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

by Peter A. A. Norris, George B. Segel, W. Richard Burack, Ulrich J. Sachs, Suzanne N. Lissenberg-Thunnissen, Gestur Vidarsson, Behnaz Bayat, Christine M. Cserti-Gazdewich, Jeannie Callum, Yulia Lin, Donald Branch, Rick Kapur, John W. Semple, and Alan H. Lazarus

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

Running title:

FcγRI and FcγRIII mediate phagocytosis in ITP

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Immunoglobulin G (IgG) anti-platelet autoantibodies are thought to play a central role in platelet destruction in immune thrombocytopenia (ITP).\textsuperscript{1} IgG autoantibodies are detected in up to 81\% of patients with ITP by the direct monoclonal antibody immobilization of platelet antigens (MAIPA) assay,\textsuperscript{2} and target several platelet glycoproteins (GP) including GPIIb/IIIa, GPIb/IX, and GPV.\textsuperscript{2,3} While anti-GPIb antibodies can mediate FcγR-independent modes of platelet clearance,\textsuperscript{4} anti-GPIIb/IIIa autoantibodies are presumed to drive FcγR-dependent platelet clearance through mononuclear phagocytes in the spleen (splenic macrophages). However, the role of specific FcγRs in the phagocytosis of autoantibody-opsonized platelets is unknown.

Here, we purified macrophages from the spleens of ITP patients and incubated them with platelets opsonized with anti-GPIIb/IIIa ITP sera to induce phagocytosis. The role of specific FcγRs was investigated by treating macrophages with individual or combined blocking antibodies against specific FcγRs. Anti-GPIIb/IIIa-specific ITP sera mediated significant phagocytosis of platelets relative to platelets incubated with sera from healthy donors. Targeting all FcγRs by combining blocking antibodies led to near complete inhibition of splenic macrophage phagocytosis. Blockade of single FcγRs revealed that FcγRI and FcγRIII, but not FcγRIIA, were responsible for phagocytosis. Further, we compared macrophages from ITP and control (trauma) spleens and identified they had similar phagocytic activity, FcγR expression, and used the same FcγRs in the phagocytosis of an unbiased target (anti-D-opsonized erythrocytes). Our results indicate that anti-GPIIb/IIIa ITP autoantibodies mediate FcγR-dependent splenic macrophage phagocytosis through FcγRI and FcγRIII.

Despite the prevailing hypothesis that anti-GPIIb/IIIa autoantibodies clear platelets through splenic macrophage FcγRs, direct demonstrations of this are lacking. McMillan et al. first observed that splenic leukocytes from patients with ITP mediated uptake and/or binding of healthy donor platelets without prior ITP serum incubation, implicating the spleen as a site of both autoantibody production and platelet clearance.\textsuperscript{5} Kuwana et al. demonstrated in vitro that peripheral blood monocyte-derived macrophages acquire antigen from ITP patient platelets through FcγRI for presentation to GPIIb/IIIa-specific T cells.\textsuperscript{6} Nakar et al. successfully treated a small cohort of ITP patients with a blocking antibody against FcγRIII, implicating FcγRIII in the clearance of platelets.\textsuperscript{7} However, the
contribution of specific splenic macrophage FcγRs to the clearance of platelets has not been directly established and the involvement of other FcγRs cannot be excluded.

To identify the FcγRs involved in splenic macrophage phagocytosis, macrophages were purified from ITP patient spleen cell suspensions by CD14 positive selection (Supplemental Figure 1). Sera from five patients with ITP positive for GPIIb/IIIa autoantibodies but negative against GPIb/IX and GPV (by the indirect MAIPA) were used individually to opsonize healthy donor platelets for phagocytosis. Available patient characteristics for the ITP spleen donors are summarized in Supplemental Table 1 and ITP sera donors in Supplemental Table 2. Platelets were fluorescently labelled with 5-chloromethylfluorescein diacetate (CMFDA) and phagocytosis was evaluated by confocal microscopy. Non-phagocytosed platelets were detected with an anti-GPIX fluorescent antibody after phagocytosis. Incubation of platelets with ITP serum led to a significant increase in splenic macrophage phagocytosis compared to normal human serum (NHS) \( (p=0.0015) \) (Figure 1 A, B). To evaluate the specific FcγRs involved, blocking antibodies against FcγRI, FcγRIIA, FcγRIIA/B/C, and FcγRIII were used. Antibodies were deglycosylated using PNGase-F to reduce non-specific blockade, and each antibody was dose-dependently examined for its ability to bind and block phagocytosis (not shown). Two representative ITP sera were selected to evaluate FcγR utilization. Platelet uptake was reduced significantly by the combination of all FcγR blocking antibodies compared to the isotype control \( (p<0.0001) \) (Figure 1 C). Using single blocking antibodies, blockade of FcγRI inhibited ITP splenic macrophage phagocytosis by 42% \( (p<0.0001) \), while blockade of FcγRIII inhibited phagocytosis by 38% \( (p<0.0001) \). Surprisingly, minimal yet non-significant inhibition was achieved with blockade of FcγRIIA (10%, \( p=0.056 \)) or all FcγRII isoforms (7%, \( p=0.15 \)).

Although antibody blockade of FcγRIII has been used to successfully treat ITP patients, unfavorable adverse events limited this approach.\(^7\) As a monovalent approach can overcome toxicity associated with bivalent FcγR blocking antibodies,\(^8\) we evaluated whether a monovalent FcγRIII blocking antibody inhibits phagocytosis with equal efficacy relative to a bivalent antibody. We generated a monovalent FcγRIII-blocking IgG1-humanized duobody composed of an anti-FcγRIII (3G8) Fab paired with an
irrelevant anti-2,4,6-trinitrophenyl Fab. The construct also encoded N297A (prevents Fc glycosylation) and PG-LALA (P329G, L234A, and L235A) mutations to completely abrogate Fc-FcγR binding. The inhibition of ITP splenic macrophage phagocytosis achieved by the monovalent anti-FcγRIII duobody was not significantly different from the bivalent and deglycosylated blocking antibody (p=0.87) (Figure 1 D).

We next compared the leukocyte composition and macrophage FcγR expression from spleens of patients with ITP relative to healthy (trauma) controls. Both ITP and control spleens contained similar percentage of B cells (CD19+), T cells (CD3+), monocyte/macrophages (CD14+), and granulocytes (CD66b+) (Figure 2 A) as evaluated by flow cytometry. Macrophage FcγR expression was also similar between the two spleen types, with a non-significant trend of increased FcγR expression observed for ITP macrophages relative to controls (Figure 2 B-C).

Lastly, we compared the phagocytic activity between ITP patient and control splenic macrophages using healthy donor erythrocytes opsonized with a commercial preparation of anti-D (Cangene) as an unbiased phagocytic target. Phagocytosis was evaluated by brightfield microscopy and non-phagocytosed erythrocytes were removed by hypotonic lysis. Erythrocytes were phagocytosed in an antibody-dependent manner and no significant difference in the phagocytic index was observed between the two spleen types (Figure 3 A-B). Blocking antibodies were next used alone or in combination to determine whether FcγR utilization may differ between ITP and control splenic macrophages for anti-D-opsonized erythrocytes. Blockade of all FcγRs led to a significant decrease in the phagocytosis of anti-D-opsonized erythrocytes, down to non-opsonized levels (Figure 3 C-D). For macrophages from ITP spleens, FcγRI blockade inhibited phagocytosis of anti-D-opsonized erythrocytes by 58% (p<0.0001), while blockade of FcγRIII inhibited phagocytosis by 29% (p<0.0001). Blockade of FcγRIIA or all FcγRII isoforms did not significantly inhibit phagocytosis (4%, p=0.62 for FcγRIIA; and 6%, p=0.20 for all FcγRII isoforms). FcγRI was also the major phagocytic receptor in control splenic macrophages, as FcγRI blockade inhibited phagocytosis by 51% (p<0.0001) while FcγRIII blockade inhibited phagocytosis by 20% (p<0.05). No
significant effect for blockade of FcγRIIA (3%, \( p=0.99 \)) or all FcγRII isoforms (4%, \( p=0.97 \)) was observed.

While both Fc-dependent and Fc-independent autoantibody-mediated platelet clearance mechanisms in ITP have been explored\(^4\), the dominant pathophysiological mechanism in most patients is thought to involve FcγR-mediated platelet clearance by splenic macrophages.\(^1\) Therapies suggested to block FcγR-dependent processes such as anti-D and IVIg are effective in many patients with ITP, and an FcγRIII-specific blocking antibody was able to rapidly raise platelet counts in a small cohort of ITP patients.\(^7\) Further, inhibition of spleen tyrosine kinase (Syk) by treatment with fostamatinib is effective clinically in ITP.\(^10\) The success of these therapies suggests that interfering with FcγR function is an effective strategy to increase platelet counts in ITP patients.

While involvement of splenic macrophage FcγRIII in anti-GPIIb/IIIa-autoantibody-opsonized platelet uptake is consistent with reports that select blockade of FcγRIII is effective clinically,\(^7\) significant involvement of FcγRI was unexpected. Although the high-affinity nature of FcγRI for monomeric IgG suggests that FcγRI is saturated with IgG in vivo, FcγRI can engage effectively with immune complexes despite saturation under conditions such as cytokine stimulation.\(^11\) Antibody targeting of FcγRI as a therapeutic strategy for ITP has been reported for a single ITP patient, leading to downmodulation of FcγRI in circulating monocytes and transient monocytopenia but not improvement in the platelet count.\(^12\) However, as the antibody used did not directly target the IgG binding region of FcγRI and was reported for a single patient,\(^12\) it remains difficult to make conclusions about this approach.

We found that control and ITP splenic macrophage levels of FcγR expression, phagocytic activity, and the specific FcγRs utilized in the phagocytosis of anti-D-opsonized erythrocytes were not significantly different. The dominance of splenic macrophage FcγRI for anti-D-opsonized erythrocytes supports the work of Nagelkerke et al., who also observed that FcγRI was the primary FcγR mediating red pulp splenic macrophage phagocytosis of anti-D-opsonized erythrocytes.\(^13\) A previous study by Audia et al. found similar FcγR expression between ITP and control splenic macrophages;\(^14\) we observed a trend of increased FcγR expression on ITP splenic
macrophages relative to controls although it did not reach statistical significance. Splenic macrophages from patients with ITP have been previously identified to have an M1-type polarization bias.\textsuperscript{15} Although we did not investigate macrophage polarization, our results indicate at least that splenic macrophage Fc\(\gamma\)R expression and phagocytic activity in patients with ITP is similar to healthy individuals.

Combined Fc\(\gamma\)R blockade may be particularly useful in patients who are refractory to splenectomy, as it may block platelet clearance mediated by macrophages residing outside of the spleen such as in the liver, marrow, and lung. Our results indicate that, to the extent that Fc\(\gamma\)R-dependent phagocytosis contributes to platelet clearance in ITP, the individual or combined blockade of Fc\(\gamma\)RI and Fc\(\gamma\)RIII is likely the most effective strategy for targeted Fc\(\gamma\)R blockade as a therapeutic modality.

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**Authorship**


Conflicts of interest disclosures: A.H.L. has patents on the use of monoclonal antibodies as replacements for IVIg and has received research funding from Rigel Pharmaceuticals Incorporated and CSL Behring. Y.L. has received research funding from Novartis and Octapharma and consulting fees from Amgen and Pfizer. P.A.A.N., G.B.S., W.R.B., U.J.S., B.B., S.N.L.-T., C.M.C.-G., G.V., J.C., D.B., R.K., and J.W.S. do not have relevant conflicts of interest to disclose.

References


Figure legends

Figure 1. ITP splenic macrophages phagocytose GPIIb/IIIa autoantibody-opsonized platelets through FcγRI and FcγRIII. A) Splenic macrophages were isolated from ITP spleens by CD14 positive selection. Healthy donor platelets were opsonized with one of five different ITP sera positive for autoantibodies to GPIIb/IIIa but negative for GPIb/IX and GPV autoantibodies (identified by symbols □, ○, △, ■, and ● as patients 1-5 respectively in Supplemental Table 2) (n=4 experiments each). A total of eight unique ITP spleens were used to perform the phagocytosis. Four normal human sera (NHS, allogeneic to the platelet donor) specimens were used to opsonize platelets as controls (n=7 experiments). Non-ops: non-opsonized (PBS only). Phagocytic index: the number of phagocytosed platelets per 100 macrophages. B) ITP splenic macrophage (left-most panel) with phagocytized anti-GPIIb/IIIa ITP serum-opsonized platelet as imaged by spinning disc confocal microscopy (63x objective). Platelets were labelled with the cytoplasmic dye CMFDA (green, middle panel). External (non-phagocytosed) platelets were identified after phagocytosis using an AlexaFluor 647 (AF647)-conjugated anti-CD42a antibody (red, right panel). Platelets were additionally defined by size (1.5 μm to 3.5 μm) to distinguish them from internalized microparticles or platelet aggregates. Arrow: one phagocytosed platelet. C) Splenic macrophage FcγRs were blocked using deglycosylated antibodies to FcγRI (clone 10.1), FcγRIIA (IV.3), FcγRIIA/B/C (AT10), or FcγRIII (3G8) as indicated. Healthy donor platelets were opsonized with one of two representative anti-GPIIb/IIIa ITP sera (depicted ● and ■) (n=3 experiments; different spleen per experiment). Isotype control: 30 μg/mL deglycosylated mouse IgG1, 10 μg/mL deglycosylated mouse IgG2b (respective to combined blocking antibodies). D) Inhibition of ITP splenic macrophage phagocytosis of anti-GPIIb/IIIa ITP serum-opsonized platelets by a deglycosylated blocking antibody to FcγRIII (clone 3G8, “FcγRIII”) or a monovalent FcγRIII-blocking IgG1-humanized duobody (“FcγRIII duobody”). The duobody was bispecific (3G8 Fab, paired with anti-2,4,6-trinitrophenyl as an irrelevant Fab) and possessed PG-LALA (P329G, L234A, and L235A) and N297A mutations. Two different ITP sera (depicted ● and ■) were evaluated (n=3 experiments; different spleen per experiment). Significance for panel A: Kruskal-Wallis test (non-parametric one-way ANOVA) with multiple comparisons against all
means with Dunn’s post-hoc test. Significance for panels C-D: one-way ANOVA with multiple comparisons with Dunnett’s post-hoc test (C) or Tukey’s post-hoc test (D). P values: **** p<0.0001, ** p=0.0015, ns = not significant. Percent phagocytosis was calculated relative to an untreated group (untreated splenic macrophages with opsonized platelets). Data error: mean ± standard deviation.

**Figure 2. Leukocyte composition and splenic macrophage FcγR expression from ITP and control spleens.** A) Splenic cells from patients with ITP or splenic trauma (controls) were analyzed by flow cytometry using fluorescent antibodies to determine the percentage of B cells (anti-CD19); T cells (anti-CD3); granulocytes (anti-CD66b); and monocyte/macrophages (anti-CD14). Four different ITP and five control spleens were assessed. Statistical significance was calculated by unpaired T tests without assuming equal standard deviations. B) FcγR expression by ITP splenic macrophages was detected using fluorescent antibodies to FcγRI (clone 10.1), FcγRIIA (IV.3), FcγRII isoforms (FcγRIIA/B/C) (AT10), and FcγRIII (3G8). Representative histograms from a single patient with ITP are depicted. Blue shade histogram = macrophages stained with fluorescent antibody; red shade histogram = unstained control. Y-axis, counts; X-axis, log10 fluorescence intensity. C) The mean fluorescence intensity (MFI) for each FcγR was determined for ITP and control splenic macrophages (n=5, different spleen per experiment). Statistical significance was calculated by unpaired two-tailed T-tests; ns = not significant. Data error: mean ± standard deviation.

**Figure 3. ITP and control splenic macrophages primarily utilize FcγRI for the phagocytosis of anti-D-opsonized erythrocytes.** A) Phagocytic activity from ITP and control splenic macrophages (MΦ) for erythrocytes opsonized with anti-D (Cangene). Non-ops = non-opsonized (PBS only). Phagocytic index was calculated as the number of erythrocytes engulfed per 100 macrophages. B) Brightfield microscopy image of ITP splenic macrophages with phagocytosed erythrocytes. Arrow indicates a splenic macrophage with one phagocytosed anti-D-opsonized erythrocyte. C-D) Splenic macrophages (MΦ) from control (C) and ITP (D) spleens were assessed for FcγR utilization in the phagocytosis of anti-D-opsonized erythrocytes (n=5 experiments; different spleen per experiment). Macrophage FcγRs were blocked using
deglycosylated antibodies to FcγRI (clone 10.1), FcγRIIA (IV.3), FcγRIIA/B/C (AT10), or FcγRIII (3G8) as indicated. Isotype control: 30 μg/mL deglycosylated mouse IgG1, 10 μg/mL deglycosylated mouse IgG2b (respective to combined blocking antibodies). Percent phagocytosis was calculated relative to an untreated group (untreated splenic macrophages with opsonized erythrocytes). Significance for panel A: one-way ANOVA with multiple comparisons against all means with Tukey’s post-hoc test. Significance for panels C-D: one-way ANOVA with multiple comparisons against isotype control with Dunnett’s post-hoc test. P values: **** p<0.0001; * p<0.05. Data error: mean ± standard deviation.
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.
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Supplemental Table 1. Characteristics of ITP patient spleen donors

<table>
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<th>ITP spleen specimen</th>
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PLT = platelet
N.A. = not available
IVIg = intravenous immunoglobulin
TPO-RA = thrombopoietin receptor agonist

Supplemental Table 2. Characteristics of ITP patient serum donors

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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.
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(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) (anti-TNP described by Dekkers et al Front Immunol. 2017 Aug 2;8:877) with the Fc region bearing N297A (prevents Fc glycosylation) and PG-LALA (L234A, L235A, and P329G) 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 K409R background, whereas the 3G8 was generated with the matching F405L 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 K409R with F405L 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-
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antimyocotic (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% CO₂ 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 (Carbacycllin) (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-GPllb/llla 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-AlexaFlour 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% CO₂ 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\textsubscript{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, Microsoft 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).