

Supplemental Data

Material and methods:

Reagents

All reagents were obtained from Sigma-Aldrich unless stated otherwise. We previously described our mAbs 10E5 (anti- α IIb cap domain),¹ and 7E3 (anti- β 3 near the MIDAS)²⁻⁴ and they were produced at the National Cell Culture Center (Minneapolis, MN). mAbs to GPIIb (6D1)⁵ and to α 2 β 1 (6F1)⁶ were produced by our laboratory. mAb PT25-2, which binds to α IIb and activates α IIb β 3 to bind ligand, was a generous gift of Professor Makoto Handa.^{7,8} A Thr receptor activating peptide (T6, SFLLRN) and the peptide RGDW were synthesized by the Rockefeller University Proteomics Resource Center; collagen type I from rat tail was purchased from Corning Inc; collagen-related peptide (CRP) was a generous gift from Dr. Richard Farndale (Cambridge University); ristocetin was obtained from Sigma-Aldrich; and von Willebrand factor and human α -thrombin (Thr) were purchased from Haematologic Technologies. All reagents were analytical grade. Acid-citrate-dextrose (2.5 g of sodium citrate, 1.5 g of citric acid, and 2.0 g of glucose in 100 ml of H₂O) was used to anticoagulate whole blood for platelet studies and HEPES modified Tyrode's buffer (HBMT) (138 mM NaCl, 2.7 mM KCl, 2 mM MgCl₂, 0.42 mM NaH₂PO₄, 5 mM glucose, 10 mM HEPES, pH 7.4) was used to wash platelets.

Human fibrinogen was obtained from Enzyme Research Labs (South Bend, IN) and fibrinogen fragments D-dimer and D98 were obtained from Haematologic Technologies. The preparation and characterization of fragments D-Dimer and D98 have been described previously.^{9,10} Purified human α IIb β 3 and soluble fibrin were obtained from Molecular Innovations.

Preparation of human platelets, platelet aggregation, and platelet interactions with polymerizing fibrin

Healthy volunteers: This study was approved by the Rockefeller University Institutional Review Board (IRB). Blood was obtained from healthy volunteers who provided informed consent and who had not taken anti-platelet medications during the 2 weeks before blood collection and anti-coagulated with one-sixth volume of acid-citrate-dextrose (2.5 g of sodium citrate, 1.5 g of citric acid, and 2.0 g of glucose in 100 ml of H₂O). Platelet-rich plasma (PRP) was prepared by centrifugation of citrated blood at 650 × g for 4 min at room temperature. To prepare washed platelets, prostaglandin E₁ was added to ACD-PRP to a final concentration of 1 μM and the preparation was centrifuged at 980 × g for 8 min at room temperature. The platelet pellet was resuspended in HBMT. The suspended platelets were allowed to regain their reactivity by incubation at 37°C for 45 min. For platelet aggregation studies and studies of platelet interactions with polymerizing fibrin, the washed platelet count was adjusted to 2 × 10⁸ platelets/mL and for studies of clot retraction the count was adjusted to 3 × 10⁸ platelets/mL.

Platelet aggregation and the interaction of platelets with polymerizing fibrin was performed with a platelet aggregation profiler PAP8 (Bio Data Corporation) platelet aggregometer. Washed platelets (250 μl) were stirred in an aggregometer cuvette at 37 °C and platelet activation was induced by adding 25 μM of T6 or 0.2 U/ml of Thr at the indicated time of injection (TI). The time to the onset of platelet aggregation was measured and reported as TA. αIIbβ3 antagonists [RGDW, γ12-like peptide (KQAGDV), eptifibatide, abciximab and, mAbs 10E5 and 7E3] were added as indicated 20 minutes prior to initiating aggregation.

Glanzmann thrombasthenia patient: Case history: The patient is a 43-year-old female who was diagnosed at infancy due to petechiae and ecchymoses at birth. Epistaxis started at 1

year of age and continued into adulthood, but with decreasing frequency low frequency. Patient also experienced gum bleeding frequently during her teen-age years, but it improved after prophylactic dental care. She also reports easy bruising and severe menorrhagia requiring hormonal treatment. She required platelet transfusions for epistaxis and after a tooth extraction. Both her parents are of Greek descent and she has 2 siblings, one of whom also has Glanzmann's thrombasthenia. Light transmission aggregometry studies demonstrated no aggregation in response to ADP, epinephrine, collagen, and arachidonic acid, but a normal response to ristocetin. Flow cytometry demonstrated normal platelet binding of an antibody to GPIb, but virtually no binding of mAb 7E3. Immunoblot analysis demonstrated the presence of <20% of normal $\beta 3$, with a faint band corresponding to pro- α IIb. Reverse transcription PCR of platelet RNA confirmed the presence of a homozygous $\beta 3$ C575R mutation.

Blood (20 ml) was obtained from the patient and washed platelets prepared as described above for healthy volunteers.

Clot retraction

A suspension of 300 μ L of washed platelets at a concentration of 3×10^8 platelets/mL was added to a glass cuvette containing a mixture of 2 mM CaCl_2 and 0.2 U/ml of Thr. The α IIb $\beta 3$ antagonist RGDW was added as indicated, to the washed platelet solution 20 min prior to initiating clot retraction. For scanning electron microscopy (SEM) analysis of clot retraction, a coverslip was first precoated with 50 μ g/ml poly-D-lysine and then after it dried, 9 μ L of an aqueous solution of 2 mM CaCl_2 , 0.2 U/ml Thr was added on top of the coverslip. Washed platelets (100 μ L; 3×10^8 platelets/mL) were then carefully added on top of the coverslip. After 15 minutes the resulting clot was fixed in 2.5% glutaraldehyde, 4% paraformaldehyde, 0.2 M sucrose, and 0.1 M sodium cacodylate, pH 7.4 for 30 min at room temperature and treated as indicated below for SEM.

Scanning electron microscopy (SEM)

The platelet/fibrin suspensions were fixed after the specified times by adding to the cuvette a 2X solution to achieve final concentrations of 2.5% glutaraldehyde, 4% paraformaldehyde, 0.2 M sucrose, and 0.1 M sodium cacodylate, pH 7.4 for 30 min at room temperature. The fixed suspension was then added to a Cytospin 3 (Thermo Shandon) sample chamber funnel and the chamber was assembled with a high-precision glass 12 mm diameter 1.5H coverslip pre-coated with 50 µg/ml poly-D-lysine. The platelet-fibrin mixture was deposited on the coverslip by centrifugation at 1000 X g for 5 min at room temperature. After centrifugation, the coverslips were rinsed twice in sodium cacodylate buffer for 10 minutes per wash and subsequently rinsed three times in deionized water. The coverslips were then dehydrated in a graded series of ethanol solutions using a Pelco Biowave (Ted Pella) at 50% EtOH, 70% EtOH, 90% EtOH, and twice at 100% EtOH. Samples were transferred to a critical point dryer (Autosamdri-815; Tousimis) and the dried coverslips were mounted onto stubs and coated with ~20 nm gold/palladium particles using a Denton Desk IV sputter coater. Images were collected with a field-emission scanning electron microscope (LEO 1550; Carl Zeiss) at 4 kV using SmartSEM, version 5 software.

Surface Plasmon Resonance

The interaction of $\alpha\text{IIb}\beta\text{3}$ with either human fibrinogen or soluble fibrin was assessed by analyzing changes in response units (RU) using a surface plasmon resonance instrument (Biacore 8K, GE Healthcare) equipped with a CM5 sensor chip (Series S, Sensor Chip CM5; GE Healthcare). The ligands (human fibrinogen or soluble fibrin, both >95% pure based on SDS-PAGE analysis) were immobilized using amine-coupling chemistry. The surfaces of all flow cells

were activated with a 1:1 mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) at a flow rate of 10 μ l/ml. The ligand fibrinogen (20 μ g/ml in 10 mM sodium acetate buffer, pH 5.5) was immobilized at a density of 6676.9 RU Channel 1, 5985.2 RU Channel 2, 6017.9 RU Channel 3 and, 5910.3 RU Channel 4, while the soluble fibrin ligand was immobilized at a density of 6579.8 RU Channel 5, 5708.8 RU Channel 6, 6529.7 RU Channel 7 and, 6214.4 RU Channel 8. All the surfaces were blocked with 1.0 M ethanolamine-HCl pH 8.5. Signals from both reference and sample channels were collected at a rate of 10 Hz. To collect kinetic binding data, the analyte α IIb β 3 (>95% pure based on SDS-PAGE analysis) in running buffer (0.02 M Tris-HCl, 0.1 M NaCl, 0.1% Triton X-100, 1 mM CaCl₂, pH 7.4) was injected over the fibrinogen and soluble fibrin flow channels at concentrations of 0.0625, 0.125, 0.250, 0.5 and 1 nM using a multi-cycle kinetics/affinity evaluation program at a flow rate of 30 μ l/minute and at a temperature of 25°C. The complex was allowed to associate and dissociate for 120 s and 660 seconds, respectively. Specific binding between the proteins was calculated by subtracting the reference sensorgram from the sample. Data were analyzed with Biacore Insight Evaluation 3.0.12.15655 software (GE Healthcare) and fit to a 1:1 interaction model, a bivalent analyte model, or a heterogeneous ligand binding model.

References

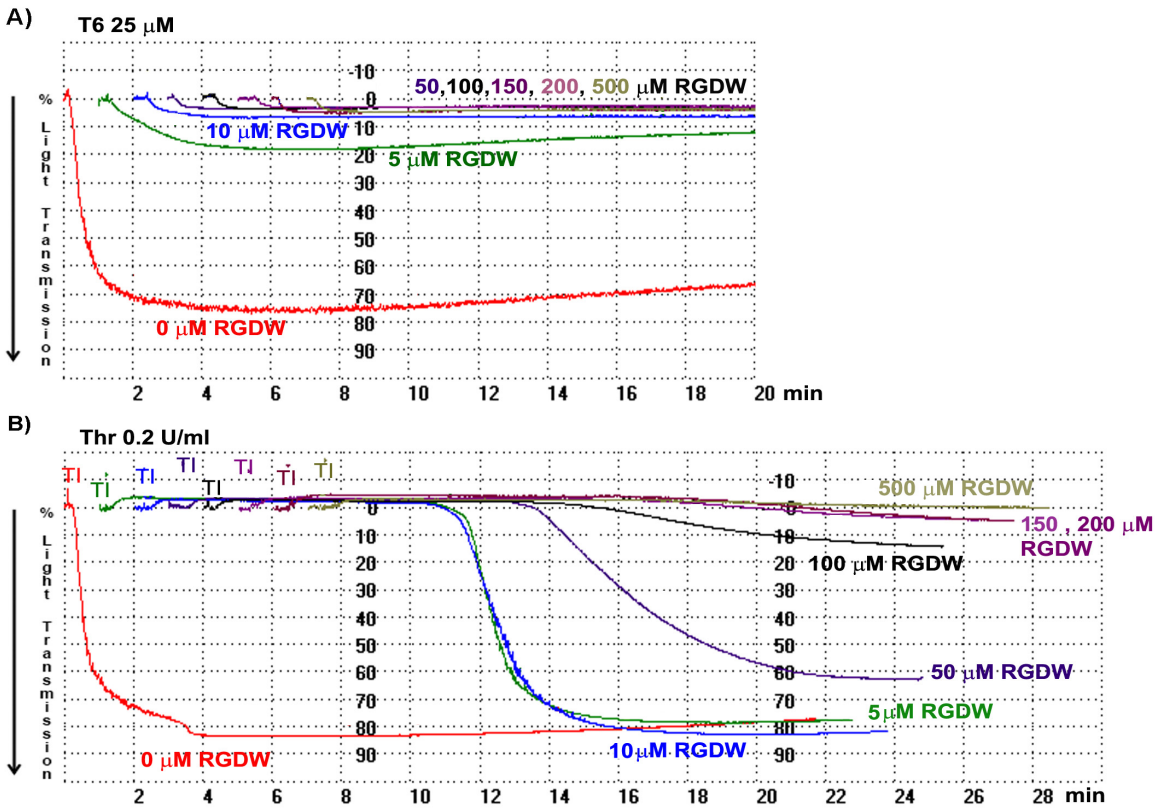
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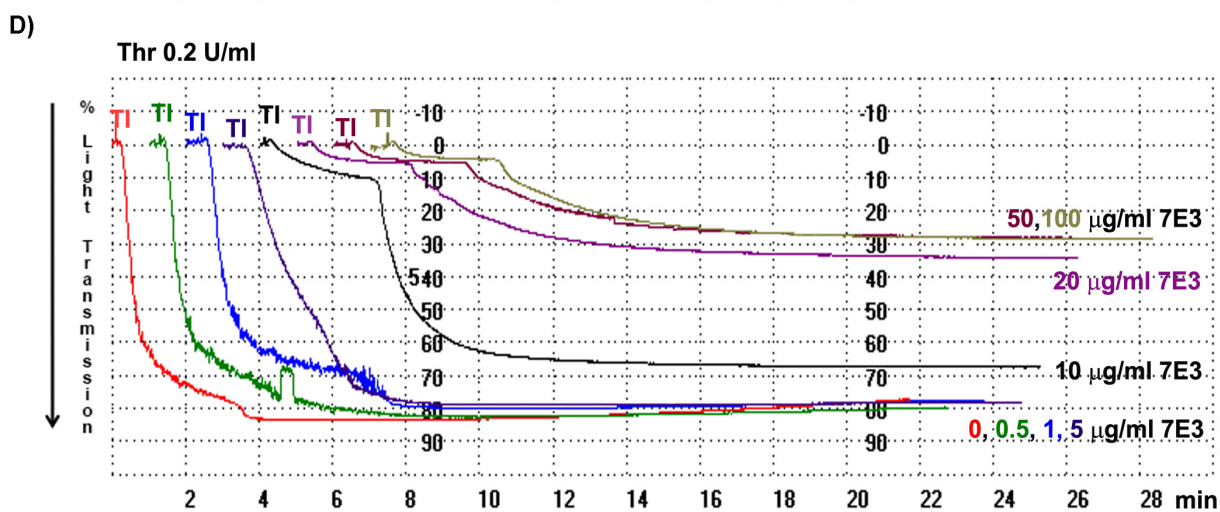
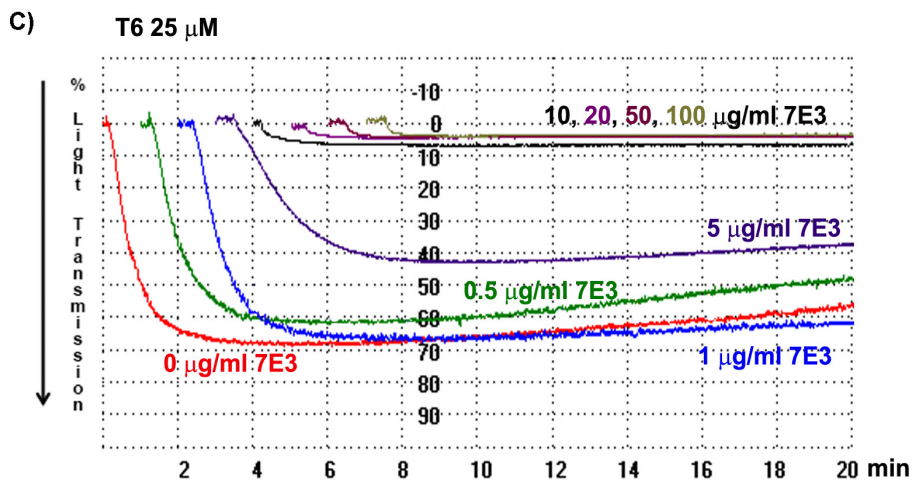
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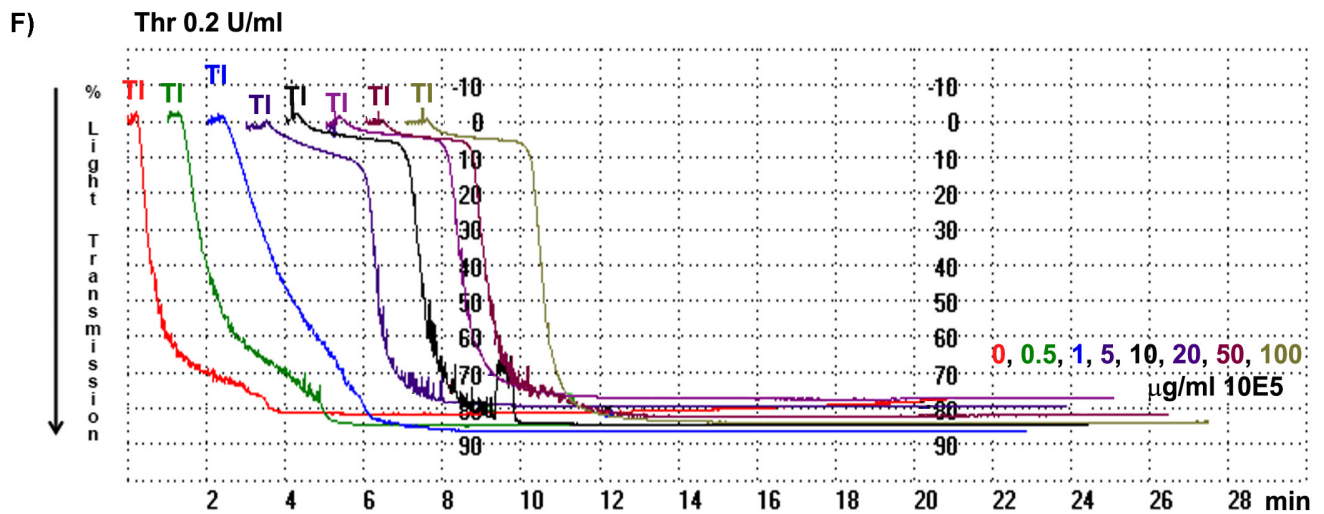
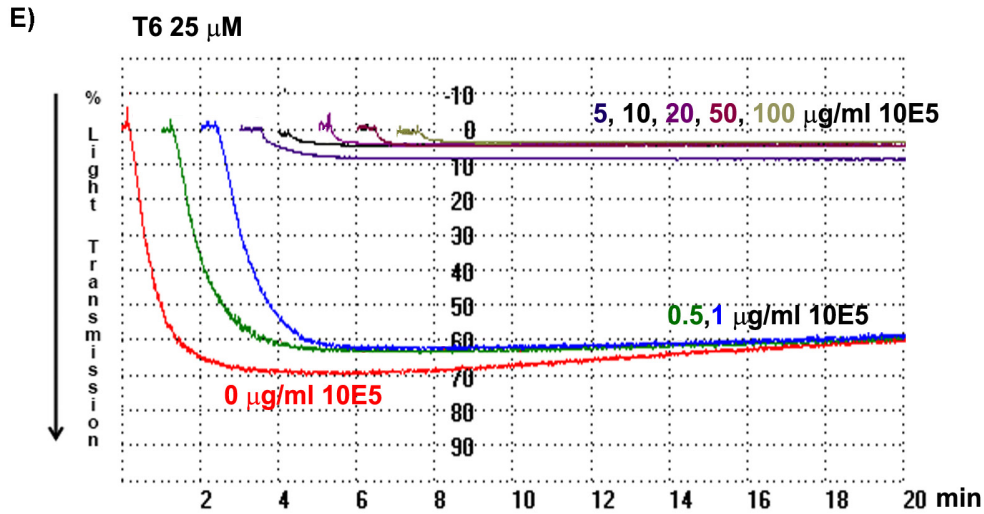
Supplemental Figures

Supplemental Figure 1

The peptide RGDW and mAb 7E3 require much higher concentrations to inhibit the delayed wave (DW) of increased light transmission produced by thrombin (Thr) than to inhibit T6-induced platelet aggregation; mAb 10E5 does not inhibit the DW despite being a potent inhibitor of T6-induced platelet aggregation. Washed platelets (2×10^8 platelets/ml) were treated with the indicated dose of RGDW, mAb 7E3, or mAb 10E5 for 20 min at room temperature and then activated with either 25 μ M T6 (A, C, E, respectively) or 0.2 U/ml Thr (B, D, F, respectively) in an aggregometer cuvette and changes in light transmission were measured at 37°C with stirring.







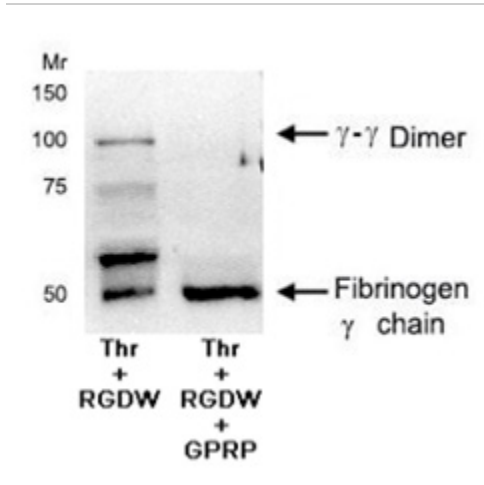
Supplemental Figure 2

A. Immunoblot analysis of the γ -chain of fibrinogen demonstrates GPRP-dependent inhibition of fibrin polymerization and cross-linking. Washed platelets (2×10^8 /ml) were treated with 150 μ M RGDW and/or the fibrin polymerization inhibitor Gly-Pro-Arg-Pro (GPRP) for 20 minutes at room temperature and then activated with 0.2 U/ml Thr in an aggregometer cuvette. The reaction was stopped by adding one-third volume of 3X SDS sample buffer. Samples were subjected to SDS-PAGE under reducing conditions and immunoblotted with a mAb specific for the fibrinogen γ -chain.

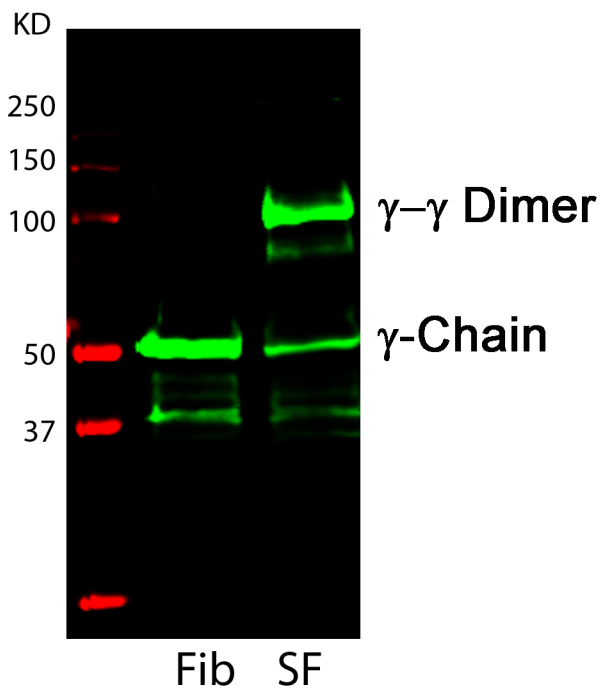
B. Immunoblot analysis of the γ -chain of fibrinogen and soluble fibrin demonstrates polymerization and cross-linking of soluble fibrin. Immunoblot data

using a mAb specific for fibrinogen γ -chain of 50 μg of human fibrinogen (Fib) or soluble fibrin (SF) solubilized with SDS sample buffer under reducing conditions. The kD values of the protein standards analyzed on the same gel are indicated on the left side of the immunoblot.

A)

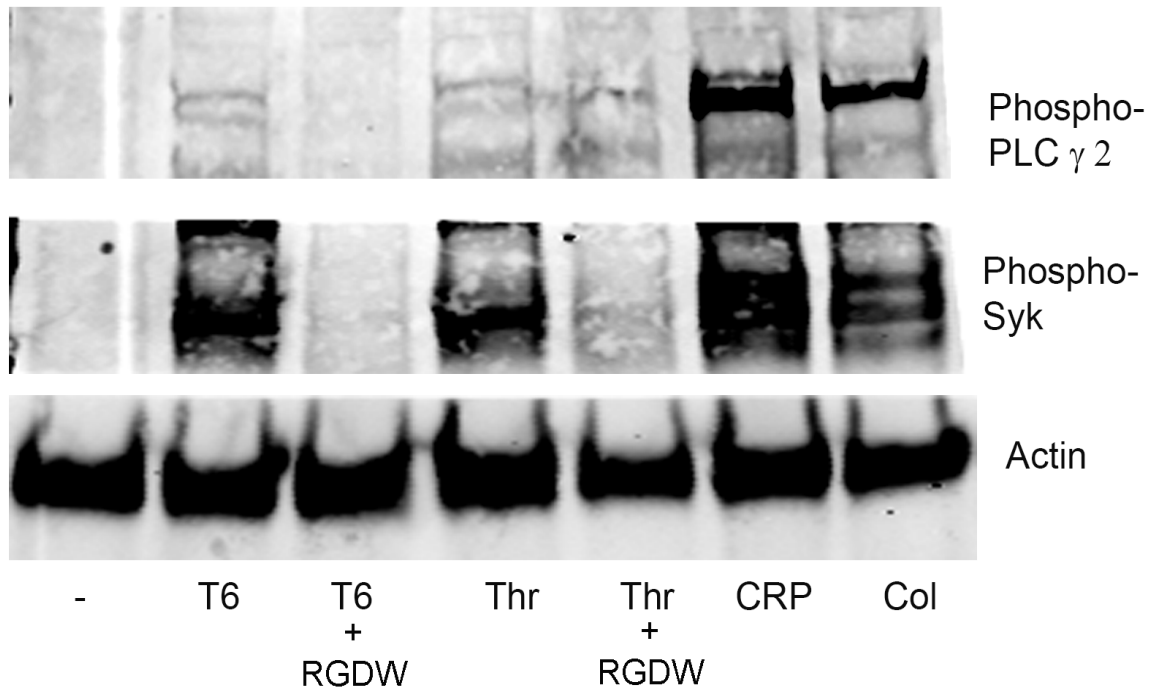


B)



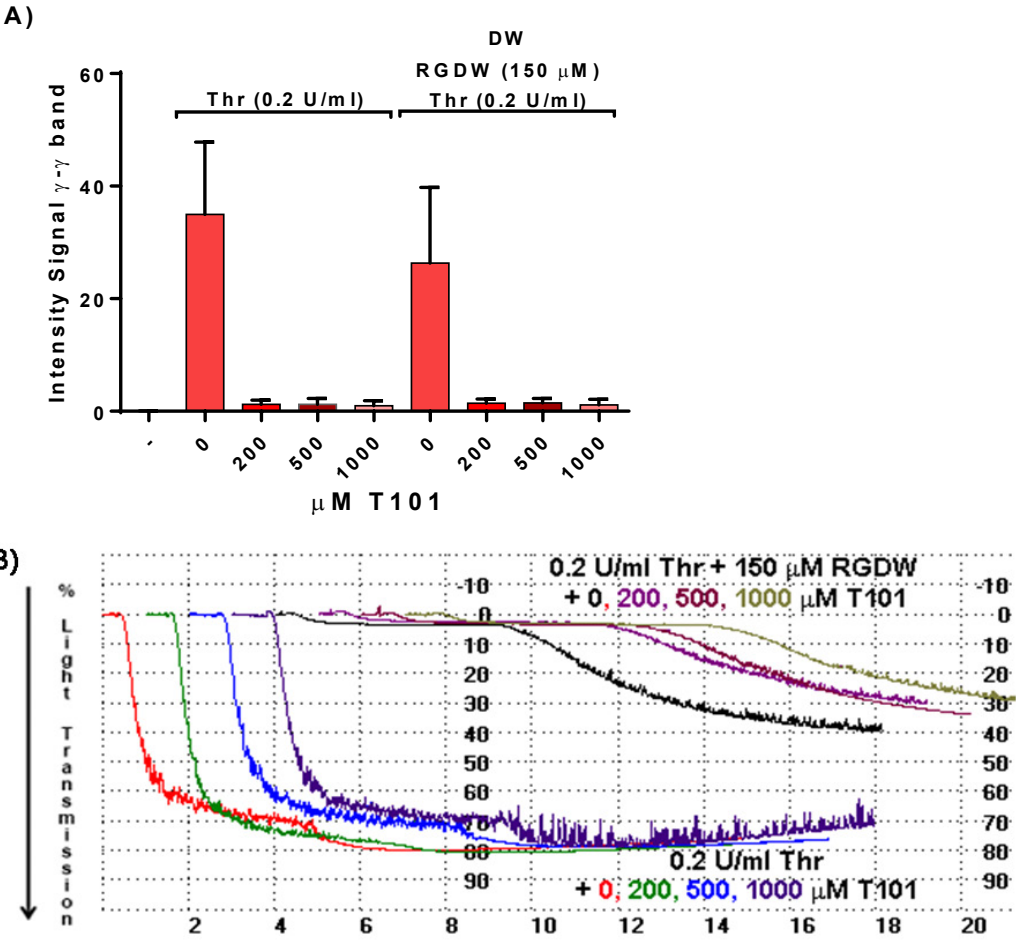
Supplemental Figure 3

GPVI-mediated signaling is minimal during the DW. Washed platelets (2×10^8 /ml) were either untreated or treated with 150 μ M RGDW for 20 min at room temperature and then activated with either 25 μ M T6, 0.2 U/ml Thr, 10 μ g/ml collagen-related peptide (CRP) or 10 μ g/ml collagen (Col) in an aggregometer cuvette. The reaction was stopped 3 minutes after initiation of aggregation or the onset of the DW by adding one-third volume of 3X SDS sample buffer. Samples were subjected to SDS-PAGE under reducing conditions and immunoblotted with the indicated antibodies. Both CRP and Col increase phosphorylation of PLC γ 2 and Syk, as do T6 and Thr. In sharp contrast, in the presence of RGDW, T6 does not increase phosphorylation of either protein after 3 minutes of aggregation, and Thr produces minimal increases in both proteins after 3 minutes of the DW.



Supplemental Figure 4

Inhibiting factor XIII (FXIII) -mediated fibrin crosslinking has little impact on the DW. Washed platelets (2×10^8 /ml) in the absence or presence of RGDW ($150 \mu\text{M}$) were treated with the FXIII trans-glutaminase inhibitor T101 at the indicated concentrations 5 minutes prior to stimulation with Thr (0.2 U/ml) in an aggregometer cuvette. **A)** Densitometry analysis of immunoblots using a mAb specific for fibrinogen γ -chain. 3 independent experiments, presented as mean \pm SD. **B)** Tracings from one of three similar experiments is shown.

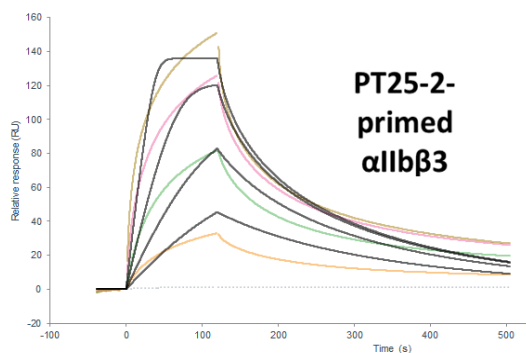
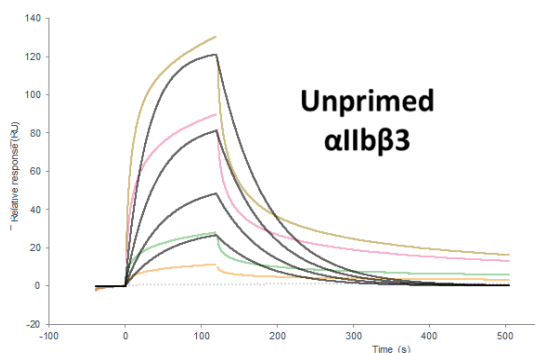


Supplemental Figure 5

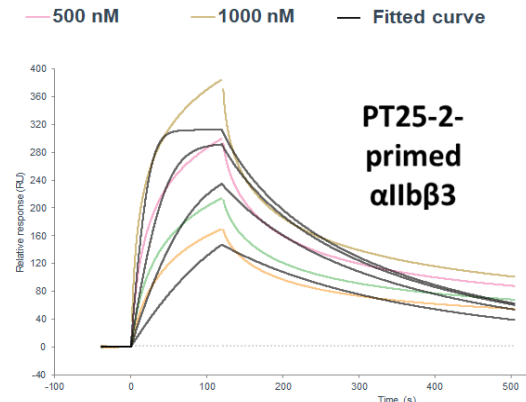
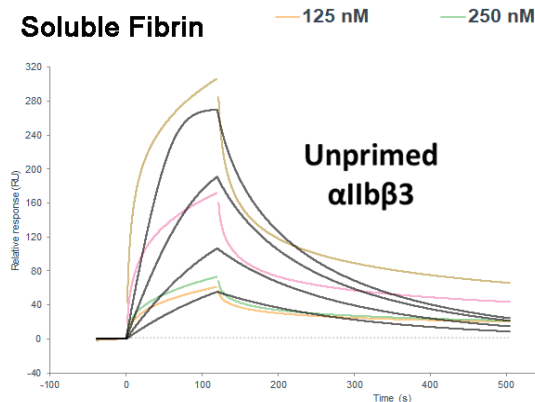
Fittings of surface plasmon resonance data of α IIb β 3 to different kinetic models: (A) Sensorgrams and result table of 1:1 binding model. (B) Sensorgrams and result table of bivalent analyte model. The colored lines indicate the observed responses and the black lines are the fitted values. Both fittings are less concordant than fitting the data to the heterogenous ligand model (main text Figure 7). Representative of 2 independent experiments.

A) 1:1 Binding model

Fibrinogen



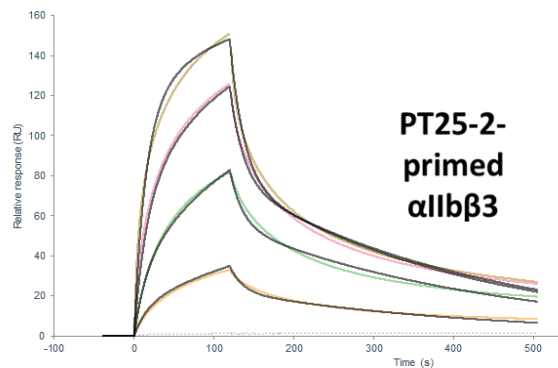
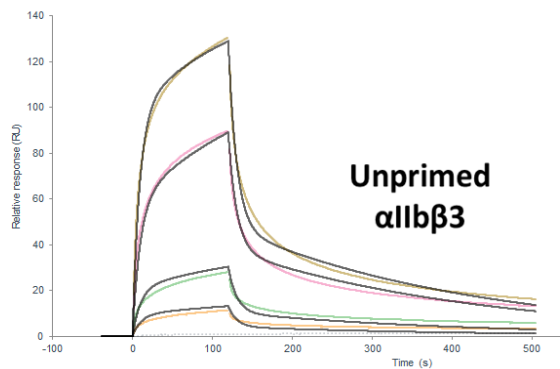
Soluble Fibrin



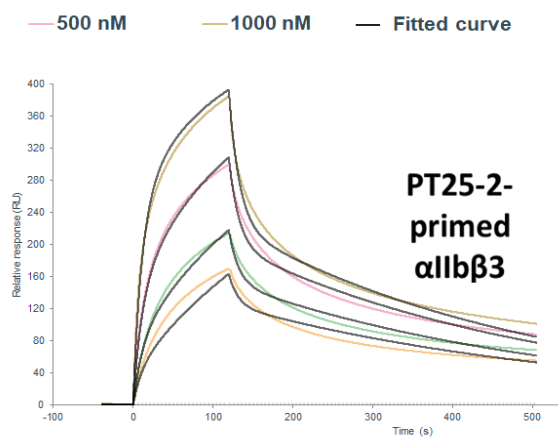
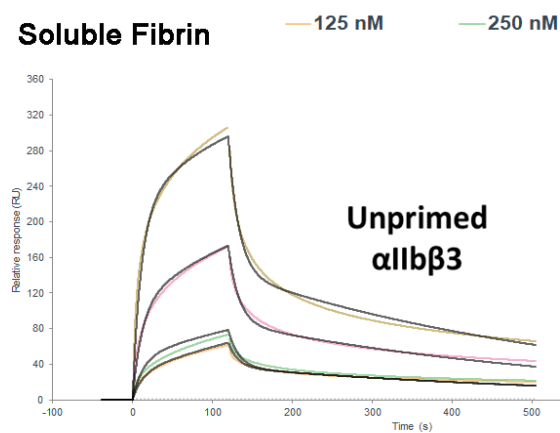
Immobilized ligand	Analyte Solution	Quality Kinetics χ^2 (RU ²)	1:1 binding k_a (1/Ms)	k_d (1/s)	KD (M)
Fibrinogen	GpIIb/IIIa	1.38e+2	1.95e+4	1.54e-2	7.88e-7
Fibrinogen	GpIIb/IIIa (primed)	6.53e+1	1.15e+6	1.61e-1	1.40e-7
Soluble Fibrin	GpIIb/IIIa	6.19e+2	7.23e+7	2.35e+1	3.26e-7
Soluble Fibrin	GpIIb/IIIa (primed)	4.75e+2	1.19e+5	8.41e-3	7.06e-8

B) Bivalent analyte binding model

Fibrinogen



Soluble Fibrin

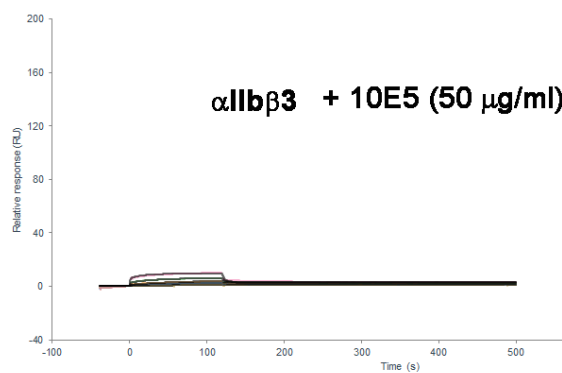
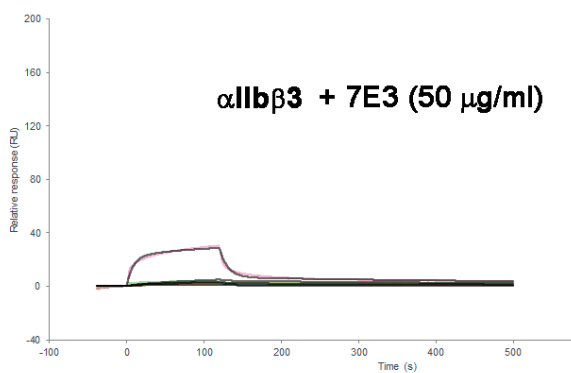


Immobilized ligand	Analyte Solution	Quality Kinetics χ^2 (RU ²)	Bivalent analyte ka_1 (1/Ms)	kd_1 (1/s)	ka_2 (1/RUs)	kd_2 (1/s)	KD1 (M)	KD2 (M)
Fibrinogen	GpIIbIIIa	4.24e0	9.98e+3	8.93e-2	1.13e-5	1.75e-3	8.95e-6	1.55e+2
Fibrinogen	GpIIbIIIa (primed)	5.24e0	9.47e+3	4.93e-2	2.71e-5	2.00e-3	5.21e-6	7.37e+1
Soluble Fibrin	GpIIbIIIa	1.71e+1	9.86e+3	6.93e-2	9.39e-6	1.23e-3	7.03e-6	1.31e+2
Soluble Fibrin	GpIIbIIIa (primed)	6.01e+1	8.96e+3	6.66e-2	1.01e-5	1.49e-3	7.42e-6	1.47e+2

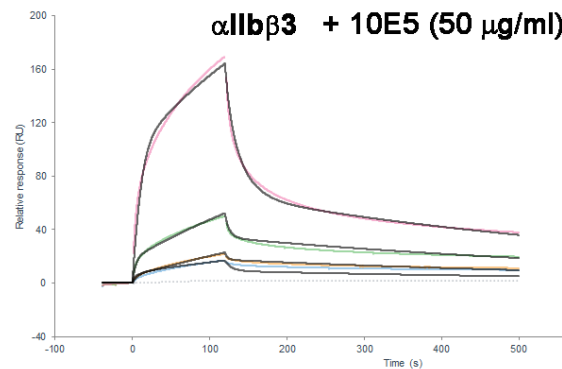
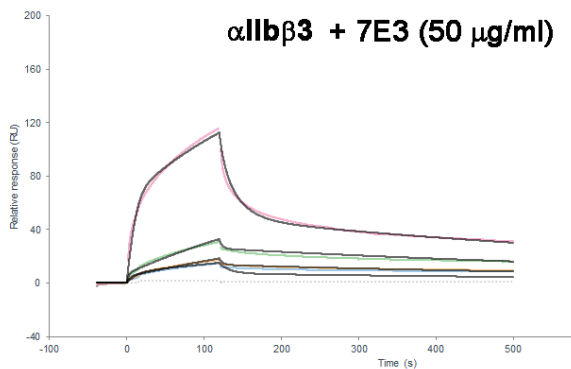
Supplemental Figure 6

Surface plasmon resonance analysis of purified human α IIb β 3 binding to fibrinogen and soluble fibrin demonstrate differential effects of mAbs 7E3 and 10E5. The binding kinetics of the α IIb β 3-fibrinogen or fibrin interaction was determined by fitting the curves to a heterologous binding reaction model (black lines). Purified integrin was incubated with mAb 10E5 or 7E3 (both at 50 μ g/ml) for 20 min at RT. **A)** SPR sensorgrams of α IIb β 3 and fibrinogen **B)** SPR sensorgrams of α IIb β 3 and soluble fibrin.

A) Fibrinogen



B) Soluble Fibrin



— 125 nM — 250 nM — 500 nM — 1000 nM — Fitted curve