

## Genetic platelet depletion is superior in platelet transfusion compared to current models

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## Supplemental methods

### Mice

C57BL/6-Gt(ROSA)26Sortm1(HBEGF)Awai/J (Stock No. 007900) (iDTR) mice expressing the simian diphtheria toxin receptor (DTR) (heparin binding EGF like growth factor – HBEGF) were crossed with mice expressing a codon improved Cre recombinase (iCre) under control of the megakaryocyte-specific PF4 promotor (Stock No. 008535)<sup>4</sup> (both The Jackson Laboratory), resulting in iDTR PF4 iCre<sup>+/-</sup> (iDTR<sup>Plt</sup>) mice. iDTR PF4 iCre<sup>-/-</sup> littermates (Cre-negative control mice) were referred to as wild-type (WT). Mice heterozygous for neurobeachin-like 2 (Nbeal2) on a C57BL/6J background were intercrossed to generated Nbeal2<sup>-/-</sup> and Nbeal2<sup>+/+</sup> mice. Genotyping was performed by direct PCR of lysed murine tissue.

Primers: iDTR-common forward: AAA GTC GCT CTG AGT TGT TAT  
iDTR-WT reverse: GGA GCG GGA GAA ATG GAT ATG  
iDTR-mutant reverse: CAT CAA GGA AAC CCT GGA CTA CTG  
PF4-iCre forward: CCA AGT CCT ACT GTT TCT CAC TC  
PF4-iCre reverse: TGC ACA GTC AGC AGG TT  
Nbeal2-WT: CAG GGA GGA TAA CGA GAT AGT CTT  
Nbeal2-mutant: CCT AGG AAT GCT CGT CAA GA  
Nbeal2-common: GTC CTG CTT GAC CTA CCG TC

### Blood Counts

Blood count and hematological parameters of ethylenediamine tetraacetic acid (EDTA) anticoagulated blood were measured by Scil Vet ABC hematology analyzer (Scil animal care company).

### Platelet isolation

For platelet isolation, 500 µl heparinized (25 U/ml) blood, supplemented with 10 % acid citrate-dextrose (ACD, Sigma-Aldrich), was diluted with 300 µl Tyrode's HEPES buffer (TH) (140 mM NaCl, 3 mM KCl, 1 mM MgCl<sub>2</sub>, 16.6 mM NaHCO<sub>3</sub>, 10 mM HEPES, pH 7.4) and centrifuged for 6 minutes at 200 g. Platelet-rich plasma and 2/3 of the erythrocyte fraction were re-centrifuged for 6 minutes at 100 g. Platelets were taken and the centrifugation step was repeated after addition of 300 µl TH to the remaining red cell fraction. Platelets were pelleted for 90 seconds at 1 000 g in the presence of 1/25 volume ACD and 0.5 U/ml apyrase (Sigma-Aldrich) and resuspended in TH.

## **Platelet aggregation**

Platelet counts were adjusted to 400 000 cells/ $\mu$ l in TH. Aggregation in response to 20  $\mu$ g/ml collagen I (Bio/Data) or 25 mU/ml thrombin (Technoclone) was measured by light transmission aggregometry (PAP-8, MöLab). Light transmission was determined between 0 % (naïve platelet suspension) and 100 % (TH buffer). Maximal aggregation was calculated by PAP-8 software.

## **Platelet activation**

Heparinized blood was diluted 1:5 with TH, supplemented with 2 mM  $\text{Ca}^{2+}$  and stimulated with 50  $\mu$ M ADP (Sigma-Aldrich), 75  $\mu$ M Protease activated Receptor 4 agonist peptide AYPGKF-NH<sub>2</sub> (PAR4-AP, Anaspec), 100 ng/ml convulxin (CVX; Santa Cruz) or TH for 15 minutes. After labelling with anti-mouse CD41-BV421 (MWReg30), anti-mouse CD62P-PE/Cy7 (RMP-1) (both Biolegend) and JonA-PE, which recognizes activated GPIIb/IIIa (emfret analytics), for 15 minutes, cells were fixed in 1 % formaldehyde and analyzed using a CytoflexS flow cytometer with Cytexpert 2.2 software (Beckman Coulter). Percentage CD62P<sup>+</sup> and JonA<sup>+</sup> events from CD41<sup>+</sup> events were determined.

## **Platelet-leukocyte-aggregate formation**

Heparinized blood was stimulated with 50  $\mu$ M ADP, 75  $\mu$ M PAR4-AP, 100 ng/ml CVX or TH. Cells were labeled with anti-mouse CD45-PerCP (30-F11), anti-mouse CD11b-PE/Cy7 (M1/70) (both Biolegend) and anti-mouse CD41-BV421 for 15 minutes, fixed for 15 minutes in 1 % formaldehyde and erythrocytes were lysed with 150 mM  $\text{NH}_4\text{Cl}$ , 10 mM  $\text{KHCO}_3$  and 0.1 mM  $\text{Na}_2\text{EDTA}$  for 10 minutes. After centrifugation for 5 minutes at 500 g, cells were resuspended in 1 % formaldehyde and analyzed using a CytoflexS flow cytometer with Cytexpert 2.2 software. Percentage CD41<sup>+</sup> events of CD45<sup>+</sup>, CD11b<sup>+</sup> events were defined as heterotypic aggregates of platelets with neutrophils or monocytes.

## **Immunofluorescence stainings**

For bone marrow stainings, femora of iDTR<sup>Pit</sup> mice were harvested at days 0, 4 and 7 of DT treatment and fixed for 1 hour in 1 % formaldehyde. Fixed bones were incubated for 24 hours in 0.5 M EDTA, 24 hours in 20 % sucrose and 24 hours in 30 % sucrose, then embedded in O.C.T.<sup>TM</sup> compound (Tissue-Tek) and frozen at -20 °C. Sections (30  $\mu$ m) were stained with 1:100 rat anti-mouse endomucin (eBioV.7C7) followed by 1:200 donkey anti-rat DyLight 555 (SA510027) (both Invitrogen), 1:50 anti-mouse CD45-AlexaFluor647 (30-F11) and 1:50 anti-mouse CD41-AlexaFluor488 (MWREg30) (both Biolegend). For peritoneal macrophages, cells were allowed to adhere on ibiTreat  $\mu$ -slide 8 well (ibidi), fixed in 1 % formaldehyde and stained with 1:50 anti-mouse CD45-AlexaFluor647, 1:50 anti-mouse F4/80-FITC (BM8,

Biologend) and Hoechst 33342 (5 µg/ml, Invitrogen). Images were taken with Nikon A1 plus confocal laser-scanning microscope, using a Plan Apo λ 20x objective (Nikon) NA: 0.75. Imaging Software: Nikon NIS-Elements Confocal 4.20.01.

### **Determination of PF4 and anti-DT antibodies**

Heparinized blood was centrifuged for 5 minutes at 3 000 g, followed immediately by 1 minute at 13 000 g. Supernatant was taken and again centrifuged for 1 minute at 13 000 g. Platelet-free plasma (PFP) was stored at -80 °C. PF4 (CXCL4) content was measured from 1:300 diluted plasma with a mouse CXCL4/PF4 DuoSet ELISA (R&D Systems) according to manufacturer's protocol. For relative quantification of anti-DT IgG, a self-made sandwich ELISA was established. Briefly, a microplate (NUNC MaxiSorp, Thermo Fisher Scientific) was coated over night with 1 µg/ml DT in PBS, washed 3 x with PBS containing 0.05 % Tween-20 (PBST), blocked (1% BSA in PBST, 2 hours) and washed again (3 x, PBST). Mouse plasma was diluted 1:1 000 in PBST and added to the plate for 2 hours before washing (3x, PBST) and incubation for 2 hours with HRP-labeled anti-mouse IgG (1:2 000 in PBST; Poly4053, Biologend). Levels of IgG were quantified using TMB substrate (Thermo Fisher Scientific) and measured at 450 nm with an EL808 Ultra Microplate Reader (Bio-Tek Instruments). A standard curve was generated using plasma obtained from an immunized and boosted (2 x 100 ng DT 4 weeks apart) mouse 14 days after boost with an initial dilution of 1:1 000, followed by serial 1:2 dilutions.

### **Recruitment of peritoneal macrophages**

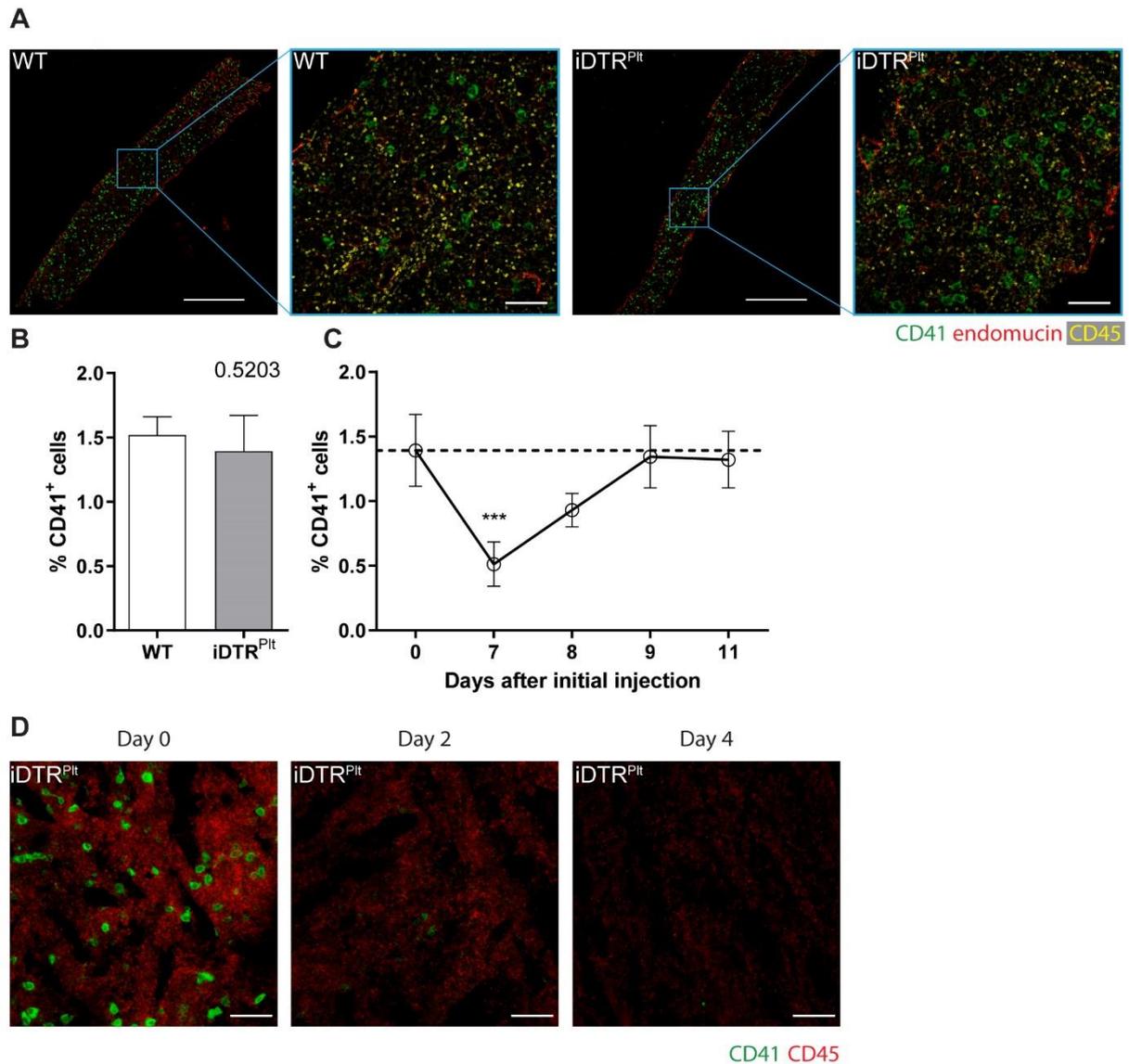
DT treatment was started 7 days and R300 treatment 12 hours prior to transfusion. Washed platelets ( $2.2 \times 10^8$ ) from WT or naïve male iDTR<sup>Pit</sup> mice were injected intravenously, followed by intraperitoneal injection of 1 ml 4 % Thioglycollate (BD bioscience). After three days, recruited macrophages were collected by peritoneal lavage and labeled with anti-mouse CD45-PerCP and anti-mouse F4/80-FITC, fixed in 1 % formaldehyde and analyzed using a CytoflexS flow cytometer with Cytexpert 2.2 software. Leukocytes were defined as CD45<sup>+</sup> and macrophages as CD45<sup>+</sup> F4/80<sup>+</sup> events. Blood collected on days -7, 0 and 3 was stained with anti-CD41-BV421 for platelet counts.

### **Intravital microscopy of FeCl<sub>3</sub> induced thrombus formation**

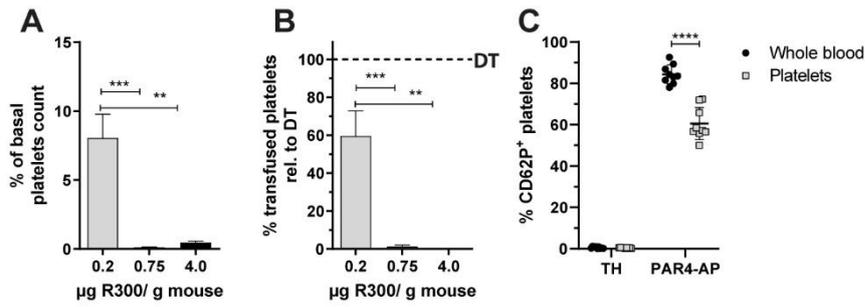
Intravital microscopy was performed as described by Deppermann et al<sup>10</sup>. Five-week-old mice were anesthetized, platelets labelled *in vivo* with anti-GPIIbβ-Dylight488 (X488, EMFRET Analytics), and mesenteric vessels were exteriorized through abdominal incision. Injury of mesenteric arterioles was induced by topical application of one drop 1 M FeCl<sub>3</sub> (Sigma-Aldrich). Thrombus formation in arterioles (35 – 60 µm) was monitored with an

Olympus IX71 microscope (Visitron Systems GmbH) using a CAch N 10x/0,25 PhP FN22 UIS-2 objective (Olympus) and iXON Life (andor) for 40 minutes and time until complete vessel occlusion was determined.

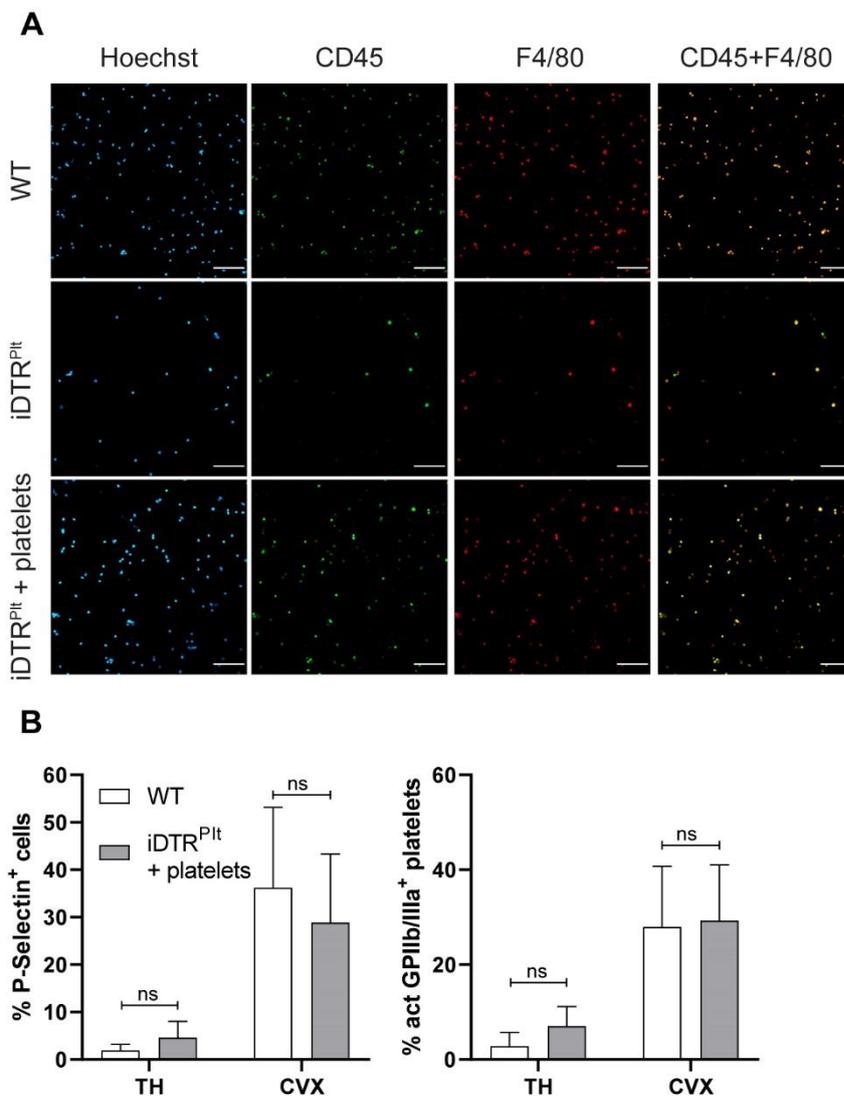
## Supplemental figures



**Figure S1. Bone marrow staining of DT-treated iDTR<sup>Pit</sup> mice.** (A) Femora of untreated WT and iDTR<sup>Pit</sup> mice, stained for megakaryocytes (CD41, green), endothelial cells (endomucin, red), and leukocytes (CD45, yellow). Scale bars: large image = 1 mm; magnification = 100  $\mu$ m. (B) Flow cytometric assessment of % CD41<sup>+</sup> bone marrow cells of untreated WT and iDTR<sup>Pit</sup> mice. (C) Analysis of recovery % CD41<sup>+</sup> bone marrow cells of iDTR<sup>Pit</sup> after treatment for 7 days with DT. n= 3-6. (D) Femora of iDTR<sup>Pit</sup> mice treated for 0, 2 and 4 days with DT. Bone marrow was stained for leukocytes (CD45, red) and megakaryocytes (CD41, green). Scale bar = 100  $\mu$ m.



**Figure S2. Comparison of different depletion antibody concentrations in depletion and transfusion.** R300-antibody was injected at indicated concentrations and (A) percentage of platelet counts, relative to initial values, as well as (B) percentage of transfused platelets, relative to respective DT treated  $iDTR^{Plt}$  mice were determined.  $n = 5-7$ . (C) Comparison of CD62P<sup>+</sup> platelets in whole blood and isolated platelets.  $n = 9$ .



**Figure S3. Activation state of transfused platelets during thioglycollate treatment.** (A) Representative images of peritoneal lavage cells, labeled with anti-CD45 (green) and anti-

F4/80 (red). DNA was stained with Hoechst33342 (blue). Scale bar = 50  $\mu$ m. (B) Comparison of percentage of CD62P<sup>+</sup> and activated GPIIb/IIIa<sup>+</sup> platelets in WT and depleted iDTR<sup>Pit</sup> transfused with platelets. n = 5-9

## Supplemental videos

**Sup Video 1. Thrombus formation is not affected in depleted iDTR<sup>Pit</sup> mice transfused with WT platelets.** Seven days after DT treatment, iDTR<sup>Pit</sup> mice received Nbeal2 WT-control (Nbeal2<sup>+/+</sup>) platelets, followed by induction of a thrombus in mesenteric arterioles by topic application of FeCl<sub>3</sub>.

**Sup Video 2. Thrombus formation is impaired in depleted iDTR<sup>Pit</sup> mice transfused with Nbeal-knockout platelets.** Seven days after DT treatment, iDTR<sup>Pit</sup> mice received Nbeal2 knockout platelets (Nbeal2<sup>-/-</sup>), followed by induction of a thrombus in mesenteric arterioles by topic application of FeCl<sub>3</sub>.