SUPPLEMENTAL MATERIALS for “Factor XIII in plasma, but not in platelets, mediates red blood cell retention in clots and venous thrombus size in mice,”
Sravya Kattula, James R. Byrnes, Sara M. Martin, Lori A. Holle, Brian C. Cooley, Matthew J. Flick, and Alisa S. Wolberg

SUPPLEMENTAL METHODS

Materials. Sigmacote® (siliconizing agent) was from Sigma-Aldrich (St. Louis, MO). Prostaglandin-I₂ was from Cayman Chemical (Ann Arbor, MI). Lipidated tissue factor (TF, Innovin) was from Siemens (Munich, Germany). The transglutaminase inhibitor T101 was from Zedira (Darmstadt, Germany). Calibrated automated thrombography reagents (fluorogenic thrombin substrate, TF/Lipid Reagents [PPP-Low, PPP-High], and thrombin calibrator) were from Diagnostica Stago (Parsippany, NJ). Laemmli 6X sample buffer containing sodium dodecyl sulfate (SDS) was from Boston Bioproducts (Ashland, MA). Tris-glycine polyacrylamide gels (10%) were from Bio-Rad (Hercules, CA). Polyvinylidene difluoride membranes were from Invitrogen, (Carlsbad, CA) and scanned on a GE Typhoon FLA-9000 Imager (GE Healthcare, Pittsburgh, PA). Odyssey Blocking Buffer was from LI-COR Biosciences (Lincoln, NE). Human α-thrombin and anti-human FXIII-A polyclonal antibody (SAF13A-AP) were from Enzyme Research Laboratories (South Bend, IN). Anti-human fibrinogen polyclonal antibody (A0080) was from Dako (Glostrup, Denmark). Alexa Fluor®-488 anti-rabbit and anti-sheep secondary antibodies were from Jackson Immunoresearch (West Grove, PA). Type 1 collagen was from Bio/Data Corporation (Horsham, PA). FXIII-A₂B₂ (Corifact) was a generous gift from CSL Behring (King of Prussia, PA). The TG-Covtest transglutaminase activity was from Covalab (Lyon, France). Cell counts were determined on a HV950FS Hemavet cell counter from Drew Scientific (Miami Lakes, FL).

Thrombin generation. Calibrated automated thrombography was performed as described.¹ Briefly, thrombin generation was triggered in platelet-poor plasma with TF, phospholipids, and CaCl₂ (1 pM, 4 µM, and 16.7 mM, final, respectively). Platelet-rich plasma (PRP) was analyzed under similar conditions, but without phospholipids.

Transglutaminase activity assay. PRP was centrifuged (400g, 5 minutes) to isolate platelets. Platelet pellets were washed, resuspended in Tyrode’s buffer (15 mM HEPES, 3.3 mM NaH₂PO₄, pH 7.4, 138 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 5.5 mM dextrose), and then incubated with thrombin (1 U/mL) for 15 minutes. Platelet lysates were prepared by adding cell lysis buffer (2% Triton X-100, 100 mM Tris, pH 7.3) to the resuspended, activated platelet pellets. Transglutaminase activity was measured using the TG-Covtest colorimetric activity microassay.

FXIII activation and fibrin crosslinking. PRP was clotted with TF and CaCl₂ (1 pM and 10 mM, final, respectively). Samples were then dissolved in 50 mM dithiothreitol, 12.5 mM EDTA, and 8 M urea at 60°C for 1 hour, diluted 120-fold in 6X reducing SDS sample buffer, boiled, separated on 10% Tris-Glycine gels, and transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1 hour at room temperature with Odyssey Blocking Buffer, incubated overnight at 4°C with primary antibodies against FXIII-A or fibrin(ogen), and then incubated with fluorescence-labeled anti-sheep and anti-rabbit secondary antibodies for 1 hour at room temperature. FXIII-A and fibrin(ogen) bands were quantified by densitometry (ImageJ 1.48v). FXIII-A’ band intensity was reported as total arbitrary fluorescence units (A.F.U.). Band intensities of fibrin γ-γ dimers and HMW crosslinked fibrin species were normalized to the fibrin(ogen) Bβ⁺+β-chain before normalizing to time zero. Identity of murine fibrinogen bands was confirmed by mass spectrometry. Briefly, bands were visualized by colloidal blue
silver stain\textsuperscript{2}, excised, and analyzed by the University of North Carolina Michael Hooker Proteomics Core using a MALDI TOF/TOF 4800 Mass Analyzer (ABSciex).

SUPPLEMENTAL REFERENCES


Supplemental Table 1. Complete blood count for $F13a^{+/+}$, $F13a^{+/-}$, and $F13a^{-/-}$ mice.

<table>
<thead>
<tr>
<th></th>
<th>$F13a^{+/+}$</th>
<th>$F13a^{+/-}$</th>
<th>$F13a^{-/-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>White blood cells (10$^6$/mL)</td>
<td>6.7 ± 0.5</td>
<td>5.5 ± 0.6</td>
<td>5.5 ± 0.8</td>
</tr>
<tr>
<td>Red blood cells (10$^9$/mL)</td>
<td>8.5 ± 0.1</td>
<td>9.0 ± 0.6</td>
<td>8.2 ± 0.4</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>11.8 ± 0.3</td>
<td>12.6 ± 1.0</td>
<td>12.2 ± 0.7</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>39.8 ± 1.2</td>
<td>41.6 ± 1.1</td>
<td>39.4 ± 0.9</td>
</tr>
<tr>
<td>Red cell distribution width (%)</td>
<td>15.6 ± 0.4</td>
<td>15.8 ± 0.4</td>
<td>16.5 ± 0.6</td>
</tr>
<tr>
<td>Platelets (10$^9$/mL)</td>
<td>748.0 ± 48.0</td>
<td>754.7 ± 90.4</td>
<td>701.0 ± 38.4</td>
</tr>
</tbody>
</table>

Data show means ± standard error of the means (N=3).
Supplemental Figure 1. Factor XIII inhibitor T101 reduces mouse whole blood clot weight. (A) Transglutaminase activity of human FXIII-A2B2 was measured in the presence of T101 at the concentrations indicated. Data show means ± standard error of the means (N=1-4). Dashed line indicates baseline activity in the assay (no FXIII-A2B2 present). (B) Whole blood from F13a<sup>+/+</sup> mice was clotted with tissue factor/CaCl<sub>2</sub> in the presence of T101 at the concentrations indicated. Contracted clots were weighed after 2 hours (N=3-4).

Supplemental Figure 2. Both human and mouse FXIII-A2B2 crosslink mouse fibrin. Clotting was triggered with tissue factor/CaCl<sub>2</sub> in F13a<sup>-/-</sup> plasma supplemented with human (A) or mouse (B) FXIII-A2B2 (62 µM, final), and reactions were quenched at the indicated time points. Fibrin crosslinking was analyzed by SDS-PAGE with western blotting (N=2).