aeruginosa* LPS (Lot #084M4013V), ADP and formic acid (Sigma).

The following antibodies were used in this study: anti-GPIbα, anti-GPIX and JON/A (all from Emfret Analytics), anti-P-selectin (BD Biosciences) anti–hIL-4R (R&D Systems), rabbit anti-BSA antibody (MP Biomedicals), goat anti-mouse $F(ab')_2$ IgG (Santa Cruz).

Methods

Mice: Wild-type, hIL-4Rα/GPIbα transgenic (Tg)[1] and *P2ry12*-deficient[2] mice were bred on a C57BL6/J background and housed in the mouse facility of the University of North Carolina at Chapel Hill. All experimental protocols were approved by The University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee (IACUC).

Murine platelet preparation: Platelet-rich plasma (PRP) was obtained from heparinized whole blood by centrifugation at 130 x *g* for 4 minutes, collecting the platelet layer with some erythrocytes and then centrifugation at 100 x *g* for 5 mins to pellet erythrocytes. Platelets in PRP were incubated in PGI_2 (2 μg/ml) for 5 minutes followed by centrifugation at 700 x *g* for 5 mins. The platelet pellet was then resuspended in modified Tyrode's buffer (137 mM NaCl, 0.3 mM Na_2HPO_4, 2 mM KCl, 12 mM $NaHCO_3$, 5 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 5 mM glucose, pH 7.3) and the platelet concentration adjusted as needed. For platelet transfusion in the rpA reaction, washed platelets were resuspended in Tyrode's buffer containing 2% BSA.

Aggregometry: Platelet aggregation was determined by light transmission aggregometry. Washed platelets were adjusted to a final concentration of 2.5×10^8 platelets/mL in modified Tyrode's buffer with 1 mM Ca^{2+}. PRP was diluted 1:1 in Tyrode's with 1 mM Ca^{2+}. Platelets were pre-treated with DMSO (0.5%) or ibrutinib for 5 minutes, and then agonists were added at the indicated concentrations and light transmission was recorded on a Chrono-log 4-channel optical aggregation system (Chrono-log, Havertown, PA).

Flow cytometry: For platelet activation, heparinized whole blood was diluted in modified Tyrode's buffer with 1 mM Ca^{2+}, pre-treated with DMSO or ibrutinib for 5 minutes, and then activated with agonist at the indicated concentration in the presence of antibodies against GPIX (anti-GPIX-AlexaFluor488), P-selectin (anti-P-selectin-AlexaFluor647) and activated αIIbβ3 (JON/A-PE). After 10 minutes of activation at room temperature, samples were diluted in PBS and analyzed immediately by flow cytometry. For platelet counts, 50 µL blood samples were collected from mice into Eppendorf tubes containing 50 µL PBS/heparin. Whole blood was stained with anti-GPIX antibody, diluted in PBS and analyzed by flow cytometry. 10 µL of the diluted samples was analyzed, GPIX-positive events were recorded and platelet counts calculated accordingly.

Platelet adoptive transfer model: Thrombocytopenia (TP) was induced in hIL-4Rα/GPIbα Tg mice by retro-orbital injection of an antibody against hIL-4Rα (1 µg/g body weight). Wild-type washed platelets were treated with DMSO (0.5%) or ibrutinib (5 µM) for 15 minutes, washed in modified Tyrode's buffer and resuspended in Tyrode's buffer with 2% BSA (for rpA reaction) or without BSA (LPS lung inflammation) before injection into recipient TP Tg mice. Endogenous vs. exogenous platelets were identified by expression of hIL-4Rα or GPIbα, respectively.

P. aeruginosa LPS-induced lung inflammation: LPS-induced lung inflammation was performed essentially as previously described[3] with some modifications. hIL-4Rα/GPIbα-Tg mice were depleted of endogenous platelets at least 30 minutes prior to LPS treatment. Mice were then inoculated intranasally with 30 µg *P. aeruginosa* LPS. After 10 minutes, DMSO- or ibrutinib-treated platelets were transfused by retro-orbital injection. After 24 hours, blood was collected for platelet counts and activation assay and then mice were sacrificed. Bronchoalveolar lavage (BAL) fluid was collected by lavaging lungs 3X with 900 µL ice cold PBS, and samples from each mouse were pooled and imaged.

Reverse passive Arthus (rpA) reaction: The rpA reaction was performed essentially as previously described[3]. Briefly, hIL-4Rα/GPIbα-Tg mice were given intradermal injections of either anti-BSA antibody (60 µg/spot in 20 µL) or Tyrode's buffer. Following intradermal injections, mice received a retro-orbital injection of platelets in Tyrode's buffer containing BSA or Tyrode's + BSA alone for non-transfused controls (final BSA concentration of 150 µg/g body weight). To avoid excessive bleeding at injection sites due to thrombocytopenia, anti-hIL-R4α depleting antibody was administered as the last step.

Hemoglobin (Hb) assay: skin biopsies (8-mm punch) were homogenized in 500 µL PBS and spun at 15,000 x *g* for 10 minutes, and the supernatant was analyzed. Formic acid was added and the OD at 405 nm was measured. Diluted bovine Hb was used to set up a standard curve.

Saphenous vein laser injury model: Platelet adhesion and bleeding time were determined using laser-induced injury of the saphenous vein as previously described[4] with some minor modifications. Mice (wild-type or *P2ry12*$^{-/-}$, male and female, 22-28g) were anesthetized by intraperitoneal injection of ketamine/xylazine. Antibodies recognizing GPIX (AlexaFluor-488 conjugated, 2.5 µg/mouse) and fibrin (AlexaFluor-647 conjugated, 4 µg/mouse) were administered by retro-orbital injection. When noted, ibrutinib (6.25 mg/kg) was administered by intraperitoneal injection. The saphenous vein was exposed, and the vessel was exposed to two maximum-strength 532-nm laser pulses (70 µJ; 100 Hz; duration, 7 ns; interval between pulses, 10 ms) (Ablate! photoablation system; Intelligent Imaging Innovations, Denver, CO, USA). Fluorescence signals were recorded for 5 minutes. Similar injuries were performed at up to five distinct locations along the saphenous vein. To avoid effects from earlier injuries, new injuries were performed upstream of the previous injury. Constant perfusion drip was maintained on the exposed saphenous vein with a physiologic salt solution containing 32 mm NaCl, 4.7 mm KCl, 1.2 mm MgSO4, 2 mm CaCl2, and 18 mm NaHCO3 (pH 7.4), which was bubbled with 5% CO_2/95% N2 for 15 min prior to the start of the experiment. The physiologic salt solution was maintained at 37 °C by perfusion through a Sloflo In-line solution heater (SF-28) (Warner Instruments, Hamden, CT, USA). Fluorescence signals within the boundaries of the saphenous vein were recorded with a Zeiss Axio Examiner Z1 microscope (Intelligent Imaging Innovations) equipped with a multicolor LED light source (Lumencor), a Hamamatsu Orca Flash CMOS camera (Hamamatsu, Japan) and a × 20/1 numerical aperture water immersion objective lens (Zeiss). All data were analyzed with Slidebook 6.0 (Intelligent Imaging Innovations).

Statistical analysis: Results are reported as mean ± SEM, and statistical significance was assessed by 2-tailed Student's *t* test or Mann-Whitney test using Prism software (GraphPad). A P value of 0.05 or less was considered significant.

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Supplemental figure legends

Supplemental Figure 1. The effect of ibrutinib on platelet aggregation is blunted in the presence of plasma. A, B) platelets in platelet-rich plasma (PRP) were diluted 1:1 in modified Tyrode's buffer with 2 mM Ca^{2+} (1 mM final) in glass cuvettes under stirring conditions. PRP was pre-incubated with 0.5% DMSO or ibrutinib (0.5 or 5 µM) for 5 minutes, followed by the addition of collagen (A) or podoplanin-Fc and anti-mouse $F(ab')_2$ IgG antibody.

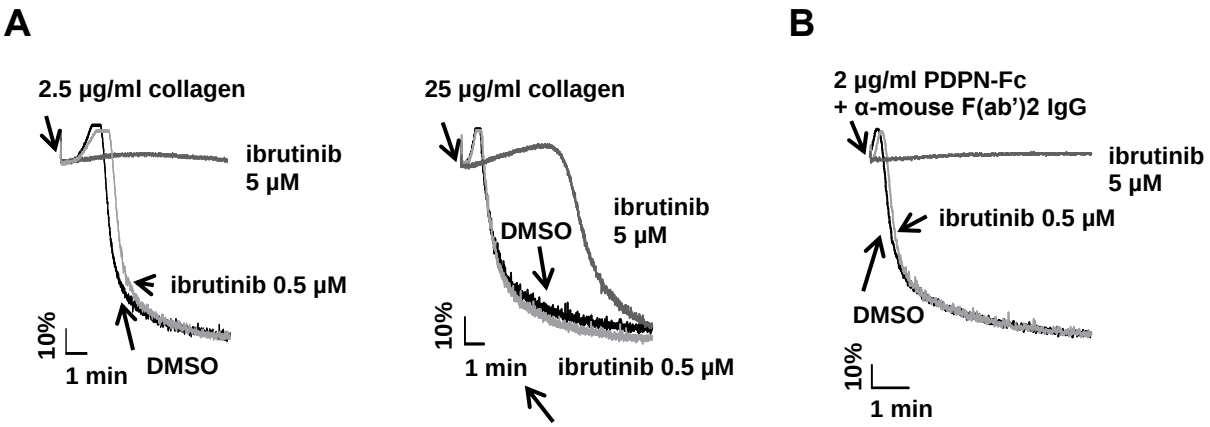
Supplemental Figure 2. Ibrutinib specifically inhibits (hem)ITAM receptor but not GPCR-mediated platelet activation. Platelets in whole blood were assayed for αIIbβ3 integrin activation (JON/A-PE) and α-granule secretion (anti-P-selectin-AlexaFluor488). Whole blood samples from wild-type mice were pre-incubated with vehicle or ibrutinib (0.5 or 5 µM) for 5 minutes, followed by addition of agonist (convulxin (Cvx), PAR4-activating peptide (Par4p) or ADP). All reactions were performed in modified Tyrode's buffer containing 1 mM Ca^{2+} in the presence of anti-GPIX-AlexaFluor647, anti-P-selectin-AlexaFluor488 and JON/A-PE antibodies for 15 minutes total (5 minutes pre-incubation, 10 minutes with agonist). Activation with the ITAM receptor agonist Cvx is shown in (A) and GPCR agonists are shown in (B). C) Representative aggregation trace showing no inhibitory effects of high-dose ibrutinib on platelet aggregation following PAR4p treatment. ** $p<0.01$ compared to control, n.s., not statistically significant.

Supplemental Figure 3. Graphical representation of adoptive platelet transfer and inflammation models. A) Graphical description of the inflammatory hemorrhage model in hIL-4Rα-Tg mice. hIL-4Rα mice are depleted of endogenous platelets by injection of an anti-hIL-4Rα antibody. Wild-type platelets are then transfused and circulate normally in the presence of the anti-hIL-4Rα antibody. To avoid bleeding from needle puncture at sites of intradermal injection, adoptive platelet transfer was performed immediately following induction of inflammation (rpA reaction or LPS-induced lung inflammation). B) Platelet counts following depletion and transfusion were comparable between mice receiving either DMSO- or ibrutinib-treated platelets. Platelet counts following transfusion reached 30-40% of initial platelet counts before depletion. n.s., not statistically significant.

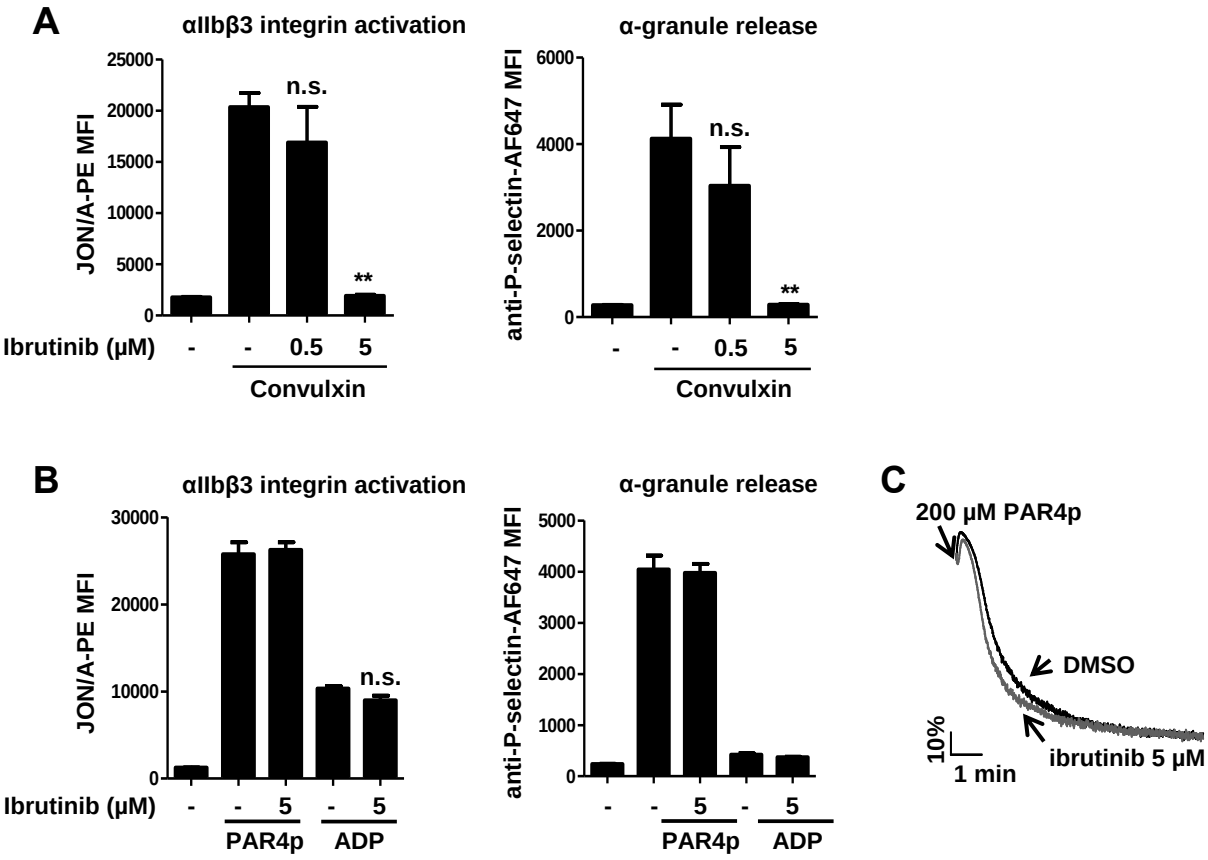
Supplemental Figure 4. Ibrutinib has no effect on hemostasis in saphenous vein needle injury model. A) Hemostasis was assessed in the saphenous vein needle injury model. 20 minutes prior to the experiment, wild-type mice were given an intraperitoneal injection of DMSO or ibrutinib (12.5 mg/kg). Mice were anesthetized, the saphenous vein exposed and a 23G needle was used to fully transect the vessel. Time to vessel occlusion was recorded. Following occlusion, the thrombus was disrupted using a 29G needle to allow for rebleeding and time to occlusion was again recorded. This process was repeated for a total of 15 minutes. Each point represents the average of all

bleeding times for one mouse. The instances of occlusion in the 15 minute time frame were not significantly different between vehicle- and ibrutinib-treated mice (not shown). B) Platelet activation in whole blood from vehicle- or ibrutinib-treated mice, showing inhibition of convulxin-induced integrin activation in platelets from ibrutinib-treated mice. * $p<0.05$, ** $p<0.01$, n.s., not statistically significant.

Supplemental Figure 1

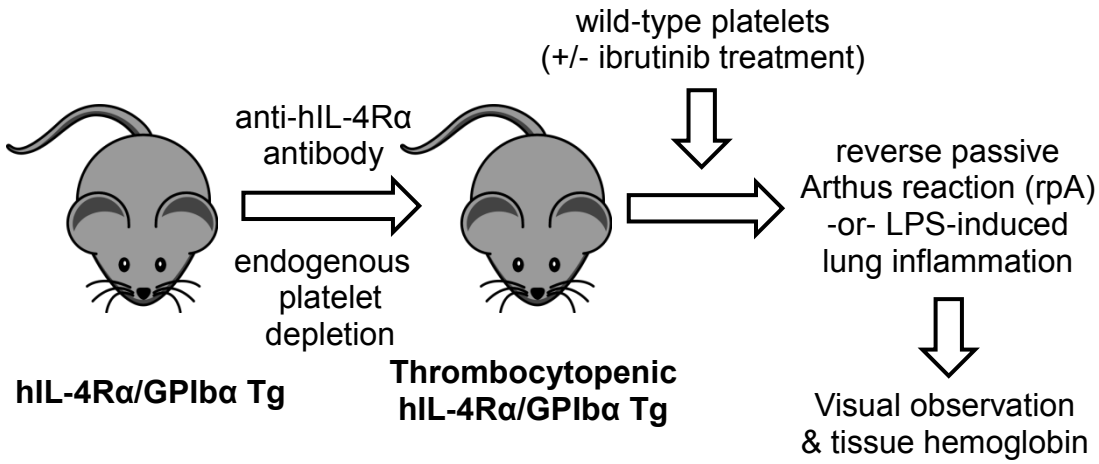


Supplemental Figure 2



Supplemental Figure 3

A



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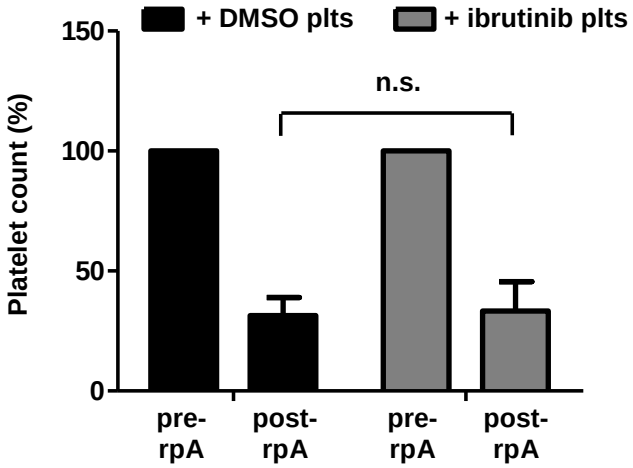

Supplemental Figure 4