Supplemental Material

Platelet serotonin aggravates myocardial ischemia/reperfusion injury via neutrophil degranulation

Brief title: Serotonin amplifies myocardial reperfusion injury

Maximilian Mauler, PhD et al.

Supplemental Methods

In vivo serotonin depletion

WT mice received drinking water supplemented with 10mg/kg fluoxetine (Flx, LKT Laboratories, Minnesota, USA) over 3 weeks.

Neutrophil depletion

WT and *Tph1*$^{-/-}$ mice were injected intravenously with either 5mg/kg anti Ly6G antibody (clone 1A8) or isotype control (clone RTK2758; both Biolegend) 12 hours prior to surgery. A second dose was injected 12 hours after surgery.

Antiplatelet therapy

WT mice treated with 10mg/kg Flx or vehicle over 3 weeks received a loading dose of 100mg/kg ticagrelor (AstraZeneca, United Kingdom) by oral gavage 2 hours prior to surgery. 50mg/kg was administered at 12 and 24 hours after surgery.

RNA extraction

Blood

RNA from isolated cells was extracted using RNeasy Micro Kit (Quiagen, Hilden, Germany) according to manufacturers´ protocol.

Tissue Samples

Excised hearts of WT and Tph1-/- mice were put in cold PBS+/+, the area apical of the suture was cut and transferred to a tube containing 500µL TriPure Isolation Reagent (TRIPURE, Roche Diagnostics, Mannheim, Germany) and incubated for 5 minutes. 100µL chloroform (Carl Roth, Karlsruhe, Germany) were added and samples incubated for 10 minutes prior to centrifugation at 12000g for 15 minutes at 4°C. The aqueous phase was transferred to a tube filled with 1µL glycogen (Roche Diagnostics), mixed with 250µL isopropanol (Carl Roth) and incubated for 10 minutes. Samples were pinned down at 12000g for 10 minutes at 4°C, mixed with 500µL 75% ethanol (Carl Roth), centrifuged again at 7500g for 5 minutes at 4°C, and supernatant carefully removed. After 25 minutes the pellet was dissolved in 25µL nuclease free water (Roche Diagnostics) and incubated for 15 minutes at 55°C. After addition of 1µL DNaseI (Quiagen, Hilden,

Germany) dissolved RNA was incubated for 30 minutes at 37°C and subsequently 15 minutes at 70°C. RNA was stored at -80°C until reverse transcription.

cDNA synthesis

Blood

Neutrophil RNA was reverse transcribed and amplified with Ovation PicoSL WTA System V2 (Nugene, Leek, Netherlands) according to manufacturers´instructions.

Tissue

RNA from tissue samples was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Braunschweig, Germany).

Platelet aggregation

Baseline light transmission of 200µL diluted platelet-rich plasma obtained by 10 min centrifugation at 300g ($2.5*10^5$/µL in $PBS^{+/+}$) was recorded on a Aggregation Profiler PAP4 (möLab, Langenfeld, Germany).After 3 minutes, 10µM ADP (möLab) was added to the suspension and aggregation was recorded for 10 minutes.

***In vitro* stimulation assays**

For *in vitro* stimulation blood was incubated with either vehicle, or 200nmol/L C5a, 100nmol/L endothelin 1.5µg/mL lipopolysaccharide (LPS), 50ng/mL epinephrine, 0.1U/mL thrombin, 500nmol/L phorbol 12-myristate 13-acetate (PMA), 10ng/mL tumor necrosis factor alpha (TNFα; all Sigma-Aldrich CHEMIE, Steinheim, Germany), or 20µM adenosine diphosphate (ADP; möLab, Langenfeld, Germany) for 15 minutes.

Quantification of serotonin, MPO, H_2O_2, and NGAL

To obtain plasma, blood was obtained from the retro-orbital venous sinus of mice into tubes containing 1.5µg/µL enoxaparine (Sanofi Aventis, Frankfurt a. M., Germany) and 2µg/mL Prostaglandin I_2 (PGI_2, Sigma-Aldrich CHEMIE, Steinheim, Germany). Human blood samples were obtained by peripheral venous puncture into tubes containing 0.1M sodium citrate (Sarstedt, Nürnberg, Germany) and incubated with 2µg/mL PGI_2. Samples were centrifuged at 600g for 5 minutes, supernatant was transferred and spinned down at 1000g for 5 minutes. Plasma serotonin

was quantified using Serotonin Research ELISA (LDN Labor Diognostika, Nordhorn, Germany) according to manufacturers instructions.
Serum was prepared from whole blood after incubation at room temperature for 1 hour and subsequent centrifugation at 100g for 5 minutes. Supernatant was transferred and centrifuged at 16000g for 15 minutes. Serum serotonin was analyzed using Fast Track Serotonin ELISA (LDN Labor Diognostika, Nordhorn, Germany) according to manufacturers instructions.
Murine MPO concentration was determined in plasma samples using MPO mouse ELISA kit (Hycult Biotech, Uden, Netherlands) according to manufacturers instructions. NGAL was analyzed with Mouse Lipocalin-2/NGAL Quantikine ELISA Kit (R&D Systems, Wiesbaden-Nordenstadt, Germany). Hydrogen peroxide was validated in plasma samples using H_2O_2 Assa Kit (Abcam, Cambridge, UK) according to manufacturers instructions.

Flow cytometry

Heart tissue

Hearts were excised after reperfusion, perfused shortly with 0.9% NaCl to remove remaining blood, and put into cold phosphate buffered saline (PBS, Lonza, Verviers, Belgium). The area apical of the suture was cut and transferred to a petri dish. 1mL of PBS containing 125U collagenase XI, 30U DNase I, 30U hyaluronidase (all Sigma-Aldrich CHEMIE, Steinheim, Germany), 5mmol/L $CaCl_2$, 450U collagenase I (both Biochrom, Berlin, Germany), and 20mmol/L HEPES (Carl Roth, Karlsruhe, Germany) was added and hearts were homogenized using a scalpel. Homogenate was transferred to an Eppendorf tube and incubated on a shaker set to 37°C, 700rpm for 1 hour. Subsequently, cell suspension was grinded through a 40µm cell strainer (Becton Dickinson, Heidelberg, Germany) into a falcon tube. 14mL FACS buffer (PBS without calcium and magnesium (PBS-/-) containing 0.1% bovine serum albumin (BSA, SERVA Electrophoresis, Heidelberg, Germany) were added to the tube and centrifuged at 4°C, 500g for 7 minutes. Supernatant was discarded, cells resuspended in 5 mL FACS buffer and spend down at 4°C, 400g for 5 minutes. After suction of the supernatant, the pellet was resuspended in 100µL FACS buffer containing 1µL anti mouse CD16/CD32 (Fc Receptor block, eBioscience, Frankfurt, Germany) and incubated on ice in the dark for 10 minutes. After addition of 200µL FACS buffer, 90µL of blocked cell suspension was transferred to tubes pre-filled with 10µL antibody mix and incubated on ice in the dark for 30 minutes. Samples were washed two times with 1mL FACS buffer (4°C, 500g for 3 minutes) and finally resuspended in 300µL FACS buffer. Data were acquired on a BD FACSCanto

II (BD Bioscience, Heidelberg, Germany) and analyzed with FlowJo v10 software (Tree Star, Ashland, OR, USA).

Antibody mix contained the following monoclonal anti mouse antibodies 1:100 diluted in FACS buffer: CD41-APC (clone eBioMWReg30), anti-CD19-PE (clone eBio1D3), anti-CD49b-PE (cloneDX5), anti-B220-PE (clone RA3-6B2), anti-NK1.1-PE (clone PK136), anti-CD3e-PE (clone 145-2C11), anti-F4/80-PECy7 (clone BM8), anti-CD45.2-PacificBlue (clone 104; all eBiosceince, Frankfurt, Germany), anti-CD90.2-PE (clone 53-2.1), anti-Ly6C-FITC (clone AL-21), anti-CD11b-APCCy7 (clone M1/70) , anti-Ly6G-PE (clone 1A8; all Becton Dickinson),anti- CD11c-AmCyan (clone N418; BioLegend, Fell, Germany), anti-MHCII-PerCPCy5.5 (clone M5114.15.2; Life Technologies, Darmstadt, Germany).

Flow-assisted cell sorting

12mL lysing solution (Becton Dickinson, Heidelberg, Germany) was added to 1mL freshly collected blood. Samples were incubated for 5 minutes at room temperature and subsequently centrifuged for 5 minutes at 300g. Supernatant was discarded and the pellet was dissolved in 500µL FACS buffer (see above) containing 10µL antibody mix and incubated for 30minutes. 5mL FACS buffer were added and cells were pelleted for 5 minutes at 300g. Supernatant was discarded and cells were resuspended in 500µL FACS buffer. Samples were sorted on a BD FACSAria Fusion III (Becton Dickinson) into RLT buffer (Quiagen, Hilden, Germany) containing 1% β-mercaptoethanol (Sigma-Aldrich CHEMIE, Steinheim, Germany).

Antibody mix contained 1:10 diluted monoclonal antibodies in FACS buffer. The following monoclonal antibodies were used: anti-CD45.2-PacificBlue (clone 104), anti-Ly6C-FITC (clone AL-21), anti-CD115-PE (clone AFS98), and CD41-APC (clone eBioMWReg30; all eBiosceince, Frankfurt, Germany), anti-CD11b-APCCy7 (clone M1/70; Becton Dickinson).

Neutrophils were identified as $CD45.2^{+}$, SSC^{+}, $CD11b^{+}$, $Ly6C^{+}$, $CD115^{-}$ and $CD41^{-}$ cells.

Cell isolation

Femura and spleen were excised after vascular perfusion with 10 ml sterile NaCl. Flushed bone marrow was strained through a 40µm cell strainer using 40mL PBS+/+. Spleens were cut in two pieces, weighted and one piece was grinded through a cell strainer using 20mL PBS+/+. 500µL were used to count total leukocytes in a Neubauer chamber. One femur contains ~5% of all bone marrow cells. Bone marrow cell counts were extrapolated accordingly. Remaining cells were spinned down at 4°C for 5 minutes at 300g. Supernatant was discarded and spleen samples were

incubated with 2mL lysis buffer for 5minutes and filled to 10mL using FACS buffer. Samples were centrifuged at 4°C for 5 minutes at 300g. Spleen and bone marrow cells were resuspended in 3mL FACS buffer. 300µL were stained with 50µL antibody mix on ice in the dark for 30 minutes. Cells were washed once with 5mL FACS buffer (4°C, 300g for 5 minutes) and finally resuspended in 300µL FACS buffer. Data was acquired on a BD FACSCanto II and analyzed with FlowJo v10 software.

Antibody mix contained the following monoclonal anti mouse antibodies 1:100 diluted in FACS buffer: anti-CD45.2-PacificBlue (clone 104), anti-F4/80-PE (clone BM8), anti-Ly6C-PerCO/Cy5.5 (clone HK 1.4), anti-CD3-FITC (clone 145-2C11), anti-CD115-APC (clone CSF-1R), anti-CD19-PECy7 (clone 1D3), and anti-CD11b-APCCy7 (clone clone M1/70; all Biolegend, Fell, Germany).

Protein transport inhibition

Murine or human blood samples were incubated with 0.6µL GolgiStop and 0.8µL GolgiPlug per 1mL Blood (both BD Bioscience, Heidelberg, Germany) and incubated for 15 minutes at room temperature prior to stimulation and processing for flow cytometry (see above).

Electron microscopy

For transmission electron microscopy blood samples treated with either vehicle, or 100µmol/L 5-hydroxytryptamine were fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.2) supplemented with 1% saccharose (Sigma-Aldrich CHEMIE, Steinheim, Germany) overnight. Samples were washed with PBS and contrasted with 2% aqueous osmium tetroxide (Sigma) for 1 hour. Increasing 1-propanol concentration series (30%, 50%, 70% and 90%; Sigma) were used to dehydrate the cells, contrasted with saturated alcoholic uranyl acetate solution (Carl Roth, Karlsruhe, Germany) for 30 minutes at 37°C and embedded in Epon resin. The sections were cut using the Ultracut UCT ultramicrotome (Leica,Wetzlar, Germany) equipped with a diamond knife (Diatome, Biel, Switzerland) and mounted on copper grids for transmission electron microscopy. Samples were analyzed at an acceleration voltage of 120 kV in a transmission electron microscope (JEOL 1400) after contrasting the sections with lead citrate for 1 min. Neutrophils with at least 8µm diameter were identified and photographed under 20000 magnification and granules were counted.

Isolation of cardiomyocytes

WT and Tph1$^{-/-}$ mice were sacrificed, hearts were carefully excised, transferred to a petri dish containing 37°C 0.9% NaCl and the aorta was cannulated using a 22G safety iV catheter with injection port, fixated and connected to the glass ware perfusion system. Temperature in the

perfusion system was set to 37°C and the flow rate was adjusted to 33µL/second. Hearts were digested for 50 minutes using digestion buffer containing (in mmol/L) 0,135 NaCl, 0,004 KCl, 0.001 $MgCl_2$, 0,005 taurine, 0.005 glucose, 0.003 HEPES, 0,01 butanedione monoxime (BDM; all Sigma-Aldrich CHEMIE, Steinheim, Germany), and 2mg/mL collagenase type II (Worthington, Lakewood, USA). After careful removal of the atria hearts were placed in stopping buffer (digestion buffer without BDM and collagenase, supplemented with 5mg/mL BSA) for 2 minutes and subsequently transferred to isolation buffer (digestion buffer without collagenase) and carefully dissembled using fine forceps. The cell suspension was filtered through a 70µm cell strainer and myocytes were allowed to sediment at 37°C for 10 minutes. Supernatant was discarded and cells were carefully resuspended in modified Krebs-Henseleit Buffer (mKHB) containing (in mmol/L) 0,5 EDTA, 10 glucose (both Carl Roth, Karlsruhe, Germany), 5.1 KCl, 0,6 $MgSO_4$, 118 NaCl, 1.2 KH_2PO_4, 1 $NaHCO_3$, and 10 HEPES (all Sigma-Aldrich), 2mg/mL BSA, and 0.4µmol/L Ca^{2+}. Sedimentation was repeated twice with mKHB supplemented with 1µmol/L Ca^{2+} and finally with mKHB containing 1mmol/L Ca^{2+}. Cell count was determined using a Neubauer chamber and 10000 cells were seeded into laminin (Sigma-Aldrich) coated culture plates and maintained in M199 medium (Life technologies, Darmstadt, Germany) supplemented with (in mol/L) 0.005 creatine, 0.005 taurine, 0.002 carnitine hydrochloride, 0.01 HEPES, 2.5% fetal bovine serum (FBS), 1% penicillin-streptomycin (all Sigma-Aldrich), and 0.1% gentamicin (Life technologies). For stimulation assays, M199 medium was supplemented with 1µmol/L or 10µmol/L Serotonin (Sigma-Aldrich). 200µL samples were taken immediately and every 9 hours over two days to evaluate cell viability using CellTiter-Glo Luminescent Cell Viability Assay (Promega, Mannheim, Germany) according to manufacturers instructions. Samples were analyzed on a GloMax 96Microplate Luminometer (Promega).

***Ex vivo* working heart perfusion**

WT and Tph1-/- mice were sacrificed, hearts were excised together with lungs and placed into Krebs-Henseleit buffer (KHB) at room temperature containing (in mmol/L) 128 NaCl, 5 KCl, 1 KH_2PO_4, 1.3 $MgSO_4$, 15 NaHCO3, 2.5 $CaCl_2$ (all from Sigma-Aldrich CHEMIE, Steinheim, Germany) and 5 glucose (Carl Roth, Karlsruhe, Germany). Lungs were carefully removed and isolated hearts were transferred to ice cold KHB. The aorta was cannulated using a 22G safety iV catheter and fixated using a 6-0 prolene suture. Perfusion was carried out using water jacketed glass hardware warmed to 38.5 °C resulting in a final myocardial perfusion temperature of 37 °C. Initial perfusion of hearts was performed in Langendorff mode (50 mmHg perfusion pressure) with KHB

solution saturated with 95% O_2 and 5% CO_2 resulting in pH 7.4. Subsequently, the left atrium was canulated within 1 minute using a 18-gauge metal cannula (B.Braun Melsungen, Melsungen, Germany) and sealed using 4/0 surgical suture (Ethicon, Norderstedt, Germany). After calibration hearts were perfused 1 hour in working heart mode adjusted to a preload of 15 mmHg and 50 mmHg afterload using KHB with 0.4 mmol/L palmitate (Sigma-Aldrich CHEMIE) bound to 3 % BSA. Pre- and afterload were adjusted by positioning the overflow of the oxygenator in a hight of 20 cm and the end of the outflow of the afterload in a hight of 68 cm above the heart.

A pressure catheter (Millar Micro-Tip, Millar Instruments, Houston, TX) was placed inside the aortic cannula to measure aortic pressure. Aortic developed pressure was calculated as the difference of systolic and diastolic pressure. Heart rate was determined by measuring the time interval between peak systolic values. Coronary flow was measured by the effluent dripping of the heart. Aortic flow was measured as by the heart transported buffer dripping into a connected syringe at the end of the afterload.. Cardiac output (ml/min) was calculated as the sum of aortic and coronary flow. Cardiac power (mW/gdHW) was calculated as the product of cardiac output and afterload per dry heart weight. Hydraulic work (J/min*g) was calculated as the product of cardiac output and aortic developed pressure per wet heart weight (WHW).

Myocardial oxygen consumption (MVO_2) was measured every 20 min during working heart mode using a fiber-optic oxygen sensor (Ocean Optics, Orlando, FL) as the difference of percent oxygen concentration in pre- (arterial (aO_2)) and postcardial (venous (vO_2) buffer samples drawn from the apparatus. MVO_2 (µl/min/g) and cardiac efficiency (%) was calculated using the following equations: MVO_2 = (arterial (aO_2) - venous (vO_2)) * (coronary flow/gWHW) * (718/760) * (1000 * Bunsen coefficient). Bunsen coefficient for plasma is 0.0212, and where 718 and 760 mmHg are atmospheric pressures in Freiburg and at sea level, respectively. Cardiac efficiency = hydraulic work/MVO_2 * 100. MVO_2 was converted to µmol/min by multiplying by the conversion factor 0.0393 and then to Joules (J/min) using the conversion of 1 µmol O2 = 0.4478 J. Rate/pressure product was calculated as the product of heart rate and developed pressure.

The flow of buffer was stopped for 20 minutes after 15 minutes of calibration using metal clamps to induce global no-flow ischemia. Subsequently, contractile recovery was monitored over 30 minutes and calculated as percentage of the mean pre-ischemic values.

Histology

WT and *Tph1*$^{-/-}$ mice were sacrificed 24 hours after surgery, hearts were excised and the left ventricle was flushed with 0.9% NaCl (B.Braun Melsungen, Melsungen, Germany). Hearts were cut

at the plane of the ligation, embedded in Tissue-Tek (Sakura Finetek Europe, Alphen, Netherlands) and stored at -20°C. Frozen Hearts were cut in 10µm sections using a Leica CM 1510S cryostat (Leica Biosystems, Nussloch, Germany). Slides were fixated in acetone (Carl Roth, Karlsruhe, Germany) for 5 minutes and subsequently washed in PBS-/- supplemented with tween 20 (PBS/T; Sigma-Aldrich CHEMIE, Steinheim, Germany). Heart sections were circled using a hydrophobic pen, incubated with 100µL 1:10 normal rabbit serum (blocking serum; Vector Laboratories, Burlingame, USA) at room temperature for 1 hour. After washing with PBS/T, 20µL 1:200 diluted anti-Ly6G antibody (clone 1A8, Becton Dickinson, Heidelberg, Germany) was added to each section and incubated at 4°C over night. Slides were washed again and 20µL 1:200 biotynilated rabbit anti rat antibody (Vector Laboratories) was added and incubated for 1 hour. After washing, slides were incubated with Vecastain ABC-AP kit and stained using Vector Red alkaline phosphatase substrate kit II (both Vector Laboratories) according to manufacturers instructions. Reaction was stopped by transferring slides to PBS/T. Cell cores were counterstained using hematoxylin (Carl Roth). Reaction was stopped using tab water and slides were washed with deionized water and embedded in Kaiser´s glycerol gelatine (Merck, Darmstadt, Germany). Microscopic analysis was performed on a Axioplan 2 imaging light microscope by AxioVision 4.8 Software (both Zeiss, Goettingen, Germany) under 20 times magnification. At least five different fields of view of the infarct were photographed in each section, neutrophils were counted and averaged for each section.

Intravital micrscopy

WT and *Tph1*$^{-/-}$ mice were injected with 200ng TNFα (Sigma-Aldrich CHEMIE, Steinheim, Germany) intraperitoneal (i.p.). After 4 hours mice were anesthetized with 100 mg/kg ketamine and 5 mg/kg xylazine and blood cells were fluorescently labeled by retroorbital injection of 50 ml rhodamine 6G (1 mg/mL, Sigma-Aldrich). After median laparotomy a loop of ileum was exteriorized. Mesenteric veins were visualized with an Axiovert 200 M inverted microscope and an AxioCam MRm camera using AxioVision Rel. 4.6 software (Carl Zeiss Microscopy, Jena, Germany). Movies were recorded for 1 minute in 8 veins/mouse and averaged. Leukocyte adhesion was defined as no visible movement for 20 seconds. Movies were analyzed using Zen software (Carl Zeiss).

Under agarose assay

3mL of 5% ultra-pure agarose (ZAGENO, Berlin, Germany) was poured into a 3mm petridish. After the gel was set, one well was punched in the middle of the gel and 4 wells in a distance of 4mm were inserted around the middle well. Gels were equilibrated at 37°C in 5% CO_2 for 1 hour prior to loading. Neutrophils of WT and *Tph1*$^{-/-}$ mice were isolated from blood using Neutrophil Isolation Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Cells were washed with Hanks balanced salt solution (HBSS; Life Technologies, Paisly, UK) and labelled with either Cell tracker green CMFDA (Invitrogen, Carlsbad, CA, USA) or 5-TAMRA-SE (AAT Bioquest, Sunnyvale, CA, USA) according to manufacturers instructions. Cells were counted and $1x10^5$ neutrophils of each genotype were loaded into the 4 outer wells of the agarose plate. 1µM LTB_4 (Sigma-Aldrich CHEMIE, Steinheim, Germany) was added to the middle well. The dish was mounted under a inverted LSM 510 NLO multiphoton microscope (Carl Zeiss Microscopy, Jena, Germany) enclosed in a custom-built environmental chamber that was maintained at 32 °C using heated air and cell movement was recorded over 4 hours.

Flow Chamber assay

Human umbilical vein endothelial cells (HUVECs, Promocell, Heidelberg, Germany) were seeded in perfusion chamber (Vena8 Endothelial+TM Biochips, Cellix, Dublin, Ireland) and grown under flow condition (KIMA pump, Cellix, Dublin, Ireland) following the manufacturer instructions. Briefly, each microchannel of the biochip was coated overnight with 100 µg/ml of human fibronectin (Promocell, Heidelberg, Germany). After the coating period, 2×10^5 cells were added in each microchannel and the biochip was placed at 37°C under 5% CO_2 for 2 hours before connection to the pump. HUVECs were then cultured under flow (alternative periods of flow, 400 µl/min for 3 min, followed by 30 min without flow) for 3 days in endothelial cell growth medium 2 (Promocell, Heidelberg, Germany). For PMNs adhesion experiments, the cells were used either unstimulated or stimulated in endothelial cell growth medium 2 by 100 µM of 5-HT for 45 minutes.

Neutrophil adhesion under flow conditions

Neutrophils at 4×10^6 /ml were perfused with an Exigo pump (Cellix, Dublin, Ireland) for 5 min at a venous shear stress of 0.5 dyne/cm^2 over the microchannels containing confluent layers of HUVECs. Channels were then rinsed with PMN flow buffer for 3 min. Phase contrast images were obtained from at least ten different microscopic fields (Mosaic). Area coverage of PMNs was analyzed off-line using Histolab software (Microvision, Evry,France).

Supplemental Tables

Supplemental Table 1 Taqman probes used to assess gene expression of pro- and anti-inflammatory cytokines in heart tissue after myocardial ischemia reperfusion injury

Name	Genomic location	Target
MPO	Mm01298424_m1	Myeloperoxidase
TNFα	Mm00443258_m1	Tumor necrosis factor alpha
IL-10	Mm00439614_m1	Interleukin-10
IL-1β	Mm01336189_m1	Interleukin-1 beta
IL-6	Mm00446190_m1	Interleukin-6
TGFβ	Mm01178820_m1	Transforming growth factor beta
Hc	Mm00439275_m1	C5a
CCL5	Mm01302427_m1	RANTES
CXCL1	Mm04207460_m1	KC
CXCL2	Mm00436450_m1	Cxcl2
VCAM1	Mm01320970_m1	vascular cell adhesion molecule 1
ICAM1	Mm00516023_m1	CD54

MPO: Myeloperoxidase; TNFα: Tumor necrosis factor alpha; IL: Interleukin; TGFβ: Transforming growth factor beta; Hc: hemolytic complement; CCL5: chemokine ligand 5; CXCL1: C-X-C motif chemokine ligand 1; CXCL2: C-X-C motif chemokine ligand 2; VCAM1: vascular cell adhesion molecule 1; ICAM1: intercellular adhesion molecule 1;

Supplemental Table 2 Sequences of mouse specific primers designed against different serotonin receptor (5-HT-R) subtypes.

Name	Sequence	Target
OMM 1	5′- TACTCCACTTTCGGCGCTTT - 3′	5-HT_{1A}-R forward primer
OMM 2	5′- GGCTGACCATTCAGGCTCTT - 3′	5-HT_{1A}-R reverse primer
OMM 5	5′- ACCTGTTGCACTGCTTCCAT - 3′	5-HT_{1B}-R forward primer
OMM 6	5′- GTGGTCGGTGTTCACAAAGC - 3′	5-HT_{1B}-R reverse primer
OMM 9	5′- TTGGCCAGATCCTGTGTGAC - 3′	5-HT_{1D}-R forward primer
OMM 10	5′- AGTCGGACATCTCCTCGTGA - 3′	5-HT_{1D}-R reverse primer
OMM 15	5′- CGACTCGAGGCTCTACCCTA- 3′	5-HT_{2A}-R forward primer
OMM 16	5′- GGACACTGCCATGATGACCA - 3′	5-HT_{2A}-R reverse primer
OMM 19	5′- AGATCCGTCAGTGGGAAACG - 3′	5-HT_{4}-R forward primer
OMM 20	5′- AGTGACAAGGCTCCACAAGG - 3′	5-HT_{4}-R reverse primer
OMM 28	5′- GCCCTTTATCTGTGGCACCT - 3′	5-HT_{7}-R forward primer
OMM 29	5′- CAAGTTTCAGGGCTTCGTGC - 3	5-HT_{7}-R reverse primer
OMM 38	5′- AATGATTGAGCCCTTGGAGCA - 3′	5-HT_{2C}-R forward primer
OMM 39	5′- CGGAAAGGTTTGGCTGGAAT - 3′	5-HT_{2C}-R reverse primer
OMM64	5′- GCACCACGTCCAATGACAT - 3′	HRP-II forward primer
OMM65	5′- GTGCGGCTGCTTCCATAA - 3′	HRP-II reverse primer

5-HT: 5-Hydroxytryptamine; HRP: Human RNA polymerase;

Supplemental Table 3: Cardiac function in WT and *Tph1*$^{-/-}$ mice over time after ischemia followed by reperfusion.

			d2					d21		
Genotype	**HR (BPM)**	**SV (µL)**	**EF (%)**	**FS (%)**	**CO (mL/min)**	**HR (BPM)**	**SV (µL)**	**EF (%)**	**FS (%)**	**CO (mL/min)**
WT	452.0±23	23.1±3†	34.9±4†	11.5±2†	9.4±1†	451.4±14	23.6±2†	39.1±3†	12.±is 1†	10.9±1†
***Tph1*$^{-/-}$**	478.3±24	42.1±4*	48.6±3*†	20.6±2*†	20.1±3*	476.1±16	31.8±3*†	48.6±2*	19.5±1*†	13.8±1
			d0					**d7**		
WT	443.9±9	41.7±3	62.8±2	39.2±2	16.2±2	432.5±9	16.0±2†	29.2±3†	9.2±2†	8.1±1†
***Tph1*$^{-/-}$**	434.3±10	47.4±2	65.1±1	37.5±2	18.2±1	460±11	27.7±2*†	42.9±3*†	18.1±2*†	12.5±1†

All values are expressed as mean±SEM; FS: Fractional shortening; EF: Ejection fraction; d: day; SV: Stroke volume; HR: Heart rate; (d): diastolic; (s): systolic; CO: Cardiac output; * denotes significant changes between different genotypes; † denotes significance compared to d0 in the respective group; two-way repeated measures ANOVA with post hoc Bonferroni test was applied;

Supplemental Table 4: Characteristics of ACS patients.

Characteristic	ACS (n=15)
Age - yr	71.8±3.3
Sex (% female)	10.5
LVEF (%)	41.4±2.5
CKmax (U/L)	1273±508
Hypertension - no.	11
Diabetes - no.	6
Smokers - no.	6
Statin	9
Serum creatinine (mg/dL)	1.18±0.07
CRP (mg/l)	37.4±12.1
Total Leukocytes (10^6/mL)	11.1±1.3
Platelets (10^3/µL)	233.8±20.4

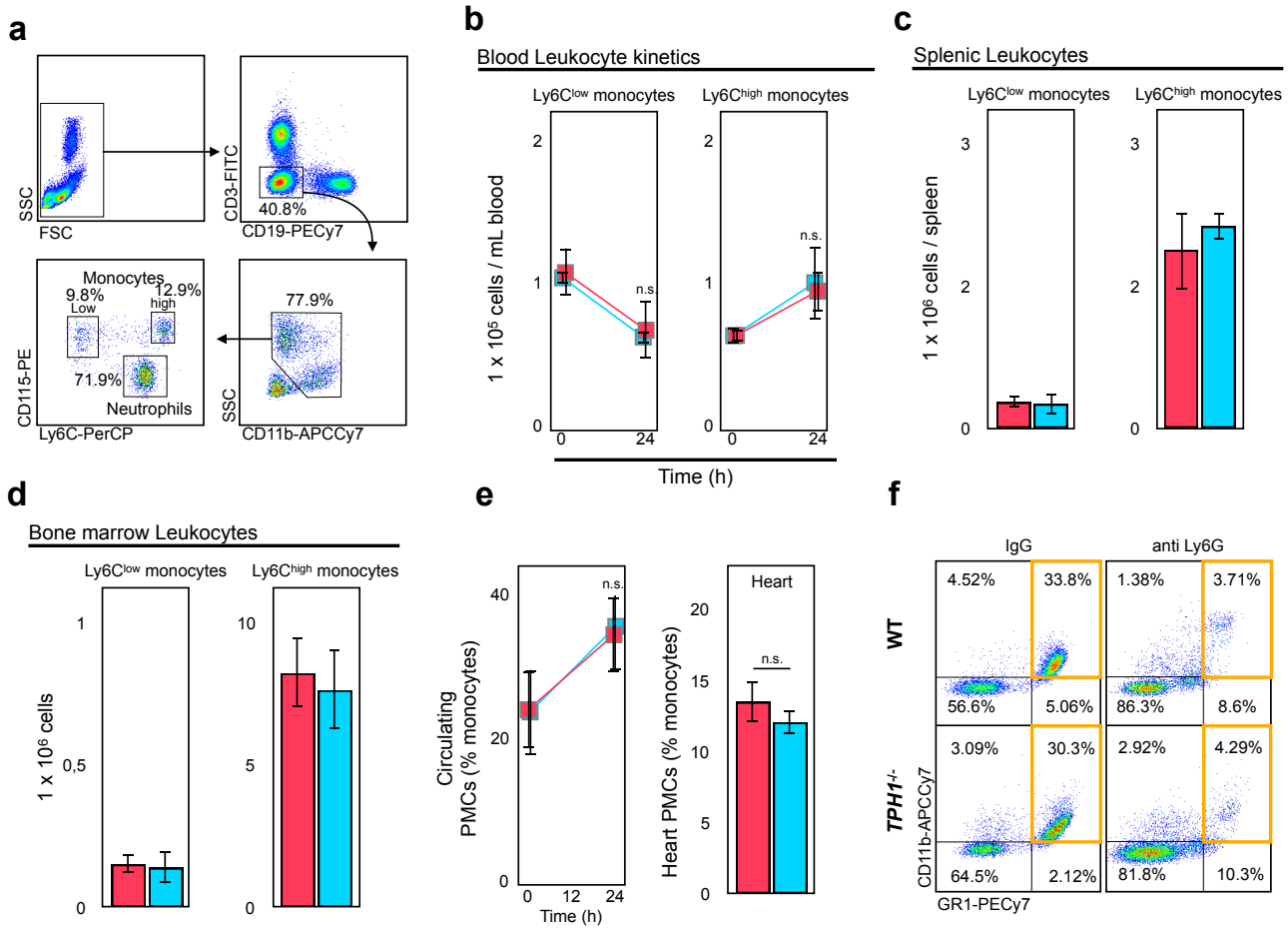
Results are presented as mean ± SD; LVEF: Left ventricular ejection fraction; CK: Creatin kinase; CRP: C-reactive protein;

Supplemental Table 5: Characteristics of depressed patients.

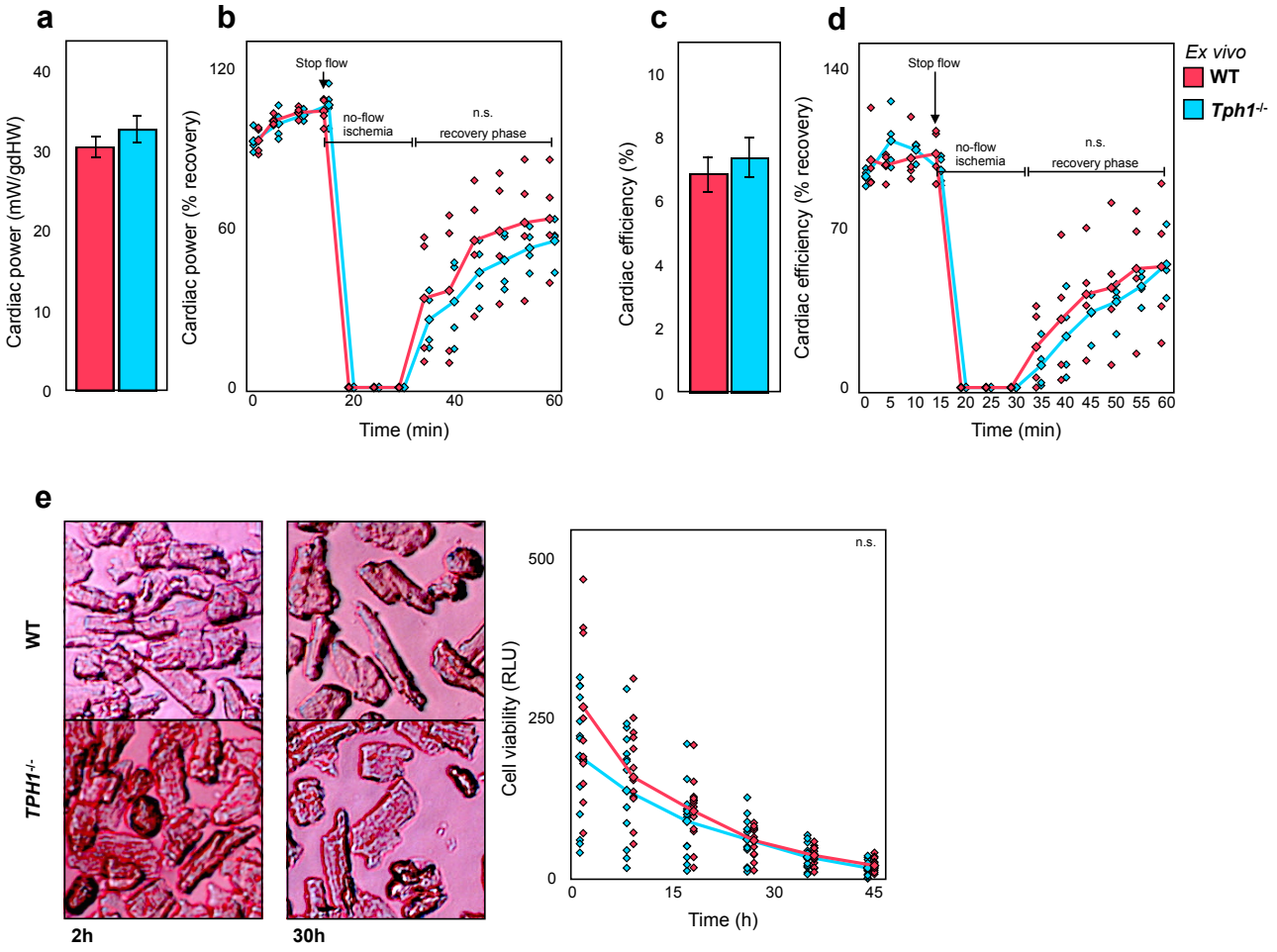
Characteristic	SRI (n=10)	no SRI (n=10)	p-value
Age - yr	46.6±13	45.3±15	n.s.
Sex (% female)	50	50	n.s.
BDI	27.9±12	33.1±8	n.s.
Hypertension - no.	3	3	n.s.
Diabetes - no.	1	1	n.s.
Smokers - no.	n.a.	n.a.	n.s.
Statin	0	0	n.s.
Serum creatinine (mg/dL)	0.88±0.1	0.85±0.1	n.s.
CRP (mg/l)	3	2,9	n.s.
Total Leukocytes (10^6/mL)	6.5±2	7.1±2	n.s.
Neutrophils (10^6/mL)	4.8±9	5.3±10	n.s.
Lymphocytes (10^6/mL)	3.8±10	3.4±9	n.s.
Monocytes (10^6/mL)	0.8±0.2	0.8±1	n.s.
Platelets (10^3/µL)	290±74	274±49	n.s.

Results are presented as mean ± SD, n.s.: not significant; Mann-Whitney U test was used to compare numerical values, Fisher exact test was used to compare categorical characteristics (Sex, Hypertension, Diabetes, Smoker, Statin use). SRI: serotonin reuptake inhibitor; BDI: Beck Depression Inventory; CRP: C-reactive protein

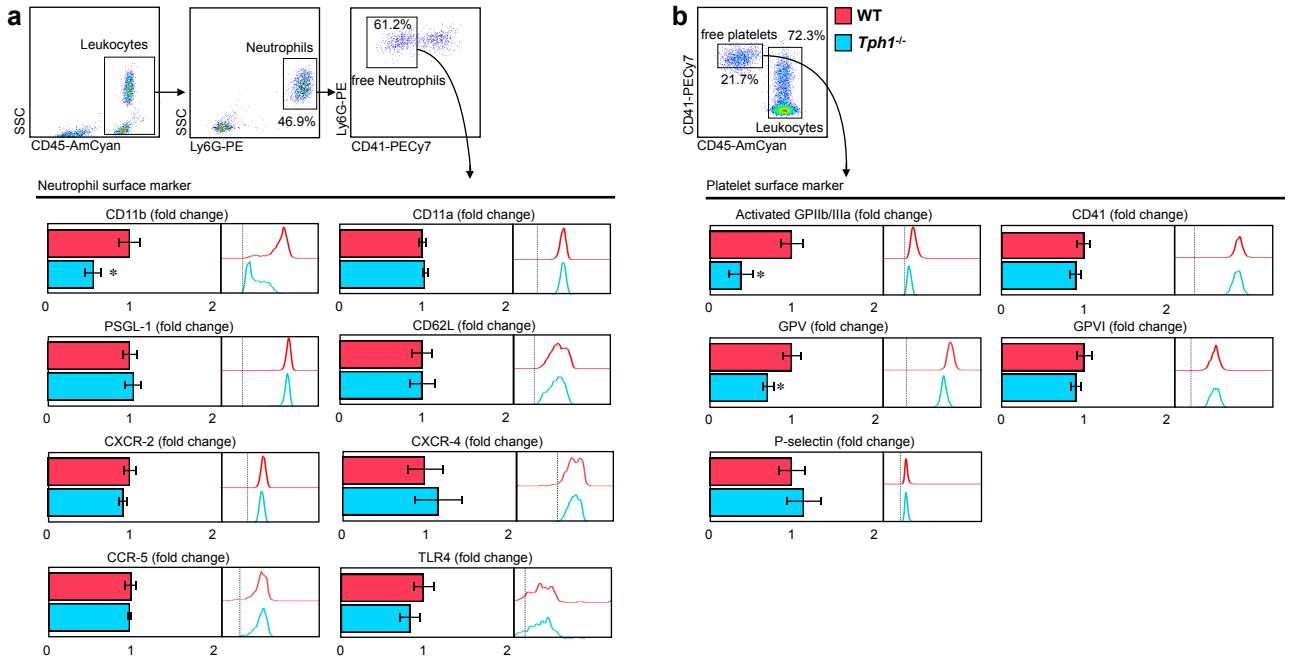
Supplemental Figures



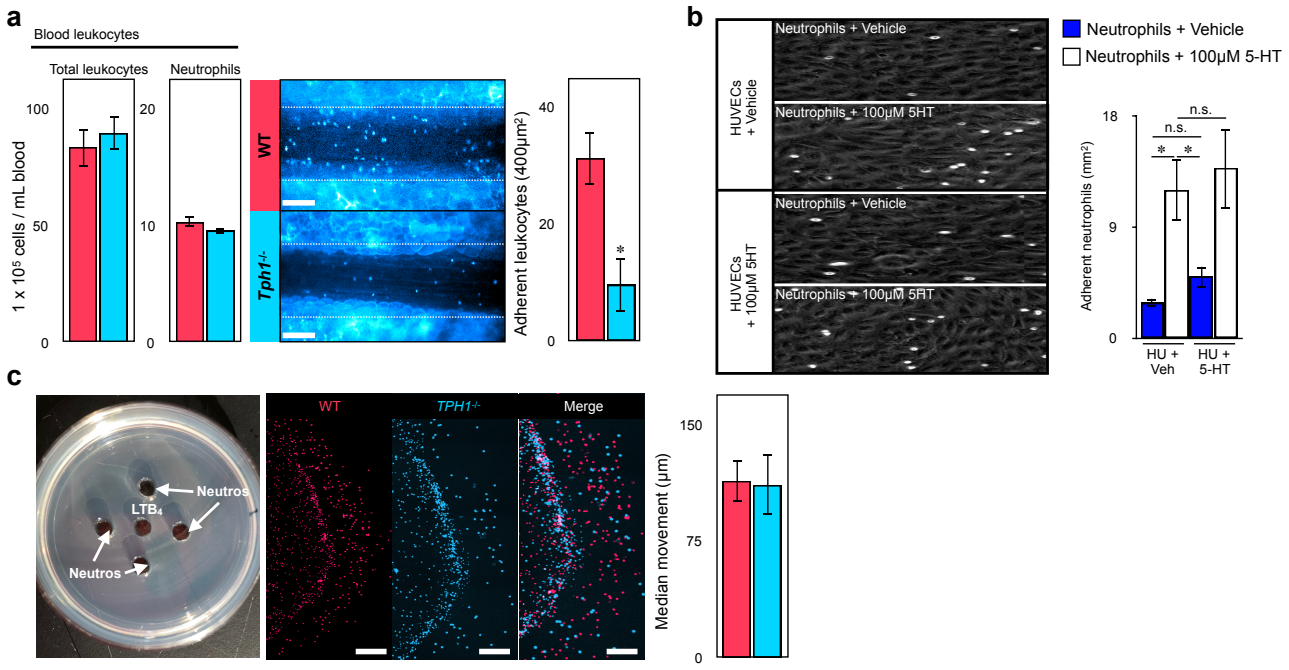
Supplemental Figure 1 Monocyte counts are not affected by TPH1 deficiency. (**a**) Dot plots of the gating strategy to identify monocyte subsets (CD115^{+}, Ly6C$^{low/high}$) in blood, spleen, and bone marrow. (**b**) Quantification of blood monocytes of WT (red) and *Tph1*$^{-/-}$ (blue) mice before and 24 hours after myocardial ischemia with reperfusion. Results are presented as mean ± SEM, n.s., two-way ANOVA with Bonferroni's multiple comparison testing, n≥5 independent animals per group and time point. (**c**) Quantification of splenic and (**d**) bone marrow derived monocytes 24 hours after myocardial I/R injury. Results are presented as mean ± SEM, n.s., Student´s t-test, n≥5 per group. (**e**) Quantification of platelet monocyte complexes (PMC) in WT (red) and *Tph1*$^{-/-}$ (blue) mice in the blood before and 24 hours after myocardial I/R injury (left panel), and in heart tissue at 24 hours after myocardial I/R injury (right panel). Results are presented as mean ± SEM, n.s., two-way ANOVA with Bonferroni's multiple comparison testing (in blood), and Student´s t-test (in the heart), n=6 independent animals per group and time point. (**f**) Representative flow cytometric dot plots of blood leukocytes following neutrophil depletion to identify blood neutrophils.



Supplemental Figure 2 *Ex vivo* heart function and myocyte viability is independent of TPH1 deficiency. (**a**) Baseline cardiac power is similar in WT (blue) and *Tph1*$^{-/-}$ (blue) mice in isolated working hearts. Results are presented as mean ± SEM, n.s., Student´s t-test, n=4 per group. (**b**) Recovery rate of cardiac power in isolated WT (red) and *Tph1*$^{-/-}$ (blue) hearts following 15 minutes of no flow ischemia. Each point represents an independent experiment, Two-way repeated measures ANOVA with Bonferroni's multiple comparisons testing, n=4 per group. (**c**) Cardiac efficiency in isolated hearts at baseline condition (**d**) and during recovery following 15 minutes of no flow ischemia. Baseline is presented as mean ± SEM, n.s., Student´s t-test, recovery phase shows independent experiments, n.s., two-way repeated measures ANOVA with Bonferroni's multiple comparisons testing, n=4 per group and treatment. (**e**) Representative images of isolated cardiomyocytes of WT and *Tph1*$^{-/-}$ hearts at 2 and 30 hours in culture (left panel) and quantification of cell viability over 45 hours. Each point presents an independent experiment, n.s., two-way repeated measures ANOVA with Bonferroni's multiple comparisons testing, n=11 per WT (red) and *Tph1*$^{-/-}$ (blue).

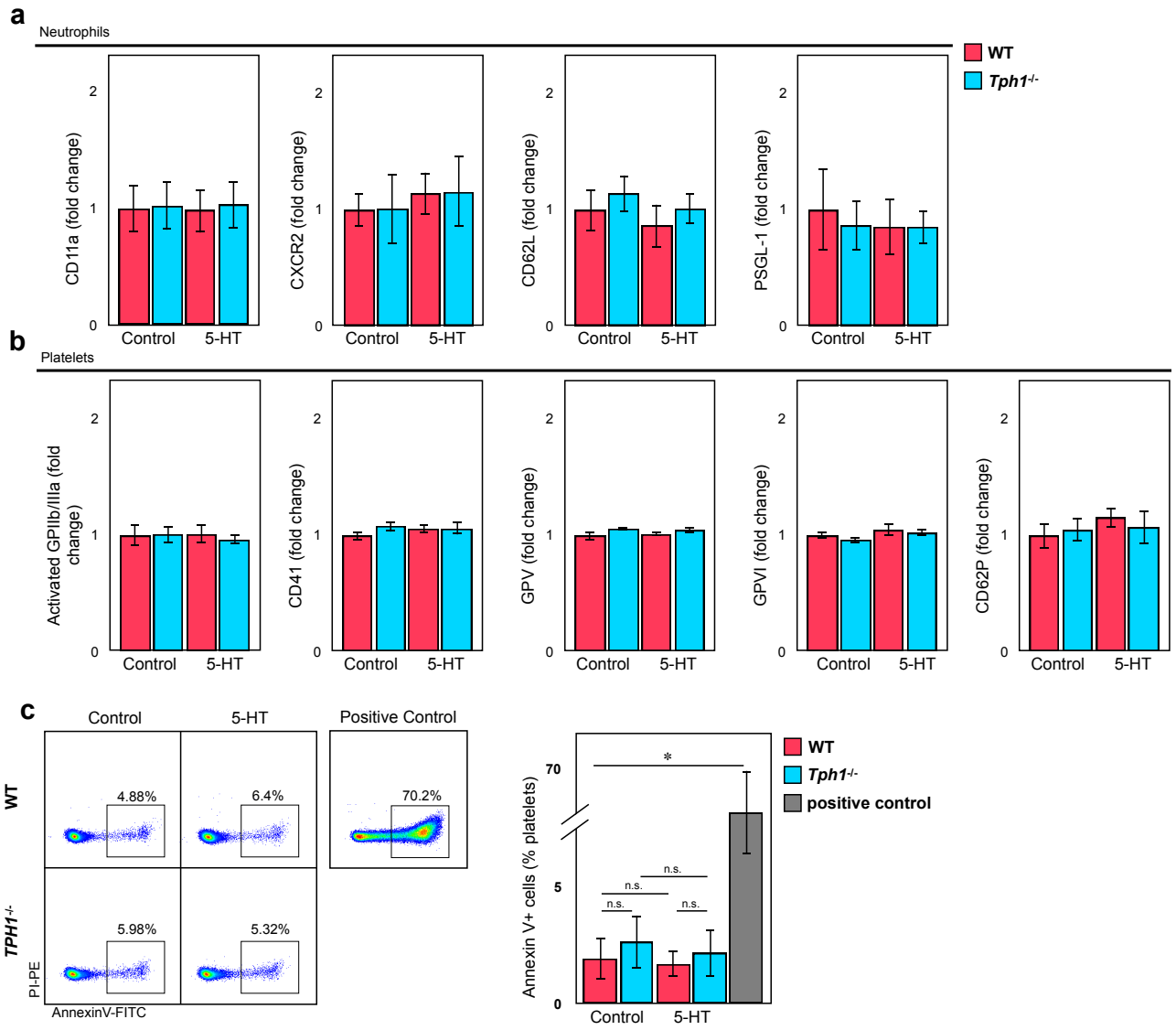


Supplemental Figure 3 Serotonin dependent expression of adhesion molecules on **c**irculating cells during myocardial reperfusion injury. (**a**) Representative dot plots showing gating strategy to identify uncomplexed (free) blood neutrophils ($Ly6G^+$, $CD41^-$)(top panel), and quantification of CD11b, CD11a, CXCR-2, CXCR-4, CCR-5, TLR-4, CD62L and PSGL-1 surface expression on free neutrophils in WT (red) and *Tph1*$^{-/-}$ (blue) mice 24 hours after myocardial I/R injury (lower panel). Results are presented as normalized mean ± SEM, *p<0.05, Student´s t-test, n=10 per group. (**b**) Representative dot plot of uncomplexed (free) platelets ($Cd41^+$, $CD45^-$) in blood (top panel), and quantification of activated GPIIb/IIIa, GPV, GPVI, and P-selectin expression in WT (red) and *Tph1*$^{-/-}$ (blue) mice 24 hours after myocardial I/R injury (lower panel). Results are presented as normalized mean ± SEM, *p<0.05, Student´s t-test, n=10 per group. Bar graphs are on the left accompanied by representative histograms of respective markers on the right.

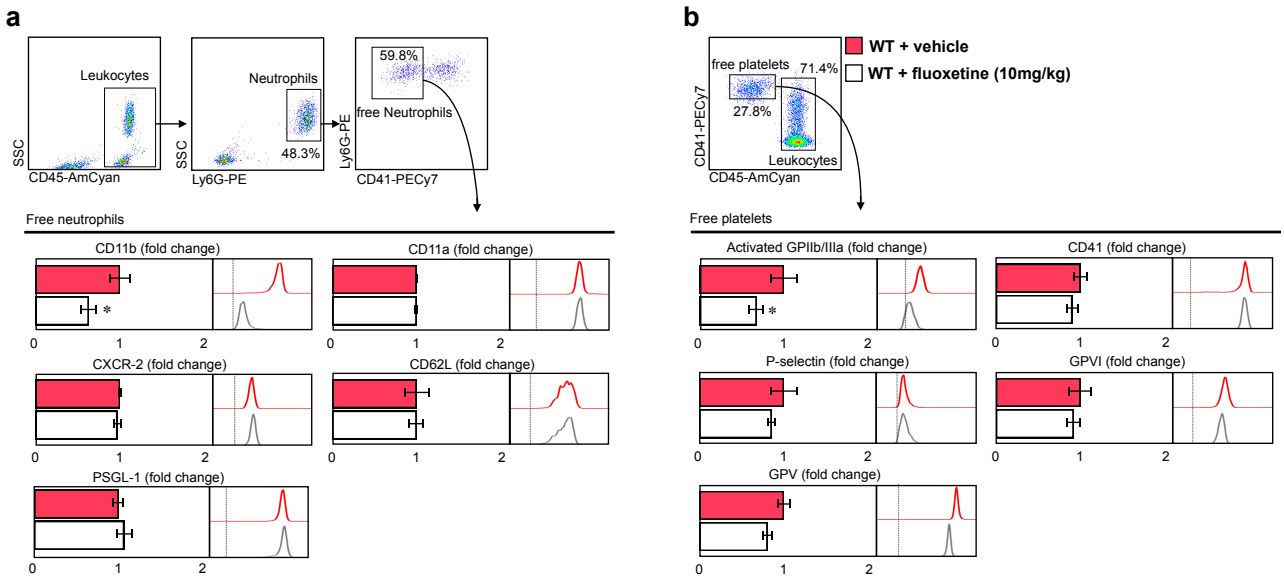


Supplemental Figure 4

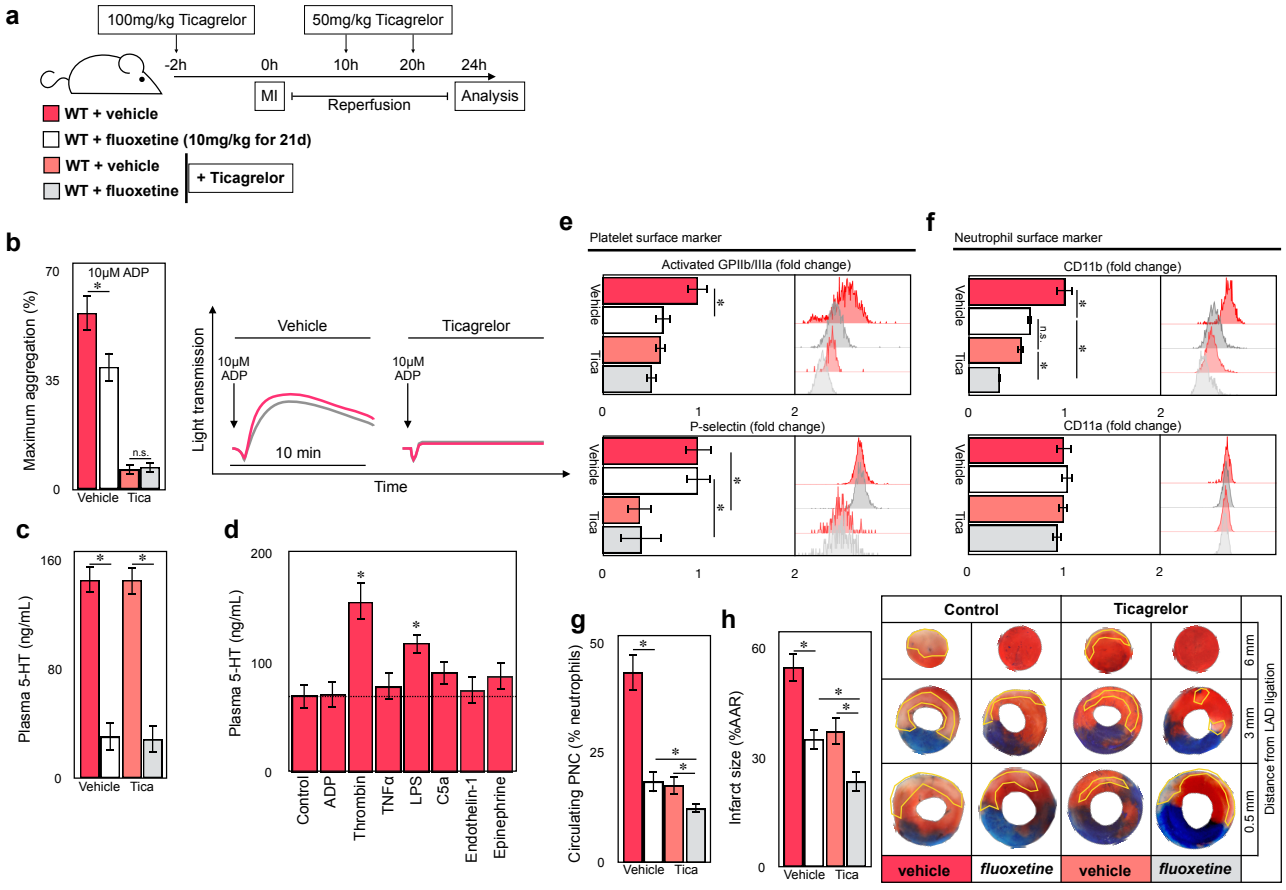
Chemoattractant capabilities of neutrophils are unaffected by serotonin depletion *in vitro*. (**a**) Total blood leukocyte and neutrophil count of WT and *Tph1*$^{-/-}$ mice 4hours after stimulation with TNFα (left panels). Representative images of mesenteric veins showing leukocyte endothelial interactions and quantification of firmly attached cells (right panel). Scale bar: 100µm. Results are presented as mean ± SEM, *p<0.05, Student´s t-test, n=12 per group from four independent experiments. (**b**) Representative images of human neutrophils (bright) adherent to endothelial cells under flow. Quantification of adherent neutrophils following stimulation of endothelial cells or neutrophils with 5-HT (white) or vehicle (blue). Results are presented as mean ± SEM, two-way repeated measures ANOVA with Bonferroni's multiple comparisons testing, n=8 per group from 6 independent experiments. (**c**) Under agarose assay and representative images of WT (red) and *Tph1*$^{-/-}$ (blue) neutrophils 4 hours after addition of the chemoattractant LTB_4 and quantification of median travel distance. Scale bar: 200µm. Results are presented as mean ± SEM, *p<0.05, two-way ANOVA with Tukey, n=24 per group from 8 independent experiments.



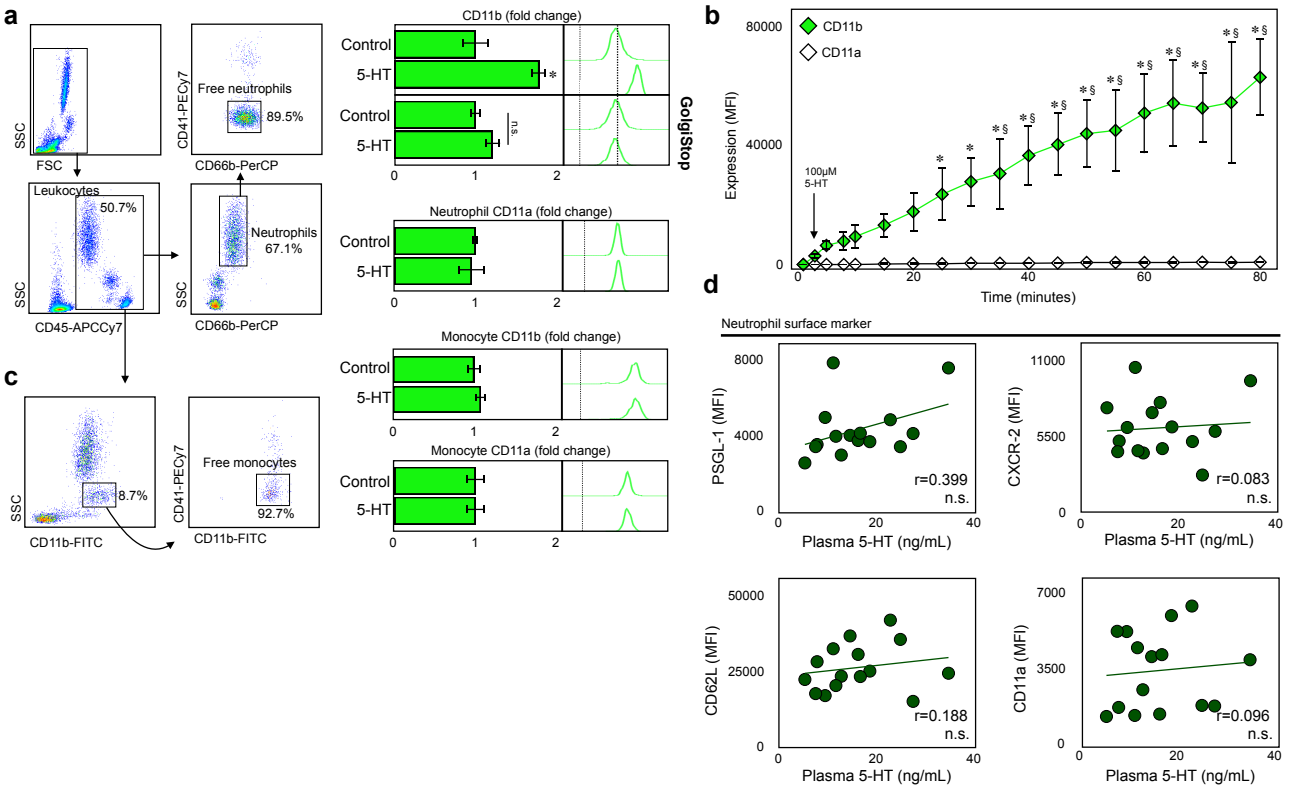
Supplemental Figure 5 Neutrophil and platelet adhesion molecules unresponsive to in vitro serotonin stimulation. (**a**) Quantification of CD11a, CXCR-2, CD62L and PSGL-1 surface expression on neutrophils by flow cytometry MFI following *in vitro* incubation of WT (red) and *Tph1*$^{-/-}$ (blue) whole blood with serotonin (5-HT) or PBS (control). Results are presented as normalized means ± SEM, n.s., two-way repeated measures ANOVA with Bonferroni's multiple comparisons testing, n=6 per group and treatment. (**b**) Expression profile of activated GPIIb/IIIa, GPV, GPVI, and P-selectin in platelet rich plasma stimulated with either PBS (control) or serotonin. Results are presented as normalized means ± SEM, n.s., two-way repeated measures ANOVA with Bonferroni's multiple comparisons testing, n=5 per group and treatment. (**c**) Representative dot plots of annexinV and PI co-staining in blood from WT and *Tph1*$^{-/-}$ (blue) mice and positive control (left panel). Quantification of platelet viability in blood of WT (red) and *Tph1*$^{-/-}$ (blue) mice after incubation with serotonin or control. Results are presented as mean ± SEM, *p<0.05 two-way ANOVA with Bonferroni's multiple comparisons testing, n=5 per group treatment.



Supplemental Figure 6 Pharmacologic depletion of peripheral serotonin mimics the effect of TPH1 deficiency on the expression of adhesion molecules after myocardial reperfusion injury. (**a**) Dot plots showing uncomplexed (free) ($Ly6G^+$, $CD41^-$) neutrophils in mouse blood (top panel), and quantification of CD11b, CD11a, CXCR-2, CD62L and PSGL-1 surface expression in vehicle (red) and Flx (white) treated mice following 24 hours of myocardial I/R injury (lower panel). Results are presented as normalized mean ± SEM, *$p<0.05$, Student´s t-test, n=5 per treatment. (**b**) Dot plot of uncomplexed (free) ($CD41^+$, $CD45^-$) platelets in blood (top panel) and quantification of activated GPIIb/IIIa, GPV, GPVI, and P-selectin surface expression 24 hours after myocardial I/R injury (lower panel). Results are presented as normalized mean ± SEM, *$p<0.05$, Student´s t-test, n=5 per vehicle (red) and fluoxetine (white) treatment. Bar graphs on the left are accompanied by representative histograms of respective markers on the right.



Supplemental Figure 7 Ticagrelor mediated cardioprotection after myocardial infarction is potentiated by fluoexetine treatment. (**a**) Working model to induce platelet ADP receptor inhibition in fluoxetine (flx, 21d)-treated mice. (**b**) Maximum platelet aggregation in vehicle and ticagrelor treated WT mice treated with either vehicle (red) or Flx (white) after I/R injury as measured by light-transmission aggregometry. Representative transmission diagrams of ADP treated PRP are shown on the right panel. Results are presented as mean±SEM, *p<0.05, two-way ANOVA with Bonferroni's multiple comparisons testing, n≥8 per group. (**c**) Plasma serotonin levels in vehicle (red) and Flx-treated (white) mice 24 hours after myocardial infarction with reperfusion. Results are presented as mean ± SEM, *p<0.05, two-way ANOVA with Bonferroni's multiple comparisons testing, n=8 independent animals per group. (**d**) Serotonin levels in supernatants of $8x10^5$ platelets stimulated with either vehicle (control), ADP, thrombin, TNFα, LPS, C5a, Endothelin-1, or Epinephrine. Results are presented as mean ± SEM, *p<0.05 compared to control, one-way ANOVA with Dunnet test, n=9 per group.(**e**) Quantification of platelet activation markers (GPIIb/IIIa and CD62P), (**f**) neutrophil alpha integrins (CD11b and CD11a), and (**g**) PNC levels in whole blood after I/R injury with respective histograms attached to bar graphs. Results are presented as mean±SEM of vehicle- (red) or Flx-treated (white) WT mice, *p<0.05, two-way ANOVA with Bonferroni's multiple comparisons testing, n≥8 per group. (**h**) Representative images of heart sections of vehicle- (red) and Flx-treated (white) WT mice after 30 minutes of LAD ligation followed by 24 hours of reperfusion. The infarct area (white tissue) was quantified as percentage of area at risk (AAR; non-blue tissue). Viable tissue within the AAR was tained in red. Results are presented as mean ± SEM, *p<0.05, two-way ANOVA with Bonferroni's multiple comparisons testing, n=8 per group.



Supplemental Figure 8 Serotonin selectively stimulates CD11b surface expression in human neutrophils. (**a**) Representative dot plots of uncomplexed (free) human blood neutrophils ($CD66b^+$, $CD41^-$)(left panel). Quantification of CD11b expression after stimulation with 100µM serotonin or PBS and co-incubated with GolgiStop/Plug reagent (right panels). Cd11a expression on free neutrophils is shown in the bottom right panel. Results are presented as mean ± SEM, *p<0.05, Student´s t-test, n=13 per treatment. (**b**) Quantification of CD11b (green) and CD11a (white) surface expression (MFI) on neutrophils over 80 minutes following initial stimulation with 100µM serotonin by continuous flow cytometry. Results from 3 independent experiments are presented as mean ± SEM, *p<0.05 denotes significant change in CD11b surface expression over time compared to time point of stimulation,, § p<0.05 denotes significant change in CD11b over CD11a at the respective time post stimulation, two-way repeated measures ANOVA with Bonferroni's multiple comparison testing. (**c**) Dot plots (left panel) of uncomplexed (free) human monocytes (SSC^{low}, $CD11b^+$, $CD41^-$) and quantification of CD11b and CD11a expression after 5HT or PBS treatment in blood of healthy volunteers(right panel). Results are presented as mean ± SEM, n.s., Student´s t-test, n=13 per treatment. Bar graphs are accompanied by representative histograms. (**d**) Correlation of serotonin plasma concentration and CXCR-2, CD62L and PSGL-1 (n.s.) surface expression on blood neutrophils of patients with acute coronary syndrome (ACS). Each point represents an individual patient. r: Pearson correlation coefficient, n=15.