Supplemental Methods

Reagents

FITC-conjugated rat anti-mouse GPVI, α2, and GPIbα, P-selectin, and anti-mouse GPVI, and PE-conjugated JON/A monoclonal antibodies (mAbs) were from Emfret Analytics (Würzburg, Germany); FITC-conjugated rat anti-mouse CD31 (PECAM-1), integrin αIIb, rat IgG, and anti-B220 and CD16/32 mAbs were from BD Biosciences (San Jose, CA,). Anti-mouse Gr-1 and CD11b, and CD31 antibodies were from eBioscience (San Diego, CA). Anti-mouse DGKζ was from Bethyl Laboratories (Montgomery, TX). Antibodies against Akt-pTyr308, Lyn-pTyr507, ERK-pThr202/Tyr204, PLCγ2-pTyr1217 were from Cell Signaling Technology (Danvers, MD). Antibodies against ERK2, Lyn, PLCγ2 were from Santa Cruz Biotechnology, Inc. (Dallas, TX). Anti-mouse Akt was from MilliporeSigma (Burlington, MA). Anit-mouse collagen I was from Novus Biologicals (Centennial, CO). Horseradish peroxidase–conjugated secondary antibodies were from Jackson Immuno Research (West Grove, PA). Collagen and ADP were from Chrono-log Corporation (Havertown, PA). Human fibrinogen was from Enzyme Research Laboratories (South Bend, IN). PAR4-activating peptide and crosslinked collagen-related peptide (CRP) were prepared by the Blood Research Institute Protein Core Laboratory. Recombinant murine SCF and TPO were from Peprotech (Rocky Hill, NJ). Sheep anti-rat IgG Dynabeads was from Invitrogen (Carlsbad, CA). R59022 was from Tocris Bioscience (Avonmouth, Bristol, UK). Cell-permeable DAG (1,2-dioctanoyl-sn-glycerol) was from Avanti Polar Lipids, Inc. (Alabaster, AL). All other reagents were from Sigma-Aldrich (St Louis, MO).

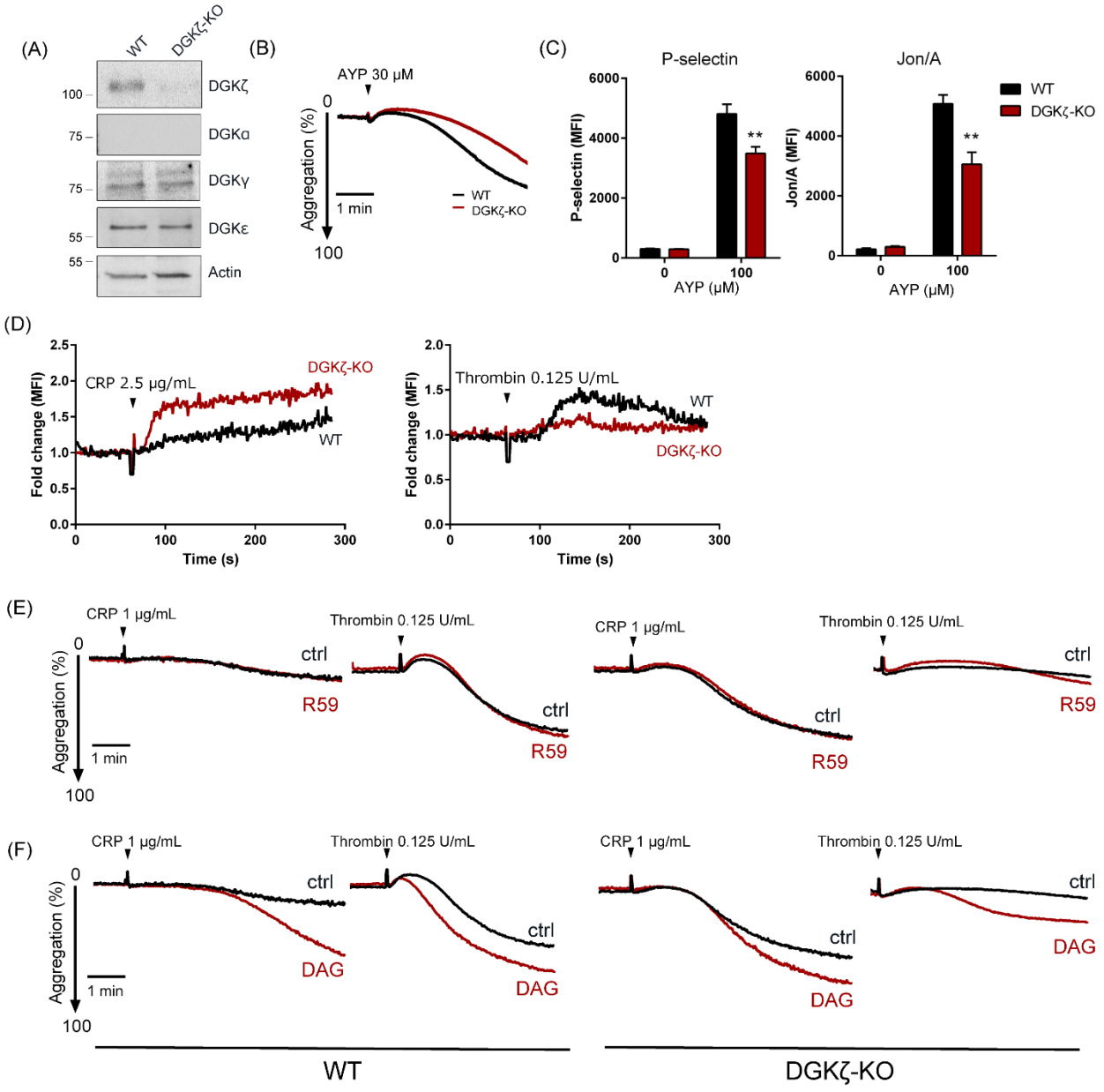
Measurement of intracellular calcium

Washed platelets were loaded with Fluo-4 AM (2.5 μg/mL) for 10 minutes at 37 °C. Calcium mobilization was analyzed in the presence of 1 mM $CaCl_2$ using an Accuri C6 plus. The calcium signal was measured for 60 seconds before stimulation and for 4 minutes after agonist stimulation. Data were analyzed using FlowJo software. Fold change in Fluo-4 MFI was calculated by normalizing MFI after stimulation with baseline MFI.

Immunohistochemistry

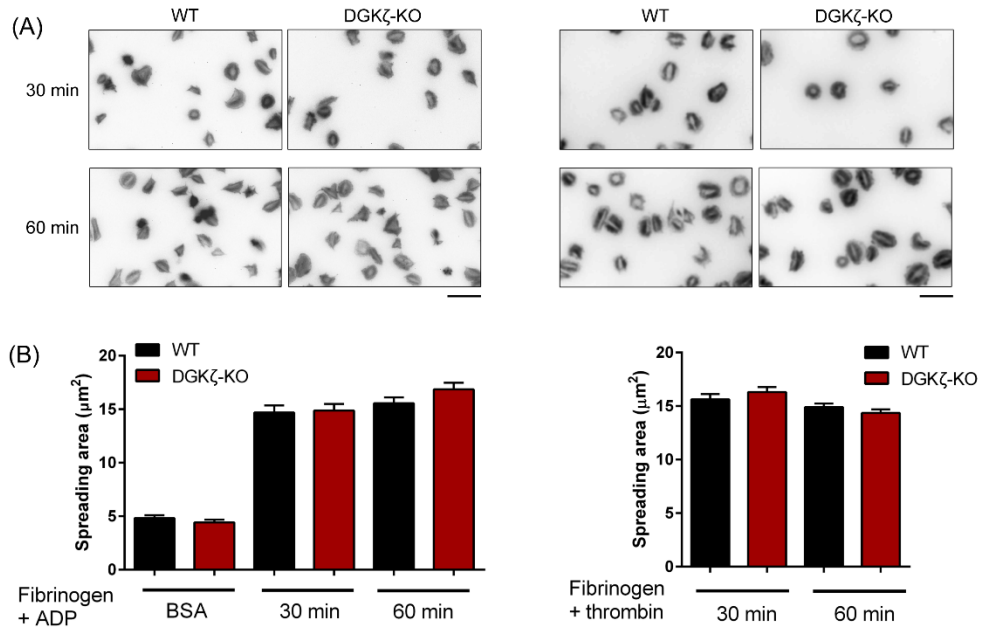
Carotid arteries treated with 10% $FeCl_3$ for 3 min were isolated, fixed and frozen in OCT compound (Sakura finetek, Torrance, CA). Sections (8 μm) were stained with anti-collagen I and -PECAM-1 followed by secondary antibody Fluor 647 anti-rat IgG and Fluor 546 anti-rabbit IgG (Invitrogen) and DAPI. Images were taken with an inverted microscope (Nikon).

Supplemental Figures

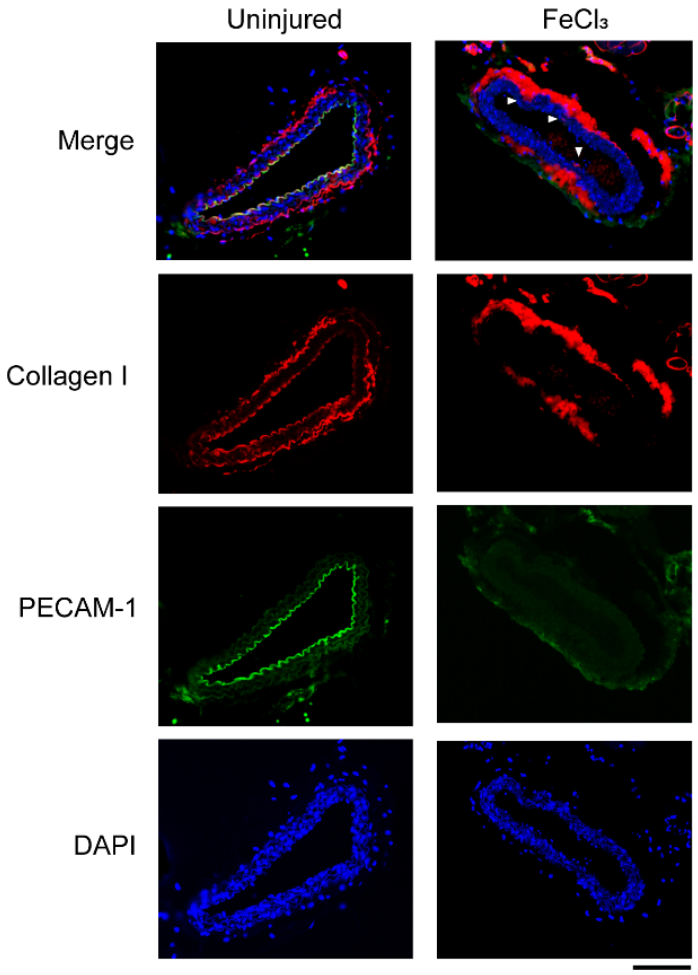


Supplementary figure 1. DGKζ-deficiency and platelet activation. (A) Whole lysates of washed platelets from wild-type (WT) and DGKζ-deficient (DGKζ-KO) mice were analyzed by western blotting for DGKζ, α, γ, ε and actin. The blot shown is representative of three independent experiments. Note that DGK isozyme expressions (α, γ, ε) are comparable between WT and DGKζ-KO platelets. **(B)** Aggregation of washed platelets from WT and DGKζ-KO mice was measured by

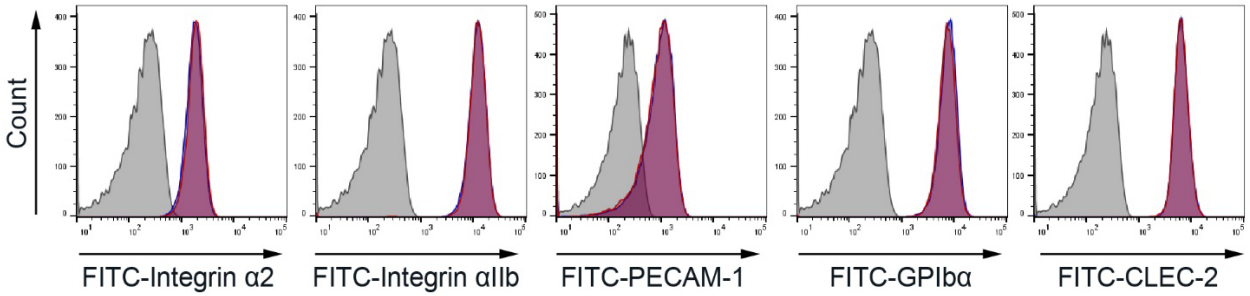
lumiaggregometry in response to PAR4 agonist peptide (AYP). Aggregation tracing is representative of three independent experiments. **(C)** P-selectin exposure (left) and Jon/A biding as a reporter of activation of integrin αIIbβ3 (right) were measured by flow cytometry after 20 minutes of stimulation with PAR4 agonist peptide at the indicated concentrations. Values represent the mean ± SEM of mean fluorescent intensity (MFI) observed in five independent experiments. **(D)** Representative tracings of calcium mobilization of Fluo-4 loaded washed platelets from WT and DGKζ-KO mice stimulated with CRP (2.5 μg/mL) and thrombin (0.125 U/mL). Baseline intracellular calcium was mesured for 1 minute followed by agonist stimulation. Increase in intracellular calcium was measured as Fluo-4 fluorescence (MFI) change normalized with baseline fluorescence. Tracing is representative of three individual experiments. **(E-F)** Aggregation of washed platelets from WT and DGKζ-KO mice was measured by lumiaggregometry in response to CRP (1 μg/mL) or thrombin (0.125 U/mL). Platelets were either pretreated with **(E)** R59022 (R59) 10 μM or **(F)** DAG 50 μM. Aggregation tracing is representative of three independent experiments. Statistical analyses were performed using an unpaired t test. $^{**}P<0.01$ of DGKζ-KO as compared to WT.



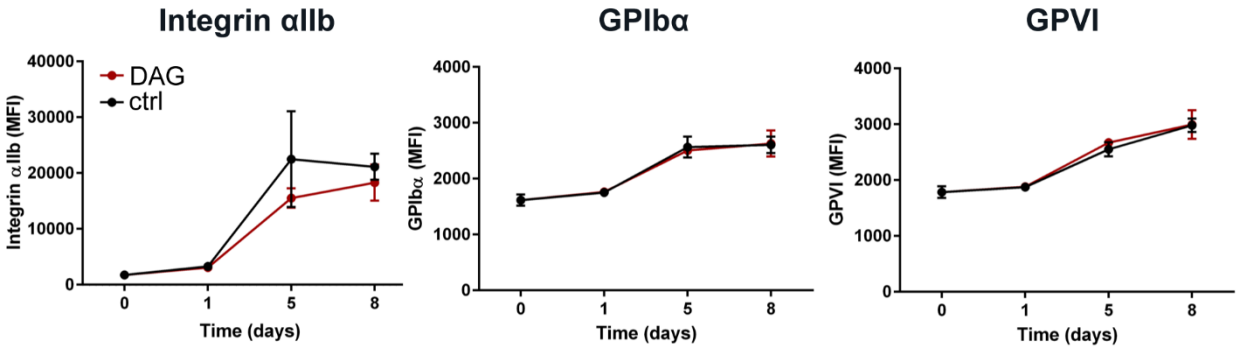
Supplementary figure 2. ADP and thrombin can normalize the platelet spreading on fibrinogen in WT and DGKζ-KO platelets. Washed platelets from wild type (WT) and DGKζ-deficient (DGKζ-KO) mice were allowed to adhere on immobilized fibrinogen (30 μg/mL) in the presence of ADP (10 μM) (left) and thrombin (1 U/mL) (right) for the indicated times at 37°C. After washing with PBS to remove unadhered platelets, adherent platelets were fixed and stained for phalloidin-TRITC. **(A)** Representative images of spread platelets from three independent experiments are shown. Bars represent 10 μm. **(B)** The avarage area covered by individual platelets was quantified from at least 4 images per sample and 250–500 platelets per time point. Spreading area is reported as the mean ± SEM. Statistical anlaysis was performed using the unpaired t test.



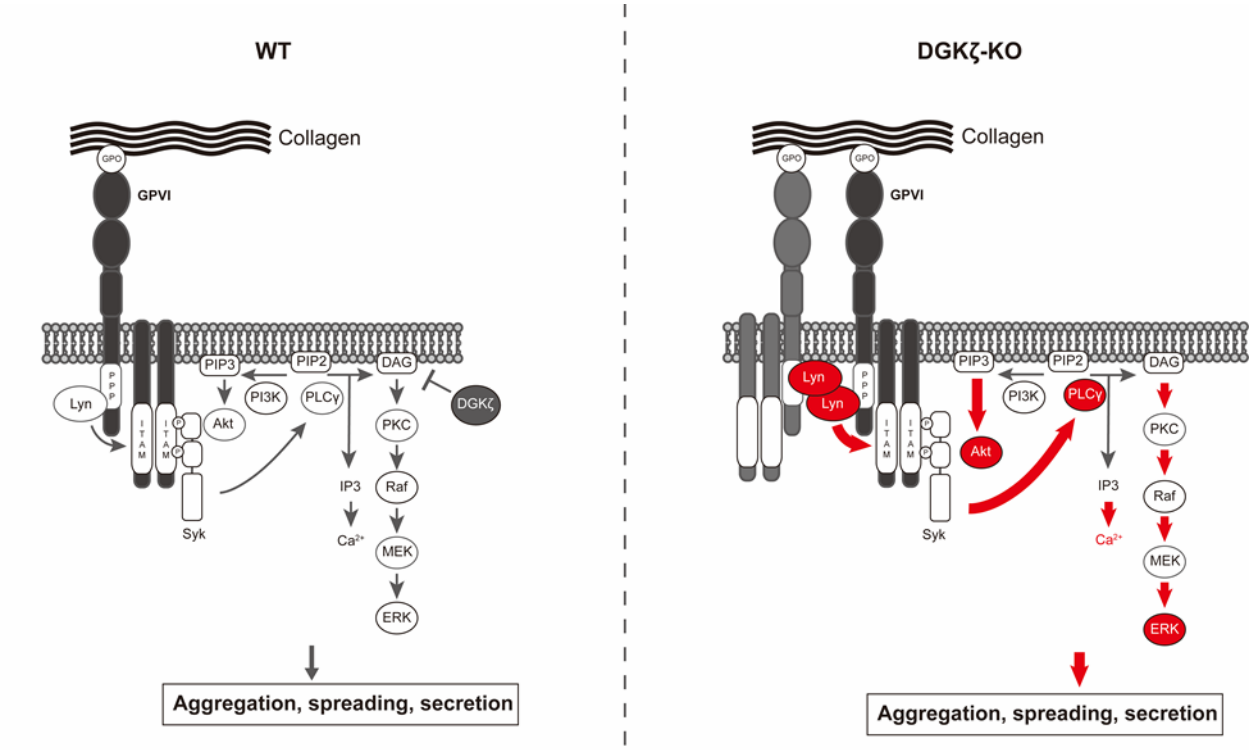
Supplementary figure 3. Collagen is exposed after $FeCl_3$ injury. Wild type (WT) mice were treated or uninjured with 10% $FeCl_3$ for 3 minutes. Carotid artery was isolated and frozen sections (8 μm) were stained with collagen I (red), PECAM-1 (green) and DAPI (blue). Bar represent 100 μm. White arrow indicates collagen deposition and expore to the lumen.



Supplementary figure 4. DGKζ deficiency does not affect platelet receptor expression other than GPVI. Platelet surface receptor expression in diluted whole blood samples from wild type (WT) and DGKζ-deficient (DGKζ-KO) mice was measured for integrin α2, integrin αIIb, PECAM-1, GPIbα, and CLEC-2 by flow cytometry.



Supplementary Figure 5. GPVI expression is not altered by DAG treatment in megakaryocytes. Bone marrow-derived hematopoietic stem cells from wild-type (WT) mice were cultured in TPO+SCF for 0, 1, 5, or 8 days with or without DAG 50 μM to obtain megakaryocytes; and surface expression of integrin αIIb, GPIbα, and GPVI was measured by flow cytometry. Mean fluorescent intensity (MFI) is reported as the mean ± SEM (n=6).



Supplemental figure 6. Proposed role of DGKζ in GPVI-mediated platelet activation. In wild-type (WT) platelets (left), DGKζ functions as a negative regulator of GPVI signaling by converting diacylglycerol (DAG) into phosphatidic acid (PA), thereby decreasing the intracellular DAG level. In DGKζ-deficient (DGKζ-KO) platelets (right), DGKζ deficiency increases the level of GPVI expression, which leads to an increase in Lyn dephosphorylation at its negative-regulatory site, and enhanced PLCγ2 and Akt activation. DGKζ deficiency also leads to an increased DAG pool within the cell, thereby resulting in enhanced activation of PKC and leading to an increase in ERK activation. These pathways ultimately lead to enhanced platelet activation, aggregation and spreading.