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

Immunoglobulin G (IgG) anti-platelet autoantibodies are thought to play a central role in platelet destruction in immune thrombocytopenia (ITP).[1] IgG autoantibodies are detected in up to 81% of patients with ITP by the direct monoclonal antibody immobilization of platelet antigens (MAIPA) assay,[2] and target several platelet glycoproteins (GP) including GPIIb/IIIa, GPIb/IX, and GPV.[2,3] While anti-GPIb antibodies can mediate FcγR-independent modes of platelet clearance,[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 types of FcγR 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 FcγR types was investigated by treating macrophages with individual or combined blocking antibodies against specific FcγR. Anti-GPIIb/IIIa-specific ITP sera mediated significant phagocytosis of platelets relative to platelets incubated with sera from healthy donors. Targeting all FcγR by combining blocking antibodies led to near complete inhibition of splenic macrophage phagocytosis. Blockade of single FcγR types revealed that FcγRI and FcγRIII, but not FcγRIIA, were responsible for phagocytosis. Furthermore, we compared macrophages from ITP and control (trauma) spleens and determined that they had similar phagocytic activity, FcγR expression, and used the same types of FcγR 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 FcγR, 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 incubation with ITP serum, implicating the spleen as a site of both autoantibody production and platelet clearance.[5] Kuwana et al. demonstrated *in vitro* that peripheral blood monocyte-derived macrophages acquire antigen from ITP patients' platelets through FcγRI for presentation to GPIIb/IIIa-specific T cells.[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.[7] However, the contribution of specific splenic macrophage FcγR to the clearance of platelets has not been directly established and the involvement of other FcγR cannot be excluded.

To identify which FcγR are involved in splenic macrophage phagocytosis, macrophages were purified from ITP patients' spleen cell suspensions by CD14-positive selection (*Online Supplementary Figure S1*). Sera from five patients with ITP who were positive for GPIIb/IIIa autoantibodies but negative for antibodies against GPIb/IX and GPV (by indirect MAIPA assay) were used individually to opsonize healthy donors' platelets for phagocytosis. Available characteristics of the ITP patients who provided spleen samples are summarized in *Online Supplementary Table S1* and while those of ITP patients who gave sera are summarized in *Online Supplementary Table S2*. Platelets were fluorescently labeled 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 the phagocytosis following incubation in normal human serum (NHS) (P=0.0015) (Figure 1A, B). To evaluate the specific FcγR types 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 1C). 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, 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 as 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 (which prevents Fc glycosylation) and PG-LALA (P329G, L234A, and L235A) mutations to completely abrogate Fc-FcγR binding.[9] The inhibition of ITP splenic macrophage phagocytosis achieved by the monovalent anti-FcγRIII duobody was not significantly different from that achieved by the bivalent and deglycosylated blocking antibody (P=0.87) (Figure 1D).

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

Lastly, we compared the phagocytic activity between splenic macrophages from ITP patients and controls using healthy donor erythrocytes opsonized with a commercial preparation of anti-D (Cangene) as an unbiased phagocytic target. Phagocytosis was evaluated by bright-field 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 3A, B). Blocking antibodies were next used alone or in combina-

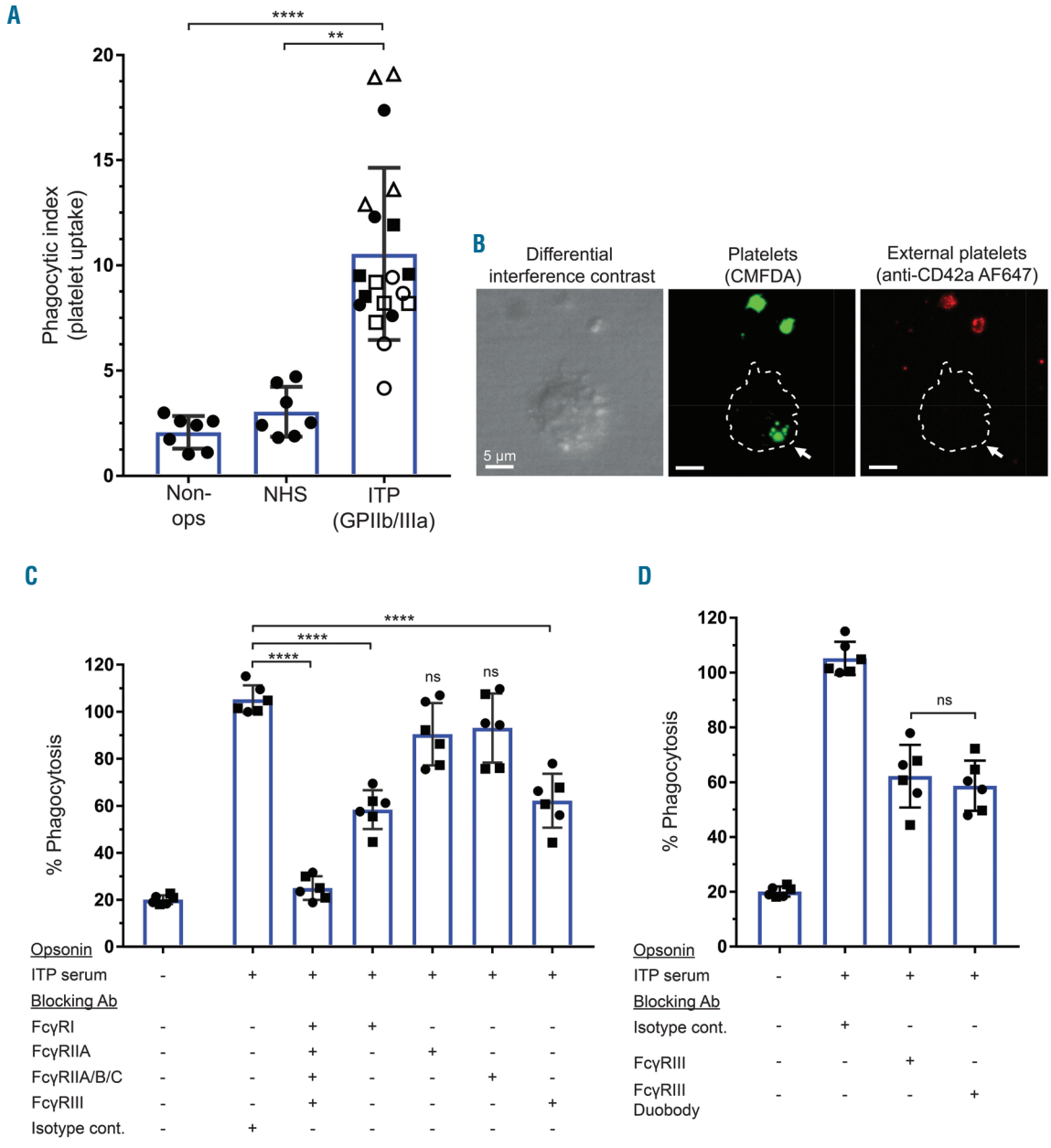

Figure 1. Splenic macrophages from patients with immune thrombocytopenia phagocytose GPIIb/IIIa autoantibody-opsonized platelets through FcγRI and FcγRIII. (A) Splenic macrophages were isolated from spleens from patients with immune thrombocytopenia (ITP) by CD14-positive selection. Healthy donor platelets 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 *Online Supplementary Table S2*) (n=4 experiments each). A total of eight unique ITP spleens were used to perform the phagocytosis studies. 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 (phosphate-buffered saline only). Phagocytic index: the number of phagocytosed platelets per 100 macrophages. (B) ITP splenic macrophage (left-most panel) with a phagocytosed anti-GPIIb/IIIa ITP serum-opsonized platelet as imaged by spinning disc confocal microscopy (63x objective). Platelets were labeled with the cytoplasmic dye 5-chloromethylfluorescein diacetate (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 FcγR 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 (represented by ● 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 ($P_{329}G$, $L_{234}A$, and $L_{235}A$) and $N_{297}A$ mutations. Two different ITP sera (represented by ● and ■) were evaluated (n=3 experiments; different spleen per experiment). Significance for (A): Kruskal-Wallis test (non-parametric one-way analysis of variance [ANOVA]) with multiple comparisons against all means with a Dunn *post-hoc* test. Significance for panels (C, D): one-way analysis of variance with multiple comparisons with a Dunnett *post-hoc* test (C) or Tukey *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.

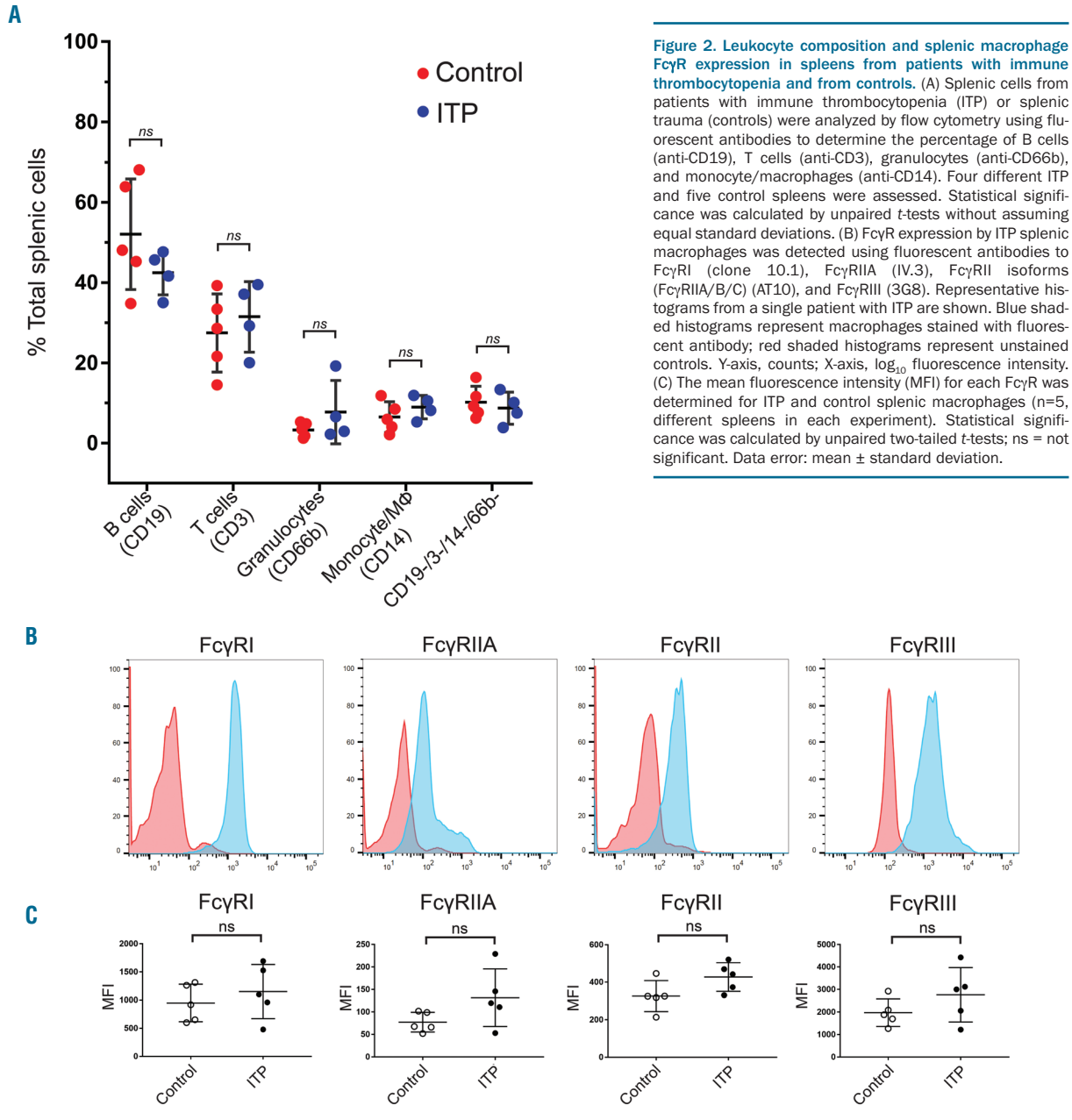

Figure 2. Leukocyte composition and splenic macrophage FcγR expression in spleens from patients with immune thrombocytopenia and from controls. (A) Splenic cells from patients with immune thrombocytopenia (ITP) or splenic trauma (controls) were analyzed by flow cytometry using fluorescent antibodies 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 shown. Blue shaded histograms represent macrophages stained with fluorescent antibody; red shaded histograms represent unstained controls. Y-axis, counts; X-axis, $\log_{10}$ fluorescence intensity. (C) The mean fluorescence intensity (MFI) for each FcγR was determined for ITP and control splenic macrophages (n=5, different spleens in each experiment). Statistical significance was calculated by unpaired two-tailed *t*-tests; ns = not significant. Data error: mean ± standard deviation.

tion to determine whether FcγR utilization may differ between ITP and control splenic macrophages for anti-D-opsonized erythrocytes. Blockade of all FcγR types led to a significant decrease in the phagocytosis of anti-D-opsonized erythrocytes, down to non-opsonized levels (Figure 3C, 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 was observed following blockade of FcγRIIA (3%, P=0.99) or all FcγRII isoforms (4%, P=0.97).

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] Furthermore, inhibition of spleen tyrosine kinase (Syk) by treatment with fostamatinib is clinically effective in ITP.[10] The success of these therapies suggests that interfering with FcγR function is an effective strategy to increase platelet counts in patients with ITP.

While involvement of splenic macrophage FcγRIII in anti-GPIIb/IIIa-autoantibody-opsonized platelet uptake is consistent with reports that selective blockade of FcγRIII is clinically effective,[7] significant involvement of

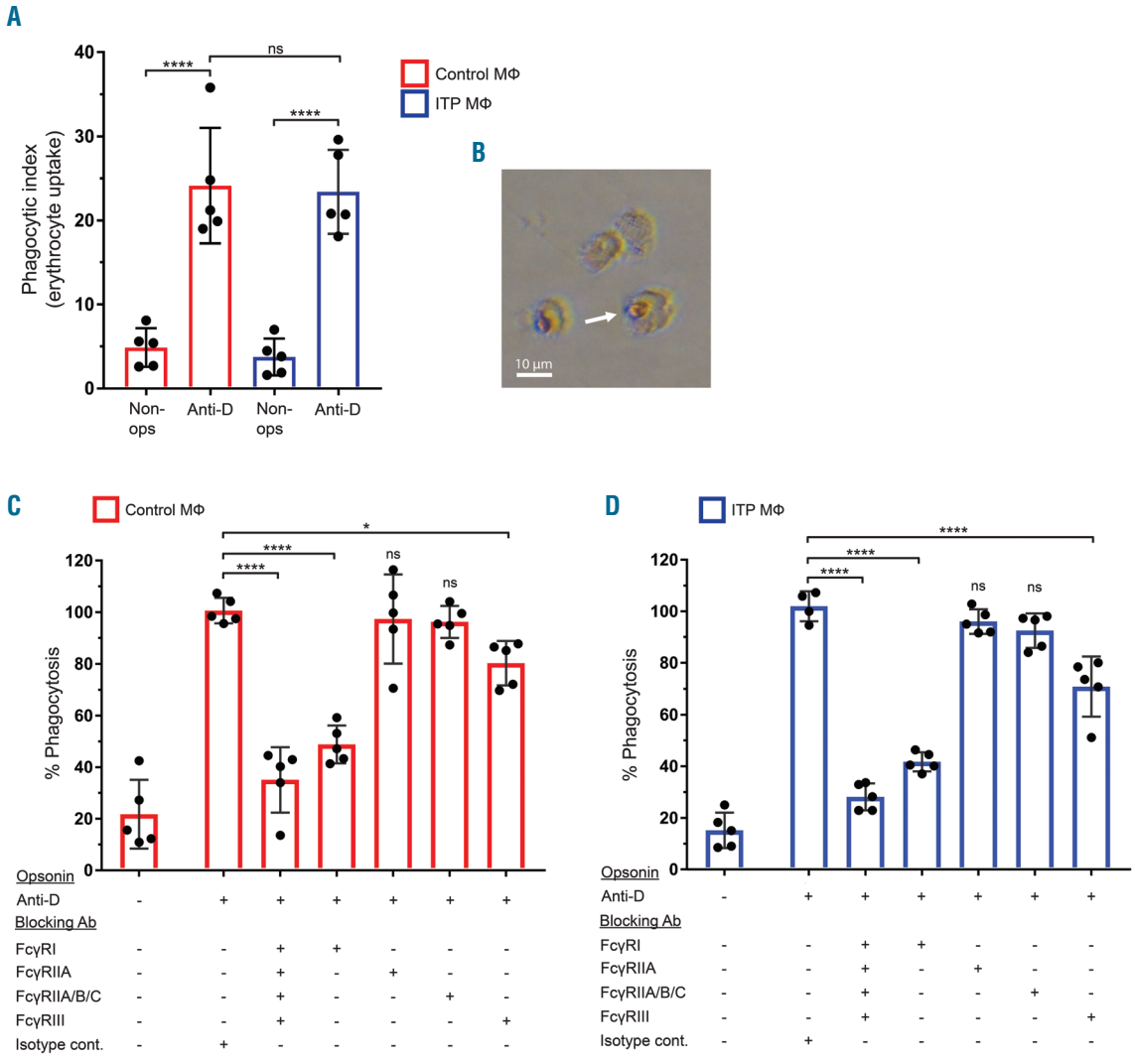

Figure 3. Splenic macrophages from patients with immune thrombocytopenia and from controls primarily utilize FcγRI for the phagocytosis of anti-D-opsonized erythrocytes. (A) Phagocytic activity of immune thrombocytopenia (ITP) and control splenic macrophages (MΦ) for erythrocytes opsonized with anti-D (Cangene). Non-ops: non-opsonized (phosphate-buffered saline only). The phagocytic index was calculated as the number of erythrocytes engulfed per 100 macrophages. (B) Brightfield microscopy image of ITP splenic macrophages with phagocytosed erythrocytes. The 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 spleens in each experiment). Macrophage FcγR 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 (with respect 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 analysis of variance (ANOVA) with multiple comparisons against all means with the Tukey *post-hoc* test. Significance for panels (C, D): one-way ANOVA with multiple comparisons against isotype control with the Dunnett *post-hoc* test. *P* values: ****$P<0.0001$; *$P<0.05$. Data error: mean ± standard deviation.

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 and led to downmodulation of FcγRI in circulating monocytes and transient monocytopenia but not to an 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 types of FcγR 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 findings 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 control macrophages although the difference did not reach statistical significance. Splenic macrophages from patients with ITP have been previously identified to have an M1-type polarization bias.[15] Although we did not investigate macrophage polarization, our results indicate at least that splenic macrophage FcγR expression and phagocytic activity in

patients with ITP are similar to those in healthy individuals.

Combined FcγR blockade may be particularly useful in patients who are refractory to splenectomy, as it may block platelet clearance mediated by macrophages residing outside the spleen such as those in the liver, marrow, and lungs. Our results indicate that, to the extent that FcγR-dependent phagocytosis contributes to platelet clearance in ITP, the individual or combined blockade of FcγRI and FcγRIII is likely the most effective strategy for targeted FcγR blockade as a therapeutic modality.

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Contributions: AHL, GBS and PAAN conceived the study; PAAN, AHL, GBS, and R.K. designed experiments; WRB, UJS, SNL-T, GV, BB, CMC-G JC, YL, Dand JWS provided reagents; PAAN performed experiments; AHL, GB and PAAN analyzed and interpreted data; PAAN and GBS wrote the manuscript; AHL, GBS, PAAN, WRB, UJS, SNL-T, GV, JC, RK and JWS edited the manuscript.

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