SUPPLEMENTARY APPENDIX

Autoantibody-mediated desialylation impairs human thrombopoiesis and platelet lifespan

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Supplementary methods

Study cohort

Patients enrolled in the study were retrospectively reviewed by two physicians to confirm immune thrombocytopenia (ITP) and exclude aplastic anemia, leukemia, lymphoma, myelodysplastic syndrome, solid tumors, liver cirrhosis, recent cardiac surgery, bone marrow/blood stem cell transplantation or sepsis. In case of disagreement a third physician was consulted. When indicated, immunoglobulin G (IgG) fractions were isolated from serum samples using a commercially available IgG purification kit (Melon^{TM}-Gel IgG Spin Purification Kit, Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions.

Antibody characterization

Detection of glycoprotein-specific autoantibodies

In order to verify the glycoprotein (GP) specificity of the autoantibodies (AAbs), free and platelet (PLT)-bound AAbs were detected by the monoclonal antibody-specific immobilization of PLT antigens (MAIPA) assay, as previously described.(1, 2) For PLT-bound AAbs, patients' PLTs ($20x10^6$) were incubated with the GP-specific monoclonal Abs (mAbs) P2 or SZ1 (both from Beckman Coulter, Krefeld, Germany) against GPIIb/IIIa and GPIb/IX, respectively. After washing, PLTs were lysed in Triton X-100 (Merck, Darmstadt, Germany) and the supernatant, containing soluble GP-mAb-complexes, was transferred into microtiter plates coated with goat anti mouse IgG (Jackson ImmunoResearch, Ely, UK). AAbs were then detected using peroxidase-labeled goat anti human IgG (Jackson ImmunoResearch) and Tetramethylbenzidine (TMB, Kementec, Taastrup, Denmark). For the detection of free AAbs, PLTs from a panel of healthy donors were incubated with patients' serum samples for 30 minutes (min) at 37°C. After washing, PLTs were incubated with the corresponding mAbs and the monoclonal Ab-specific immobilization of PLT antigens (MAIPA) assay was

performed as described above (supplementary table 1). For both variants of MAIPA, test results were read using a photometer at 450 nm (photometer infinite F50, Tecan Group Ltd., Männedorf, Switzerland). An optical density of greater than 0.15 was considered positive as previously described.(3)

Assessment of the desialylation ability of autoantibodies

The AAb-mediated changes in the sialylation of GPs were analyzed by flow cytometer (FC, Navious, Beckman Coulter) using FITC-labeled ricinus communis agglutinin (1 µg/mL, RCA) and erythrina crista galli lectine (0.5 µg/mL, ECL, both from Vector laboratories, Burlingame, CA, USA) that binds to ß-Galactose and N-Acetylglucosamine residues, respectively. $8x10^6$ washed PLTs or $5x10^4$ megakaryocytes (MKs) were resuspended in phosphate-buffered saline (PBS) and incubated with serum or IgG fraction (1:1, v/v) for 2 hours (h) at room temperature (RT). Thereafter, samples were fixed with 2% paraformaldehyde (PFA, 20 min at RT), washed twice with PBS and then analyzed by FC. Cutoffs were calculated by testing 20 sera from healthy donors and determined as mean of fold increase (FI) compared to buffer+2xstandard deviation (SD). Representative dot plots and histograms of the lectine binding assay are reported in supplementary figure 1.

Analysis of antibody-mediated destruction of human platelets

The impact of desialylation on the survival of human PLTs was analyzed using the NSG mouse model as described previously.(4, 5) Sex- and age-matched animals were used in this study (8-16 weeks old NOD-scid IL2Rg^{null}; Stock No. 005557, purchased from The Jackson Laboratory Bar Harbor, ME, USA via Charles River, Research Models and Services Sulzfeld, Germany). Freshly isolated human PLTs ($2x10^9$/mL in 200 µL), with or without preincubation (30 min at RT) with 50 µM of oseltamivir acid (Santa Cruz biotechnology, Dallas, USA), were injected into the lateral mouse tail vein. 30 min after PLT injection a blood sample was collected by tail vein punctuation to set the baseline of human PLTs circulating in the mice (100%). Thereafter, IgG fractions isolated from sera of ITP patients or healthy

donors were injected into the other lateral tail vein. The survival of human PLTs was assessed by taking blood samples at 60, 120 and 300 min after baseline. After collection, samples were prepared using a commercially available kit (PerFix-nc Kit, Beckman Coulter, Marseille, France) according to the manufacturer's instructions. Briefly, ~10 µL blood samples were collected into 30 µL of citrated buffer and fixed for 15 min at RT. Red blood cells were lysed using 100 µL of lysis buffer. PLTs were then stained with anti-human CD41-PE-Cy5 (Beckman Coulter) and anti-mouse CD41-FITC (BD Biosciences, San Diego, CA, USA) for 30 min at RT and analyzed using FC.

Assessment of antibody-mediated apoptosis

The tetramethylrhodamine ethyl ester (TMRE) assay kit (Abcam, Cambridge, United Kingdom) was used to detect the mitochondrial inner transmembrane potential (ΔΨm). In brief, washed PLTs were incubated with IgGs from ITP patients for 2 h at RT and treated with 10 µM TMRE for 30 min at RT, in the presence or in the absence of an ionophore uncoupler of oxidative phosphorylation (FCCP, 1 h at RT). Thereafter, PLTs were directly assessed by FC to determine ΔΨm. Data were analyzed as FI compared to IgG from healthy donors.

Platelet adhesion assay

Chamber slides (Corning, New York, USA) were coated with 250 µg/mL human serum albumin (HSA, Kedrion, Barga, Italy), 100 µg/mL of fibrinogen (Sigma Aldrich, Munich, Germany) or 5 U/mL von Willebrand factor (vWF, Baxalta, Vienna, Austria) and put on a shaker (Philips, Brussels, Belgium) overnight at 4°C. After washing, the coated coverslips were blocked with HSA (1 h at RT). Washed PLTs ($2x10^6$/well) were incubated with IgG fractions from ITP patients or control sera for 2 h at RT under rotation and then allowed to adhere to the coated slides for 1 h at RT. To verify the impact of sialylation of PLT adhesion, cells were preincubated with 50 µM of oseltamivir acid (Santa Cruz biotechnology) for 30 min at RT followed by incubation with IgG fractions from healthy donors or ITP patients. Afterwards, cells were fixed with 4% PFA (20 min at RT) and the slides were washed 3 times

with PBS. Images of adherent cells were taken from 7 randomly chosen microscopic fields (x100, Olympus IX73, Olympus GmbH, Hamburg, Germany). The number of adherent cells in each field was calculated and normalized to control as 100%.

In vitro generation of human megakaryocytes

Megakaryopoiesis *in vitro*

CD34+ cells were isolated from peripheral blood in order to produce MKs, as previously described. (6) In brief, blood samples were diluted 1:1 in EDTA-PBS buffer (PBS with 2 mM EDTA, Invitrogen Life Technologies, New York, USA) and layered on a density gradient medium (Ficoll-Paque, GE Healthcare, Uppsala, Sweden). After centrifugation (400g, 40 min at RT, without brake) the supernatant was discarded and the mononuclear cell layer was collected and washed 3 times with EDTA-PBS buffer (200g, 10 min at RT). Cells were incubated with FcR blocking reagent and CD34-MicroBeads (CD34 MicroBead UltraPure Kit, Miltenyi Biotec, Bergisch Gladbach, Germany) for 30 min at 4°C. After washing (300g, 10 min at RT), cells were collected and applied to the column on a magnetic separator and washed 3 times with EDTA-PBS buffer. After removal of the column from the separator the CD34+ cells were flushed out and resuspended in EDTA-PBS buffer. For expansion, CD34+ cells were then cultured in StemSpan SFEM culture medium (Stem Cell Technology, Vancouver, Canada) supplemented with 40 ng/mL stem cell factor (SCF), 20 ng/mL interleukin 3 (IL3) and 30 ng/mL thrombopoietin (TPO, all from Miltenyi Biotec). Cells were cultured under controlled conditions for 6-7 days (Binder, Tuttlingen, Germany, 37ºC and 5% CO_2). Medium was changed every 3-4 days. After expansion, CD34+ cells were seeded on 8 well chamber slides ($5x10^4$ cells/well, Corning) and cultured for differentiation into MKs in StemSpan SFEM medium supplemented with 50 ng/mL of TPO. After 2 weeks, MKs and PLTs were collected and washed once with PBS (650g, 7 min at RT) for further investigations.

Characterization of megakaryocytes

After 14 days of *in vitro* differentiation, MKs were characterized by microscopy and FC. In order to identify the viable cells, Calcein (Thermo Fisher, Germany) staining (1 µM, 20 min at 37°C) was always performed during FC measurements. The analysis of the ploidy phenotype of the MKs was based on propidium iodide (PI, Sigma Aldrich, St. Loris, USA) staining. Briefly, cells were fixed with 2% PFA (Morphisto) and washed with PBS (650g, 7 min at RT). Prior PI staining (50 µg/mL, 15 min at RT), cells were incubated with 10 µg/mL of RNase (Sigma Aldrich) for 10 min at RT. A FC gating strategy based on side scatter (SSC), forward scatter (FSC), markers expression (CD41 and CD42a), Calcein signal and PI staining was used, as previously described. (6) MKs were defined as a population of large cells (SSC/FSC) with a high DNA content, quantified by positive PI staining. See supplementary figure 2.

Flow cytometry analysis of the thrombopoiesis

The total number of PLTs and MKs was determined by FC using a commercially available cell quantification kit (Coulter Flow-Count Fluorospheres, Beckman Coulter), according to the manufacturer's instructions, as previously described. (6) In this kit, fluorospheres with a fixed size are labeled with dye with a fluorescent emission between 525 nm and 700 nm, upon excitation at 488 nm. As reported in the manufacturer's instructions, all fluorospheres have uniform fluorescence intensity and a defined concentration which allowed a direct quantification of absolute cell count. Cells were mixed with the fluorospheres at a 1:1 ratio and the fluorescent emission was immediately measured by FC. MKs were identify as reported in the "Characterization of megakaryocytes" paragraph. In order to define and quantify viable PLTs and MKs, after 14 days of differentiation, a FC gating strategy was developed based on SSC, FSC, markers expression (CD41 and CD42a), Calcein signal and PI staining. MKs were defined as a population of large cells (SSC/FSC) with a high DNA content (PI positive). While, PLTs were detected as small and anucleated cells (PI negative). See supplementary figure 2.

Quantification of proplatelet formation and platelet release

Prior to quantification of proplatelet (proPLT) formation, each MK cell line was characterized by FC according to the ploidy status (PI staining), CD41 and CD42a expression (see supplementary methods “Flow cytometry analysis of the thrombopoiesis”) as well as by cell morphology. The proPLT formation was assessed, as previously described.(7) In brief, MKs were incubated with IgGs from ITP patients or healthy donors for 24 h at 37°C and 5% CO_2. Cell images from 7 randomly chosen microscopic fields were captured using bright field microscopy (x40, Olympus IX73). Only cells with at least 10µm of diameter were considered MKs and included in the quantification analysis. The percentage of proPLT forming MKs was determined as the number of MKs displaying at least one cytoplasmic extension out of the total number of MKs in each field and normalized to control conditions. PLT release from MKs was quantified after gentle resuspension of the culture medium. 10 µL samples were collected and diluted in 100 µL PBS. PLTs were gated in FC depending on SSC/FSC and Calcein positive and PI negative signal. CD41, CD42a-positive events were then counted using a commercially available kit according to the manufacturer’s instructions (Coulter Flow-Count Fluorospheres).(6)

Immunofluorescence microscopy of proplatelet extensions

CD34+ cells were seeded on 8 well chamber slides ($5x10^4$ cells/well, Corning) and cultured in StemSpan SFEM medium in the presence of 50 ng/mL of TPO. After 2 weeks, MKs were incubated (24 h, 37ºC and 5% CO_2) with IgG fractions of sera from ITP patients or healthy donors. After washing, cells were fixed with 2% PFA for 8 min at RT. After 3 washing steps, cells were permeabilized with Triton X-100 (0.25%, Sigma Aldrich) for 5 min at RT. Thereafter, the cells were blocked with 5% bovine serum albumin (BSA, 30 min at RT) before staining with anti-human Filamin Ab (1:50, Santa Cruz, Dallas, USA) overnight at 4°C and a secondary FITC-labeled anti-mouse Ab (1:500, Invitrogen, Munich, Germany) in combination with DAPI (1:100, Life Technologies, Darmstadt, Germany) for 1 h at RT. After one wash with

PBS, a cover glass was mounted onto the slide and images were taken using the Olympus IX73 microscope and analyzed with the CellSens Standard software (Olympus).

Megakaryocyte interaction with extracellular matrix proteins

8 well chamber slides (Corning) were coated with 250 µg/mL HSA (Kedrion), 100 µg/mL fibrinogen (Sigma Aldrich), 5 U/mL vWF (Baxalta) or 100 µg/mL collagen (Collagen-Horm, Takeda, Linz, Austria). MKs ($1.8x10^6$) were incubated with IgG fractions from ITP patients or healthy donors for 2 h at RT under rotation. After blocking the coated coverslips with HSA (1 h at RT), cells were allowed to adhere for 1 h at RT. Subsequently, cells were fixed with 2% PFA (5 min at RT) and washed 3 times with PBS. Images of adherent MKs were taken from 7 different microscopic fields (x40, Olympus IX73). The number of adherent MKs in each field was calculated and normalized to control as 100%.

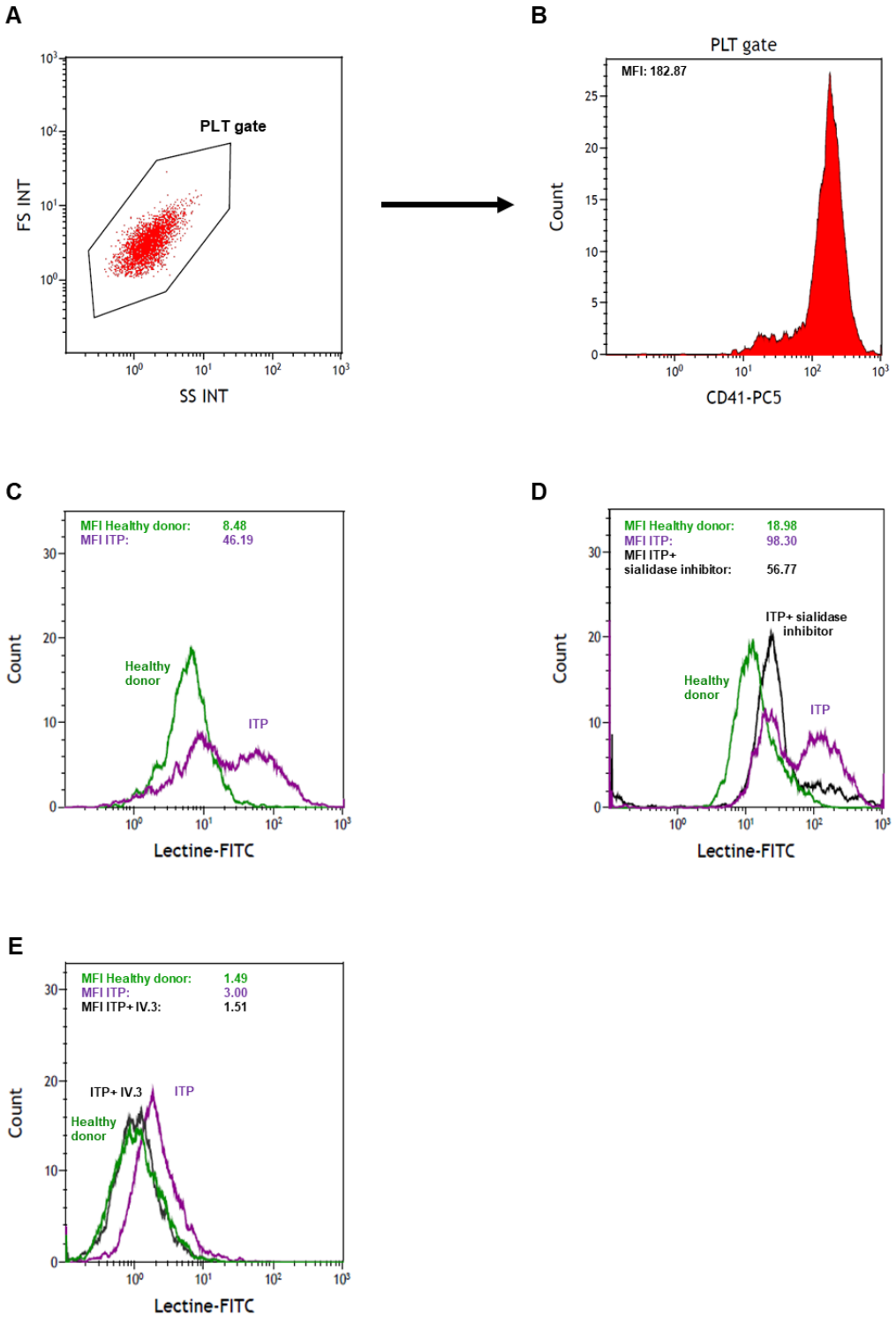
Supplementary tables and figures

Supplementary table 1: Free and platelet-bound autoantibodies (MAIPA)

AAB against	GP IIb/IIIa n=23	GP Ib/IX n=5	GP IIb/IIIa+GP Ib/IX n=23	
			IIb/IIIa	Ib/IX
Free (n,%)	16/23 (70%)	5/5 (100%)	19/23 (83%)	18/23 (78%)
Bound (n,%)	14/23 (61%)	0/5 (0%)	9/23 (39%)	9/23 (39%)
Both (n,%)	7/23 (30%)	0/5 (0%)	5/23 (22%)	4/23 (17%)

Abbreviations: AAB: autoantibodyy; GP: glycoprotein. Free: positive indirect MAIPA; bound: positive direct MAIPA; both: positive indirect and direct MAIPA.

Supplementary figure 1

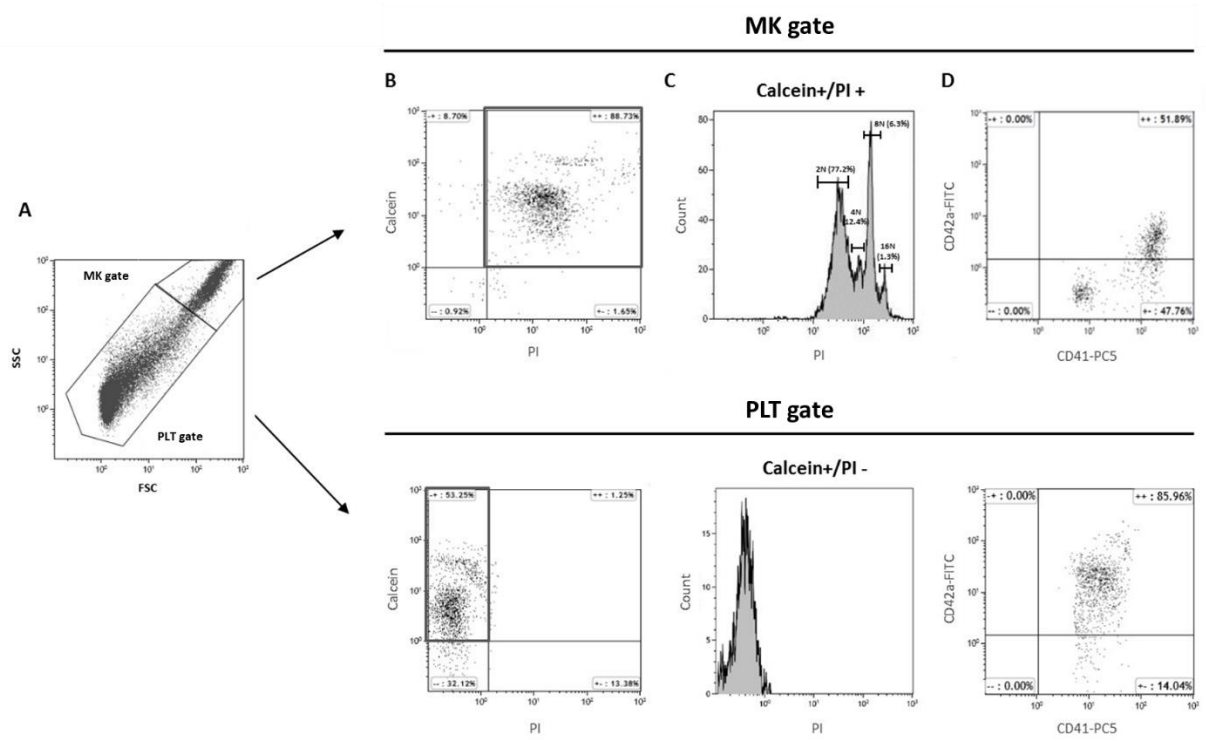


Representative flow cytometry dot plot and histograms of lectin binding assay

(A) Washed platelets (PLTs) were gated and (B) the expression of CD41 in this cell population was analyzed. The exposure of ß-galactose in the presence of (C) sera from

healthy donors or ITP patients in combination with (D) sialidase inhibitor or (E) IV.3 antibody was measured using FITC-labeled lectine. (MFI: mean fluorescence intensity).

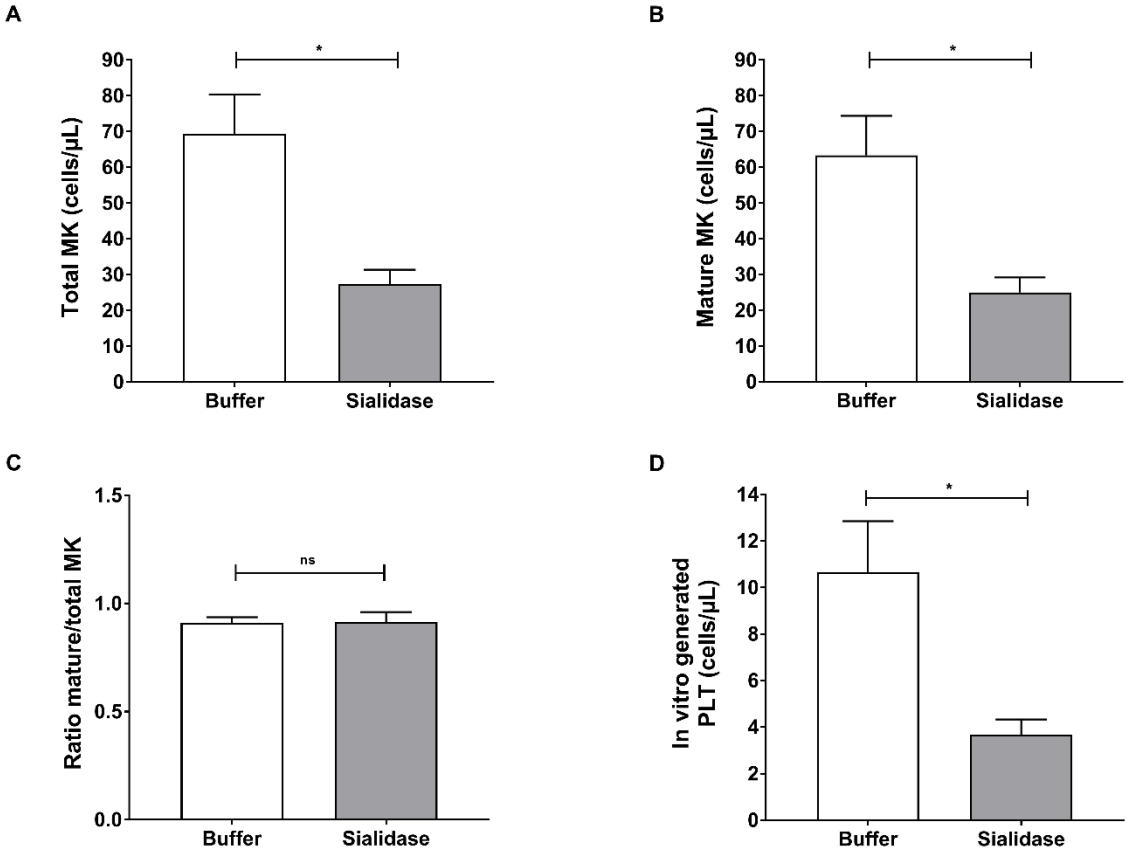
Supplementary figure 2



Gating strategy for the characterization of megakaryocytes and platelets by flow cytometry

After 14 days of differentiation megakaryocytes (MKs) and platelets (PLTs) were gated according to (A) the cell size, (B-C) Calcein signal and DNA content (propidium iodide, PI). (B) For each cell population the percentages of viable cells (Calcein positive) as well as the DNA content using PI staining were quantified. (C) MKs, characterized by a polyploid status, were defined as double positive Calcein+/PI+ while PLTs, as anucleated cells, Calcein positive and PI negative (Calcein+/PI-). (D) The markers expression (CD41 and CD42a) in both cell types was analyzed.

Supplementary figure 3



The impact of sialylation on *ex vivo* megakaryopoiesis

To verify the role of glycoprotein sialylation in megakaryopoiesis and thrombopoiesis, buffer (white columns) and exogenous sialidase (grey columns) was added to culture medium at the beginning of the differentiation stage. Total (A) and mature (B) megakaryocytes (MKs) were quantified by flow cytometry using a commercially available kit and the ratio of mature/total MKs was calculated (C). (D) Released platelets (PLTs) were counted by quantification of CD41+/CD42a+ events in the supernatant using a commercially available kit. Data are shown as mean of cells/µL±standard error mean of three independent experiments performed testing three MK cell lines; ns, not significant, *p<0.05.

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