FcγRI and FcγRIII on splenic macrophages mediate phagocytosis of anti-glycoprotein IIb/IIIa autoantibody-opsonized platelets in immune thrombocytopenia

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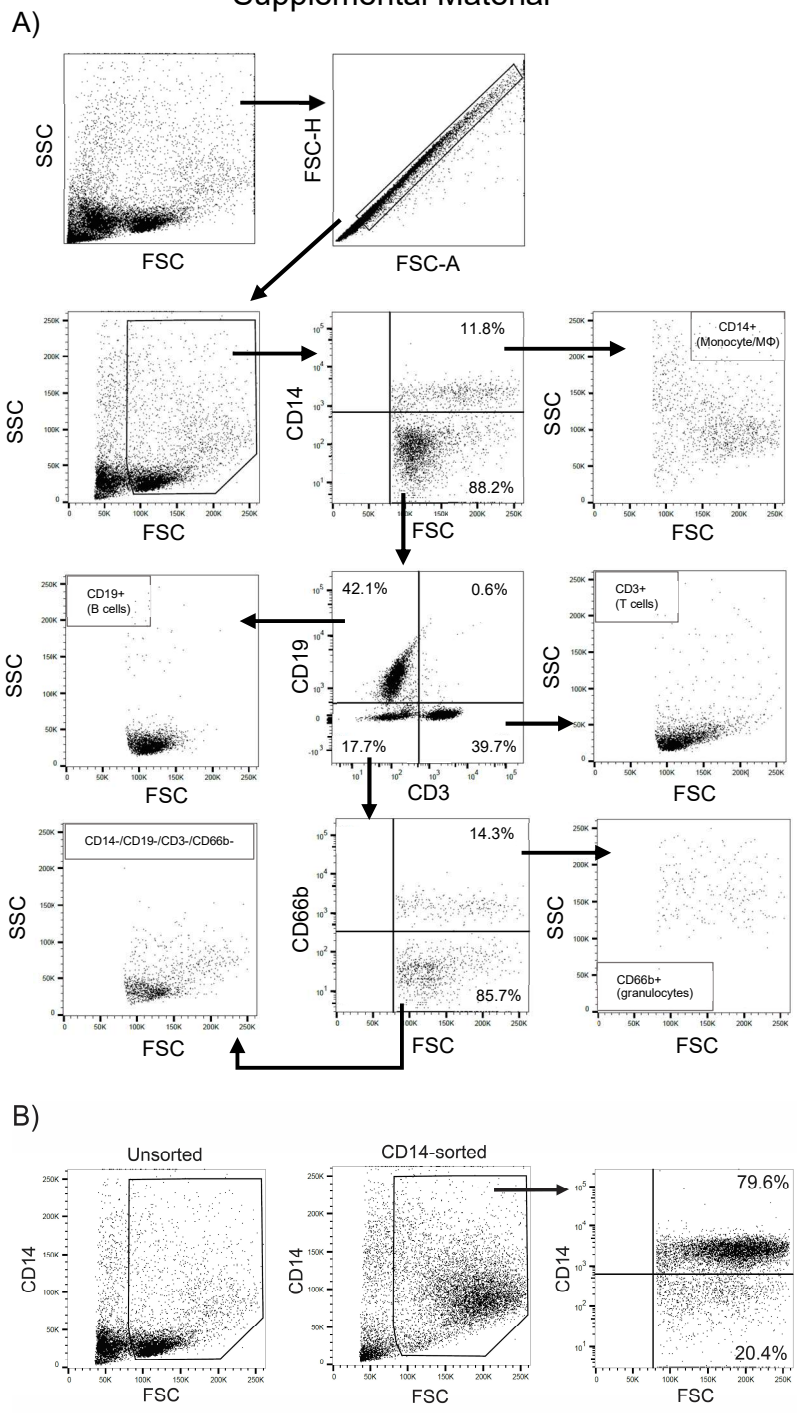
Supplemental Material



Supplemental Figure 1. Flow cytometric identification of splenic leukocyte populations. A) Flow cytometric dot plots to identify splenic leukocyte populations from a frozen single-cell suspension of a representative ITP patient spleen. Fluorescent

antibodies were used to determine the percentage of B cells (anti-CD19); T cells (anti-CD3); granulocytes (anti-CD66b); and monocyte/macrophages (monocyte/MΦ) (anti-CD14). The remaining cells were defined as CD14-/CD19-/CD3-/CD66b-negative. B) Splenic macrophages were isolated from an ITP patient spleen cell suspension by CD14 positive selection. Depicted is the same splenic specimen before (leftmost panel) and after (two rightmost panels) positive selection. The remaining CD14-negative cells after positive selection were predominantly T & B lymphocytes, and the percentage of granulocytes was less than 5%. Macrophage purity was further increased by adherence and washing with PBS to remove remaining lymphocytes and other non-adherent cells. Flow cytometry was performed using a Fortessa X-20 (Beckton Dickson) and data analysis was performed using FlowJo v10 (Beckton Dickson). SSC = side-scatter; FSC = forward-scatter.

Supplemental Table 1. Characteristics of ITP patient spleen donors

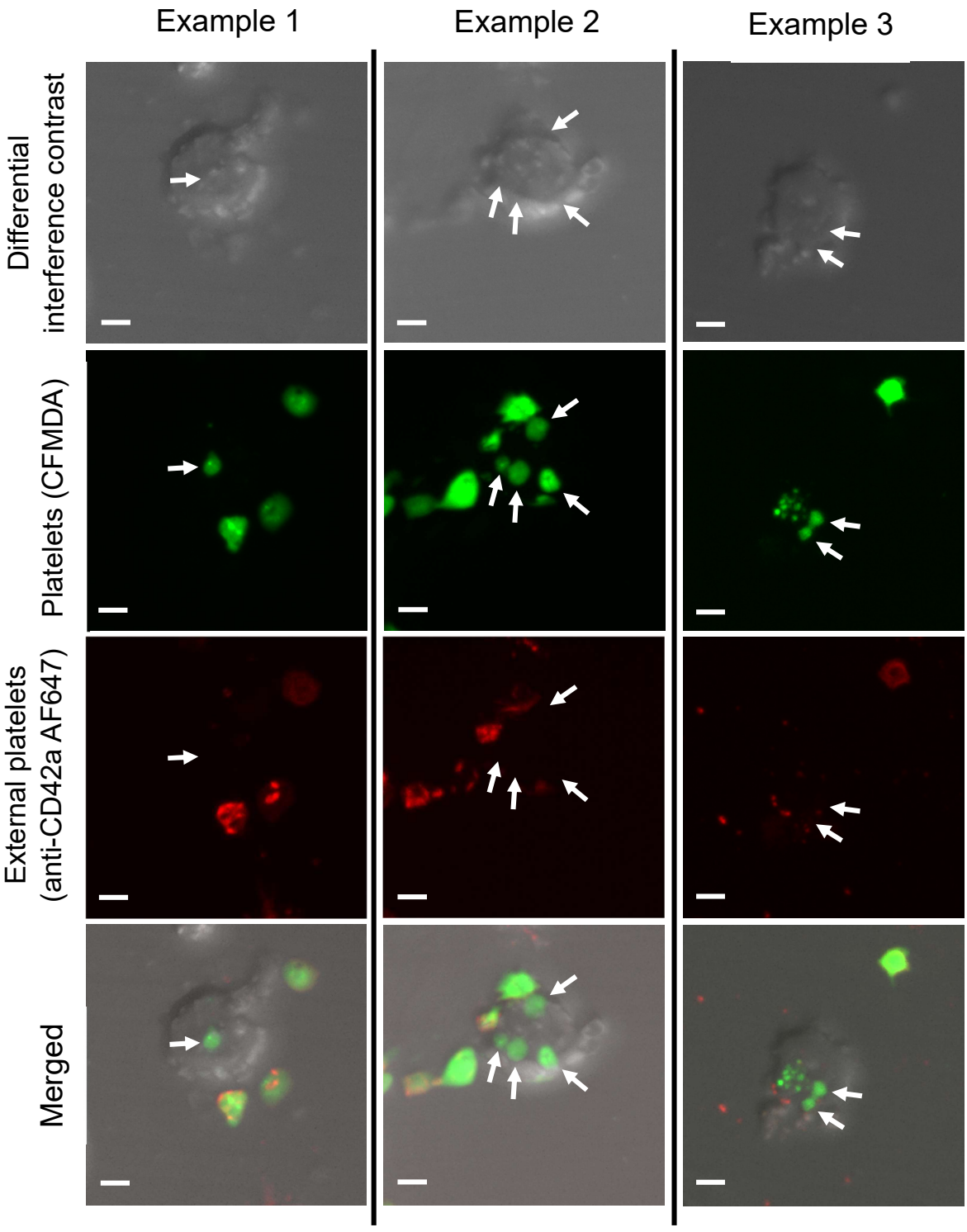
ITP spleen specimen	PLT count pre-splenectomy (10^9/L)	Highest reported PLT count post-splenectomy (10^9/L)	Prior treatments	Age	Sex
A	32	887	Corticosteroids, IVIg, TPO-RA	69	Male
B	113	548	Corticosteroids, IVIg	48	Female
C	20	365	Corticosteroids, IVIg	28	Female
D	51	517	Corticosteroids, IVIg, TPO-RA	41	Male
E	N.A.	N.A.	N.A.	54	Female
F	N.A.	N.A.	N.A.	60	Male
G	N.A.	N.A.	N.A.	50	Female
H	N.A.	N.A.	N.A.	42	Female

PLT = platelet
N.A. = not available
IVIg = intravenous immunoglobulin
TPO-RA = thrombopoietin receptor agonist

Supplemental Table 2. Characteristics of ITP patient serum donors

ITP Serum Specimen (Symbol, Figure 2)	PLT count (x10^9/L) *	Treatment *	Indirect MAIPA O.D. (GPIIB/IIIA)†	Direct MAIPA O.D. (GPIIB/IIIA)†
1 (□)	2	Prednisolone	0.867	n.t.
2 (○)	91	Prednisolone	2.827	0.437
3 (△)	1	none	0.642	n.t.
4 (■)	12	none	1.767	2.488
5 (●)	8	none	3.568	n.t.

PLT = platelet
* at time of serum collection
O.D. = optical density
† = sera were negative (O.D. <0.15) for autoantibodies to GPIb/IX and GPV
n.t. = not tested (insufficient platelet count for test)



Supplemental Figure 2. Additional examples of splenic macrophages with phagocytosed anti-GPIIb/IIIa ITP serum-opsonized platelets. Platelets were labelled with the cytoplasmic dye CMFDA (green). External (non-phagocytosed) platelets were detected after stopping phagocytosis and macrophage fixation using an AlexaFluor 647

(AF647)-conjugated anti-CD42a (GPIX) antibody (red). Splenic macrophages were observed by spinning-disc confocal microscopy under 63x objective oil immersion (numerical aperture 1.47) with differential interference contrast and laser fluorescence (488, 647 nm excitation) on a Quorum multi-modal imaging system (Quorum Technologies, Ontario, Canada). At least four images were taken at the centre of each well with Z-stacking every 0.33 µm. Z-stacked images were 3D reconstructed to assist in discriminating internalized from external platelets using Imaris v8.0.2. Platelets were additionally defined by size (1.5 µm to 3.5 µm) to distinguish them from internalized microparticles, microvesicles, or platelet aggregates; arrow indicates a phagocytosed platelet for each example. Scale bar = 3 µm.

Materials and Methods

Antibodies

FcγR-blocking antibodies: purified anti-human FcγRI (clone 10.1, murine IgG1, catalogue number 305016) and anti-human FcγRIII (clone 3G8, murine IgG1, catalogue number 302014) were obtained from BioLegend (San Diego, California, USA); purified anti-human FcγRIIA (clone IV.3, murine IgG2b, catalogue number 60012) was from Stemcell Technologies (Vancouver, Canada); and purified anti-human FcγRIIA/B/C (clone AT10, murine IgG1, catalogue number LS-C187457) was obtained from LifeSpan Biosciences (Seattle, Washington, USA). PE-Cy7 anti-human CD66b, APC anti-human CD14, Brilliant Violet 605 anti-human CD19, and Pacific Blue anti-human CD3 were obtained from BioLegend (San Diego, California, USA). AlexaFluor 647 anti-human CD42a (GPIX) was obtained from BioRad Laboratories (Hercules, California, USA). Mouse IgG1 isotype control and mouse IgG2b isotype control antibodies were obtained from Bio X Cell (West Lebanon, New Hampshire, USA). PE-Cy7 anti-human FcγRI (clone 10.1) and Brilliant Violet 421 anti-human FcγRIII (clone 3G8) were obtained from BioLegend (San Diego, California, USA). AlexaFluor 647 anti-human FcγRIIA/B/C (clone AT10) was obtained from LifeSpan Biosciences (Seattle, Washington, USA). FITC anti-human FcγRIIA (clone IV.3) was obtained from Stemcell Technologies (Vancouver, Canada).

Deglycosylation of FcγR-blocking antibodies

FcγR blocking antibodies and isotype controls (mouse IgG1, mouse IgG2b) at a concentration of 1 mg/mL in phosphate-buffered saline (pH 7.4) were fully deglycosylated using recombinant glycerol-free PNGase F (New England Biolabs, Ontario, Canada) by adding 8 units PNGase F per µL antibody for mouse IgG1 antibodies (10.1, AT10, 3G8, mouse IgG1 control) or 40 units PNGase F per µL antibody for mouse IgG2b antibodies (IV.3 and mouse IgG2b control). Antibodies were incubated for 48 hours at 37°C then separated from the glycans and PNGase F using a 50 kDa Vivaspin 6 molecular weight cut-off column concentrator (Millipore Sigma Canada) with repeated phosphate-buffered saline (PBS) washing. Final antibody concentration was determined using a Nano Drop ND-1000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). Deglycosylation of FcγR blocking antibodies was confirmed by SDS-PAGE under reducing conditions by a decrease of ~3 kDa in the heavy chain apparent molecular weight compared to the parental (unmodified) blocking antibody heavy chain.

Monovalent anti-FcγRIII blocking duobody generation

A monovalent and bispecific duobody with one Fab specific for FcγRIII (clone 3G8) and other Fab specific for 2,4,6-trinitrophenyl (anti-TNP) (ant-TNP described by Dekkers et al Front Immunol. 2017 Aug 2;8:877) with the Fc region bearing $N_{297}A$ (prevents Fc glycosylation) and PG-LALA ($L_{234}A$, $L_{235}A$, and $P_{329}G$) mutations to completely eliminate FcγR binding was generated using a human IgG1 duobody platform. Bi-specific (heterodimer) antibody formation was done using a “knob-into-hole” system; anti-TNP was generated in the $K_{409}R$ background, whereas the 3G8 was generated with the matching $F_{405}L$ background. The two antibodies were then mixed in equimolar with 25 mM 2-mercaptoethylamine for 1.5h at 37°C, allowing heavy chain exchange for heterodimer formation of $K_{409}R$ with $F_{405}L$ backgrounds, and dialyzed overnight in PBS as described by Labrijn et al. (Nat Protoc. 2014;9(10):2450-63). The antibodies were produced in HEK freestyle cells and purified by protein A as previously described (Dekkers et al Front Immunol. 2017 Aug 2;8:877).

Detection of free anti-GPIIb/IIIa-specific autoantibodies in ITP sera

Anti-platelet IgG autoantibodies in ITP patient sera were detected by the indirect monoclonal antibody-specific immobilization of platelet antigens (MAIPA) assay as described by Kiefel et al. (Blood. 1987;70(6):1722-1726). Assay sensitivity was controlled by the use of anti-HPA-1a WHO standard (NIBSC, Potters Bar, UK) and results were read using a photometer at 492 nm. An absorbance/optical density (O.D.) ≥0.15 was the threshold for positivity. Five ITP sera positive for GPIIb/IIIa autoantibodies and negative for GPIb/IX and GPV autoantibodies (available characteristics summarized in Supplemental Table 2) were used to opsonize of platelets for induction of phagocytosis. The study was conducted in accordance with the Declaration of Helsinki, and the collection of patient sera was approved by the local ethics committees in Giessen, Germany, and Toronto, Canada.

Splenic macrophage isolation

Spleen cell suspensions were obtained from a tissue bank at the University of Rochester, School of Medicine (Rochester, New York). The collection of splenic specimens was approved by the ethics committee at the University of Rochester Medical Center. ITP spleens or spleens removed for splenic trauma (controls) were manually separated and pressed through a 100 µL sieve with calcium- and magnesium-free PBS washing. Erythrocyte lysis was performed using a dilute ammonium chloride buffer solution and single cell suspensions were washed by centrifugation and resuspended in CryoStor CS5 cryopreservation medium (Stemcell Technologies, British Columbia, Canada). Samples were placed in pre-cooled rate-controlled freezing tubes in a -80°C freezer overnight before moving to liquid nitrogen for long-term storage. A total of eight unique spleens from ITP patients and five from controls were used a source of splenic macrophages for experiments (ITP patient characteristics are summarized in Supplemental Table 1).

On the day of an experiment, spleen cell suspensions from ITP patients were retrieved from liquid nitrogen storage and thawed in a 37°C water bath. Cell suspensions were washed with complete RPMI pH 7.4 (10% fetal bovine serum (Wisent Bioproducts, Ontario, Canada), 10 mM HEPES (MilliporeSigma Canada, Ontario, Canada), antibiotic-

antimycotic (catalogue # 15240096; Thermo Fisher Scientific, Massachusetts, USA), and supplemented L-glutamine (Thermo Fisher Scientific, Massachusetts, USA)) and resuspended in cold PBS pH 7.4 with 1% FBS and kept on ice for the rest of the separation procedure. Splenic macrophages were separated by CD14 positive selection according to the manufacturer's protocol using PBS-resuspended lyophilized human CD14 MicroBeads (sodium azide-free) (Miltenyi Biotec, Germany) with an autoMACS Pro Separator (Miltenyi Biotec, Germany). Isolation and quality of splenic macrophage isolation was confirmed by flow cytometry by forward-side scatter, FcγR expression, and percentage of CD14-positive cells (average 80%). For phagocytosis, $1x10^5$ CD14 positively-selected macrophages were seeded into wells for phagocytosis and allowed to recover and adhere for 2 hr in complete RPMI in a 37°C, 5% CO_2 incubator. Splenic macrophages were further enriched after adherence by washing with PBS to remove non-adherent lymphocytes and other non-adherent cells before phagocytosis.

Flow cytometric analysis of splenic cells

The percentage of B cells (anti-CD19); T cells (anti-CD3); granulocytes (anti-CD66b); and macrophages (anti-CD14) were determined for splenic specimens using fluorescent antibodies before and after CD14-positive selection (both CD14-positive and CD14-negative fractions). Antibodies were incubated at 1/400 dilution in PBS-1% FBS with splenic macrophages for 20 minutes on ice before washing with PBS-1% FBS and centrifuged at 300xg for 5 minutes. Flow cytometry was performed using a BD LSRFortessa X-20 (Beckton Dickson, Franklin Lakes, New Jersey, USA) and data analysis was performed using FlowJo v10 (Beckton Dickson).

Splenic macrophage FcγR expression

FcγR expression of CD14 positively selected macrophages was determined using fluorescent antibodies to FcγRI (clone 10.1), FcγRIIA (clone IV.3), FcγRIIA/B/C (clone AT10), and FcγRIII (clone 3G8). Antibodies were incubated at 1/400 dilution in PBS-1% FBS with splenic macrophages for 20 minutes on ice before washing with PBS-1% FBS and centrifugation (300xg for 5 minutes). Flow cytometry and data analysis was performed as done for the flow cytometric analysis of splenic cell populations.

Splenic macrophage phagocytosis of ITP sera-opsonized platelets

Citrated whole blood was obtained from healthy donors and platelet-rich plasma was obtained by centrifugation. Platelet concentration was determined using a Z Series Coulter Counter (Beckman Coulter) and adjusted to $150x10^9$/L. Carbocyclic PGI_2 (Carbacyclin) (Santa Cruz Biotechnology, Texas, USA) was added to the platelet-rich plasma to 1 µg/mL to inhibit platelet activation. Platelets were fluorescently labelled by adding Cell Tracker Green 5-chloromethylfluorescein diacetate (Thermo Fisher Scientific, Massachusetts, USA) at 20 µM to the platelet-rich plasma and incubated at room temperature for 45 minutes under gentle agitation. Platelets were washed by adding sterile PBS to a volume of 10 mL and centrifuged for 8 minutes at 800xg and resuspended in PBS without carbocyclic PGI_2. Platelets were incubated 1:1 with anti-GPIIb/IIIa ITP sera (ITP sera), normal human serum (NHS), or PBS (non-opsonized) for 30 minutes at room temperature. Splenic macrophages isolated by CD14 positive selection as outlined in section “splenic macrophage isolation” were seeded into wells of a 24-well polystyrene plate (Corning Incorporated, New York, USA) on 12 mm poly-D-lysine round coverslips (Corning Incorporated, New York, USA), and treated with individual or all FcγR blocking antibodies at 10 µg/mL each to FcγRI (10.1), FcγRIIA (IV.3), FcγRIIA/B/C (AT10), FcγRIII (3G8), or an isotype control (mouse IgG1 at 30 µg/mL and mouse IgG2b at 10 µg/mL; respective to all blocking antibodies) diluted in complete RPMI for 30 minutes at 37°C in a 5% CO_2 incubator. Splenic macrophages were washed three times with PBS, and 50 µL platelet-serum suspension was added to each well followed by 200 µL of warm complete RPMI (platelet to macrophage ratio of 37.5:1). Phagocytosis proceeded at 37 °C in a 5% CO_2 incubator for 40 minutes. Phagocytosis was stopped on ice, and wells were washed three times with ice-chilled PBS, and splenic macrophages were fixed with 4% paraformaldehyde-PBS solution (Electron Microscopy Sciences, Pennsylvania, USA) for 15 minutes. Macrophages were then washed three times with PBS and surface-bound, non-phagocytosed platelets were identified by immunofluorescence using a mouse anti-human GPIX-AlexaFluor 647 antibody (Bio-Rad Laboratories, California, USA) with incubation in PBS for 20 minutes at room temperature. Splenic macrophages were washed twice and mounted on to glass slides (Thermo Fisher Scientific, Massachusetts, USA) with Dako

Fluorescence Mounting Medium (Agilent Technologies, California, USA). Splenic macrophages were observed by spinning-disc confocal microscopy under 63x objective oil immersion (numerical aperture 1.47) with differential interference contrast (DIC) and laser fluorescence (488, 647 excitation) on a Quorum multi-modal imaging system (Quorum Technologies, Ontario, Canada) equipped with 50 micrometer pinhole spinning disc and ORCA-Flash 4.0 V2 PLUS sCMOS camera. At least four images were taken at the centre of each well with Z-stacking every 0.33 µm with >30 stacks. Z-stacked images were 3D reconstructed for analysis using Imaris v8.0.2 (Bitplane, United Kingdom). Surface-bound (non-phagocytosed) platelets were identified by staining for AlexaFluor 647-conjugated anti-GPIX (incubated after stopping macrophage phagocytosis and fixation), and 3D reconstructions of Z-stacked images allowed us to confirm phagocytosed platelets as being within the macrophage body. Platelets were additionally defined by size (1.5 µm to 3.5 µm) in Imaris v8.0.2 to distinguish them from internalized microparticles or platelets aggregates. Phagocytic index was calculated as (engulfed platelets counted / splenic macrophages counted) x 100.

Splenic macrophage phagocytosis of anti-D-opsonized erythrocytes

Citrated whole blood from donors was washed by mixing 100 µL blood with 900µL PBS and centrifuged at 300xg for 3 minutes. Pelleted erythrocytes were resuspended at a concentration of $1x10^8$ erythrocytes/mL with PBS and opsonized with anti-D (WinRho, Cangene corporation, Winnipeg, Canada) at 1/250 dilution (94 µg/mL) or PBS (control) and incubated for 30 minutes at room temperature. Erythrocytes were washed by centrifugation and resuspended in PBS to $1x10^8$ erythrocytes/mL for phagocytosis. Splenic macrophages isolated by CD14 positive selection as outlined in section “splenic macrophage isolation” were seeded into wells of a 12-well polystyrene plate (Corning Incorporated, New York, USA), and treated with individual or all FcγR blocking antibodies at 10 µg/mL each to FcγRI (10.1), FcγRIIA (IV.3), FcγRIIA/B/C (AT10), FcγRIII (3G8), or isotype control (mouse IgG1 at 30 µg/mL and mouse IgG2b at 10 µg/mL) diluted in complete RPMI for 30 minutes at 37°C in a 5% CO_2 incubator. Macrophages were washed twice with PBS and 300 µL complete RPMI was added back. Anti-D opsonized or non-opsonized erythrocyte solutions (100µL) were added to

appropriate wells and mixed briefly (erythrocyte to macrophage ratio of 100:1). Phagocytosis proceeded for 40 minutes at 37 °C in a 5% CO_2 incubator and was stopped on ice. Media and excess erythrocytes were aspirated from the wells and non-internalized surface-bound erythrocytes were removed by hypotonic lysis by adding 900µL ice-cold water for 90 seconds, followed by 100µL of 10x-PBS to return isotonicity. Wells were washed once more with PBS before fixation with iced 4% paraformaldehyde solution for 15 minutes. Phagocytosis of erythrocytes was determined by microscopy using a Nikon Eclipse TS100 inverted microscope and subsequent analysis using Fiji (ImageJ). Phagocytic index was calculated as (engulfed erythrocytes counted/ splenic macrophages counted) x 100.

Data Analysis

Data error is presented as mean ± standard deviation. All statistical calculations as indicated were performed using GraphPad Prism (San Diego, California, USA) version 7.04. All data was tested for normal distribution by the Shapiro-Wilk normality test. Figures were prepared using GraphPad Prism (San Diego, California, USA) version 7.04, Microscoft PowerPoint 2016, and Adobe Illustrator version CC 2020 (24.0) (Adobe Incorporated, Mountain View, California, USA). Flow cytometry data was analysed using FlowJo version 10 (Beckton Dickson, Franklin Lakes, New Jersey, USA. Microscopy images were analysed using Imaris version 8.0.2 (Bitplate, United Kingdom) and Fiji (ImageJ).