Supplemental information

Crucial roles of red blood cells and platelets in whole blood thrombin generation

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Supplemental Materials and Methods

Materials

Recombinant tissue factor (TF; Innovin) was purchased from Siemens Healthineers (Marburg, Germany). Fluorogenic thrombin substrate Z-Gly-Gly-Arg-aminomethylcoumarin (ZGGR-AMC) was purchased from Bachem (Basel, Switzerland). Glycoprotein VI (GPVI) agonist, collagen-related peptide (CRP-XL) was from CambCol (Cambridge, United Kingdom). TG calibrator (α_2-macroglobulin-thrombin complex) was prepared in house, as described.[1] Annexin A5 and mutant annexin A5 were obtained from Tau Technologies (Kattendijke, The Netherlands).[2] Atopaxar was purchased from Axon Medchem (Groningen, The Netherlands), vorapaxar from MedChem Express (Princeton, NJ, USA), and BMS-986120 from ChemeGen (Riverside, CA, USA). Integrin αIIbβ3 inhibitor tirofiban came from Bayer (Essen, Germany), Syk inhibitor PRT-060318 from ApexBio (Houston, TX, USA). Aptamer 1 blocking thrombin exosite I (HD1), aptamer 2 blocking thrombin exosite II (HD22), aptamer 1+2 blocking thrombin exosite I and II (combined HD1 and HD22),[3] and FITC-conjugated annexin A5 were obtained from ThermoFisher Scientific (Eindhoven, The Netherlands). Russell's viper venom factor X activator (Rvv-X, 0.189 OD) was purified, as described before.[4] Active-site inactivated FVIIa (FVIIai) came from Novo Nordisk (Bagsvaerd, Denmark). Ionomycin was from Calbiochem (San Diego, CA, USA). Human thrombin, factor Xa and factor XIa were produced by Synapse Research Institute (Maastricht, The Netherlands).

Blood preparation and separation,

Blood samples were collected into 3.2 % trisodium citrate Vacuette tubes (Greiner Bio-One, Alphen a/d Rijn, The Netherlands). Corn trypsin inhibitor (CTI) was added, if indicated. Platelet-rich plasma (PRP) at autologous platelet count was obtained by centrifugation of blood at 220 g for 15 minutes at room temperature.[2] The collected blood WB and PRP were used within 4 hours after preparation. Platelet-poor plasma (PPP) was obtained through two runs of centrifugation at 2840 g for 10 minutes at room temperature. Washed RBC were prepared by double centrifugation at 890 g for 15 minutes in Hepes buffer pH 7.35 (136 mM NaCl, 2.7 mM KCl, 10 mM Hepes, 2 mM $MgCl_2$, 0.1% w/v bovine serum albumin and 0.1% w/v glucose).[5] Where indicated, autologous PPP or PRP were combined with RBC to generate reconstituted samples with 20-40% hematocrit.

The collection of blood samples from healthy controls and patients on citrate suppresses eryptosis induced by high Ca^{2+} levels.[6,7] Before use, all blood samples were checked on the absence of clots. Yet, we cannot fully exclude phosphatidylserine expression due to sample handling, although such effects would be similar for control subjects and patients.

Blood cell parameters

Blood cell parameters were measured with a coulter counter analyzer (Beckman Coulter, Woerden, The Netherlands), or were determined with a Cell-Dyn Emerald 22 (Abbott Medical, Sesto San Giovanni, Italy).

Pre-incubation of blood and plasma samples

When required, samples in wells were pre-incubated for 10 minutes at 37°C with vehicle control medium, PAR1 inhibitor atopaxar (0.04-10 µM), PAR1 inhibitor vorapaxar (0.04-10 µM), PAR4 inhibitor BMS-986120 (0.04-10 µM), thrombin exosite inhibitors aptamer 1 (0.5-15 µM), aptamer 2 (0.5-15 µM) or aptamer 1+2 (0.5-15 µM).[3] Other wells were preincubated with integrin αIIbβ3 inhibitor tirofiban (0.3-5.0 µg/mL) or Syk kinase inhibitor PRT-060318 (0.04-10 µM). Vehicle controls were run at the same concentration of DMSO solvent or Hepes buffer pH 7.35.

Blood mixing and calibration in for thrombin generation experiments

Citrated WB, PRP or PPP was mixed with substrate solution (ZGGR-AMC dissolved into BSA60 buffer, containing 20 mM Hepes, 6% w/v bovine serum albumin, pH 7.35) and trigger solution (coagulation trigger, 11 mM $CaCl_2$ and 5.5 mM $MgCl_2$ dissolved into BSA5 buffer, containing 20 mM Hepes, 140 mM NaCl, 0.5% w/v bovine serum albumin, pH 7.35). The final volume ratio of WB or plasma, trigger solution and substrate solution was 3:2:1. The optimized final concentration of ZGGR-AMC was 417 µM.

The procedure using 96-well plates were as follows. Trigger solution 120 µL (row A) and the mixture of 120 µL WB or plasma plus 40 µL substrate solution (row B) were prewarmed at 37°C for 10 minutes. Then, 80 µL trigger solution was transferred from row A to row B, followed by 8 times mixing using a multi-channel pipette. Subsamples were transferred to the recording wells (rows D-F), and measurements were started. In calibration wells, the trigger solution was replaced by α_2-macroglobulin-thrombin complex (corresponding to 320 nM thrombin activity).

Red blood cell annexin A5 treatment and wash

Washed RBC (35% hematocrit) were preincubated with annexin A5 (18.5-75 µg/mL) in the presence of 1 mM $CaCl_2$ for 15 minutes at room temperature. After the addition of 25x Hepes buffer plus 1 mM $CaCl_2$, the cells were centrifuged at 800 g for 15 minutes. The wash step in the presence of $CaCl_2$ was repeated three times. This procedure retained the annexin A5-binding to the phosphatidylserine-exposing RBC.

The washed RBC were then resuspended in the presence of 1 mM $CaCl_2$ at required hematocrit for TG measurements. Since the binding of annexin A5 is Ca^{2+}-dependent,[2] care was taken to keep $CaCl_2$ present in all steps of RBC treatment. Procedures with 96 well plates were modified as follows. Trigger solution 120 µL plus RBC sample 90 µL (row A), as well as autologous PRP or PPP 60 µL plus substrate solution 40 µL (row B) were prewarmed for 10 minutes. Subsequently, 140 µL from row A was transferred to row B with 8 times mixing using a multi-channel pipette. Subsamples were transferred to the recording wells (rows D-F), and fluorescence measurements were started.

Red blood cell treatment and reconstitution

To block exposed phosphatidylserine, washed RBC (35% hematocrit) in isotonic Hepes buffer (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 5.5 mM glucose, 0.1% BSA, pH 7.35) supplemented with 1 mM $CaCl_2$ were preincubated with annexin A5 (18.5-75 µg/mL) or vehicle in the presence of 1 mM $CaCl_2$ for 15 minutes at room temperature. After incubation, the cells were triple washed with Hepes buffer plus 1 mM $CaCl_2$.

Flow cytometric analysis

Freshly washed RBC (10% hematocrit) were preincubated with ionomycin (10 µM) with or without unlabeled annexin A5 (7.50 µg/mL) in the presence of 2 mM $CaCl_2$ for 10 minutes at 37°C. Cells were then labeled with FITC-conjugated annexin A5 in the presence of 2 mM $CaCl_2$ for 15 minutes in the dark. After 20x dilution in Hepes buffer pH 7.35 containing BSA, glucose and 2 mM $CaCl_2$, fluorescence was measured with an Accuri C6 flow cytometer (BD Biosciences, Flanklin Lakes, NJ, USA).[8]

Light transmission microscopy

Freshly washed RBC (10% hematocrit) were preincubated with ionomycin (10 µM) or vehicle in the presence of 2 mM CaCl2 for 10 minutes at 37°C. The samples were diluted 3 times with Hepes buffer pH 7.35 containing 2 mM CaCl2, and examined by an inverted light transmission microscope with 63x objective (Leica DFC 3000 G, Wetzlar, Germany).

Measurement of factor Xa activity

To measure RBC-dependent factor X activation,[9] 30 µL of washed RBC (10% hematocrit) were mixed with 20 µL substrate solution (containing 417 µM ZGGR-AMC and 2 mM $CaCl_2$), and 70 µL purified factor Xa (20 µM, f.c.). Fluorescence development was measured for 70 minutes at fluorescence wavelengths of λex 355 nm and λem 460 nm using Fluoroskan Ascent Software (version 2.6). The cells were preincubated with annexin A5 and/or ionomycin in the presence of 2 mM $CaCl_2$ (10 minutes at 37°C), as indicated.

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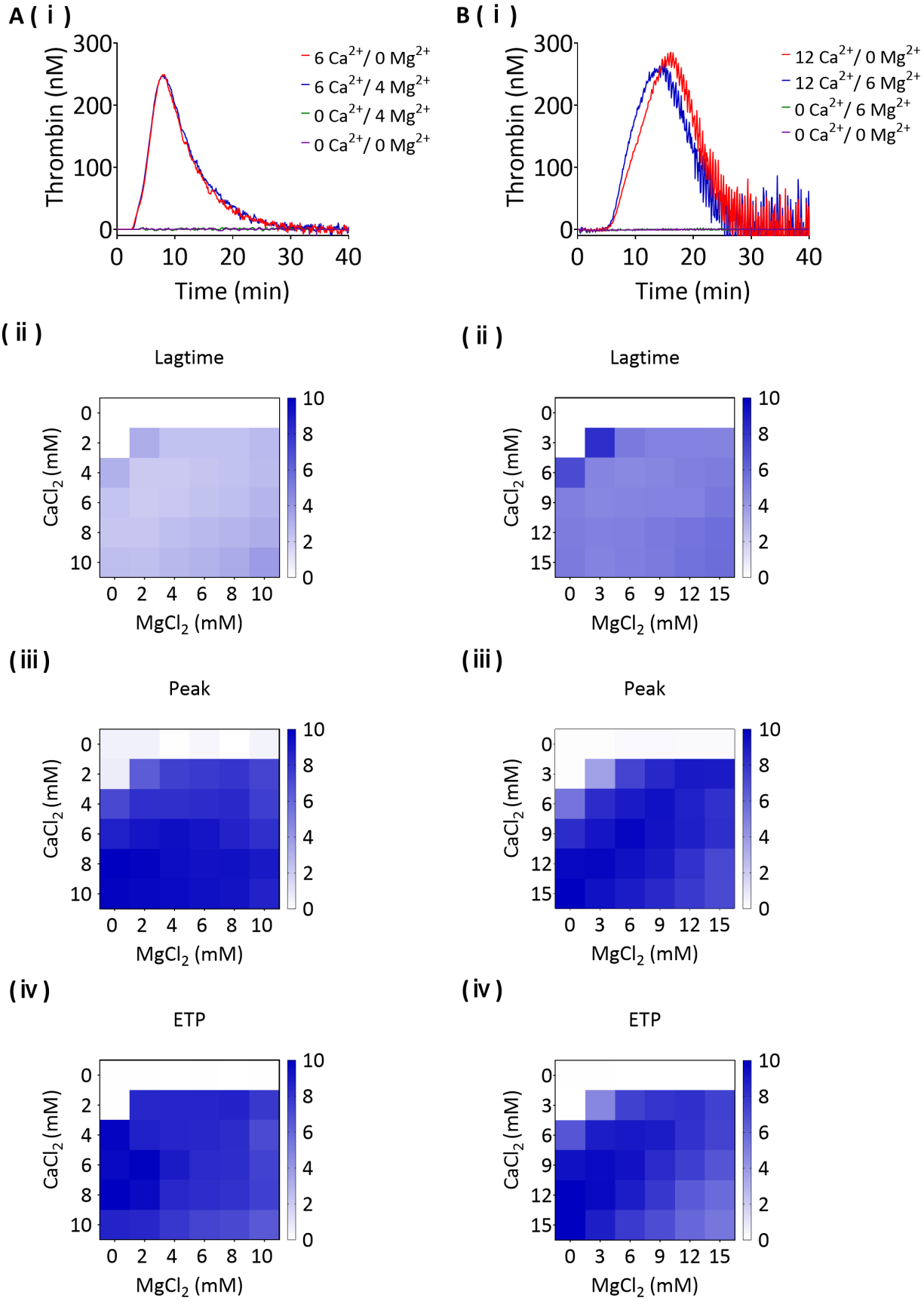
A (i)
Thrombin (nM)
300
200
100
0
0 10 20 30 40
Time (min)
6 Ca2+/ 0 Mg2+
6 Ca2+/ 4 Mg2+
0 Ca2+/ 4 Mg2+
0 Ca2+/ 0 Mg2+
B (i)
Thrombin (nM)
300
200
100
0
0 10 20 30 40
Time (min)
12 Ca2+/ 0 Mg2+
12 Ca2+/ 6 Mg2+
0 Ca2+/ 6 Mg2+
0 Ca2+/ 0 Mg2+
(ii)
Lagtime
CaCl2 (mM)
0 2 4 6 8 10
MgCl2 (mM)
0 2 4 6 8 10
10 8 6 4 2 0
(ii)
Lagtime
CaCl2 (mM)
0 3 6 9 12 15
MgCl2 (mM)
0 3 6 9 12 15
10 8 6 4 2 0
(iii)
Peak
CaCl2 (mM)
0 2 4 6 8 10
MgCl2 (mM)
0 2 4 6 8 10
10 8 6 4 2 0
(iii)
Peak
CaCl2 (mM)
0 3 6 9 12 15
MgCl2 (mM)
0 3 6 9 12 15
10 8 6 4 2 0
(iv)
ETP
CaCl2 (mM)
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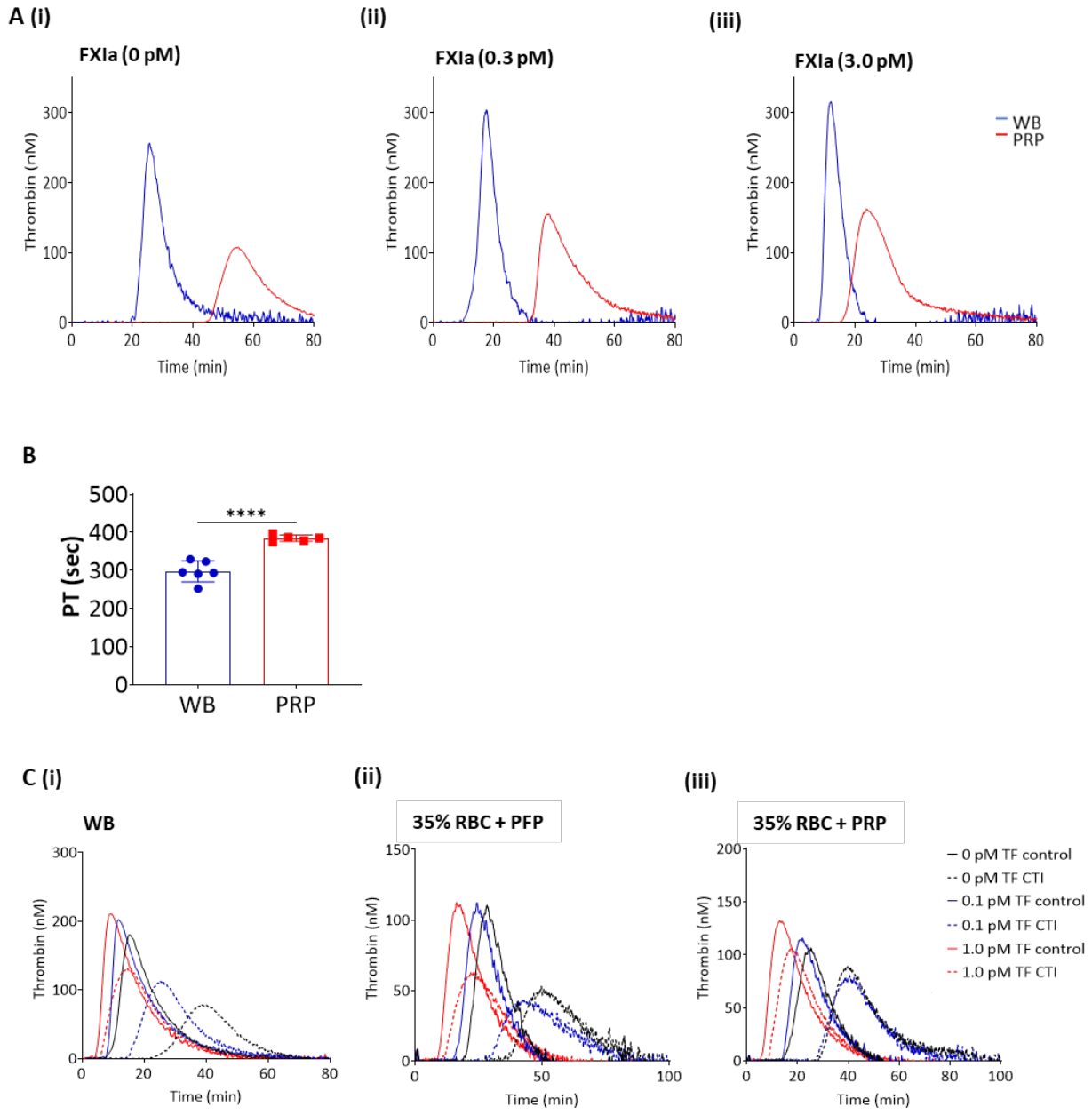
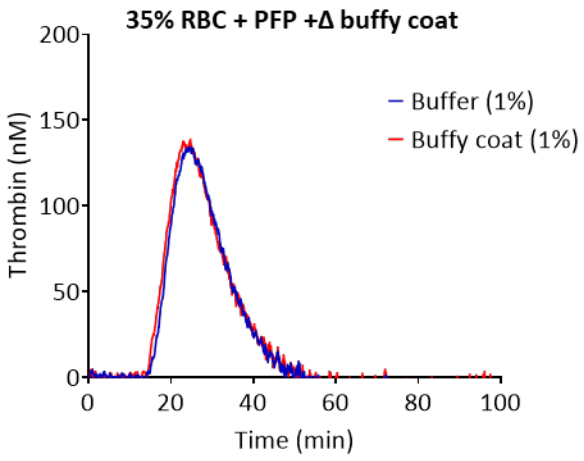
Supplemental Figure 1. Optimal recalcification with $CaCl_2$ and $MgCl_2$ for thrombin generation in citrated whole-blood and PRP. Parallel samples of citrated whole blood (**A**) and PRP (**B**) were supplemented with indicated concentration of $CaCl_2$ (0-15 mM) and/or $MgCl_2$ (0-15 mM), after which TG was triggered with tissue factor (TF, 1 pM). Shown are representative TG curves (**i**) at indicated conditions, as well as heatmapped values of the curve parameters, lagtime (**ii**), thrombin peak level (**iii**) and ETP (**iv**). For the comparisons, we assumed a 33% hematocrit, meaning that 10 mM in whole blood equaled 15 mM in PRP. For heatmapping, parameter values were scaled 0-10 across conditions. Data are means from three experiments. Note that higher concentrations of $MgCl_2$ initially stimulated and then decreased the peak and ETP parameters (panels **A ii-iv**).



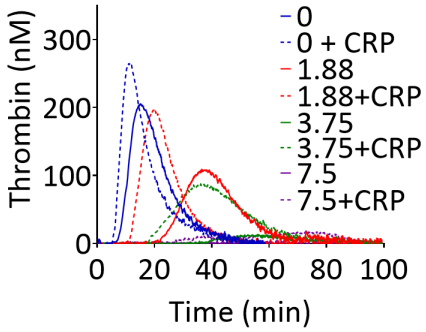
Supplemental Figure 2. Trigger-induced thrombin generation and clotting in (reconstituted) whole blood and in corresponding plasma samples. A, Thrombin generation in whole blood (WB, blue lines) or autologous PRP (red lines) was triggered in the presence of $CaCl_2/MgCl_2$ with vehicle medium or factor XIa (FXIa, 0.3-3.0 pM). Representative curves are shown (n=3). **B**, Recalcified WB or autologous PRP was triggered with 0.1 pM tissue factor, and mechanical prothrombin times (PT) were measured. Data are presented as means ± SD (n=6), ****P<0.0001 (t-test). **C**, Whole-blood on citrate was pretreated with corn trypsin inhibitor (CTI, 50 µg/mL) or left untreated (control). Samples were used for the collection of RBC, PRP and PFP, followed by reconstitution of the plasmas with RBC at 35% hematocrit. Thrombin generation was measured in parallel in whole-blood and the reconstituted blood. Coagulation was triggered upon recalcification with $CaCl_2/MgCl_2$ by 0.1 pM or 1.0 pM TF, as indicated. Representative calibrated TG curves are shown (n=3).



Supplemental Figure 3. Contribution white blood cells to phosphatidylserine-dependent thrombin generation. Autologous PFP was reconstituted with washed RBC (35% hematocrit) plus 1 vol% of buffer or buffy coat (corresponding to the buffy coat volume in whole blood). Thrombin generation was triggered with $CaCl_2$/$MgCl_2$ and a low dose of 0.1 pM TF. Shown are TG traces, representative for 3 experiments. Final counts for buffer (buffy coat): RBC 3.53 (3.82) x 10^{12}/L, WBC 2.70 (5.70) x 10^9/L, platelets 4.1 (191.3) x 10^9/L.

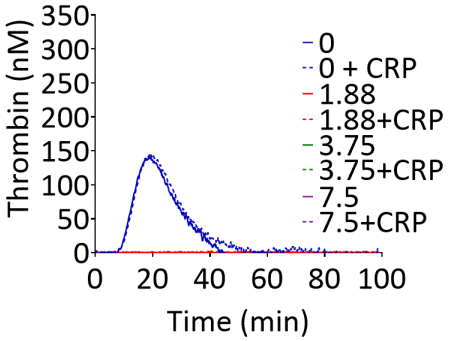
A (i)

WB + Δ A5/CRP)

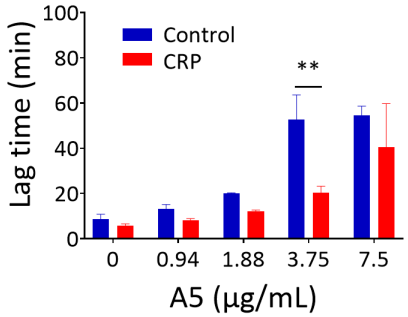


B (i)

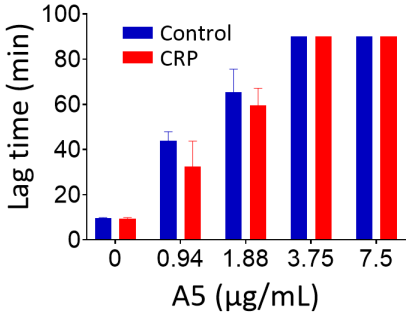
35% RBC + PPP + Δ A5/CRP



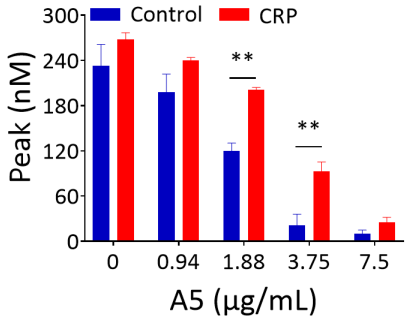
(ii)



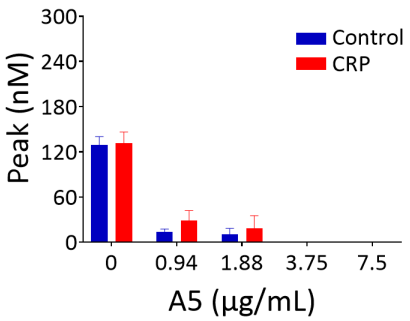
(ii)



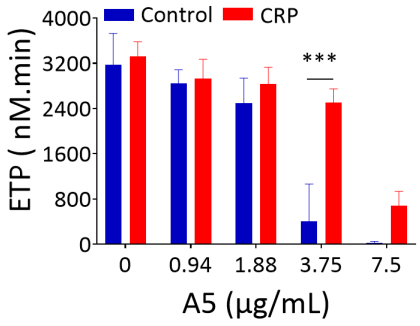
(iii)



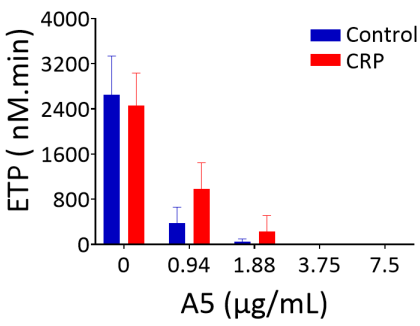
(iii)



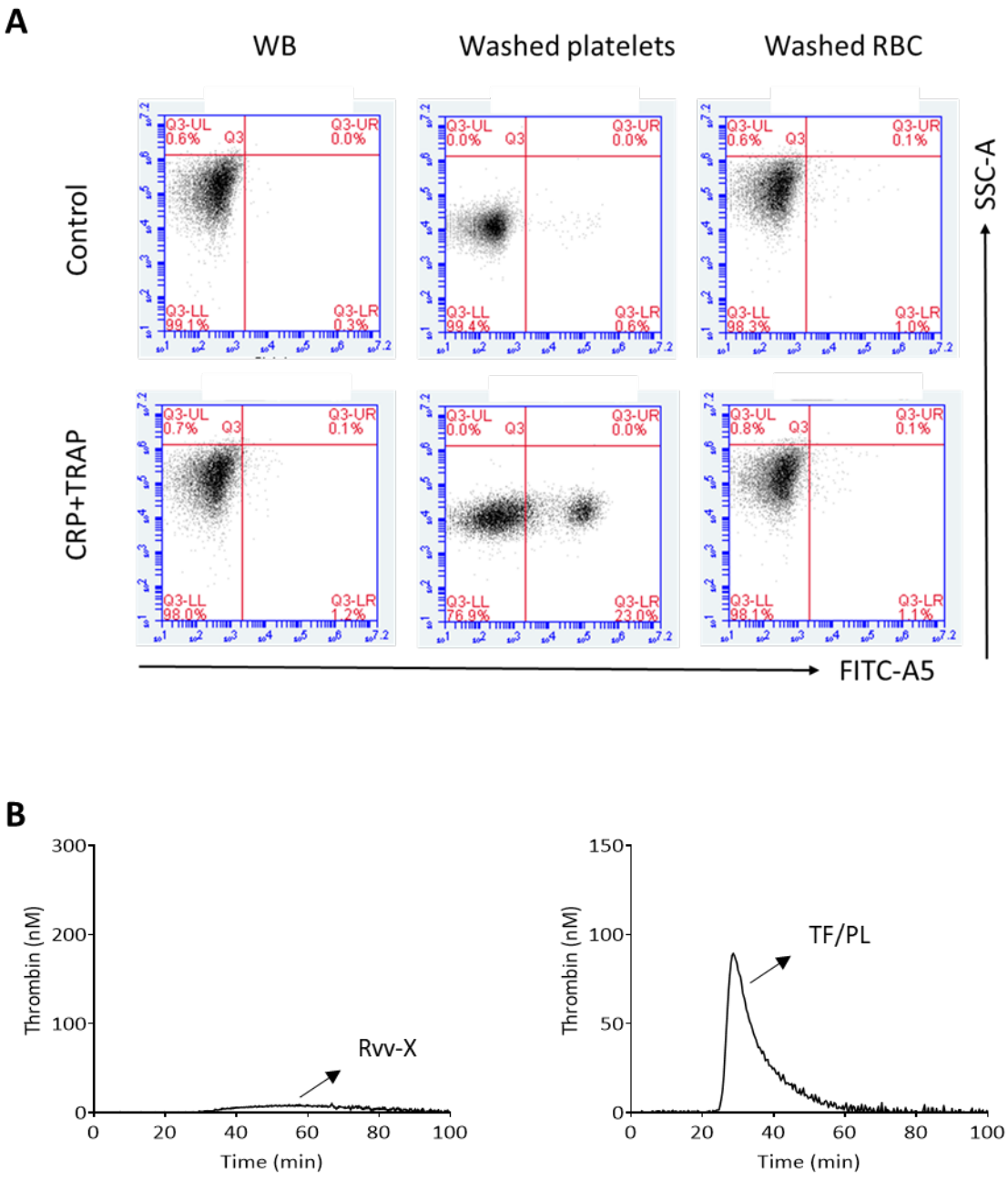
(iv)



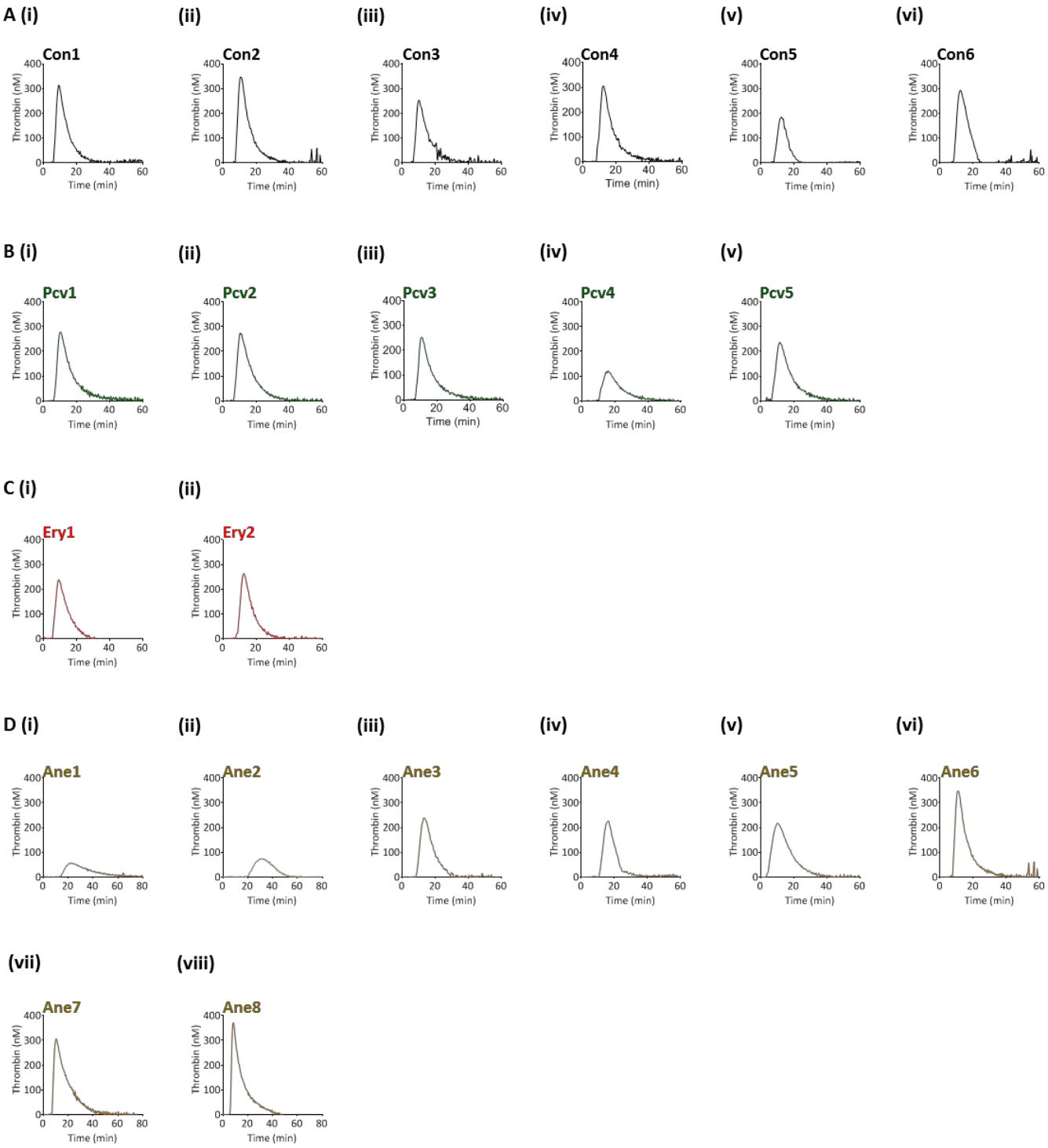
(iv)



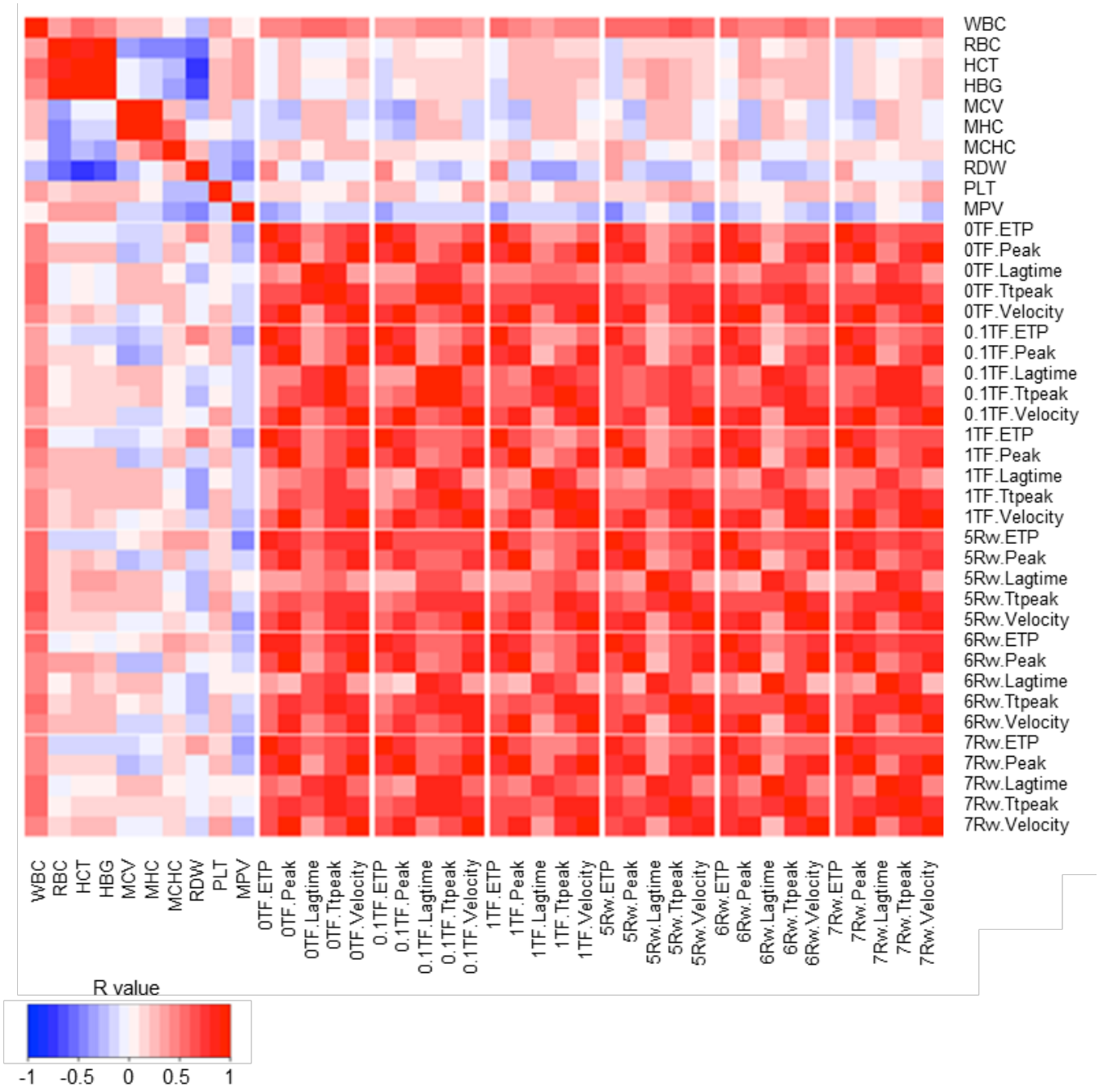
Supplemental Figure 4. Effect of annexin A5 (A5) addition on thrombin generation in whole-blood and platelet-rich plasma. Whole-blood of 35% hematocrit (**A**) or RBC reconstituted with plasma (**B**) was pre-incubated with CRP-XL (25 μg/mL) and/or annexin A5 (0-7.5 μg/mL) as indicated, and subsequently triggered with 0.1 pM tissue factor for measurement of TG. Shown are representative TG curves, indicating the effects of annexin A5 and CRP-XL (**i**). Furthermore, quantified lag times (**ii**), thrombin peak levels (**iii**) and ETP values (**iv**), as a function of the added annexin A5 concentration. Data are means ± SD (n=3), one-way ANOVA, **P<0.01 and ***P<0.001.



Supplemental Figure 5. Phosphatidylserine expression on blood cells and extracellular vesicles. A, Flow cytometric analysis of phosphatidylserine exposure of freshly isolated whole blood, autologous washed platelets and washed RBC. Blood cell preparations were pre-labeled with FITC-annexin A5. Where indicated, the preparations were pretreated for 10 minutes with 25 µg/mL CRP and 10 µM TRAP6. SSC = side scatter. Representative data from >3 experiments. **B**, Platelet-free plasma (PFP) was isolated from citrate-anticoagulated blood samples (healthy subjects). Thrombin generation was after recalcification with $CaCl_2$/$MgCl_2$. Triggering was either by Rvv-X (dilution 1e-6) or by tissue factor/phospholipids (TF, 1 pM TF, phospholipids 4 µM). Curves are representative for three experiments. Note the essential absence of TG with Rvv-X.



Supplemental Figure 6. Thrombin generation curves with blood from investigated patients with RBC abnormalities. Blood samples from individual day-control subjects (Con, **A**), or from patients with polycythemia vera (PCV, **B**), erythrocytosis (Ery, **C**) or anemia (Ane, **D**) were investigated for TG in response to 0.1 pM tissue factor. Representative curves are shown.



Supplemental Figure 7. Multivariate Spearman correlation. Analysis was performed of hematological parameters and six conditions of thrombin generation (TG) parameters for cohort of 21 control subjects and patients with altered red blood cell traits**.** For incorporated subjects and variables, see Figure 7A. Note the overall high consistency of all TG parameters independent of trigger. Color code: blue, negative relation; red, positive relation.

Supplemental Figure 8. Principal component matrix of TG and blood cell traits in investigated patients. Shown is the relative contribution to dimensions 1-21 of the principal component analysis of combined TG and hematological parameters for cohort of 21 control subjects and patients. For incorporated subjects and variables, see Figure 7A. Visualized is per dimension the relative contribution of each variable. Positive correlations are shown in blue, negative correlations in red.