Thrombospondin 1 requires von Willebrand factor to modulate arterial thrombosis in mice

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**Supplementary methods**

**Platelet preparation**

Blood was isolated from anesthetized mice and diluted with tri sodium citrate (1.9% w/v) in 1:9 ratio. Platelet-rich-plasma (PRP) was obtained by centrifugation at 100x g for 5 min. The PRP and buffy coat containing some RBCs were gently transferred to fresh polypropylene tubes and re-centrifuged at 100x g for 5 min. PRP was incubated with prostaglandin I₂ (PGI₂, 20 ng/mL) at 37°C for 5 minutes. PRP was further centrifuged at 600x g for 5 min and obtained pellets were resuspended in 1 mL modified Tyrode-HEPES buffer (137 mM NaCl, 0.3 mM Na₂HPO₄, 2 mM KCl, 12 mM NaHCO₃, 5 mM HEPES, 5 mM glucose, 0.35% BSA, pH 7.2) containing PGI₂ (20 ng/mL) for 5 minutes at 37°C. Platelets were washed twice and finally resuspended in modified Tyrode-HEPES buffer. For intravital microscopy experiments platelets were differentially either fluorescently labeled with calcein green, AM or calcein red-orange, AM at a concentration of 2.5 µg/mL (Molecular Probes, Eugene, OR) for 10 min at room temperature.

**Flow cytometry**

15 µl of washed platelets (3-4 X 10⁸) diluted 1:10 in modified Tyrode’s buffer (supplemented with 1mM Ca²⁺, 5mM glucose and 0.35% bovine serum albumin) were incubated with either antibody against GPIbα (5 µl of PE-anti-CD42b; Emfret) or those against integrin αIIbβ3 in
active conformation (5 µl of PE-JON/A; Emfret) and P-selectin (5 µl of FITC-anti-CD62P; Emfret) for 15 minutes in the dark at 37°C. The reaction was quenched by adding 250 µl of buffer (FACSSheath; BD Biosciences) and analyzed using flow cytometer (FACSCalibur; BD Biosciences). 10000 events were acquired in platelet gate for each sample.

Platelet Aggregation

Platelets rich plasma and washed platelets were prepared as described above. The final platelet count was adjusted to 3-4 x 10^8/ml with platelet-poor plasma or Tyrode’s buffer. Aggregation was initiated by adding agonists in 250 µl of platelet suspension and recorded in light transmittance aggregometer (Chrono-Log Corporation).
Supplementary Figures

Figure S1

Flow cytometric analysis of Tsp1−/− and WT platelets. (A) The upper left panel shows representative histogram overlay for GPIbα expression detected using PE-labeled anti-CD42b antibody. The lower panel shows quantitative data represented as mean fluorescence intensity (n = 6). (B) The upper middle and right panels respectively show histogram overlays for P-selectin exposure (FITC-CD62P antibody) and integrin activation (PE-JON/A antibody) in Tsp1−/− and WT platelets with or without thrombin (0.1 U/ml) stimulation. The lower panels show corresponding quantitative data represented as mean fluorescence intensity (n = 6). Data is presented as mean ± SEM.
Aggregation assays of $Tsp1^{-/-}$ and WT platelets. Representative tracings and corresponding bar diagrams showing aggregation responses induced by (A) Thrombin 0.1 U/ml in washed platelets (n=6) (B) ADP 2µM in platelet-rich plasma (n=5) or (C) Collagen 5µg/ml in platelet-rich plasma (n=4). Data is presented as mean ± SEM.
Hematopoietic cell-derived TSP1 modulates arterial thrombosis. (A) Genomic DNA PCR analysis for the Tsp1 gene in peripheral blood mononuclear cells from transplanted Tsp1−/−BM→ WT mice and WT-BM→ WT mice. (B) Quantification of first thrombus (>20 µm). (C) Thrombus growth kinetics (fold increase). *P<0.05; WT-BM in WT mice vs. Tsp1−/−-BM in WT mice. (D) Mean time to complete occlusion. Data represent mean ± SEM. N=8 mice/group.