Efficiently restored thrombopoietin production via AMR and IL-6R induced JAK2-STAT3 signaling early after partial hepatectomy

Friedrich Reusswig[1], Nastaran Fazel Modares[2], Marius Brechtenkamp[1], Leonard Wienands[1], Irena Krüger[1], Kristina Behnke[2], Melissa M. Lee-Sundlov[3], Diran Herebian[4], Jürgen Scheller[2], Karin M. Hoffmeister[3], Dieter Häussinger[5], Margitta Elvers[1]*

Supporting Material and Methods

CHEMICALS

Platelets were activated by ADP (Sigma-Aldrich), Thrombin (Roche), CRP (Richard Farndale, University of Cambridge, United Kingdom), PAR-4 activating peptide (AYPGKF, JPT Peptide Technologies) and U46619 (Enzo Life Sciences). Human fibrinogen (Sigma-Aldrich), heparin (Ratiopharm), prostacyclin (Calbiochem), apyrase (Sigma-Aldrich), Complete protease inhibitor cocktail (Roche) were purchased.

PARTIAL HEPATECTOMY

PHx of mice is performed by using a modification of the method of Greene and Puder with two separate ligatures and removal of the gallbladder [1]. To reduce the mild pain from operation mice were treated with Buprenorphine 0,1mg/kg (Temgesic; Indivior UK) i.p. before surgery. Mice were placed into an acrylic glass chamber for anaesthesia induction with an inflow of 3% isoflurane and an oxygen flow rate of 1 l/min. After induction, at a sufficient depth of anesthesia, monitored by pinching the foot, mice were fixed in a supine position on a heated surgical pad. The head was placed in a mask, which is connected to an evaporator with an inflow of 2% isoflurane and an oxygen flow rate of 1 l/min. The abdomen of the

mouse was clipped using an electric shaver and subsequently disinfected. This was followed by an approximately 3 cm long, median laparotomy starting distal and ending briefly proximal of the xiphoid process, thus opening the skin and muscle layers of the abdomen in 2 separate steps. During surgery, the liver is manipulated using saline wetted Q-tips to avoid injury. At first, the *Ligamentum falciforme* is cut using microdissecting scissors. As the second step the left lateral lobe as well as the left and right segments of the median lobe containing the gallbladder are ligated using 4-0 polyester suture tie (B. Braun Surgical, S.A., Rubi, Spain) resulting in 2/3 partial hepatectomy. After ligation, the lobes were resected, and the abdomen was irrigated with 5 ml of preheated sterile saline ensuring the removal of blood as well as decreasing contamination of the abdominal cavity. Sham operated control mice underwent the same surgical procedure without ligating and removing of liver tissue. After the removal of 2/3 of the liver, the abdominal cavity was closed using an interlocking running stitch with 5-0 polyester suture tie. Hypoderm and skin were likewise closed. Subsequently, the animals were weighed to be able to determine a later increase or decrease in weight. The animals received every 6h post operation Buprenorphine 0,1mg/kg via i.p. injection or drinking water (conc. 0.009 mg/ml H_2O).

ADMINISTRATION OF HYPER-IL-6

To induce IL-6 trans-signaling, C57Bl6 mice were injected once with of 20 µg IL-6/IL-6R (hyper-IL-6) protein 24 hours before PHx as described before [2]. As vehicle control group, C57Bl6 mice were injected with a comparable amount of PBS.

PREPARATION OF MOUSE TISSUE

The animals were anesthetized by intraperitoneal administration of ketamine (100 mg / kg body weight) and xylazine (5 mg / kg body weight). As soon as no more pain reflexes were detectable, the abdominal cavity and the thorax were opened. At first the right atrial auricle of

the exposed heart was incised and in a second step ice-cold heparin solution [20 U/ml in PBS – 10 ml] was administered via puncture of the left ventricle to flush the entire circulatory system. Liver and spleen were dissected directly, put in liquid nitrogen, and stored at 80°C for protein and RNA isolation. For histologically purpose parts of the liver, spleen and the femur were also prepared and stored in 4% paraformaldehyde for at least 24h. Subsequently the femur was put into 10% EDTA solution for at least 1 weak for decalcification. Finally, the organs were transferred to paraffin via an ascending alcohol series and stored for histology.

PLATELET PREPARATION AND TOTAL BLOOD CELL COUNT

Platelets were prepared as previously described [3, 4]. Briefly, murine blood from retro-orbital plexus was collected in 300 µl heparin solution [20 U/ml in PBS] and total blood cell counts were analyzed by a hematology analyzer (Sysmex, Norderstedt, Germany). The blood samples were centrifuged at 250 g for 5 minutes at room temperature. To obtain platelet-rich plasma (PRP), the supernatant was centrifuged at 50 g for 6 min. plasma samples were taken after an additional centrifugation step with 650 g for 5 min at room temperature. Sedimented platelets were washed twice at 650 g for 5 min at room temperature and pellet was resuspended in Tyrode's buffer [136 mM NaCl, 0.4 mM Na_2HPO_4, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.1% glucose, 0.35% bovine serum albumin (BSA), pH 7.4] supplemented with prostacyclin (0.5 µM) and apyrase (0.02 U/ml). Before use, platelets were resuspended in the same buffer and incubated at 37°C.

AGGREGOMETRY

Aggregation was measured as percentage light transmission compared to Tyrode's buffer (as=100%) using Chrono-Log dual channel lumi-aggregometer (model 700) at 37 °C stirring at 1000 rpm.

WESTERN BLOTTING

Vasodilator-stimulated phosphoprotein (VASP) phosphorylation was determined in platelets lysates that had been incubated with the platelet guanylyl cyclase activator sodium nitroprusside (SNP), the platelet adenylyl cyclase activator prostaglandin I_2 (prostacyclin, PGI_2) or in vehicle alone (rest). Each sample had a total number of $40x10^6$ platelets, which were sedimented by centrifugation and lysates were prepared, separated on SDS-polyacrylamide gel and transferred onto nitrocellulose blotting membranes. Subsequently, the membrane was blocked using 5% powdered skim milk in TBST (TBS with 0.1 % Tween 20) and probed with appropriate antibody. VASP phosphorylation was detected with phosphorylation specific antibodies (phospho-VASP (Ser157, #3111 Cell Signaling Tech.) and phospho-VASP (Ser239, #3114, Cell Signaling Tech.)); total VASP was detected with polyclonal antibody (#3112, Cell Signaling Tech.). Antibody against β-Actin (#4979, Cell Signaling Tech.) served as loading control.

Liver samples were homogenized following the manufacturer's recommendations for soft tissue in RIPA-buffer (150 mM NaCl; 50 mM Tris-Base; 0.5% NaDeoxycholat; 0.1% SDS; 1% Triton-X; Na_3OV_4 1 mM; 1x protease inhibitor complete (Roche, Germany) pH = 8,0) using a tissue homogenisator (Precellys® 24) and Precellys Lysing Kit (P000918-LYSK0-A). Western Blot was performed as described above, while loading 50 µg total protein per lane, determined by Bradford Assay standard procedure. STAT5 phosphorylation was detected with a phosphorylation specific antibody (Tyr694, #9351, Cell Signaling Tech.); total STAT5 was detected with a polyclonal antibody (#9363, Cell Signaling Tech.). STAT3 phosphorylation was detected with a phosphorylation specific antibody (Tyr705, #9145, Cell Signaling Tech.); total STAT3 was detected with a monoclonal antibody (#12604, Cell Signaling Tech.). JAK2 phosphorylation was detected with a phosphorylation specific antibody (Tyr1007/1008, #3771, Cell Signaling Tech.); total JAK2 was detected with a monoclonal antibody (#3230, Cell Signaling Tech.). Detection of total thrombopoietin was performed using a monoclonal antibody (sc-398525, Santa Cruz.) while GAPDH (14C10, # 2118, Cell Signaling Tech) served as loading control for appropriate protein loading.

The antibody incubation was performed following the manufacturer´s manual by use of peroxidase-conjugated anti-rabbit and anti-mouse IgG (GE Healthcare, Code: NA9340, 1:2500). Immobilon Western Chemiluminescent HRP substrate solution (BioRad) and the Vilber Fusion-FX6-EDGE V.070 system and for quantification of the chemiluminescent signals Evolution-Capt EDGE software (Version 18, 02) were used for visualizing protein bands.

FLOW CYTOMETRY

Flow cytometry analysis was performed as described elsewhere. Briefly, two-colour analysis of murine platelet activation was performed using fluorophore-labeled antibodies for P-selectin expression (Wug.E9-FITC, Emfret Analytics) and the active form of αIIbβ3 integrin (JON/A-PE, Emfret Analytics). Heparinized blood was diluted in Tyrode's buffer and washed twice. Blood samples were mixed with antibodies after addition of 2 mM $CaCl_2$ and stimulated with indicated agonists for 15 min at room temperature (RT). Reaction was stopped by the addition of PBS and samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences). For analysis of glycoprotein surface expression blood samples were mixed with antibodies (GPVI, JAQ1-FITC; GPIb/CD42, Xia.G5 – PE; Integrin α5/CD49e, Tap.A12 – FITC; Emfret Analytics) and incubated for 15 min at room temperature. Up-regulation and activation of integrin expression was detected with antibody against integrin β3 chain (GPIIIa, CD61, Luc.H11 - FITC, Emfret analytics). For determination of platelet size, the geometric mean of the CD42 positive platelets in their forward scatter profile was used.

For detection of the PS exposure Cy™5 AnnexinV (BD Biosciences)–staining was performed while binding buffer (10mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) was used instead of PBS and CD42 was used as platelet specific marker.

~~For detection of vWF binding on platelets, platelets were isolated as described above and diluted to a concentration of 30.000 platelets/µL. Subsequent incubation with anti-vWF antibody (Cat. No. P150-1; Emfret Analytics), anti-GPIbα (GPIb/CD42, Xia.G5 – PE; Emfret Analytics) antibody and botrocetin (Sigma, Cat. No. V5625) was performed for 30 min at 37°C.~~

For detection of desialylated platelets a lectin binding assay was performed. *R. communis* agglutinin-1 (RCA-1, Cat. No.: FL-1081, Vector Laboratories) coupled with FITC was used. The samples were treated for 15 min at 37°C with neuraminidase (Neuraminidase from *Vibrio Cholerae*, Roche) as positive control before incubation with RCA-1 for 15 min. The lectin binding was stopped by the addition of Tyrode's buffer and samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences).

For detection of Neu-1 expression on platelets, unwashed ACD anti-coagulated murine blood samples were diluted 1:2 with Tyrodes buffer after addition of 2 mM Ca^2Cl. Samples were stimulated with indicated agonists or anti-GPIbα antibody (GPIb/CD42, Xia.G5 – FITC; Emfret Analytics) to inhibit vWF-GPIb interaction for 15 min at 37°C in the dark. Platelets were gated according to their specific forward- and side-scatter profile and the MFI of Neu-1-PE antibody (Cat. No. sc-166824; Santa Cruz Biotechnologies) binding was analyzed.

For detection of reticulated platelets, blood samples were prepared as described above. Washed blood samples were incubated with Thiazole Orange (ReticCount, BD Biosciences) for 30 min at RT while CD42 was used as a platelet specific marker. CD42 positive platelets were gated accordingly to their forward- and side-scatter profile and reticulated platelets were analyzed as described in Wu *et al [5].* For analyzing and processing of the FACS data FlowJO Single Cell Analysis v10 Software was used.

THROMBUS FORMATION ASSAY

Cover slips (24 x 60 mm) were coated with 200 µg/ml fibrillary type I collagen (Nycomed) and blocked with 1% BSA. Mice were anesthetized with isoflurane and blood was taken from the retro-orbital plexus and collected in a tube containing 300 µl Heparin (20 U/ml in PBS). Subsequently, the heparinized whole blood was filled in a 1 ml syringe and perfused over a collagen-coated surface at shear rate of 1000 and 1700 s^{-1} and platelet adhesion and aggregate formation were evaluated in six different microscopic areas (x 40, Carl Zeiss).

QUANTIFICATION OF PLASMA/SERUM PARAMETERS BY ELISA

Prostacyclin (PGI_2) and NO metabolites nitrate and nitrite in heparinized plasma of BDL mice and sham controls was determined 1, 3, 7 and 14 days after partial hepatectomy following the manufacturer's protocol (PGI_2-ELISA: Cat. No. CSBE13698m, CUSABIO BIOTECH Co., LTD (); NO-ELISA: Cat.No. KGE001, R&D Systems Europe, Ltd., UK) TPO in heparinized plasma and serum of wildtype and IL-$6r^{-/-}$ mice was measured in naive and partial hepatectomized mice at indicated time points following the manufacturer's protocol (Cat.No. DY488, R&D Systems Europe, Ltd., UK). The concentration of vWF as well as the bioactivity of neuraminidase-1 (Neu-1) was tested in ACD-anti coagulated plasma of sham and PHx treated mice following the manufacturer's protocol (Mouse Von Willebrand Factor A2 ELISA-Kit, Cat. No. ab208980 (abcam); Neuraminidase Assay Kit, Cat. No. ab138888, (abcam)).

QUANTIFICATION OF SERUM BILE ACID LEVELS

Bile acids and their glycine and taurine derivatives were analyzed by UPLC-MS/MS. The system consisted of an Acquity UPLC-I Class (Waters, UK) coupled to a Xevo-TQS tandem mass spectrometer (Waters, UK) which is equipped with an ESI source in the negative ion mode. Data were collected in the multiple reaction monitoring (MRM) mode.

QUANTITATIVE REAL-TIME PCR

For transcriptome analysis total RNA was isolated from livers of WT, *IL-6r*$^{-/-}$ and *Asgr2*$^{-/-}$ mice before and after PHx at indicated time points. Liver tissue samples were homogenized in 500µl cold TRIzol as described above. 100µl chloroform was added to the lysates and they were centrifuged for 15 min at 18000 g and 4°C for phase separation. The upper aqueous phase was collected and mixed with 1.5 volume of 100% ethanol for DNA precipitation. Afterwards, the samples were loaded on RNeasy columns and the procedure was followed according to the manufacturer's protocol of the RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA concentration was measured with an Eppendorf BioPhotometer® D30.

A total of 200 ng RNA was used for cDNA generation (InPromII Reverse transcription System; Promega; #A3800). Quantitative Real-time PCR was performed by using Fast Sybr Green Master Mix (Life Technologies, Carlsbad, CA, United States) following the manufacturer's protocol. The expression level of the target genes was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels as housekeeping gene.Following oligonucleotide primers were used: GAPDH for′GGTGAAGGCGGTGTGAACG′; GAPDH rev′CTCGCTCCTGGAAGATGGTG′; TPO for'CACAGCTGTCCCAAGCAGTA; TPO rev'CATTCACAGGTCCGTGTGTC'; IL-6R for'CTGCCAACCTTGTGGTATCAG'; IL-6R rev'GCAGCAAGTAGTAACTCGGGT; ASGR2 for'GCTCAGTGGCGATGATGAAC'; ASGR2 rev'AGAGGCGCTGAGGAAAGG'; ASGR1 for'ACGTGAAGCAGTTAGTGTCTG'; ASGR1 rev'CCTTCATACTCCACCCAGTTG'. The evaluation of the data was carried out as relative quantification according to the ΔΔCt method.

HISTOLOGY

For the analysis of platelets in the liver, megakaryocytes (MKs) in the spleen and in the bone marrow of the femur of partial hepatectomized mice, five-micron sections of paraffin-embedded tissue were prepared. For the MK's, hematoxylin and eosin staining was

performed using a common HE-staining protocol. For each mouse (n=1) at least three spleen and bone marrow sections were stained and three to five pictures per section were taken and the MKs per visual field were counted. For immunofluorescence staining of platelets, paraffin-embedded liver sections of PHx and sham operated mice were stained with an anti-GPIbα monoclonal antibody (rat anti mouse GPIbα (CD42b), Emfret Analytics, # M042-0, 1:100) followed by an Alexa-Fluor 594 labeled secondary antibody (goat anti rat, invitrogen, 1:250). Nuclei were identified using DNA staining with 4,6 diamidino-2-phenylindole dihydrochloride (DAPI, Roche, 1:3000). For platelet sequestration into the spleen, a GPIX (rabbit anti mouse GP9, biorbyt, Cat. No.: orb167288, 1:50) polyclonal antibody followed by an Alexa-Fluor 555 labeled secondary antibody (1:200) was used.

BLEEDING TIME

The animals were anesthetized by intraperitoneal administration of ketamine and xylazine as described above and the tail was cut off at 3 mm from the tip with a scalpel. The tail was immersed in saline (37°C). The time from incision to cessation of bleeding was recorded (no blood flow for 1 min) as described previously [6].

$FECL_3$-INDUCED INJURY OF THE CAROTID ARTERY

The right *A. carotis communis* was prepared and placed into a flow probe (Transonic Systems, 0.5 PSB, AD Instruments). Before starting the measurement, the artery and the surrounding area were moisturized with 0.9% NaCl and blood flow was measured in ml/min. Then a small pad was placed under the artery below the measuring head and the environment of the artery was dried. A 0.5 x 1 mm filter paper (Whatmann No.1) saturated with 10% $FeCl_3$ (Sigma-Aldrich) was placed at the area of the carotid artery below the measuring head for 3 min. After removing the filter paper, the environment of the artery was moisturized with 0.9% NaCl again. Thrombus formation and occlusion of the artery were

recorded by the software until 5 min after a firm occlusion (stopped blood flow) or if there is no occlusion until the measurement reached a duration of 30 min.

~~**STATISTICAL ANALYSIS**~~

~~Data are provided as arithmetic means ± SEM, statistical analysis was made by one way or two way ANOVA and student's paired t test, where applicable using GraphPad Prism Version 7.02. If not stated different, significant differences are indicated in the graphs, while * specifies the difference between PHx versus sham and wildtype versus knock out, respectively, and # specifies the difference between experimental time points of one group.~~

DATA AVAILABILITY

The authors declare that the data supporting the findings of this study are available within the paper and from the authors on request.

Supporting Figure Legends

Supporting Fig. 1. Platelet activation and thrombus formation are unaltered 7 after PHx. (A) Activation of αIIbβ3 integrin on the platelet surface and (B) externalization of P-selectin on the platelet surface 7 days after PHx (n= 6 sham, 9 PHx). (C) Externalization upon platelet stimulation of the β3 integrin subunit 7 days post PHx (n = 3 sham, 5 PHx). (D) Expression of indicated glycoproteins 7 days post PHx on the surface of platelets measured via MFI in flow cytometric analysis (n= 9 sham, 11 PHx). (E) Thrombus formation on a collagen matrix under arterial shear rates with representative pictures ($1000s^{-1}$ and $1700s^{-1}$ n= 3 sham, 5 PHx).

Supporting Fig. 2. Platelet activation and thrombus formation are unaltered 14 days after PHx. (A) Activation of αIIbβ3 integrin and (B) externalization of P-selectin on the platelet surface with indicated agonists (n= 6 sham, 9 PHx). (C) Externalization upon platelet stimulation of the β3 integrin subunit 14 days post PHx (n= 3 sham, 4 PHx). (D) Expression of indicated glycoproteins 14 days after PHx on the surface of platelets measured via MFI in flow cytometric analysis (n= 10 sham, 12 PHx). (E) Thrombus formation on a collagen matrix under arterial shear rates with representative pictures (n= 5/group).

Supporting Fig. 3. Inhibited platelet function via phosphorylation of VASP one day after PHx. (A) Immunoblots show phosphorylation of vasodilator-stimulated phosphoprotein (VASP) at serine 157 and serine 239 in platelets stimulated with 5 µM PGI_2 or 1 µM sodium nitroprusside (SNP) 1 day after PHx. (B) Immunoblots show phosphorylation of vasodilator-stimulated phosphoprotein (VASP) at serine 157 and serine 239 in platelets stimulated with 5 µM PGI_2 or 1 µM SNP 3 days after PHx.

Supporting Fig. 4. Taurin-conjugated bile acids have no effect on platelet aggregation. (A) Composition of total bile acid concentration of C57BL6/J mice 1 day after PHx (n=6). Aggregation experiments were performed using isolated platelets, pre-incubated for 30 min with the indicated bile acids with a concentration of 50 µM for each at 37°C without agitation. (B) Maximum aggregation amplitude 5 min after stimulation with different PAR4 peptide concentrations (n= 5). (C) Maximum aggregation amplitude 5 min after stimulation with different CRP concentrations. (n= 7 for TC/TCDC, n=3 for TDC/TLC). Representative aggregation curves for (D) PAR4 peptide and (E) CRP stimulation are shown.

Supporting Fig. 5. PHx results in enhanced plasma vWF levels accompanied by enhanced vWF-platelet binding and neuraminidase-1 (Neu-1) expression on platelets 24h after operation (A) Expression of Neu-1 was investigated using the specific FSC and SSC scatter profile of platelets by flow cytometry in unwashed whole blood (n=6). (B) Plasma activity of

Neu-1 revealed no differences 24h after PHx compared to sham controls (n=6). (C) PHx leads to enhanced plasma levels of vWF compared to sham controls 24h after operation (n=6). Depicted are mean values + s.e.m.;* indicates statistical difference between sham and PHx, *P<0.05 using ~~*paired*~~ *students t-test.*

Supporting Fig. 6. PHx induces phosphorylation of JAK2 and STAT3 1 day after operation. (A) Immunoblots of total liver cell lysates showing enhanced pJAK2 signal predominantly 1 day after PHx using anti-pJAK2 and anti-JAK2 antibodies. (B) Immunoblots of total liver cell lysates showing enhanced pSTAT3 signal predominantly 1 day after PHx using anti-pSTAT3 and anti-STAT3 monoclonal antibodies. GAPDH was used as loading control.

Supporting Fig. 7. Genetic deletion of IL-6 receptor leads to altered STAT signaling but enhanced TPO expression leading to enhanced splenic platelet sequestration. (A) Immunoblots of total liver cell lysates using anti-pJAK2 and anti-JAK2 antibodies and anti-pSTAT3 and anti-STAT3 monoclonal antibodies. (B) Immunoblots of total liver cell lysates using anti-pSTAT5 and anti-STAT5 antibodies and anti-TPO monoclonal antibody. GAPDH was used as loading control (C) platelet sequestration was examined in spleen sections of WT and *IL-6R*$^{-/-}$ mice in a native state and 1d post PHx. As platelet specific marker GPIX was combined with DAPI staining to distinguish between red and withe pulp of the spleen. (D) Total GPIX positive fluorescence area [IF_{GPIX}/ µm²] of the red pulp of the spleen sections was determined (n= 5) (E) Calculated spleen weight ratio of native and PHx operated *IL-6R*$^{-/-}$ mice (n = 5) (F) Gene expression of *Il-6r* in native *Asgr2*$^{-/-}$ mice was analysed using the $2^{-\Delta\Delta Ct}$ method (n = 5). Depicted are mean values + s.e.m.; # indicates for statistical difference between time points,* indicates statistical difference between WT and *IL-6R*$^{-/-}$; ~~*P<0.05~~ using *Two-Way ANOVA* with *Sidak's post-hoc test (d+e) and* ~~*paired*~~ *students t-test (f).*

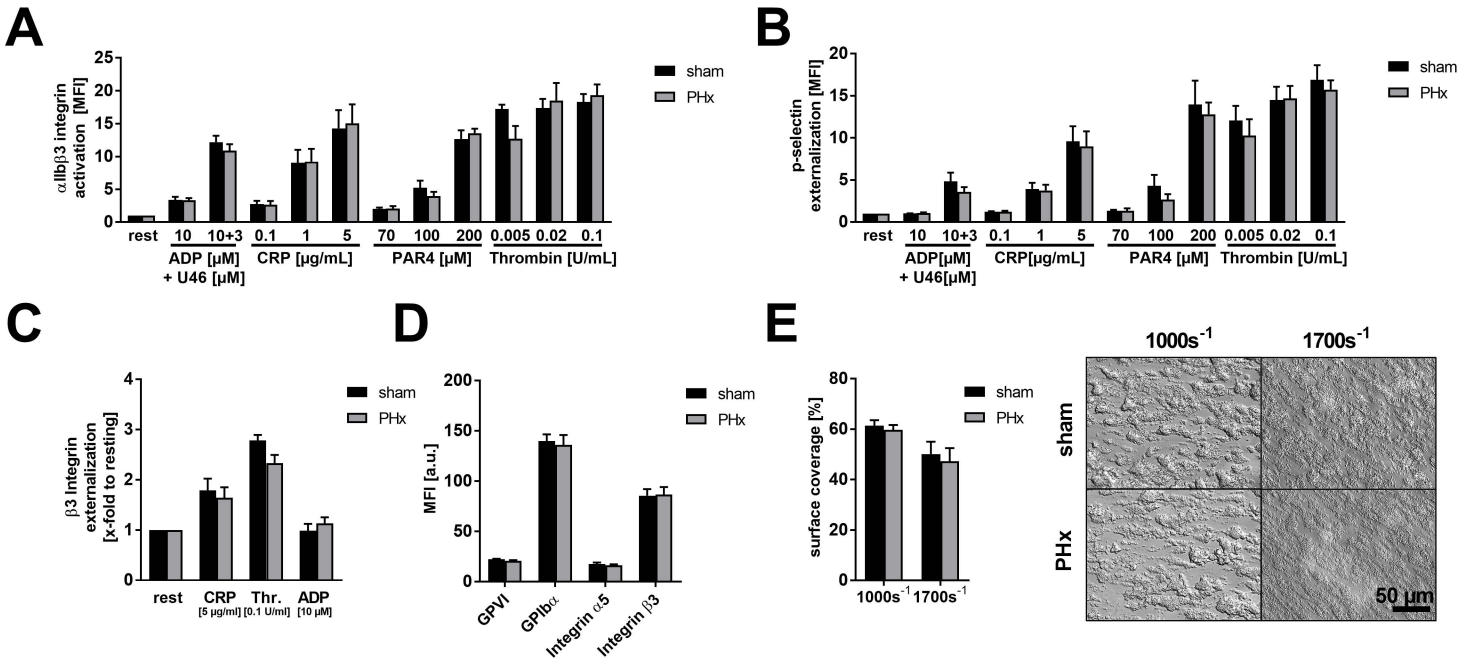
Supporting Fig. 8. Pre-operative administration of hyper-IL-6 results in enhanced TPO plasma concentration and megakaryopoiesis 24h after PHx. (A) Platelet counts of C57Bl6

mice after hyper-IL-6 administration. Mice undergoing PBS administration served as controls (n = 6). (B) Platelet size was measured via flow cytometry using the geometric mean of the FSC signal of GPIb positive platelets 24h after PHx treated with or without hyper-IL-6 (n = 6). (C) Plasma concentration of TPO in PBS controls and hyper-IL-6 mice 24 after PHx (n = 6). (D) Calculated spleen weight ratio of PHx mice after administration of hyper-IL6 or PBS (n = 6). (E) Number of megakaryocytes in paraffin embedded spleen tissue (n = 6) and (F) bone marrow of the femur (n = 6). (G) Representative images from spleen and bone marrow tissue stained with HE. Depicted are mean values + s.e.m.;* indicates statistical difference between experimental groups; *P<0.05 using ~~*paired*~~ *students t-test.*

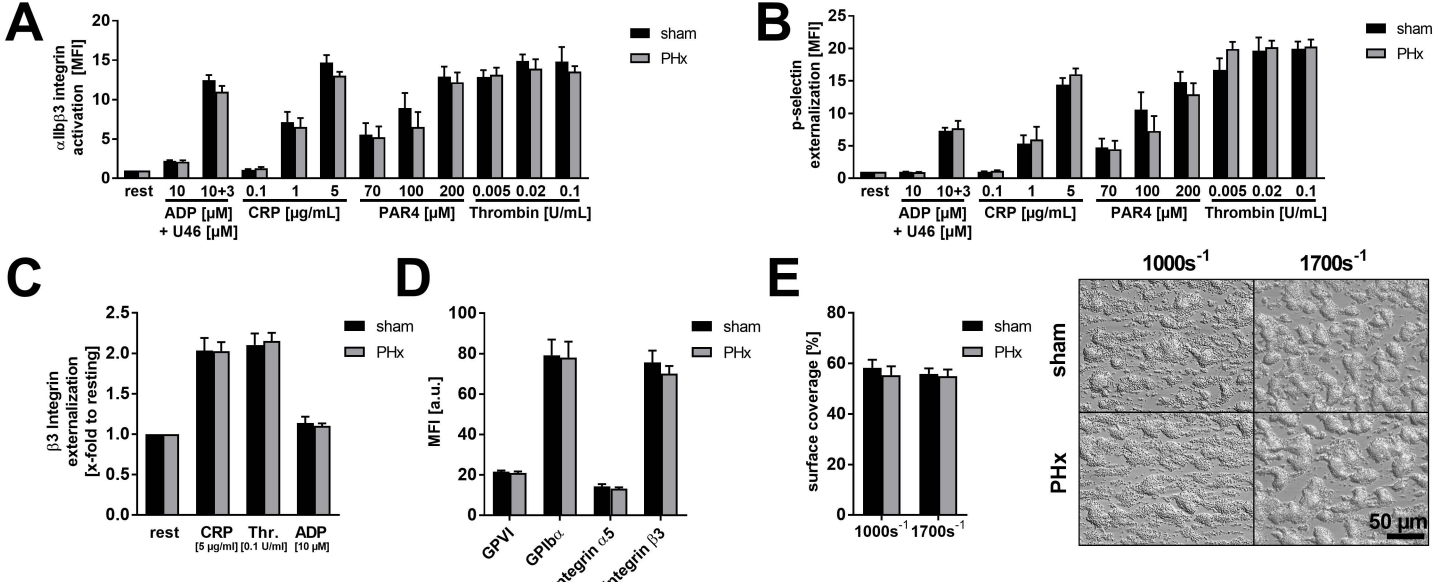
References

1. Greene, A.K. and M. Puder, *Partial hepatectomy in the mouse: technique and perioperative management.* J Invest Surg, 2003. **16**(2): p. 99-102.
2. Behnke, K., et al., *B Cell-Mediated Maintenance of Cluster of Differentiation 169-Positive Cells Is Critical for Liver Regeneration.* Hepatology, 2018. **68**(6): p. 2348-2361.
3. Elvers, M., et al., *Platelet hyperreactivity and a prothrombotic phenotype in mice with a gain-of-function mutation in phospholipase Cgamma2.* J Thromb Haemost, 2010. **8**(6): p. 1353-63.
4. Donner, L., et al., *Platelets contribute to amyloid-beta aggregation in cerebral vessels through integrin alphaIIbbeta3-induced outside-in signaling and clusterin release.* Sci Signal, 2016. **9**(429): p. ra52.
5. Wu, Q., et al., *An elevated percentage of reticulated platelet is associated with increased mortality in septic shock patients.* Medicine (Baltimore), 2015. **94**(19): p. e814.
6. Fotinos, A., et al., *Loss of oligophrenin1 leads to uncontrolled Rho activation and increased thrombus formation in mice.* J Thromb Haemost, 2015. **13**(4): p. 619-30.

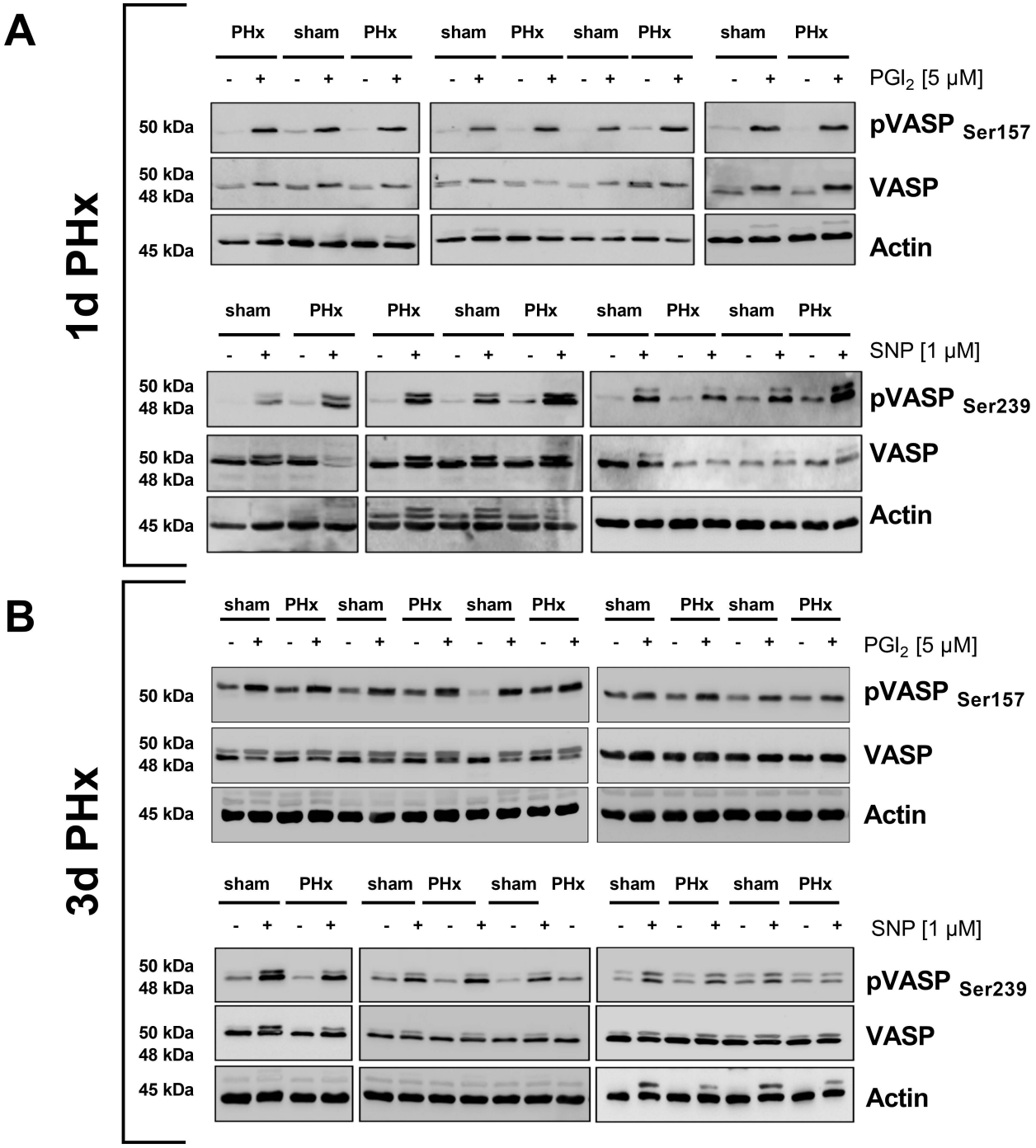
Supporting Figure 1



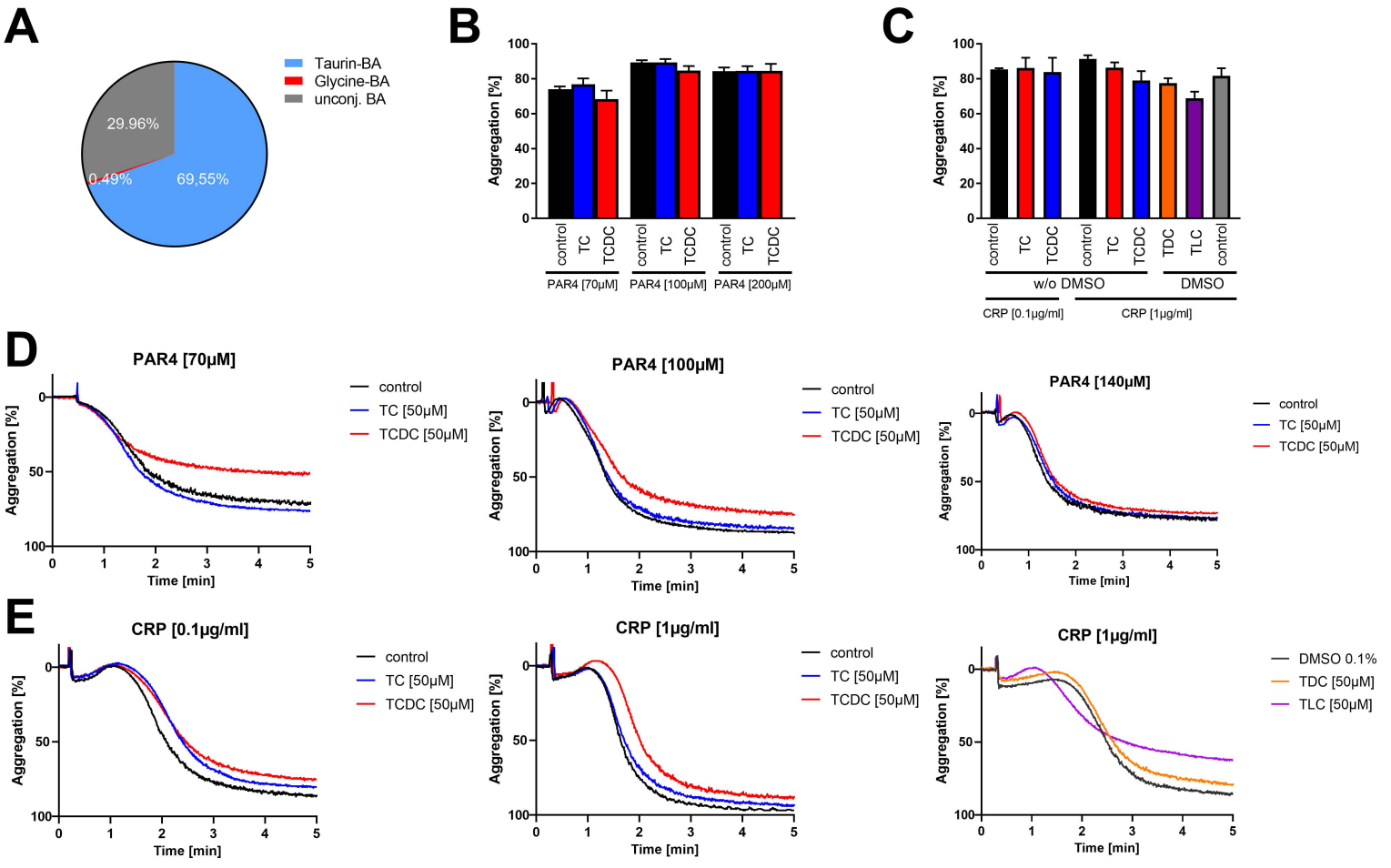
Supporting Figure 2



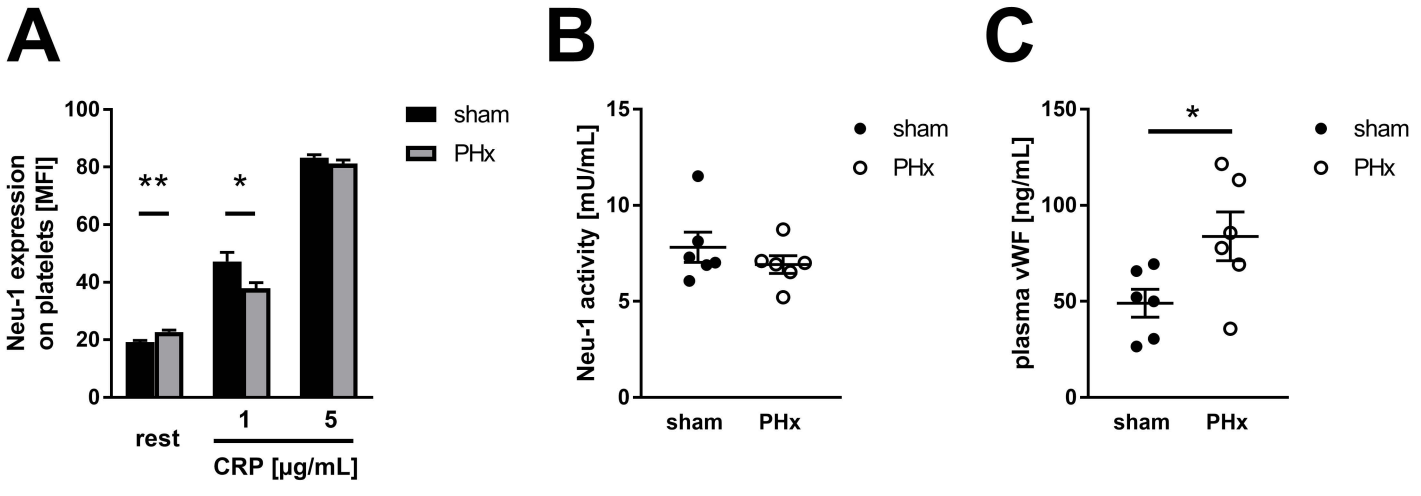
Supporting Figure 3



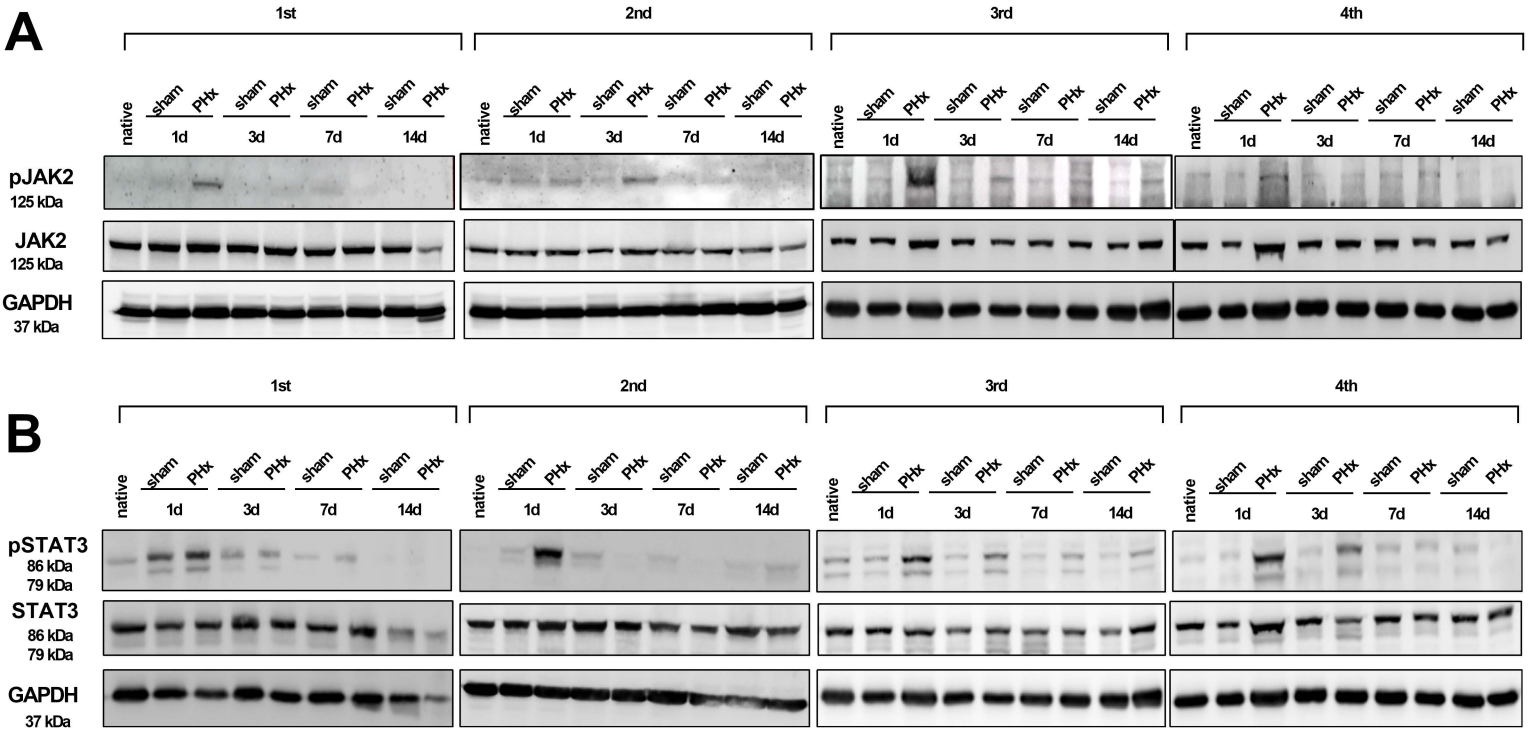
Supporting Figure 4



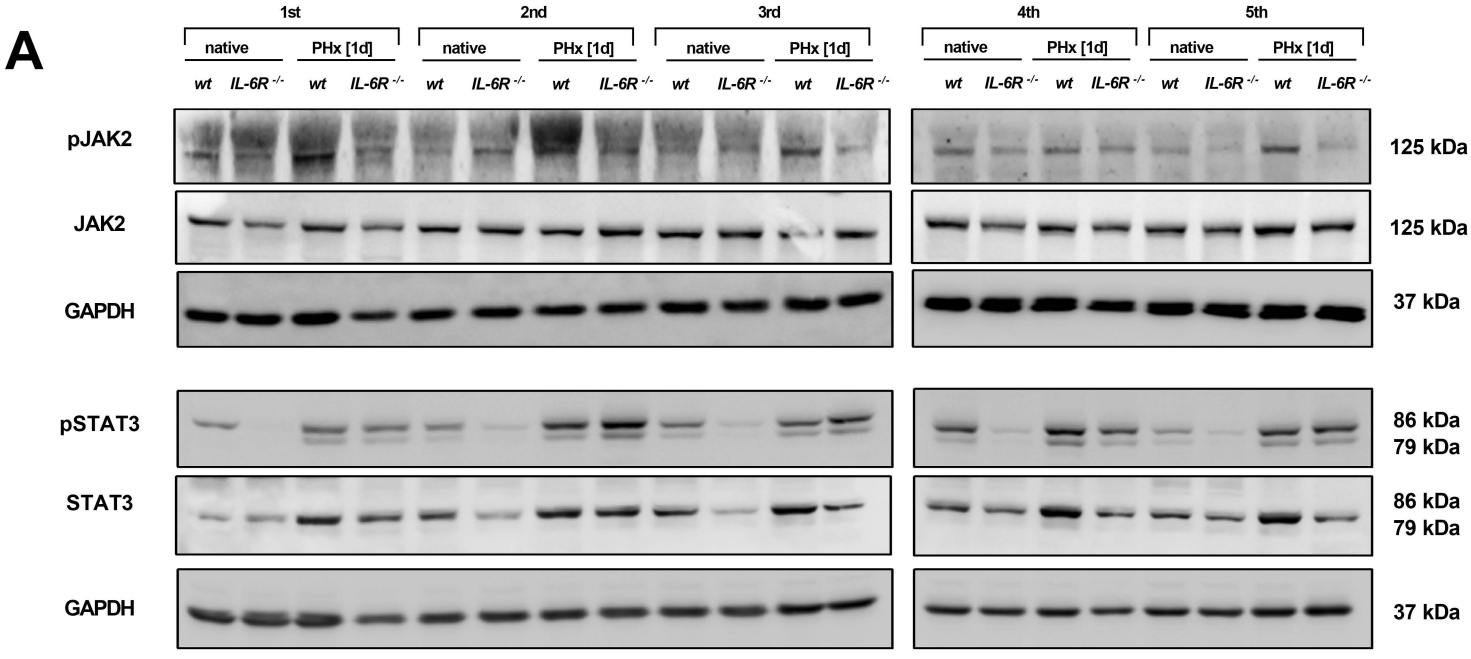
Supporting Figure 5

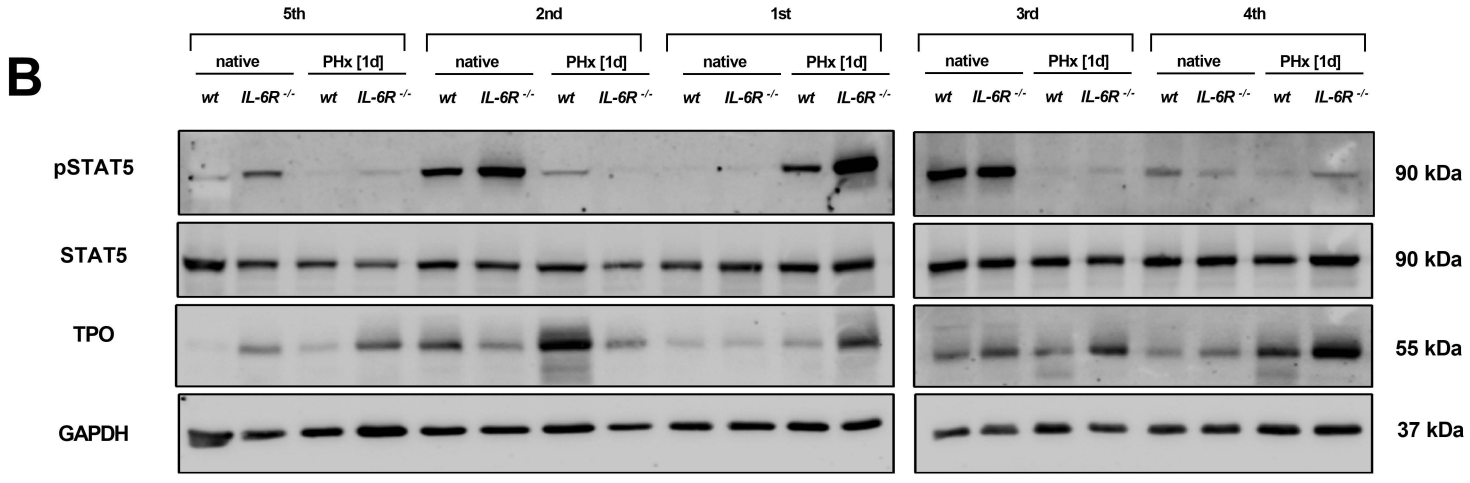


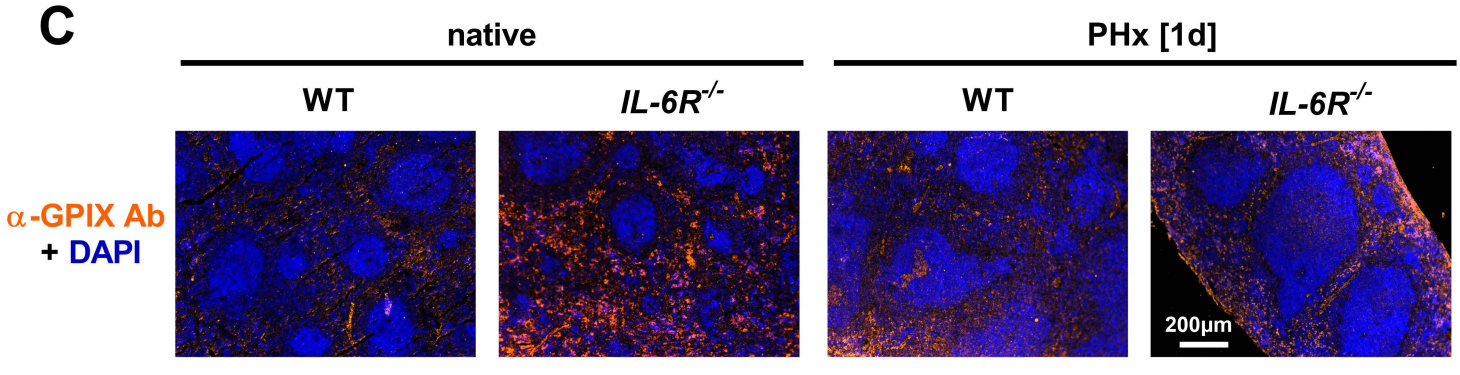
Supporting Figure 6

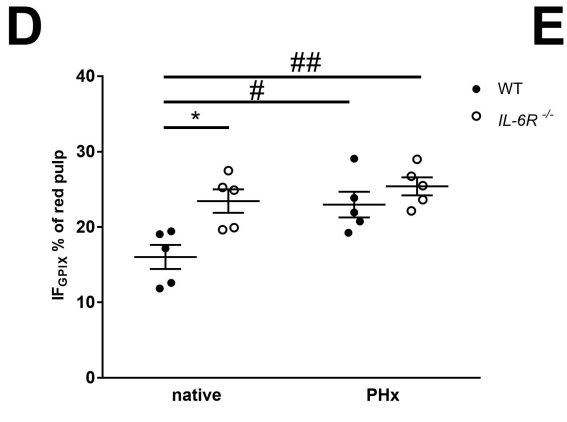


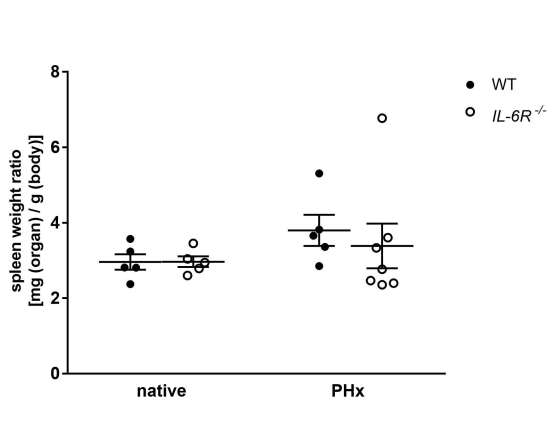
Supporting Figure 7

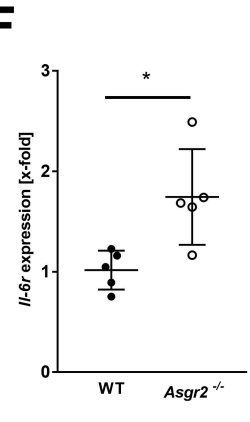












Supporting Figure 8