Dual roles for hepatic lectin receptors in the clearance of chilled platelets.

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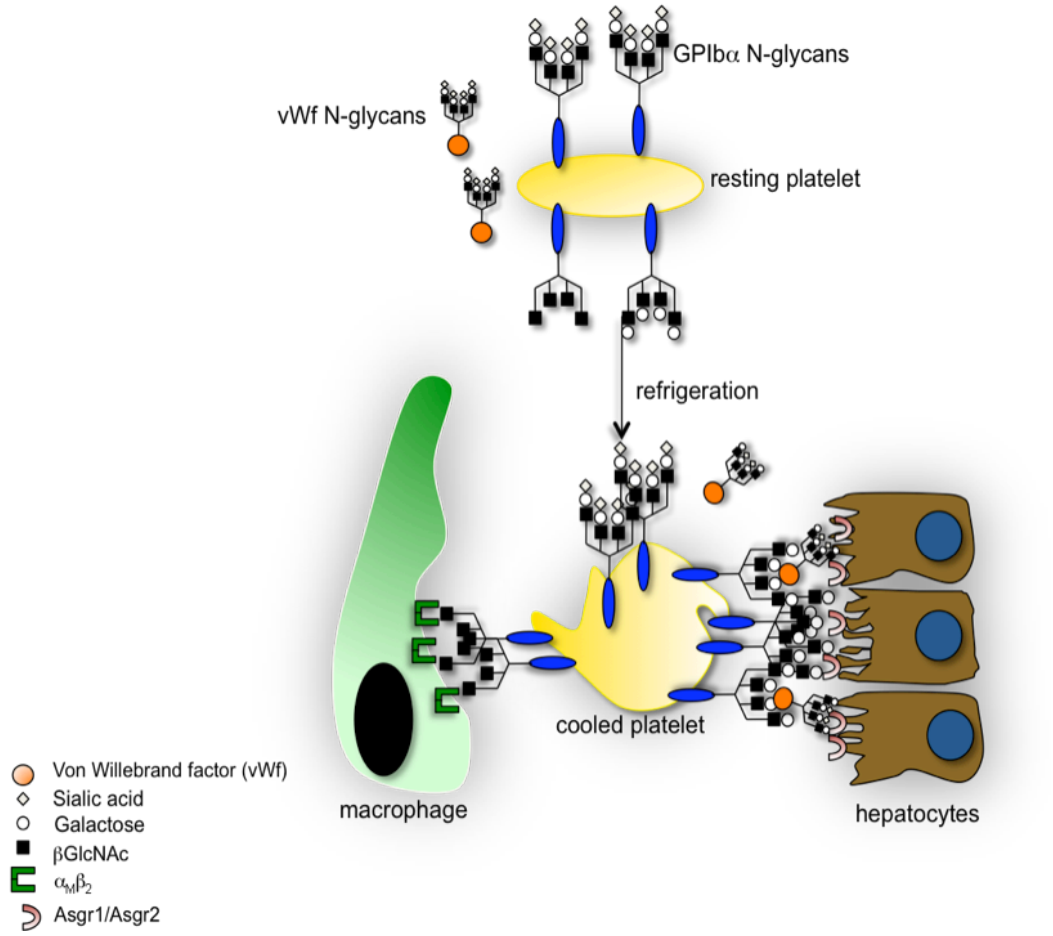

Figure S1 Lectin receptors mediating the clearance of cooled platelets. Short-term platelet cooling induces irreversible βGlcNAc exposure and clustering and recognition by macrophages bearing the $\alpha_M\beta_2$ integrin. Prolonged platelet refrigeration increases βgalactose exposure, hyper-aggregation of GPIbα molecules, and vWf binding to platelets. These changes induce clearance by asialoglycoprotein receptors on hepatocytes (Ashwell-Morell receptors) and macrophages (integrin $\alpha_M\beta_2$).

SUPPLEMENTARY METHODS

Materials. We obtained: Biotin-NHS and *Streptavidin phycoerythrin* conjugate from Calbiochem; Streptavidin - POD Conjugate from Roche; ImmunoHisto™ Peroxidase Detection Kit from PIERCE; Fetal calf serum Type-I fetuin, Fetal calf serum Type-I Asialofetuin, Cytochalasin D, PGE_1, and *Clostridium histolyticum* collagenase from Sigma-Aldrich, Inc.; paraformaldehyde from Bio Products; FITC-conjugated succinyl *Triticum vulgae* lectin (sWGA), FITC-conjugated *Ricinus communis*-1 (RCA I) and FITC-conjugated *Erythrina cristagalli* lectin (ECA) from EY Laboratories Inc.; Human Hepatocellular Carcinoma cells from ATCC; CellTracker™ Orange CMTMR and CellTracker Green CMFDA were from Molecular Probes, Inc.; Avertin was from Fluka Chemie; Biotin-NHS was from Fisher Scientific. Mocarhagin was provided by Dr. M. Berndt, Monash University, Melburne Victoria 3181, Australia; *O*-sialoglycoprotein endopeptidase was from Cerladane; Clodronate liposomes were purchased from Dr. Nico van Rooijen, Vrije Universiteit, Amsterdam, The Netherlands.

Antibodies. FITC, or PE conjugated- or unconjugated-rat antibody to mouse GP1bα (clones Xia.G7, Xia.B2 and Xia.G5), rat antibody to mouse CD61 (clone 2C9G2 and LUCA5) and rabbit antibody to mouse vWf were from Emfret Analytics; Rabbit antibody to human von Willebrand Factor was from Dako; Peroxidase-conjugated AffiniPure goat antibody to rat IgG and peroxidase-conjugated AffiniPure goat antibody to rabbit IgG were from Jackson ImmunoResearch Laboratories Inc.; FITC-conjugated mouse antibody to human integrin β3 (CD 61) was from Becton Dickinson; Mouse antibody to human

Asialoglycoprotein subunit 1 (N-18) and mouse antibody to human Asialoglycoprotein subunit 2 (G-17kk) were from Santa Cruz Biotechnology, Inc.; FITC-conjugated antibody to human GPIbα (SZ2) was from Immunotech.

Mouse platelet preparation. We obtained murine blood by retroorbital eye bleeding into 0.1 volume of Aster-Jandl anticoagulant from mice anesthetized with 3.75 mg ml^{-1} of Avertin. The anti-coagulated blood was centrifuged at 268 x *g* for 8 min at RT to obtain PRP. Platelets were separated from plasma proteins as described [2]. Platelets were suspended at a concentration of 5 x 10^9 ml^{-1} in platelet buffer or plasma for injection into recipient mice.

Human and mouse platelets were labeled with 1.8 μM CellTracker™ Orange CMTMR, or 2.5 μM CellTracker Green CMFDA[47] for 20 min at 37 °C [48] before storage for 0 to 48 h at 4 ºC. Unincorporated dye was removed by centrifugation[2]. Platelets were resuspended to 3 x $10^9$$ml^{-1}$ in platelet poor plasma (PPP) before storage at 4 °C for 48 h.

Mouse platelets were biotinylated by incubation with 1 μg ml^{-1} Biotin-NHS for 1 h at RT. Unbound biotin was removed by centrifugation [2].

Mouse platelet recovery, survival and fate. We injected 1x10^8 CMFDA-labeled mouse platelets, 0 °C, 4 ‘C or 22 °C maintained into retroorbital venous plexus of syngeneic recipient mice. For recovery and survival determinations, blood samples were collected immediately (2 min), and at 5 min, 10 min, 0.5 h, 2 h, 24 h, 48 h, and 72 h. Whole blood

analysis using flow cytometry (Becton Dickinson Biosciences) was performed and the percentage of CMFDA positive platelets determined [47]. 50,000 events were collected from each sample. In experiments using asialofetuin/fetuin and platelet co-transfusions the 2 min values of transfused platelets were set at 100%. In all other experiments, the values of transfused platelets measured at 5 min were set at 100 %. The survivals and recoveries were calculated as described [2,47]. For biotinylated platelets the recoveries were 78.0 % ± 12 and 60.2 ± 9.25 (mean ± s.e.m.) for RT and long-term cooled platelets, respectively; the survivals were 72.34 ± 5.4 and 59.48 ± 3.9 for 22 °C and long-term refrigerated platelets, respectively. All other calculated platelet recoveries and survivals are summarized in table 1.

To evaluate the fate of platelets, tissues were harvested from recipient mice 1 h after the injection of 0.4 x 10^9 ^{111}Indium-labeled (NEN Life Science Products) platelets. Platelets were radiolabeled after storage as decribed before [2]. Organ-weight was measured and radioactivity was determined from γ-count. Results are expressed as % of radioactivity per g organ relative to the total radioactivity injected. For recovery and survival determinations of radioactive platelets, blood samples were collected immediately (< 2 min), and at 0.5 h, 1 h and 24 h after platelet infusion and γ-count determined [49].

For recovery and survival determinations of biotinylated platelets, blood samples were collected after 5 min, 2 h, 24 h, 48 h and 72 h by intraorbital bleeding. PRP was separated from whole blood by centrifugation, and biotinylated platelets were detected

after labeling with Streptoavidin-PE (1:1000). The percentage of biotinylated platelets was determined by flow cytometry. 50,000 events were collected in each sample.

Enzymatic galactosylation of human and mouse platelets. Mouse CMFDA-labeled platelets (10^9 ml^{-1}) were suspended in mouse plasma incubated with 1.2 mM uridine 5'-diphosphogalactose (UDP-Gal) for 60 min at 37 °C in platelet rich plasma prior to storage at 4 °C [10].

Platelet clearance inhibition studies using asialofetuin and fetuin. A bolus of 10 mg asialofetuin or fetuin was injected via the retroorbital venous plexus into WT C57/Bl6 mice 2 min before the platelet transfusion. Following platelet transfusion, 5 mg asialofetuin or fetuin was injected *i.v.* at time points of 10, 20, 30 and 60 min. The recipient animals were bled at 2, 10, 30, 90 min and the survival of labeled platelets determined by flow cytometry, as described above.

***In vitro* HepG2 based platelet ingestion assay.** We maintained human HepG2 hepatocarcinoma cells in αMEM (GIBCO Invotrigen), 2% heat-inactivated bovine calf serum (BCS), 3% standard fetal bovine serum (FBS), and 1% of a penicillin and streptomycin solution composed of 10,000 U ml^{-1} penicillin G and 10 mg ml^{-1} streptomycin sulphate. HepG2 cells were not passaged > 2 times before use. For assays, the HepG2 cells were transferred to 24-well plates (10^6 per well), allowed to adhere for 24 h, and starved for 30 min by replacing αMEM media without serum. Cytochalasin D (SIGMA) was diluted into αMEM media at the indicated concentrations and added to the

HepG2 cells. DMSO was used as control. $1x10^8$ CM-Orange-labeled platelets (fresh 22 °C platelets or platelets cooled at 0°C or 4 °C) were added per well, with or without cytochalasin D. Hepatocytes and platelets were incubated for 5-30 min at 37 °C with gentle agitation. After the incubation period, the HepG2 monolayers were washed 3 times by removing and changing the buffer. HepG2 cells were dissociated from the wells with 0.05% trypsin, 0.53 mM EDTA in HBSS (GIBCO Invitrogen) at 37 °C for < 10 min. CM-Orange-labeled platelet ingestion was quantified by flow cytometry. HepG2 cells were gated according to their forward and side scatter characteristics. HepG2 with ingested platelets acquired orange fluorescence. Platelets adherent to HepG2 cells label with the FITC conjugated antibody to human CD61. Only single CM-orange positive events are shown, with the exception of Fig 2 A. 10, 000 events were acquired for each sample.

Removal of the extracellular domain of GPIbα. GPIbα was enzymatically cleaved from the surface of human or mouse platelets in platelet buffer containing 1 mM Ca^{2+} and 10 μg ml^{-1} of the snake venom metalloprotease mocarhagin [38] or 10 μg ml^{-1} *O*-sialoglycoprotein endopeptidase, respectively. After the enzymatic digestion, the platelets were washed by centrifugation, resuspended in platelet poor plasma (PPP), and allowed to rest for 30 min at 37 °C before storage at 4 °C. The extent of GPIbα removal was monitored by flow cytometry using 5 μg ml^{-1} of the FITC-conjugated antibody to human GPIbα (clone SZ2) or 5 μg ml^{-1} of the PE-conjugated antibody to mouse GPIbα (clone Xia.G5).

Von Willebrand factor binding. Platelet bound vWf was detected by incubating 3 μl of FITC-conjugated antibody to human or mouse vWf, or with non-immune rabbit polyclonal IgG (control), with 2.5 x10^6 human or mouse platelets in PRP for 20 min at RT. Labeled samples were analyzed by flow cytometry after dilution into buffer B. Data is expressed as % positive cells determined for vWf compared to the appropriate IgG negative control.

Measurement of glycan exposure on platelet surfaces. Fresh 22 °C platelets or platelets stored in platelet buffer for 2 h at 0 °C, or in plasma for 48 h at 4 ºC, were diluted 4-fold with platelet buffer, collected by centrifugation at 830 x *g* for 5 min, and resuspended in platelet buffer at 1x10^7 ml^{-1}. Surface galactose was analyzed by flow cytometry following incubation with FITC-conjugated RCA I or ECA at 0.1 and 10 μg ml^{-1}, respectively. Surface βGlcNAc was measured by incubation with FITC-conjugated s-WGA at 0.1 μg ml^{-1}. Samples were incubated at RT for 20 minutes and diluted 5 x with platelet buffer before analysis by flow cytometry. A total of 10,000 events were analyzed using the Cell Quest software (Beckton Dickinson). All experiments were done in triplicate.

Surface labeling of platelet GPIbα. Mouse platelets, maintained at RT or chilled, were applied to glass coverslips by centrifugation at 280 x *g* in PBS containing 0.05% glutaraldehyde, fixed with 0.5% glutaraldehyde in PBS for 10 min, quenched with 0.1% sodium borohydride in PBS and washed extensively with PBS containing 1% BSA.

GPIbα was labeled with a mixture of three rat anti-mouse GPIbα monoclonal antibodies, each at 10 μg ml^{-1} for 1 h, followed by incubation with 10 nm gold conjugated goat anti-rat IgG for 3 h. The coverslips were washed, fixed with 1% glutaraldehyde and prepared for electron microscopy as described [50].

Gold particles were quantified on the replica surface of RT or chilled platelets. Images were scanned at 1600 dpi resolution and 8-bit depth using Adobe Photoshop 7.0. Files were saved as TIFF format. An area of 800 x 800 pixels was selected and pasted into a new file. The background was removed from the image by using the Brightness/Contrast tool to prevent false positives. Gold size calibration was done in Image J (J 1.32j). Threshold range was 0-170. Center of mass and area was recorded to 8 decimal places. Particles at a size between 20 and 150 nm^2 were used for nearest neighbor analysis. All particles at a size of >20 nm^2 were used for area analysis. The areas of more than 100 single gold particles were measured as a size control.

Immunoblot analysis and immunoprecipitation. Prior to lysis, platelets were washed 1x by centrifugation as described above and resuspended in platelet buffer at 4 x 10^8 platelets ml^{-1}. For immunoblotting, platelets were lysed with 1/3 volume of 3x lysis buffer (3% Nonidet P-40, 150 mM Tris/HCl, pH 7.4, 450 mM NaCl, 3 mM EGTA, 3 mM PMSF, 3 mM Na_3VO_4, 30 μg ml^{-1} leupeptin and 30 μg ml^{-1} aprotinin) [52] and by the addition of 1/4 volume of 4X SDS-PAGE loading buffer containing 5% β-mercaptoethanol [53].

For immunoprecipitation experiments, platelets were lysed by the addition of 0.5 volume of 3x lysis buffer (3% Nonidet P-40, 150 mM Tris/HCl, pH 7.4, 450 mM NaCl, 3 mM EGTA, 3 mM PMSF, 3 mM Na_3VO_4, 30 μg ml^{-1} leupeptin and 30 μg ml^{-1} aprotinin) [52]. Insoluble material was removed by centrifugation at 14,000 x *g* for 10 min and the soluble fraction was immunoprecipitated with 2 μg of the antibody to mouse GPIbα (clone Xia G7) and antibodies bound to protein G-Sepharose beads (Pharmacia). Immune complexes were collected and solubilized in SDS-PAGE buffer containing 5% β-mercaptoethanol.

Proteins were displayed by SDS-PAGE on 7.5% precast polyacrylamide gels (Lonza) and transferred onto Immobilon-P membrane (Millipore). Membranes were blocked using 1% BSA in 100 mM NaCl, 20 mM Tris/HCl, pH 7.4 and probed with a mixture of 1 μg ml^{-1} of the antibodies to mouse GPIbα (XiaG7, Xia B2 and Xia G5) followed by the appropriate peroxidase-tagged secondary antibodies, or with 1 μg ml^{-1} of peroxidase conjugated-sWGA or 0.4 μgml^{-1} RCA I, or 1 μg ml^{-1} of rabbit anti human vWf antibody followed by peroxidase-tagged secondary antibodies. Detection was performed with an enhanced chemiluminescence system (Pierce).