SUPPORTING ONLINE MATERIALS

MATERIALS

Generation and characterization of platelet-specific STAT3 null mice

C57BL/6J and B6.129S4-*Gt(ROSA)26Sor*^{tm1Sor} (ROSA26-lacZ) mice were purchased from the Jackson Laboratories (Bar Harbor, ME); B6.C-Tg(CMV-cre)1Cgn/J (CMV-Cre) and B6.129X1-*Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$/J (ROSA26-eYFP) were obtained from colonies of the Genetically Engineered Mouse Facility of MD Anderson Cancer Center where they have been backcrossed multiple times into C57BL/6 background.

To generate the transgenic C57BL/6-Tg(CXCL4-cre) (PF4-Cre) mouse, the DNA transgenic construct consisted of the 387-bp of the promoter region of human PF4 (nucleotides -312 to +75) followed by the cDNA of Cre recombinase cloned at the start codon of the native gene, and the polyA sequence of the human β-globin gene. Transgenic animals were generated by pronuclear injection of fertilized C57BL/6 oocytes. Colonies were founded from 9 transgenic mice and screened for Cre activity. The line with the most robust and selective expression was backcrossed into C57BL/6J 8 times to dilute undesirable genomic modification introduced by the genetic manipulation.

To evaluate the tissue specificity of Cre recombinase expression, PF4-Cre transgenic mice were crossed with ROSA26-lacZ and ROSA26-eYFP reporter

mice. In these reporters, the cDNA for lacZ or eYFP respectively was introduced into the *Gt(ROSA)26Sor* locus preceded by a STOP sequence flanked by two loxP sites. In cells expressing Cre, recombination at the two loxP sites excises the intervening sequence allowing the expression of β-galactosidase or eYFP. Bone marrow and different tissues from PF4-Cre;ROSA26-lacZ were fixed and processed for X-gal staining, and blood from PF4-Cre;ROSA26-eYFP was collected and processed for flow-cytometry after labeling. We used F4-Cre, ROSA26-lacZ, and ROSA-eYFP mice as negative controls, and CMV-Cre;ROSA26-lacZ and CMV-Cre;ROSA26-eYFP mice as positive controls.

Megakaryocytes/platelet-specific STAT3 deletant mice ($pSTAT3^{\Delta/\Delta}$) were generated by crossing PF4-Cre with mice carrying STAT3 alleles flanked by loxP sites ($STAT3^{F/F}$; provided by Dr. Shizuo Akira, Department of Host Defense, Osaka University (*22*)). Colonies were expanded by crossing PF4-Cre;$STAT3^{F/F}$ ($pSTAT3^{\Delta/\Delta}$) mice with $STAT3^{F/F}$ mice. These mice were genotyped via PCR by amplifying a fragment of the STAT3 gene flanked by loxP sites (primers: 5′-CCT-GAAGACCAAGTTCATCGTTGTGA-3′ and 5′-CACACAAGCCATCAAACTCTG-GTCTCC-3′). For data verification, a second strain of $pSTAT3^{\Delta/\Delta}$ mice was also generated using a commercial strain of PF4-Cre mice (Stock #008535, Jackson laboratories, Bar Harbor, ME) and used to repeat experiments conducted using $pSTAT3^{\Delta/\Delta}$ mice produced by crossing with the home-generated Cre line.

Mouse experiments were performed according to the provisions of the Animal Welfare Act, PHS Animal Welfare Policy, the principles of the NIH Guide for the Care and Use of Laboratory Animals, and the policies of Baylor College of Medicine and the University of Texas MD Anderson Cancer Center.

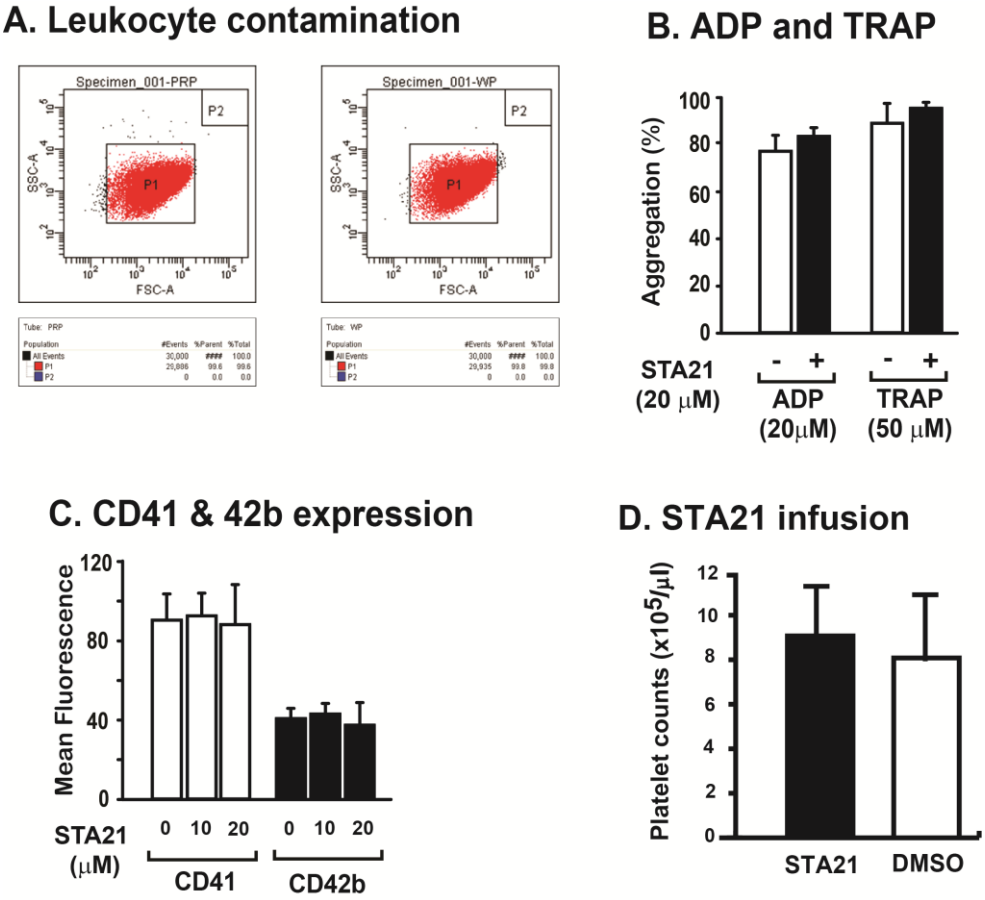
Histochemistry for β-galactosidase

After euthanasia of mice with CO_2, the IVC was ligated and sectioned distally. The animal was perfused via the right ventricle first with 10 ml of PBS and then 10 ml of 0.2% glutaraldehyde in Buffer A (EGTA 5 mM, $MgCl_2$ 2 mM, 0.1M K-Phos buffer pH 7.3). Femora and tibiae were dissected and bone marrow was flushed and smeared onto slides, and air dried. The kidney, intestines, spleen, liver, and heart were dissected and fixed overnight in 0.2% glutaraldehyde solution at 4ºC. Fixed tissues were dehydrated in 30% sucrose, migrated into OTC, and then frozen in OTC. Cryosections (10 μm) and bone marrow smears were incubated 5 min at room temperature in 0.2% glutaraldehyde solution, washed in sodium deoxycholate (0.01%) and Nonidet O-40 (0.02%) in Buffer A, and then incubated overnight at 37ºC in Buffer A containing 0.5 mg/ml of X-gal, 10 mM of $K_3Fe(CN)_6$, 10 mM of $K_4Fe(CN)_6$, 0.01% of sodium deoxycholate, and 0.02% of Nonidet O-40. After washings, slides were counterstained with eosin and nuclear fast red and mounted.

Mouse model of thrombosis

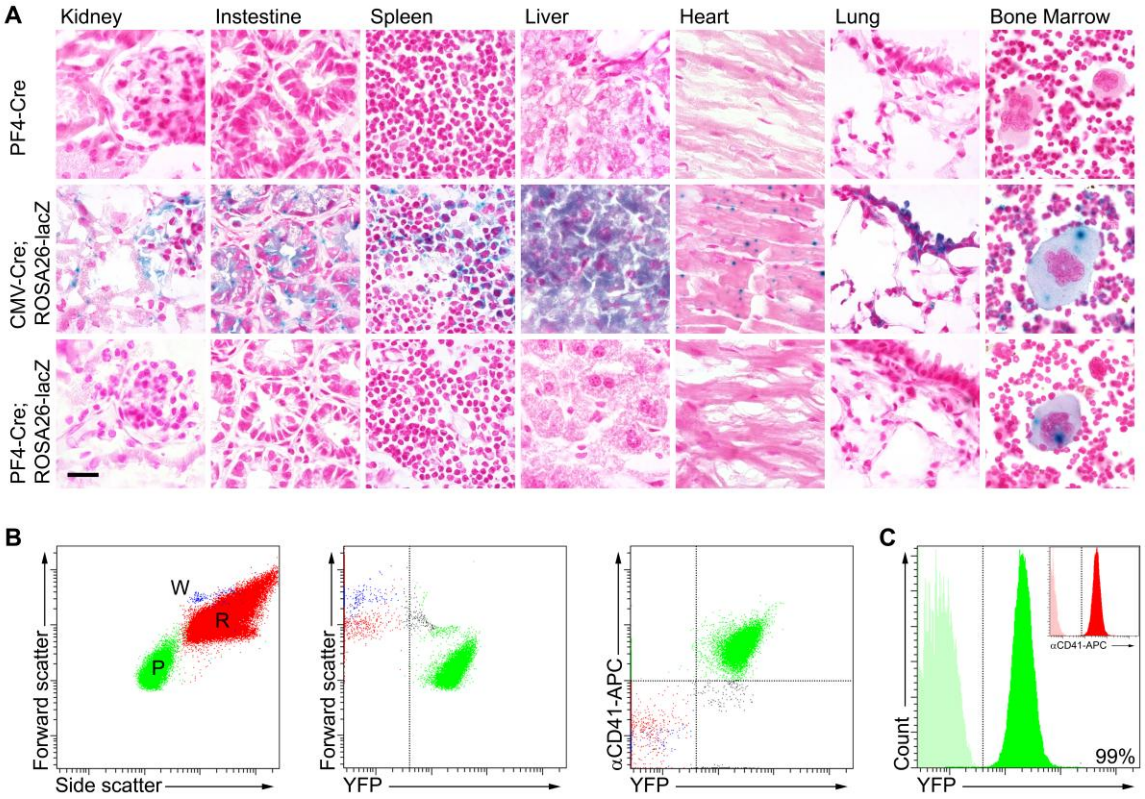
Male C57BL/J6 mice, ~25 – 30g in weight and 12 – 16 wks of age, were anesthetized with pentobarbital (50 mg/kg), underwent tracheotomy, received a jugular venous catheter, and were positioned on a custom tray to carefully expose the cremaster muscle under an upright microscope (Olympus BX50, 40X NA 0.8 water-immersion objective) (*34, 35*). Following a 30-min equilibration period, FITC-dextran (150 kD, 10 ml/kg of a 5% solution) was injected via the venous catheter and allowed to circulate for ~10 min. After vascular diameter (NIH Image 1.6 public domain software) and mean blood velocity (Optical Doppler velocimeter, Cardiovascular Research Institute, Texas A&M University) were recorded, photochemical injury was induced by exposing ~100 μm of vessel length to epi-illumination (0.6 W/cm^2). Time to onset of forming a platelet thrombus and time to flow cessation due to thrombotic vessel occlusion were recorded. The investigator performing the animal experiments was blinded with regard to the treatments or mouse strains.

SUPPLEMENTAL FIGURES AND LEGENDS



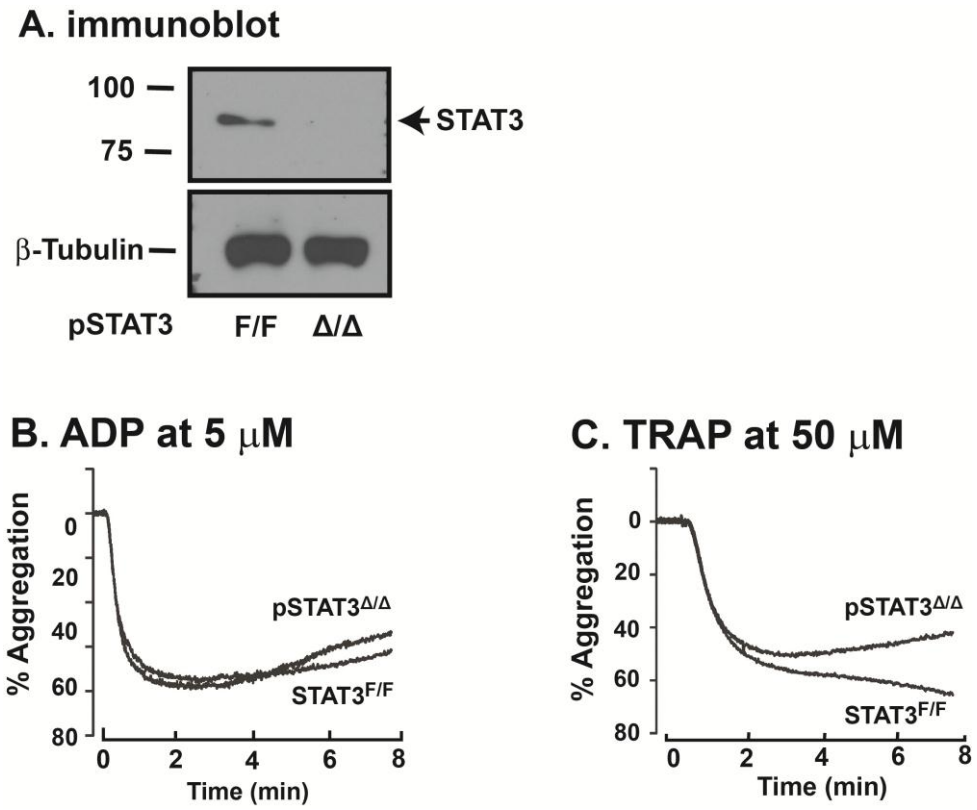
Supplemental Figure 1: The potential contamination of leukocytes in PRP and washed platelet preparations were determined by flow cytometry. Platelets were first incubated with CD45 for 10 min at room temperature and analyzed first for CD45 positivity and then on particle size **(A)**. Window P2 shows CD45 positive cells that are the size of leukocytes on a forward scatter plot. The bottom panels show counts of platelets (PI window) and leukocytes (P2 window). The plots were representatives PRP (left) and washed platelets (right). Platelet aggregation was induced by ADP or TRAP in the presence of STA21 (**B**, n = 6). The surface expression of $\alpha IIb\beta_3$ and GP Ibα on resting platelets treated with STA21 for 60

min at 37°C was quantitatively measured by flow cytometry **(C)**. Platelet counts of mice infused with 20 μM of STA21 daily for 3 days were measured immediately before aggregation and thrombus formation assays (**D**, n = 8).



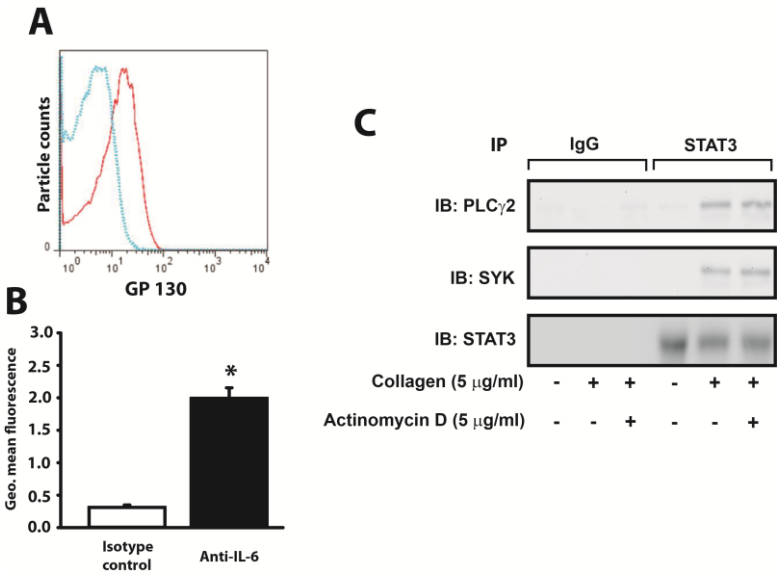
Supplemental Figure 2: Characterization of PF4-Cre mice: PF4-Cre and CMV-Cre mice were crossed with the reporter lines ROSA26-lacZ and ROSA26-eYFP in which β-galactosidase or eYFP was expressed only in cells with Cre activity. (**A**) Bone marrow smears and tissue sections from ROSA26-lacZ, PF4-Cre (top row, negative control), CMV-Cre;ROSA26-lacZ (middle row, positive control) and PF4-Cre;ROSA26-lacZ (bottom row) were incubated with X-gal. β-galactosidase activity reflecting Cre activity was absent in negative controls, ubiquitous in positive controls, and restricted to megakaryocytes in PF4-Cre;ROSA26-lacZ mice (bar = 20 μm). (**B**) Whole blood from ROSA26-eYFP (negative control), CMV-Cre;ROSA26-eYFP (positive control) and PF4-

Cre;ROSA26-eYFP mice was incubated with α-mCD41-APC, α-mCD45-PE-Cy7 and α-mTER119-APC-Cy7 and analyzed by flow-cytometry. The left scattergram shows $CD41^+$ (platelets, P, green), $CD45^+$ (white blood cells, W, blue), and $mTER119^+$ (RBC, R, red) cells. The middle scattergram shows most cells expressing eYFP being platelets. The right scattergram shows most $CD41^+$ cells being $eYFP^+$ (99.3%) and most $eYFP^+$ cells being $CD41^+$ (98.8%). (**C**) Flow-cytometry of PRP from PF4-Cre;ROSA26-eYFP mice (green) shows that most cells are $eYFP^+$ when compared to PRP from ROSA26-eYFP mice (light green). The insert shows that almost all cells were platelets ($CD41^+$, red). The images are representative of 6 mice per line.

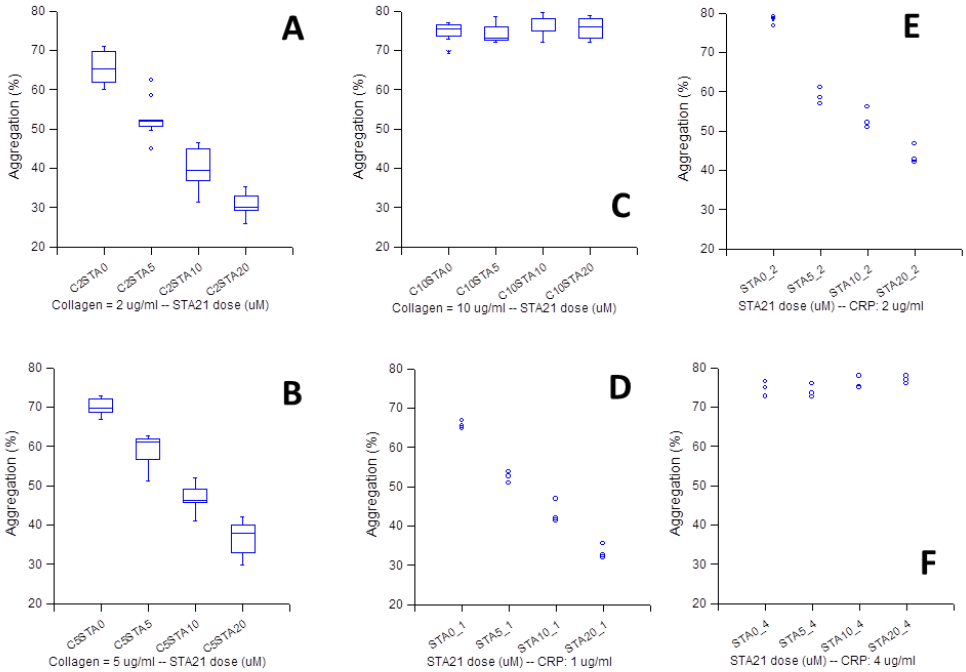


Supplemental Figure 3: Platelet functions of pSTAT3$^{\Delta/\Delta}$ mice:

Platelets from pSTAT3$^{\Delta/\Delta}$ and STAT3$^{F/F}$ mice were lysed, separated on 10% of SDS-PAGE, and probed for STAT3 with a monoclonal antibody; β-tubulin was used as a loading control **(A).** The aggregation of platelets from pSTAT3$^{\Delta/\Delta}$ and STAT3$^{F/F}$ mice was induced by ADP (**B**, n = 26) or TRAP (**C**, n = 26).



Supplemental Figure 4: (A) GP 130 was detected on the surface of human platelets by flow cytometry using a mouse anti-human GP 130 antibody (red line). The isotype control antibody (blue line) was a mouse IgG2. **(B)** Detection of IL-6-sIL-6R complex on human platelets in citrate PRP after incubation with the complex for 10 min at room temperature (Mann-Whitney Rank Sum Test, n = 5, *p<0.001). **(C)** The effect of Actinomycin D on collagen-induced STAT3 interaction with Syk and PLCγ2 in platelets. Washed platelets were first incubated with 5 μg/ml of actinomycin D for 2 hrs at 37^{o}C and then stimulated with 5 μg/ml of collagen for 10 min. Platelets were lysed and platelet lysates were incubated with a STAT3 antibody followed by protein A beads for immunoprecipitation as described in the method section. Co-immunoprecipitated proteins were immunoblotted for Syk and PLCγ2. Non-immune IgG was used as negative control.



Supplemental Figure 5: Dose responses of STA21 on aggregation of human platelets induced by fibrillar collagen at 2 μg/ml **(A)**, 5 μg/ml **(B)**, and 10 μg/ml **(C).** STA21 was also tested for platelet aggregation induced by 1 μg/ml **(D)**, 2 μg/ml **(E)**, and 4 μg/ml **(F)** of CRP.