

## Variable impairment of platelet functions in patients with severe, genetically linked immune deficiencies

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### **Supplemental Methods**

#### **Materials**

Horn type-I collagen was purchased from Nycomed. Convulxin was purified to homogeneity from the venom of *Crotalus durissus terrificus* (Latoxan). Human  $\alpha$ -thrombin came from Kordia Life Science. Thapsigargin was from Santa Cruz Biotechnology. Annexin A5 labeled with Alexa Fluor-568 (AF568) and Fura-2 acetoxymethyl ester were from Invitrogen. FITC-labeled anti-fibrinogen monoclonal antibody (mAb), staining for activated integrin  $\alpha_{IIb}\beta_3$ , was from BD Bioscience; AF647-labeled anti-human CD62P (P-selectin) mAb was from BioLegend. Other chemicals were obtained from sources, as described.<sup>1</sup>

#### **Blood collection and preparation of platelets**

Blood was collected in trisodium citrate anticoagulant. Samples from patients and travel controls were collected under sterile conditions and supplemented with 10 mM glucose, to extend the life time of platelets and red blood cells. Transportation within 24 h was in thermal isolation material to avoid temperature changes. A part (1-2 mL) of the samples was used for whole-blood flow-perfusion experiments. The remaining blood was used

for preparation of platelet-rich plasma (PRP) and  $\text{Ca}^{2+}$  responses.<sup>2</sup> The platelets were finally suspended in HEPES buffer pH 7.45 (10 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 2 mM  $\text{MgCl}_2$ , 5 mg/mL glucose and 1 mg/mL bovine serum albumin) at a concentration of  $2 \times 10^8/\text{mL}$ . Where indicated, remaining (unloaded) platelets were used for flow cytometry.

### **Platelet $\text{Ca}^{2+}$ responses**

To measure changes in cytosolic  $\text{Ca}^{2+}$  concentration, platelets in plasma were incubated with fluorescent Fura-2 acetoxymethyl ester (2.5  $\mu\text{M}$ ) for 45 min at ambient room temperature under gentle rotation, under standardized conditions as described before.<sup>3</sup> Loaded platelets were washed, and activated with indicated agonists under magnetic stirring (37 °C). Fluorescence changes were recorded by 340/380 nm calibrated ratio fluorometry, using appropriate settings of  $R_{\text{max}}$  and  $R_{\text{min}}$ .<sup>3</sup> Calcium responses are presented as nM rises in cytosolic  $[\text{Ca}^{2+}]_i$ .<sup>4</sup> Agonist-induced changes in  $[\text{Ca}^{2+}]_i$  were compared of platelets from patients and from two cohorts of healthy control subjects (home controls and travel controls).

### **Flow cytometry**

Washed platelets ( $2 \times 10^8/\text{mL}$ ) were activated in the presence of 2 mM  $\text{CaCl}_2$  with agonists for 5-30 min. Multicolor flow cytometry was used for measurements of surface exposure of phosphatidylserine (with AF647-labeled annexin A5), activated integrin  $\alpha_{\text{IIb}}\beta_3$  (with FITC-labeled PAC1 mAb), and  $\alpha$ -granule secretion (with FITC-labeled anti-CD62P mAb), as described before.<sup>1, 5</sup> Analysis was with a FACScan flow cytometer (BD Accuri Cytometers).

### **Thrombus formation on microspots under flow**

Whole-blood was assayed for thrombus formation under defined flow conditions, essentially as described before.<sup>6</sup> In brief, citrate anticoagulated blood samples were recalcified with 3.75 mM MgCl<sub>2</sub> and 7.5 mM CaCl<sub>2</sub> (final concentrations) in the presence of PPACK (40 μM) and fragmin (40 U/mL). Samples were then directly perfused over a glass coverslip coated with 1-3 microspots (spot 1: collagen type I, spot 2: VWF/rhodocytin, spot 3: VWF/fibrinogen), mounted onto a transparent parallel-plate perfusion chamber. Perfusion was at a laminar wall-shear rate of 1600 s<sup>-1</sup> for 3.5 min. Spot 1 with strongest thrombogenic activity was located most downstream of the flow direction, in order to prevent cross-reactivity between microspots.<sup>6</sup> Remaining blood samples were recalcified and perfused over microspots at lower shear rate of 150 s<sup>-1</sup> for 6 min. Thrombi formed on the spotted surfaces were immediately post-stained with a mixture of FITC-labeled anti-fibrinogen mAb (1:100), FITC-anti-CD62P mAb (25 μg/mL) and AF647-annexin A5 (0.25 μg/mL) in HEPES buffer pH 7.45 containing CaCl<sub>2</sub> (2 mM) and heparin (1 U/mL). Series of phase-contrast and fluorescence images were then captured for analysis of surface area coverage by adhered platelets or by activated platelets (*i.e.*, with integrin α<sub>IIb</sub>β<sub>3</sub> activation, α-granule release or exposed phosphatidylserine).<sup>7</sup> Three random images were taken per spot and stain. Image analysis was performed blinded to the condition, using Fiji software,<sup>8</sup> and predefined scripts.

For comparative analysis, average values from 7 thrombus parameters were scaled over a range from 0-10 (over all 3 microspots), and heatmaps were generated. These data were further processed to obtain subtraction heatmaps in comparison to the

normalized mean data from a cohort of normal control subjects.<sup>6</sup> Filtering of the subtraction data was based on differences in comparison to control values outside the range of mean  $\pm$  2 SD; and a relevance level arbitrarily set at 20%.

Coding of microspots: Sp1, type I collagen; Sp2, VWF/rhodocytin; Sp3, VWF/fibrinogen. Coding of outcome parameters: V1, thrombus morphological score (scale 0-5); V2, platelet surface area coverage (% SAC); V3 thrombus contraction score (scale 0-3); V4, thrombus multilayer score (scale 0-3); V5, PS exposure (% SAC); V6, P-selectin expression (% SAC); V7, integrin  $\alpha_{IIb}\beta_3$  activation (% SAC). Scoring of V1-3 was based on predefined reference images.

## **Statistics**

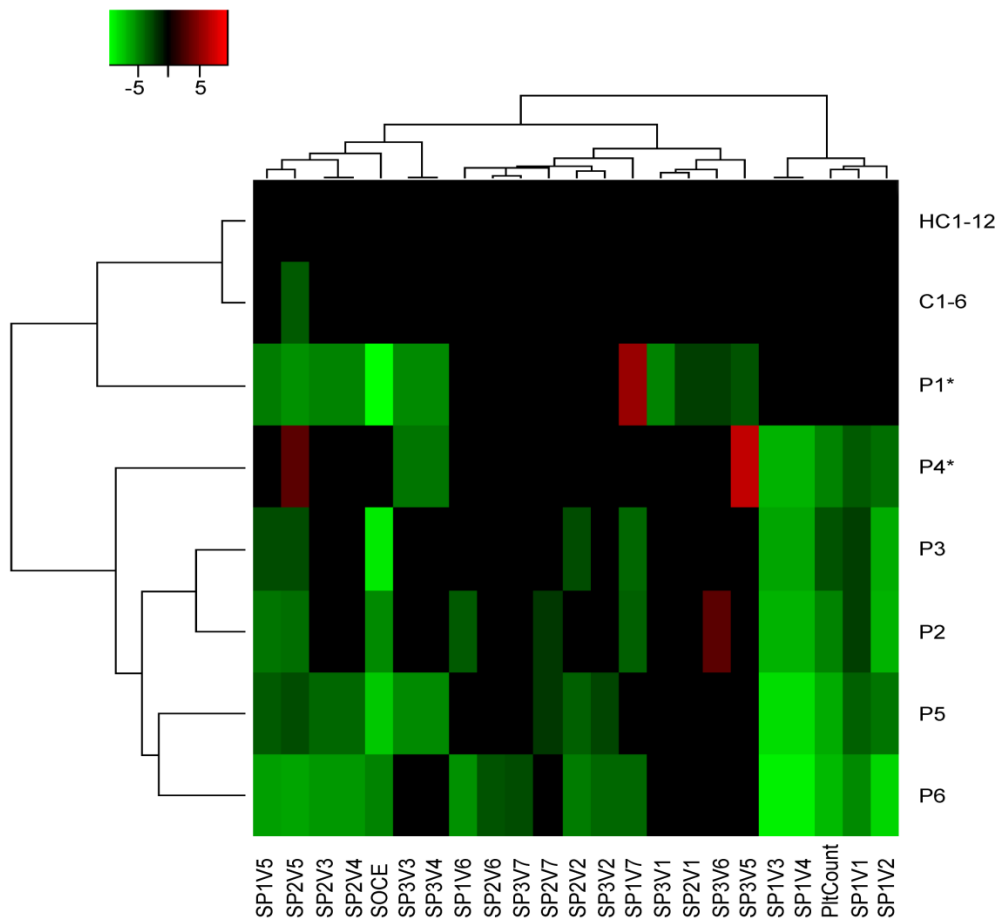
The size of control groups (HC1-12, C-16) with  $n \geq 6$  was pre-assessed by power analysis, based on expected effects on PS exposure.<sup>6</sup> Significance of differences was determined with the paired sample *t* test (intervention effects) and principal component analysis (PCA) using the statistical package for social sciences (SPSS, version 11.0).

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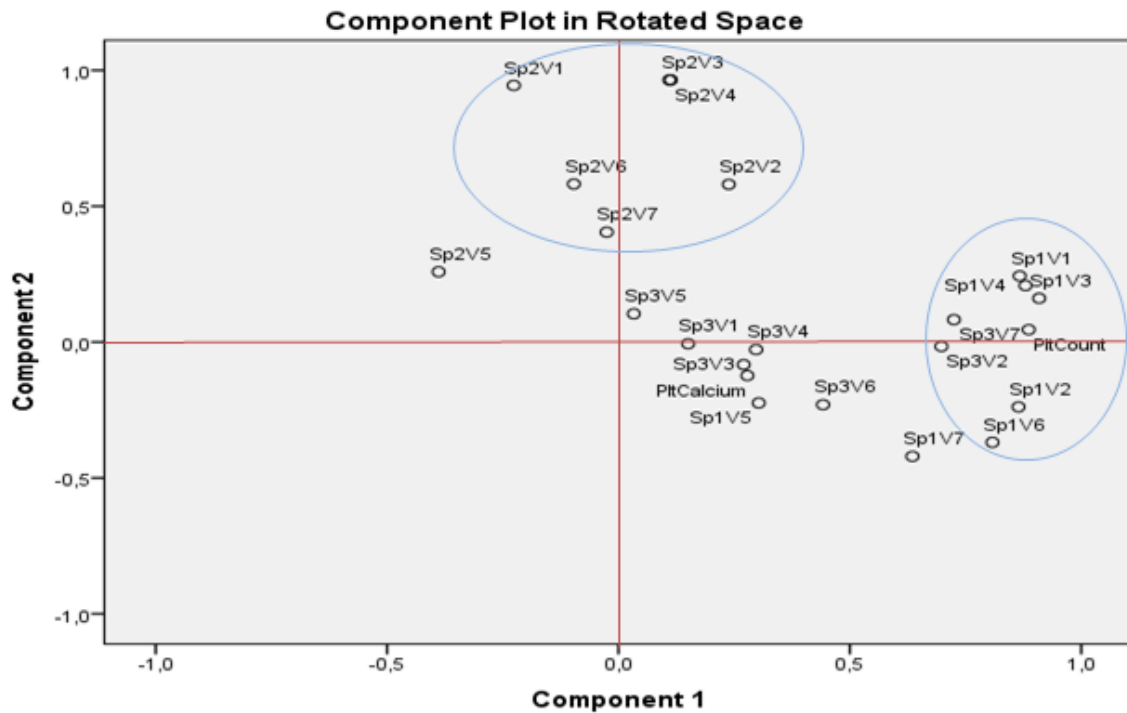
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## Supplemental Figures



**Supp. Figure 1. Clustering of parameters of thrombus formation on three microspots with SOCE and platelet count.** Unsupervised clustered heatmap for 21 parameters of thrombus formation at high shear rate, SOCE and platelet count (columns) for cohorts of healthy controls (HC1-12 and C1-6) and patients/relatives P1-5 (rows). Clustering reveals a clear distinction between parameters linked to SOCE or platelet count.

**A**

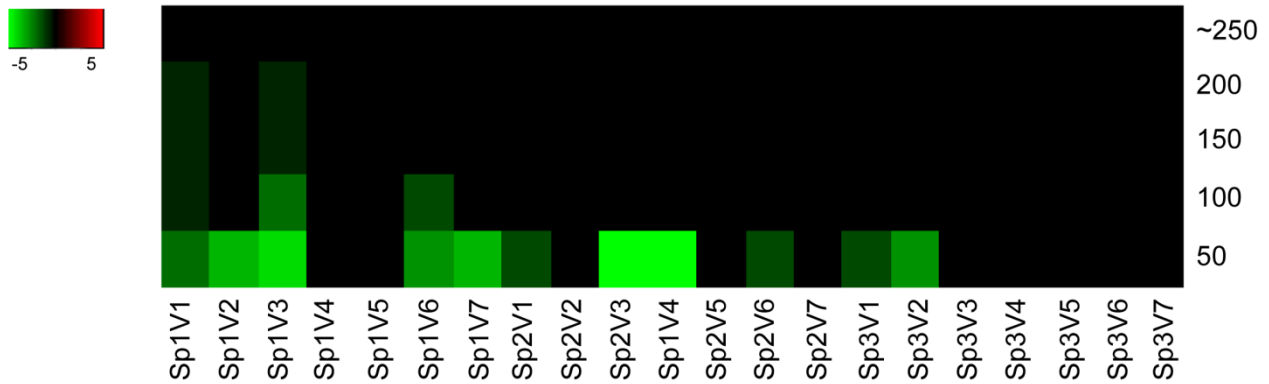


**B**

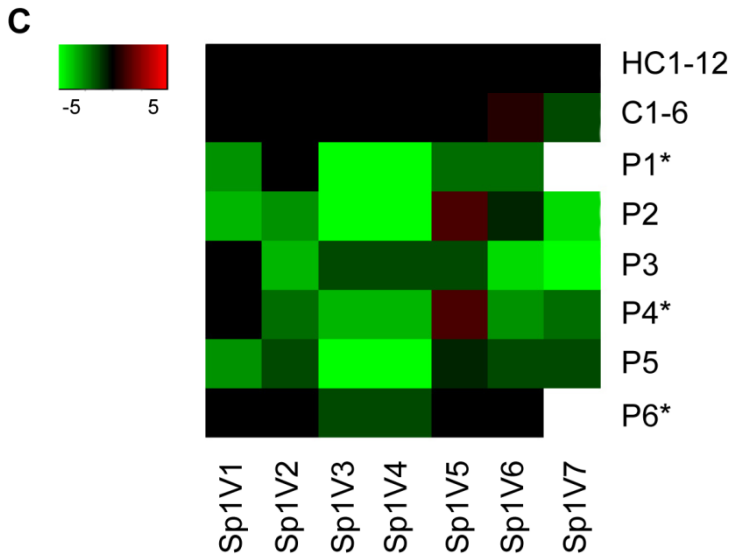
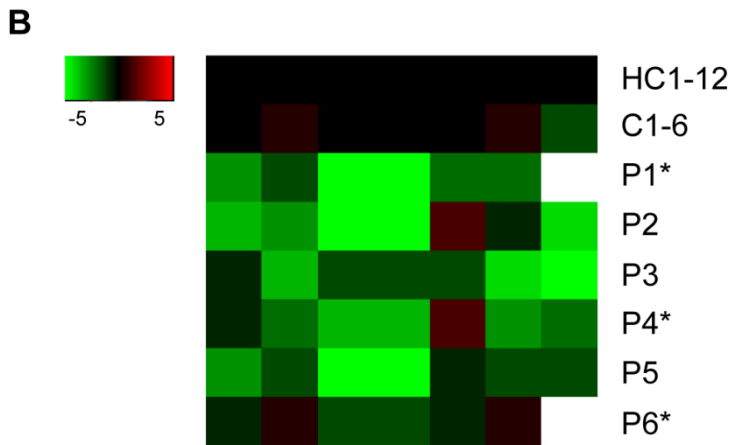
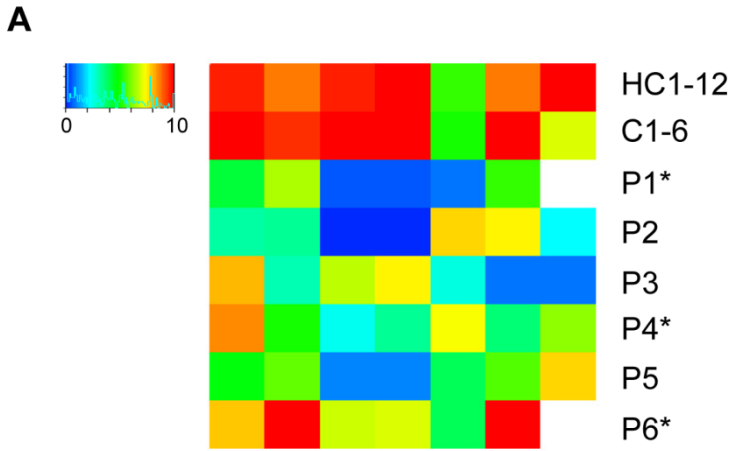
	Parameter	R <sup>2</sup>
<b>PltCount</b>	Sp1V1	0.734**
	Sp1V2	0.678**
	Sp1V3	0.796**
	Sp1V4	0.756**
	Sp1V6	0.516*
	Sp3V2	0.611*
	Sp3V7	0.726**
<b>PltCalcium (SOCE)</b>	Sp1V5	0.536*
	Sp3V5	0.577*

**Suppl. Figure 2. Principal component analysis (PCA) of parameters of thrombus formation, SOCE and platelet count at high shear rate. (A) PCA biplot with parameter values projecting the first two principal components. (B) Tabled R<sup>2</sup> values (Pearson correlation coefficient) and significance of correlations with platelet count and platelet SOCE. Statistical analysis was performed in SPSS; \*P<0.05, \*\*P<0.01.**



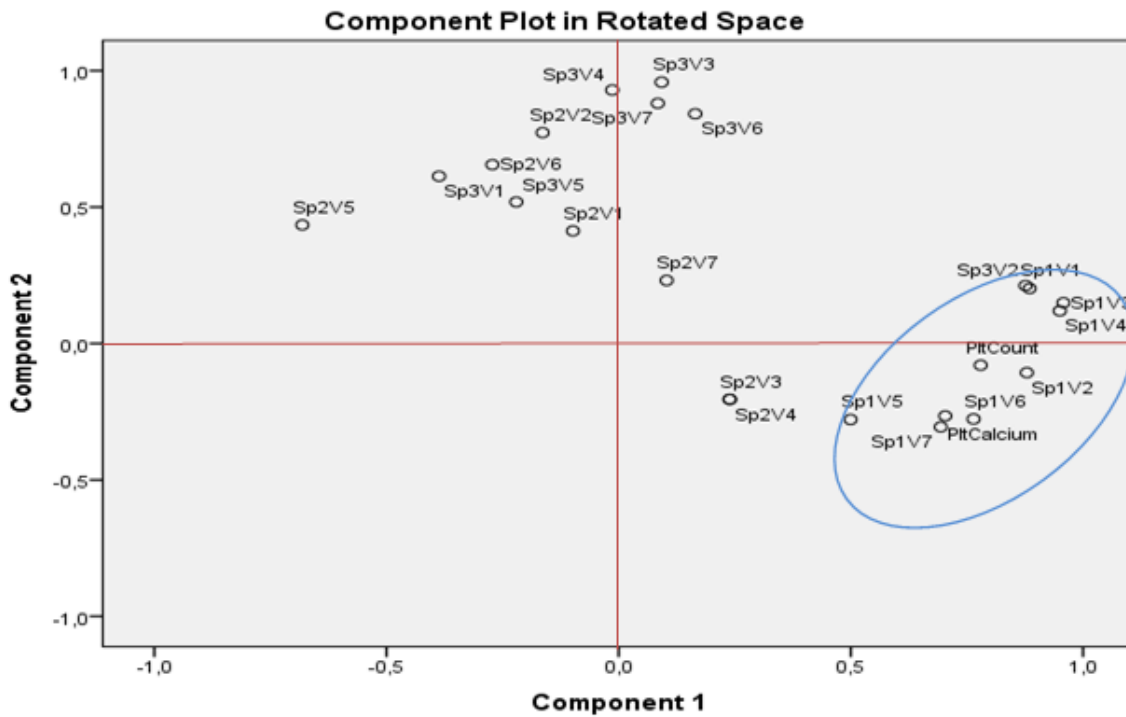


**Suppl. Figure 3. Dependency of thrombus formation on platelet count.** Blood was reconstituted using washed red blood cells, platelet-rich plasma and platelet-free plasma at hematocrit levels of 0.40 and platelet counts of 50, 100, 150, 200 or 250 x 10<sup>9</sup>/L, as indicated. Samples were perfused over 3 microspots (Sp1-3) at shear rate of 1600 s<sup>-1</sup> (n=3-5), as described for Figure 4. Parameters of thrombus formation were determined (V1-7), and scaled for a subtraction heatmap relative to values for 250 x 10<sup>9</sup> platelets/L, as in Figure 5. Subtracted values were then filtered, based on significant differences in comparison to original platelet count of 250 x 10<sup>9</sup>/L. Note the limited reduction of thrombus parameters on Sp1 at 100 x 10<sup>9</sup>/L, and more extended changes on Sp1-3 at the lower count of 50 x 10<sup>9</sup>/L.



**Suppl. Figure 4. Altered thrombus formation of blood from patients with *ORAI1* or *STIM1* mutations at low shear rate.** Whole blood from control subjects or indicated patients was perfused over collagen type I surface at low shear rate of  $150 \text{ s}^{-1}$ . **(A)** Heatmap of normalized parameters of thrombus formation for control subjects (HC1-12, C1-6) and patients with genetic mutations in *ORAI1* (P1-5) or in *STIM1* (P6). **(B)** Subtraction heatmap of parameters in comparison to control cohort (HC1-12). **(C)** Filtered subtraction data based on differences in comparison to controls outside the range of mean  $\pm$  2 SD. \*Subject with reduced SOCE.

**A**



**B**

	Parameter	R <sup>2</sup>
<b>PltCount</b>	Sp1V1	0.609**
	Sp1V2	0.725**
	Sp1V3	0.654**
	Sp1V4	0.643**
	Sp1V6	0.692**
	Sp1V7	0.659*
<b>PltCalcium (SOCE)</b>	Sp1V1	0.704**
	Sp1V3	0.714**
	Sp1V4	0.737**

**Suppl. Figure 5. Principal component analysis (PCA) of parameters of thrombus formation, SOCE and platelet count at low shear rate. (A) PCA biplot with parameter values projecting the first two principal components. (B) Tabled R<sup>2</sup> values (Pearson correlation coefficient) and significance for correlations with platelet count and platelet SOCE. Statistical analysis was performed in SPSS; \*P<0.05, \*\*P<0.01**

	<b>Subject</b>	<b>Spot1</b>	<b>Spot2</b>	<b>Spot3</b>
<b>PS exposure (%)</b>	Control	9.4-13.44	6.84-13.88	2.12-8.06
	P1	4.09	1.97	0.09
	P2	7.02	5.69	7.25
	P3	4.53	3.83	7.28

**Suppl. Table 1. Reduced PS exposure of platelets from patient P1.** Whole blood from control subjects and indicated patients P1-3 was perfused over three microspots, as in Figure. Data are shown of platelet PS exposure (%SAC) at spots 1-3.