

Platelet Munc13-4 regulates hemostasis, thrombosis and airway inflammation

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SUPPLEMENTAL METHODS

Sample isolation

Under anesthesia with isoflurane, blood was collected from the inferior vena cava into a citrated syringe (50 μ l of 4% Na citrate; 21 G needle), mixed with an equal volume of Tyrode's buffer (in mM: 5.56 glucose, 140 NaCl, 12 NaHCO₃, 2.7 KCl, 0.46 NaH₂PO₄), and used as whole blood. This sample was centrifuged (60 RCF, 10 min) and the supernatant was used as platelet-rich plasma (PRP). PRP was centrifuged (635 RCF, 10 min), the pellet was washed with PBS and resuspended in Tyrode's buffer (2.5×10^8 platelets/ml) and used as washed platelets. Cell counts were obtained from whole blood using a VET abc hematology analyzer (Scil), and from washed platelets with a Z2 counter (Beckman Coulter).

Expression studies

For qPCR, washed platelets were lysed (DNA/RNA Shield; Zymo Research). Total RNA was isolated (EZNA Total RNA Kit I; Omega Bio-tek), concentrated and cleaned (RNA Clean & Concentrator; Zymo Research), and reverse-transcribed (qScript cDNA SuperMix; Quanta Biosciences). cDNA was amplified (PerfeCTa qPCR ToughMix; Quanta Biosciences) and the abundance of Munc13-1 (Mm01340418_m1), Munc13-2 (Mm01351419_m1), Munc13-3 (Mm00463432_m1), Munc13-4 (Mm01252625_m1) and β -actin (Mm00607939_s1) transcripts were relatively quantified using hydrolysis probes (TaqMan Gene Expression Assays; Life Technologies) on a ViiA7 RTPCR System (Applied Biosystems). For immunoblotting, tissue and platelet lysates were run under denaturing conditions on 10% SDS polyacrylamide gels, transferred to nitrocellulose and probed with anti- β -actin (ab119716; abcam) and anti-Munc13-4 antibodies.¹

Secretion and activation assays

For ATP, 600 μ l of whole blood were diluted 5-fold in Tyrode's buffer and stirred (1200 rpm, 37 °C, 5 min; 700 Lumi-Aggregometer) in the presence of luciferin/luciferase, and collagen or thrombin, while recording ATP release (Aggro/Link8; all from Chrono-Log). For PF4, 5×10^7 washed platelets were stimulated for 5 min with thrombin with or without ADP in the presence of 0.7 mM CaCl₂, and PF4 in the supernatants was measured by ELISA (ELM-PF4; RayBiotech). For P-selectin, LAMP-1 and integrin $\alpha_{IIb}\beta_3$, 2.5×10^6 washed platelets were incubated in 40 μ l of PBS with 10 μ g/ml of FITC-anti-P-selectin antibody (RB40.34; BD Pharmingen) or 10 μ g/ml of FITC-anti-LAMP-1 antibody (1D4B; BD Pharmingen) or 10% PE-anti- $\alpha_{IIb}\beta_3$ antibody (Jon/A; Emfret Analytics) for 10 min. Platelets were then stimulated with thrombin or collagen for 10 min in the presence of 0.7 mM CaCl₂. Then, samples were placed on ice, diluted with 1 ml of PBS and analyzed by flow cytometry (LSR II; BD Biosciences), using the scattergram to select mostly single-particle events (the window was set using unstimulated platelets). The difference between baseline and stimulated values of mean fluorescence intensity (MFI) represents the gain in MFI (Δ MFI).

Electron microscopy and stereology

Washed platelets were resuspended in Tyrode's buffer with 0.7 mM CaCl₂. Resting and activated (thrombin 0.1 U/ml, 2 min) platelets were fixed in 2.5% glutaraldehyde with 0.1 M Na-cacodylate (2 h), post-fixed in 1% OsO₄ (1 h, both at room temperature), pelleted, and embedded in 3% low-melting agarose. The agarose blocks were dehydrated through an acetone series before

embedding in Embed 812 resin.² Sections of 100 nm were stained with uranyl acetate and lead citrate prior to acquiring images with a Tecnai 12 (FEI) transmission electron microscope (8200 \times , 100 KeV). For stereology, 10 fields per sample were analyzed with a grid consisting of 81 line pairs (line width = 2 pixels [0.0318 μ m] and T-bar = 5 pixels [0.0795 μ m]) in STEPanizer³ to obtain the volume occupied by platelet granules with respect to the whole platelet volume (volume density, Vv) using a point-count system.⁴

Aggregometry and flow-chamber assay

Aggregometry was conducted in a 700 Lumi-Aggregometer. Platelet-rich plasma (500 μ l) was stirred (800 rpm, 37 $^{\circ}$ C, 10 min) in the presence of 0.7 mM CaCl₂ and collagen. Flow assays were conducted using a microfluidic BioFlux System (Fluxion Biosciences) with plates coated with type I collagen (25 μ g/ml; Helena). Whole blood was anticoagulated with 80 μ M PPACK (d-phenylalanyl-prolyl-arginine chloromethyl ketone) and labeled with 10 μ M mepacrine (37 $^{\circ}$ C, 20 min) before being perfused over the collagen-coated surfaces at fixed shear stress. Thrombus buildup was monitored with a fluorescence microscope every 10 s for 200 s, and analyzed using BioFlux Montage.⁵ Final measurements were used for statistical comparisons.

Ferric chloride–induced thrombosis

Mice (13 \pm 1 weeks) were anesthetized with pentobarbital 50 mg/kg i.p. A common carotid artery was exposed and a 1 \times 2 mm piece of filter paper soaked with 10% FeCl₃ was applied to its surface for 3 min. After removing the filter paper and rinsing with saline, the time to vessel occlusion was recorded using a Doppler flow probe (Transonic Systems). All animals were euthanized after blood flow cessation or at 30 min. All vessels that failed to occlude were assigned a value of 30 min for statistical comparisons.

Airway mechanics

Mice were anesthetized with urethane (2 mg/g i.p.), tracheotomized (18 G cannula), connected to a SCIREQ-flexiVent (f = 150/min, VT = 10 ml/kg, PEEP = 3 cm H₂O) and paralyzed with succinylcholine (10 mg/kg/min i.p. continuous infusion). Increasing concentrations of methacholine (Mch; 0, 3, 10, 30 and 100 mg/ml) in PBS were administered via an in-line ultrasonic nebulizer (10 s per dose). After each dose, two consecutive deep inflations (27 cm H₂O, 6 s) were applied and the total resistance of the respiratory system (Rrs) was recorded 12 times using a 1.25 s, 2.5 Hz single-frequency forced oscillation maneuver (SnapShot-150 perturbation). We report the ratio of the highest Rrs measured for each dose over the baseline Rrs. Additionally, we fitted a 4-parameter sigmoidal curve to each individual dose response curve and derived the PC₁₀₀₀ (provocation concentration causing a 10 \times or 1000% increase in baseline Rrs) for each animal.

Histology and mucin quantification

After cannulating the trachea (20 G) and dissecting the lungs, they were inflated and fixed (10% formalin, 20 cm H₂O). A 3 mm-thick transversal section at the entry point of the left lobar bronchus into the left hilum was embedded in paraffin.⁶ Transversal sections (5 μ m) were stained with hematoxylin and eosin for histology and eosinophil counts, or PAFS reagent for airway mucin quantification.⁷ Under a dual fluorescence filter (Ex 500/573 nm, Em 531/628 nm) basement

membrane, nuclei and cytoplasm were detected in the green spectrum and mucins in the red. The area of intracellular mucin stores (A) and the length of the basement membrane (L) were obtained from ten 40x micrographs from the left axial bronchus (ImagePro Plus), and mucin volume density was calculated as $A/(L \times 4/\pi)$.⁶

Bronchoalveolar lavages

Lungs were perfused through the right ventricle (PBS, 5 ml) and 1 ml of PBS was flushed and then aspirated through the tracheal cannula, twice. The lavage was processed for cell counts (hemocytometer) and differentials (cytospins, modified Wright-Giemsa).

Statistical analysis

For continuous variables, we compared the means of all groups by 1-way ANOVA. We used 2-way ANOVA when assessing the effect of two factors (e.g. genotype and agonist dose). If a significant difference was found we applied Holm-Sidak's test for multiple comparisons against the control group. For variables not following a normal distribution, we compared the medians of all groups by Kruskal-Wallis test, followed by Dunn's test for multiple comparisons against the control group. Significance was set at $p < 0.05$.

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SUPPLEMENTAL FIGURES

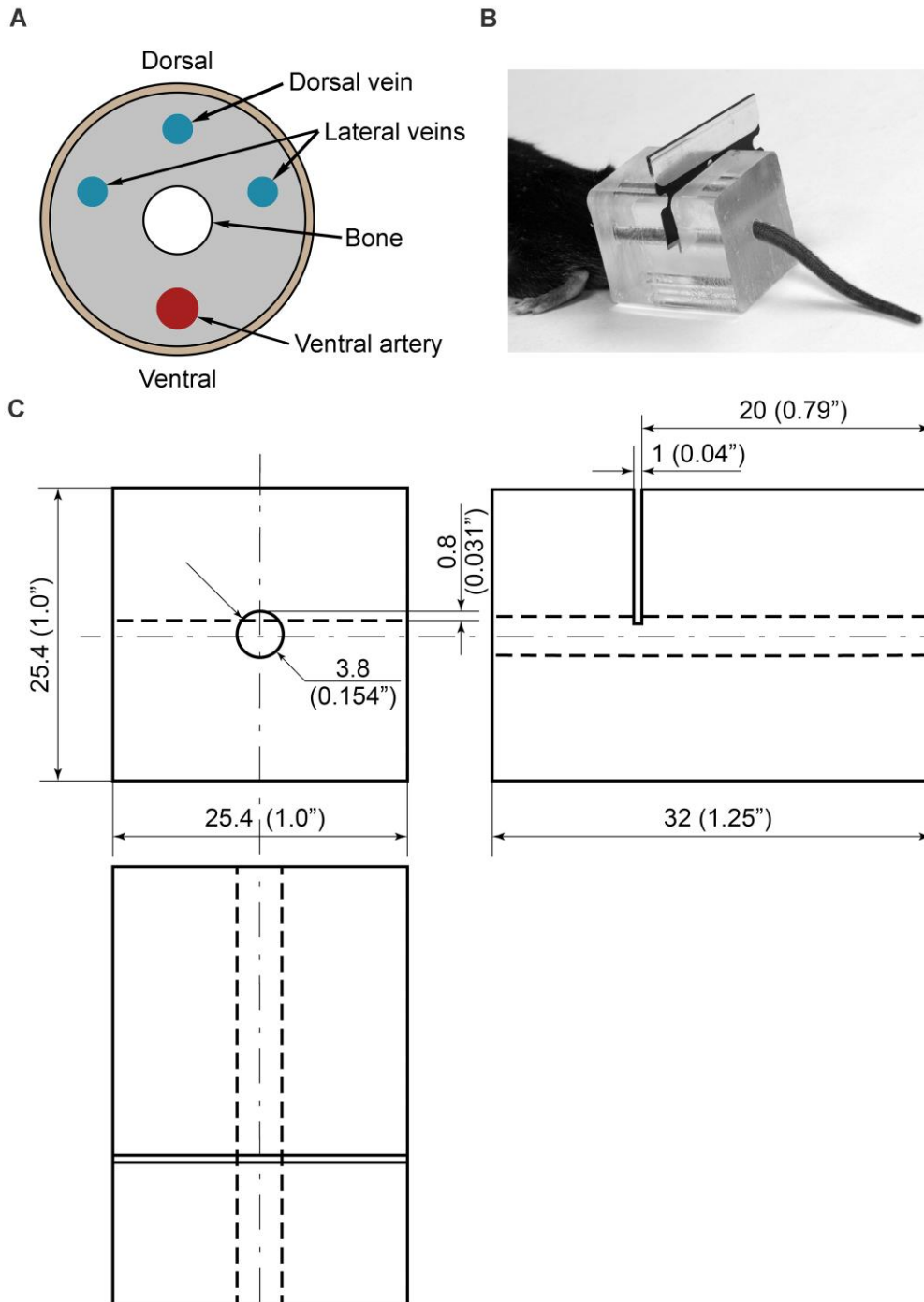


Figure S1. Tail dorsal vein bleeding device. (A) Cross-section diagram of a mouse tail. **(B)** Picture of the bleeding device in use. **(C)** Multiview projection of the device. Measurements are in mm (and inches). A 3D-printer file is attached.

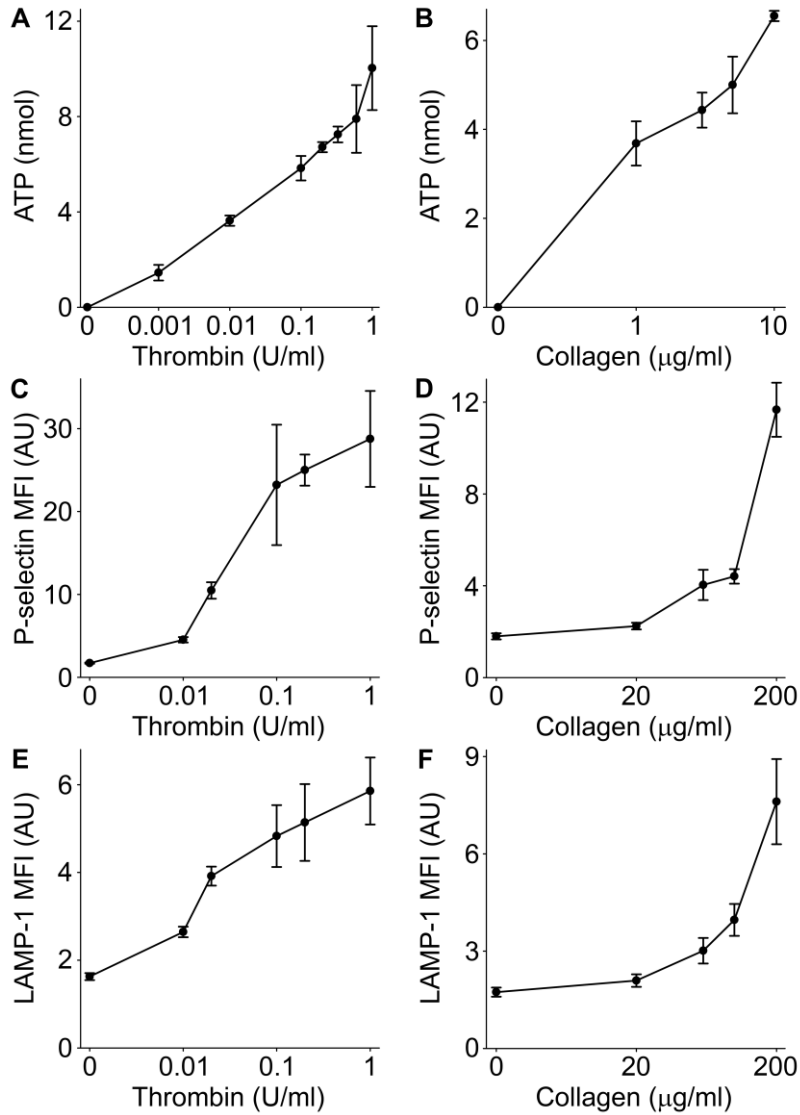


Figure S2. Dose-response curves for platelet granule exocytosis. Samples from wild type mice were stimulated with different doses of thrombin or collagen. **(A-B)** mean release of ATP measured by luminometry in whole blood. Mean fluorescence intensity (MFI) of P-selectin **(C-D)** and LAMP-1 **(E-F)** translocated to the surface of activated washed platelets measured by flow cytometry. N = 3. Circle = mean; error bar = SEM.