**SUPPLEMENTAL DATA**

**Supplementary Methods**

**Antibodies and reagents**

Prostacyclin, apyrase grade III, human fibrinogen, adenosine diphosphate (ADP), indomethacin were from Sigma-Aldrich, high-molecular-weight heparin (Ratiopharm), U46619, convulxin (Enzo Life Sciences), thrombin, midazolam, mini protease inhibitor cocktail were all from Roche, medetomidine (Pfizer), fentanyl (Janssen-Cilag), JON/A-PE antibody (Emfret Analytics) were purchased from the respective companies. Mouse anti-Grb2 antibody (clone 3F2), rabbit anti-Gads antibody and mouse anti-phosphotyrosine antibody 4G10 (all Merck Millipore), anti-actin, anti-GAPDH (Sigma-Aldrich), anti-PLCγ2 (Santa-Cruz Biotechnology) antiphospho-PLCγ2 (Y759), anti-phospho-LAT (Y191), anti-Syk, anti-phospho-Syk (Y519/520) (all Cell Signal Technologies) were purchased from the respective companies. Collagen-related peptide (CRP) was generated as previously described.[1] Rhodocytin was isolated as described.[2] All other antibodies were generated and modified in our laboratory as previously described.[3]

**Platelet preparation**

Mice were bled from the retro-orbital plexus under isoflurane anesthesia and blood collected in a tube containing 300 µL (20 U/ml in 1x TBS) heparin as described [4]. The platelet-rich plasma (PRP) was fractionated from whole blood by 2 cycles of centrifugation at 300 *g* for 6 min at room temperature (RT). The upper phase (PRP) was collected and centrifuged at 800 *g* for 5 min at RT to obtain platelets as a pellet. Platelets were re-suspended in modified Tyrodes-HEPES buffer (134 mM NaCl, 0.34 mM Na2HPO4, 2.9 mM KCl, 12 mM NaHCO3, 5 mM HEPES, 1 mM MgCl2, 5 mM glucose, 0.35 % BSA, pH 7.4) supplemented with prostacyclin (0.5 µM) and apyrase (0.02 U/mL) to prevent platelet activation and washed twice by centrifugation at 800 *g* for 5 min at RT. Platelet count was measured by hematology analyzer (Sysmex KX-21TM) and adjusted to 5 × 105 platelets/µL by re-suspending in modified Tyrodes-HEPES buffer containing 0.02 U/mL apyrase, unless otherwise stated.

**Preparation of platelet lysates and Western blotting**

For detection of Grb2 and Gads, washed platelets were adjusted to a count of 2 × 106 platelets/µL and lysed with RIPA buffer (150 mM sodium chloride, 0.1 % Triton-X 100, 0.5 % sodium deoxycholate, 0.1 % SDS, 50 mM Tris pH 8.0) containing protease inhibitors, for 20 min on ice. Cell debris were removed through centrifugation at 18,400 g for 10 min at 4 °C. The protein concentration of the supernatant was determined by Bradford method and it was mixed with 4x Laemmli buffer (reducing or non-reducing). Samples were boiled at 95 °C for 5 min. Proteins were separated on a 10% SDS-PAGE gel and transferred onto a PVDF membrane by semi-dry transfer. The membrane was blocked with 5 % non-fat milk for 1 h at RT and subsequently incubated with the primary antibody overnight at 4 °C. Proteins were visualized following incubation with HRP-conjugated secondary antibody for 1 h at room temperature and using the ECL detection kit.

For tyrosine phosphorylation studies [5], washed platelets were prepared as described, except that the second wash step was performed using Tyrodes buffer without BSA supplementation, the platelet count was adjusted to 7 × 105 platelets/µL and the suspensions were treated with 10 µM indomethacin, 2 U/mL apyrase and 5 mM EDTA to prevent platelet activation by second wave mediators and platelet aggregation. The platelet suspensions were then taken into cuvettes and continuously stirred at 1,000 rpm at 37 °C. A quarter of the sample volume was withdrawn as 0 s time point and immediately added to an equal volume of ice-cold 2x lysis buffer (300 mM NaCl, 20 mM Tris, 2 mM EGTA, 2 mM EDTA, 10 mM NaF, 4 mM Na3VO4, 1% IGEPAL, 1% protease inhibitor cocktail). The remaining platelet suspension was stimulated with the indicated agonist (convulxin or rhodocytin), and samples were taken at 30 s, 90 s and 300 s post stimulation, and immediately added to equal volumes of ice-cold 2x lysis buffer. The platelet lysates were centrifuged at 18,400 rpm for 10 min at 4 °C and the supernatants collected. The platelet lysates were mixed with 4x SDS reducing sample buffer, incubated at 70 °C for 10 min. Proteins were separated on NuPAGE Novex 4-12 % gradient Bis-Tris gels using 1x NuPAGE MOPS SDS Running buffer at 4°C. Further steps were performed as described above, using the indicated antibodies. Quantification of band intensities was performed with ImageJ.

**Flow cytometric analysis of platelet activation**

Heparinized whole blood was washed twice in Tyrode-HEPES buffer and then diluted in Tyrode-HEPES buffer containing 2 mM CaCl2. Aliquots of 50 µL of this diluted blood were stained with antibodies JON/A-PE (to determine activation of integrin αIIbβ3) and anti P-selectin-FITC (as a measure of platelet degranulation response as P-selectin is stored in alpha-granules of platelets) and activated with the indicated concentration of agonists for 8 min at 37 °C and further 8 min at RT. The reaction was stopped by addition of 500 µL 1x PBS. Unstimulated samples were used as resting controls. The samples were analyzed on a FACSCalibur (BD Biosciences) and the data obtained was analyzed on FlowJo version 7.6.

**Platelet aggregation studies**

The aggregation response of platelets on agonist stimulation was measured on a Fibrintimer 4 channel aggregometer (APACT Laborgeräte und Analysensysteme) [6]. Aliquots of 50 µL of washed platelets were diluted with 110 µL Tyrodes-HEPES buffer supplemented with 2 mM CaCl2 and 100 µg/mL human fibrinogen (except in the case of thrombin stimulation where fibrinogen was not used). The platelet suspension was continuously stirred at 1,000 rpm. 30 s after starting the measurement, the indicated agonists were added and the aggregation curve recorded for 10 min.

**Supplemental Table 1**

*Grb2fl/fl Pf4-Cre +/-* and *Grb2fl/fl* mice on a *WT* (Mating 1) or *Gads-/-* background (Mating 2) were crossed and offspring was genotyped.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **x**  ***Grb2fl/fl Pf4-Cre-/-***  ***Grb2fl/fl Pf4-Cre+/-***  **Mating 1:** | | |  | | |
|  | **EXPECTED** | **OBTAINED** |  | **EXPECTED**  **Mating 2:**  ***Gads-/-/Grb2fl/fl Pf4-Cre-/-***  ***Gads-/-/Grb2fl/fl Pf4-Cre+/-***  **x** | **OBTAINED** |
| *WT* | 50% | 32 (50.8%) | Gads KO | 50% | 48 (47.1%) |
| Grb2 KO | 50% | 31 (49.2%) | DKO | 50% | 54 (52.9%) |
| **Total** |  | 63 | **Total** |  | 102 |

**Supplemental Figures**



Supplemental figure 1. Platelet glycoprotein expression in Grb2 KO, Gads KO and Grb2/Gads double knockout (DKO) mice.

Expression of major glycoproteins in *WT*, Grb2 KO, Gads KO or Grb2/Gads DKO mice were measured by flow cytometry. 8 mice per group from 2 independent experiments. *\*P*<0.05, *\*\*P*<0.01, *\*\*\*P*<0.001; one-way ANOVA with Dunnett post-hoc test.



Supplemental Figure 2. Impaired global tyrosine phosphorylation in Grb2/Gads DKO platelets.

The tyrosine phosphorylation pattern in Grb2 KO, Gads KO and DKO platelet lysates in comparison to *WT* platelet lysates was determined using the pan anti-phosphotyrosine antibody 4G10 (p-Tyr).The β3 subunit of platelet integrin αIIbβ3 or GAPDH were used as a loading control. (The black lines marking the lanes for samples from the four different genotypes are only for the purpose of clarity; samples from all four genotypes were run on the same gel). Representative of at least 3 independent experiments.

**Supplemental References**

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