Dynamin 2 is required for GPVI signaling and platelet hemostatic function in mice

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Supplemental Methods

Ex vivo perfusion assay

Vena8Fluor+ biochips (Cellix) were coated overnight at 4°C in a humidifier chamber with 1:20 type I collagen (Chrono-log), then blocked with 1% BSA/PBS for 1 h and replaced with PBS.[1] Mouse blood was collected by retro-orbital plexus bleeding and was anticoagulated in 25 mM PPACK. Platelets in whole blood were incubated with 10 mM DiOC6(3) (Thermo Fischer Scientific) for 10 min. Perfusion was performed for 4 min on the Venaflux Microfluidic Perfusion Platform at 37°C with an arterial shear rate of 1500 s^{-1}. Platelet adhesion to the collagen-coated chip was monitored with an Axioscope A.1 Inverted Microscope (Carl Zeiss) at 20x through a Digital CCD Camera C10600-10B (Hamamatsu Photonics), with excitation using an X-cite Series 120 Mercury Arc Lamp (Excelitas Technologies), viewed through a fluorescein isothiocyanate (FITC) filter. Images were collected at a frame rate of one frame per second and analyzed using ImageJ v.1.52a (National Institutes of Health). Images were brightness corrected and converted to a binary mask with software provided auto-thresholds. Binary Mask Particles were then analyzed to determine thrombus count and surface area. Venaflux data was image processed using Imaris (Bitplane) software. The time it took a platelet to move greater than half its diameter (about 3 μm) after its initial attachment was defined as the dwell time.

Immunofluorescence microscopy

For fibrinogen and von Willebrand factor (VWF) content, platelets were seeded onto 5% BSA-coated glass, fixed in 4% paraformaldehyde for 30 min at 37°C, permeabilized with 0.5% Triton-X for 20 min at room temperature (RT), and blocked overnight in 5% BSA/PBS at 4°C.[2,3] For platelet spreading, platelets were seeded onto human fibrinogen (Sigma-Aldrich)-coated glass and incubated 30 min at 37°C in 1 μg/ml CRP or 0.01 U/ml thrombin before fixation. Samples were probed with antibodies directed toward proteins of interest for 2 hours, then with appropriate Alexa Fluor secondary antibodies for 1 hour at room temperature. Spread platelets were further stained with Oregon Green 488 Phalloidin (Invitrogen) for 20 min at room temperature before mounting in Aqua Poly/Mount (Polysciences). Samples were imaged on Nikon Structured Illumination Microscopy (N-SIM, NIS-Elements AR v4.40.00 software) and

Olympus Confocal FV1000-MPE (FluoView software) platforms under 100x oil objectives. Data was processed using Imaris software.

For platelet spreading, individual platelets were segregated into one of three morphologically distinct categories based upon the presence or absence of filopodia and/or lamellipodia: “rest” platelets retained visibly intact marginal bands and/or lacked any filopodia or lamellipodia; “filopodia” platelets developed one or more filopodium without the formation of lamellipodia; and “spread” platelets were defined by the major or complete development of lamellipodia, thereby yielding a flat, rounded appearance with few or no distinct filopodium.

References

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