REGULAR ARTICLE

blood advances

Human Fc gamma receptor IIIA blockade inhibits platelet destruction in a humanized murine model of ITP

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Key Points

- Human FcγRIII monovalent blocking as a 1-armed antibody or albumin fusion protein are effective in a humanized model of ITP.
- The monovalent albumin fusion protein showed no AEs, whereas the Fc-impaired 1-armed antibody induced minor changes in body temperature.

Fc gamma receptor (FcγR) IIIA is an important receptor for immunoglobulin G (IgG) and is involved in immune defense mechanisms as well as tissue destruction in some autoimmune diseases including immune thrombocytopenia (ITP). FcγRIIIA on macrophages can trigger phagocytosis of IgG-sensitized platelets, and prior pilot studies observed blockade of FcγRIIIA increased platelet counts in patients with ITP. Unfortunately, although blockade of FcγRIIIA in patients with ITP increased platelet counts, its engagement by the blocking antibody drove serious adverse inflammatory reactions. These adverse events were postulated to originate from the antibody's Fc and/or bivalent nature. The blockade of human FcγRIIIA in vivo with a monovalent construct lacking an active Fc region has not yet been achieved. To effectively block FcγRIIIA in vivo, we developed a high affinity monovalent single-chain variable fragment (scFv) that can bind and block human FcγRIIIA. This scFv (17C02) was expressed in 3 formats: a monovalent fusion protein with albumin, a 1-armed human IgG1 antibody, and a standard bivalent mouse (IgG2a) antibody. Both monovalent formats were effective in preventing phagocytosis of ITP serum–sensitized human platelets. In vivo studies using FcγR-humanized mice demonstrated that both monovalent therapeutics were also able to increase platelet counts. The monovalent albumin fusion protein did not have adverse event activity as assessed by changes in body temperature, whereas the 1-armed antibody induced some changes in body temperature even though the Fc region function was impaired by the Leu234Ala and Leu235Ala mutations. These data demonstrate that monovalent blockade of human FcγRIIIA in vivo can potentially be a therapeutic strategy for patients with ITP.

Introduction

The mononuclear phagocyte system plays an important role in the clearance of antibody-sensitized cells to maintain normal host defenses against microorganisms and tumor cells.[1] However, this capability

Submitted 13 November 2023; accepted 22 January 2024; prepublished online on *Blood Advances* First Edition 8 February 2024; final version published online 10 April 2024. https://doi.org/10.1182/bloodadvances.2023012155.

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Data are available on reasonable request from the corresponding author, Alan H. Lazarus (alan.lazarus@unityhealth.to).

The full-text version of this article contains a data supplement.

also allows for the innate immune system to contribute to various immune-mediated diseases, such as cytopenias, in which immunoglobulin G (IgG)–coated erythrocytes and platelets are destroyed.[2,3] Immune thrombocytopenia (ITP) is an example of 1 of these autoimmune cytopenias, in which autoantibodies bind to platelet glycoproteins, clearing them through the mononuclear phagocyte system.

It is widely accepted that IgG-sensitized platelets can be recognized and phagocytosed through Fc gamma receptors (FcγRs) expressed on macrophages. Thus, FcγR blockade was hypothesized to be a potential strategy to ameliorate ITP. One of the first FcγRs to be targeted was murine FcγRIII. Studies performed with the monoclonal antibody 2.4G2, which blocked the murine low-affinity FcγRIII (as well as FcγRIIB), demonstrated a dramatic reduction in the clearance of antibody-sensitized cells.[4] The first human application of FcγR blockade was using a mouse antihuman FcγRIIIA antibody called 3G8 in ITP.[5] In later studies, a humanized version of this antibody (called GMA161) raised platelet counts in some patients, but both 3G8 and GMA161 induced severe adverse events, thus limiting further use.[6,7] In 2016, we demonstrated that monovalent blockade of murine FcγRIII could increase platelet counts in murine ITP without triggering adverse inflammatory reactions.[8] This strategy consisted of using the variable regions of the 2.4G2 antibody expressed as a single-chain variable fragment (scFv) fused to murine albumin, which was performed to increase half-life. Although this approach successfully raised platelet counts without triggering adverse inflammation, expressing the antibody as a scFv dramatically reduced its affinity. Human FcγRIIIA-blocking activity of a similarly designed scFv using 3G8 also had dramatically lower FcγR-blocking activity,[8] and our work demonstrated this showing that a 3G8-albumin construct could not effectively treat ITP in a FcγR-humanized murine ITP model (not shown). Thus, successful blockade of human FcγRIIIA and consequent therapeutic activity required the generation of a new product.

Here, we demonstrate an effective first-in-class monovalent blocking therapeutic against human FcγRIIIA for potential use in ITP. Using phage display, a monovalent scFv capable of blocking human FcγRIIIA was generated. The scFv, called 17C02, was expressed in 3 forms: a monovalent fusion protein with albumin, a 1-armed human IgG1 antibody, and a standard bivalent mouse IgG2a antibody. All 3 molecules effectively prevented phagocytosis of ITP serum–sensitized human platelets in vitro. However, only the monovalent therapeutics ameliorated ITP in FcγR-humanized mice, notably at a very low dosage as well (2 mg/kg). Inflammatory reactions were not provoked by the monovalent albumin fusion protein. However, the 1-armed antibody induced some changes in body temperature despite impairing the Fc region function by Leu234Ala and Leu235Ala mutations (commonly referred to as LALA mutations). These data demonstrate that monovalent blockade of human FcγRIIIA may be a potential therapeutic for patients with ITP, with caution that the 1-armed antibody, but not the albumin fusion, can lead to inflammatory activity.

Materials and methods

For full details, see the supplemental Materials and Methods.

Cell line, FcγRs, and mice

THP-1-CD16A cells[9] were maintained in a 37°C, 5% CO_2 environment in complete RPMI medium. The extracellular domain of human FcγRs fused with a polyhistidine tag at the C-terminus were purchased from Sino Biological (Chesterbrook, PA). FcγRI (CD64, catalog number 10256-H08H), FcγRIIIA (CD16A, catalog number 10389-H27H), and FcγRIIIB (CD16B, catalog number 11046-H08C), as well as biotinylated-FcγRIIA (catalog number 10374-H27H-B) and biotinylated-FcγRIIIA (catalog number 10389-H27H1-B) were also purchased from Sino Biological. Recombinant cynomolgus FcγRIIIA (cynoFcγRIIIA; catalog number 9224-FC) was purchased from R&D (Minneapolis, MN).

FcγR humanized (H-2^d) mice[10] were kindly donated by Jeffery Ravetch from the Rockefeller University. These mice express human FcγRI, FcγRIIA, FcγRIIB, FcγRIIIA, and FcγRIIIB but are genetically deficient for mouse FcγRI, FcγRIIB, FcγRIII, and FcγRIV. The mice were bred in-house, and both male and female mice aged 7 to 14 weeks were used. Female BALB/c (H-2^d) mice (aged 4-5 weeks) were purchased from Jackson Laboratories (CA). All mice were maintained in a specific pathogen-free facility at Keenan Research Centre for Biomedical Science, St. Michael's Hospital. All studies were approved by the St. Michael's Hospital Institutional Animal Care and Use Committee.

Generation of the 17C02 scFv by phage display library

Briefly, for the construction of a scFv-phage display library to obtain scFv blocking human FcγRIIIA, messenger RNA from spleen cells of FcγRIIIA-immunized mice were used. Universal primers were used to amplify the genes that encode the heavy and light chain variable regions (V_H and V_L) by polymerase chain reaction. A G4S sequence was introduced as a linker to connect the V_H and V_L, with final constructs cloned into the phagemid vector pADL-23c. *Escherichia coli* (TG1 electrocompetent cells; Agilent) were transformed with the phagemid vector to generate the scFv-phage display library. Multiple rounds of positive and negative selection were performed to select the best binders to FcγRIIIA with low or no binding to FcγRI, FcγRIIA, FcγRIIB, and FcγRIIIB. The selected scFv clones were purified, and additional screening steps and criteria were applied to obtain scFvs with dramatically improved binding to FcγRIIIA, compared with the 3G8-based construct.

The final scFv designated as 17C02 was expressed as a monovalent fusion protein linked to human albumin using a G4S linker. In addition, 17C02 was also assembled as a 1-armed monovalent human IgG1 antibody with the LALA mutation (17C02-$IgG1_{OA}$) and a standard bivalent mouse (IgG2a) antibody.

Antibody deglycosylation

Antibodies 3G8, 10.1, AT10, IV.3, 17C02-IgG2a, and isotypes controls (IgG1 and IgG2a) at 0.5 mg/mL in phosphate-buffered saline (PBS; Gibco) were fully Fc region deglycosylated as previously described.[11]

Flow cytometric analysis

The binding capacity of 17C02-albumin, 17C02-IgG2a, and 17C02-$IgG1_{OA}$ to different sources of cells expressing the FcγRIIIA was analyzed by flow cytometry using 3G8 and 3G8-albumin as positive controls and albumin, mouse IgG (mIgG1 and mIgG2a), and human IgG (hIgG1) as negative controls. Molecules were diluted

(to concentrations of 0.055-274 μM) in PBS 1% BSA (Millipore Sigma, Canada) solution and incubated with cells for 30 minutes on ice. Cells were washed and incubated with the corresponding secondary antibodies: FITC-conjugated goat antihuman albumin (Bethyl Laboratories, Boston, MA), APC-conjugated F(ab)'2 goat antimouse IgG-Fcγ specific (Jackson ImmunoResearch, West Grove, PA), or AF647-conjugated donkey antihuman IgG (H+L) (Jackson ImmunoResearch); washed and analyzed on a BD LSRFortessa X-20 (Beckton Dickson). Data analysis was performed using FlowJo v10 (Beckton Dickson).

Phagocytosis of antibody-opsonized platelets

Phagocytosis of antibody-opsonized platelets was performed as previously described.[9] Briefly, THP-1-CD16A cells were seeded on sterile glass coverslips (Thermo Fisher Scientific, Canada) in a 24-well polystyrene plate at 2×10^5 cells per well in complete RPMI and differentiated to macrophages by treating them with PMA (BioShop, Canada). Whole blood collected in citrate-dextrose solution (BD) was used to obtain platelet-rich plasma. Platelets were maintained in the presence of 100 ng/mL of Prostaglandin E1 (Sigma-Aldrich) to prevent activation. The platelet-rich plasma was adjusted to 4×10^8 platelets/mL in PBS. Afterward, platelets were labeled with 20 μM 5-chloromethylfuorescein diacetate (Thermo Fisher Scientific) for 45 minutes and washed with PBS, and 50 μL of the platelet solution was sensitized with 50 μL of serum from patients with ITP or normal human serum for 30 minutes. Concurrently, macrophages were treated with FcγR-blocking antibodies or controls at a concentration of 10 μg/mL in complete RPMI for 30 minutes at 37°C, 5% CO_2. Afterward, macrophages were washed and ITP serum–sensitized platelets were added to the macrophages at a ratio of 100:1 (platelet:macrophage). Phagocytosis proceeded for 60 minutes at 37°C before stoppage on ice. The wells were then washed with ice-cold PBS and fixed using 400 μL of 4% formaldehyde diluted in PBS. An anti-CD42a (GPIX)-AlexaFluor 647 antibody (GP5) was added at a concentration of 0.5 μg/mL for 30 minutes to distinguish non-phagocytosed surface-bound platelets. After washing with PBS, coverslips were mounted onto glass slides (Thermo Fisher Scientific) with Dako Fluorescence Mounting medium (Agilent Technologies). Wells were imaged using a spinning-disk confocal microscopy under 63× objective oil immersion (numerical aperture 1.47) with differential interference contrast and laser fluorescence (488 nm, 647 nm excitation) on a Quorum multimodal imaging system (Quorum Technologies) equipped with 50 micrometer pinhole spinning disk and ORCA-Flash 4.0 V2 PLUS sCMOS camera. Phagocytic index (PI) was calculated as:

$$\text{PI} = [(\text{Total number of platelets internalized}) \,/\, (\text{Total number of macrophages counted})] \times 100$$

In vivo evaluation of 17C02-based molecules in a passive model of ITP

ITP was passively induced with a rabbit antiplatelet serum (Cedarlane, CLA31440) in FcγR-humanized mice using a method previously described in detail.[12] Briefly, all treatments were administered IV through the lateral tail vein. To examine the in vivo effect of 17C02-albumin and 17C02-IgG2a, mice were treated with an equimolar amount (540 μM/mouse) of these molecules as well as human albumin as a negative control. For clarity, 2 different concentrations of these FcγRIIIA blockers as well as the albumin control were used in the blocking studies. The same equimolar amount of 3G8 (mouse IgG1) was also evaluated as a reference reagent. Body (rectal) temperature was then monitored using a rectal temperature probe (Kent Scientific) to assess adverse events. After 2 hours, the animals were bled via the saphenous vein to count platelets (as described in detail[13]), and ITP was induced with 15 μL of the rabbit antiplatelet serum. Animals were bled again 2 hours later via the saphenous vein, and the platelet number was enumerated by a Multisizer 3 particle counter (Beckman Coulter, Canada) as described in detail.[12]

Statistical analysis

Prism version 8.00 for Windows (GraphPad Software, San Diego, CA) was used for statistical analysis. Data normality was verified using D'Agostino-Pearson test, and homogeneity of variance was checked using Bartlett test. Nonparametric tests were used for further analysis whenever data were not normally distributed even after transformation. Parametric analyses of >2 groups were performed using a 1-way or 2-way analysis of variance with Tukey posttest or Sidak posttest. Nonparametric analyses of >2 groups were performed using the Kruskal-Wallis test and Dunn multiple comparison test.

Results

Development of a scFv-phage display library against the human FcγRIIIA

To develop a scFv-phage display library against the human FcγRIIIA (overview of the strategy is shown in Figure 1), BALB/c mice were immunized with the recombinant human FcγRIIIA adjuvanted with aluminum and CpG ODN. Although all mice developed an antibody response against the antigen, spleens from the 3 best responders were used to purify total messenger RNA by reverse transcription, and the V_H and V_L were amplified by polymerase chain reaction. Regions V_H and V_L were then linked using a G4S sequence and cloned into the vector pADL-23c. *E coli* (TG1 strain) was then transformed with the final construct to generate the 6.92×10^8 colony-forming unit scFv-phage display library.

Selection of FcγRIIIA binding clones with reduced FcγRIIA cross-reactivity

Rounds of selection were performed to select phages with high binding affinity to FcγRIIIA and low or no cross-reactivity with the other FcγRs (supplemental Table 1). Phages obtained from each round of selection were used to infect TG1 cells, and individual colonies were selected to obtain the scFvs secreted into the periplasm of the bacteria (peripreps). One-hundred peripreps from each round (500 clones in total) were assayed for their binding to FcγRIIIA by ELISA. As a result, 170 clones had similar or better binding capacity than 3G8-scFv or 3G8-albumin, used as controls (supplemental Figure 1). The binding of these 170 clones to FcγRIIIA on NK cells was assessed. These clones were also assayed for inhibiting the binding of hIgG to FcγRIIIA, by homogeneous time-resolved fluorescence. Eighteen of the clones bound well to human NK cells (supplemental Figure 2A), and 10 of them (16A12, 17D10, 17C02, 17F07, 14F04, 17F11, 15B07, 17E03, 10E07, and 17A09) inhibited >30% of binding of hIgG to FcγRIIIA (supplemental Figure 2B). The negative controls (Her-scFv, albumin, and mIgG) did not inhibit the binding (supplemental Figure 2B).

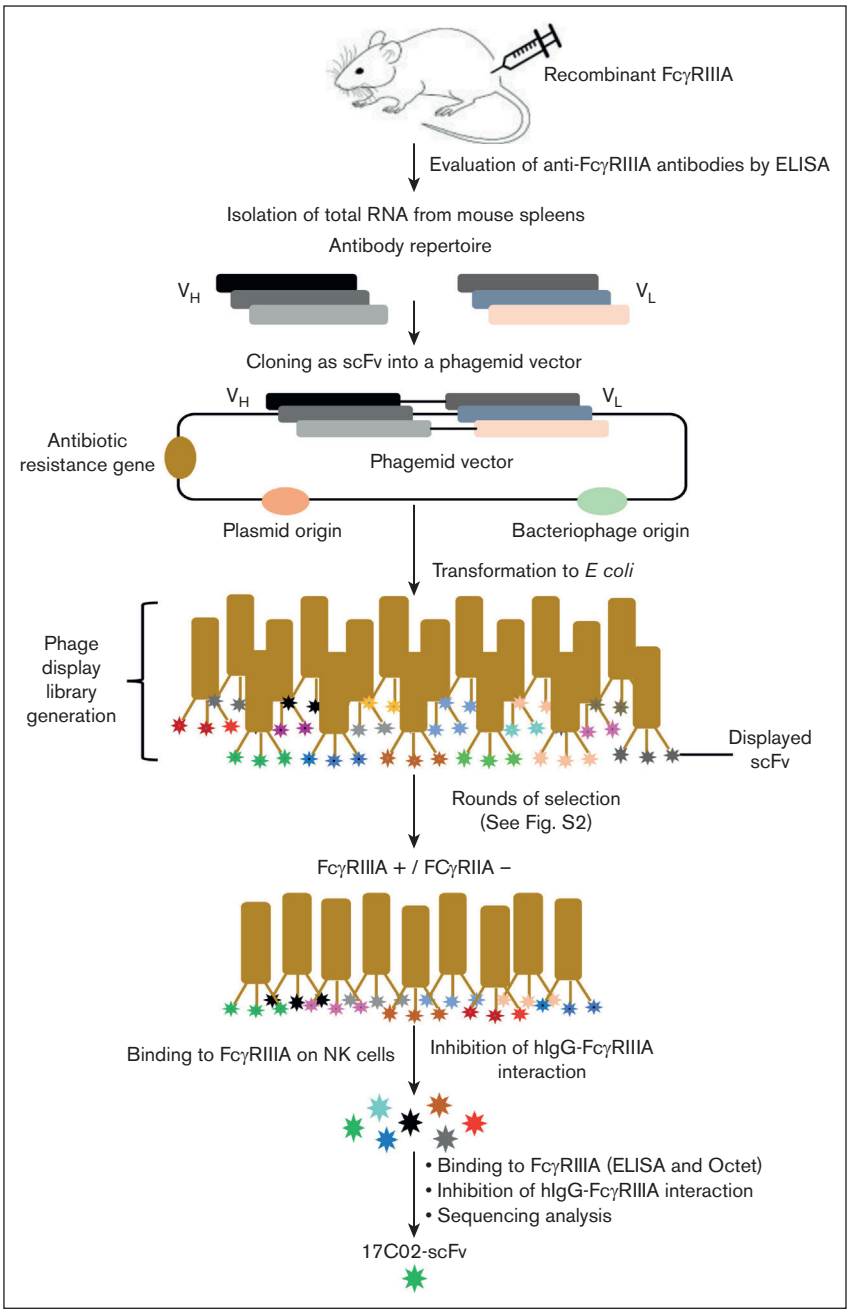

Figure 1. Schematic representation for obtaining scFvs that bind and block FcγRIIIA. BALB/c mice were immunized with the recombinant human FcγRIIIA, total splenic RNA was isolated, and genes encoding the V_H and V_L chains were amplified. A second polymerase chain reaction stitched V_H and V_L with a linker, and the products were cloned into a phagemid vector via Gibson assembly. *E coli* was then transformed with the constructs and a scFv-phage display library obtained. Five rounds of selection (R1, R2A, R2B, R3A, and R3B) were performed to select phages bound to FcγRIIIA with minimal cross-reactivity with FcγRIIA (supplemental Figure 1). Selection of scFv was based on binding to NK cells by flow cytometry and inhibition of hIgG-FcγRIIIA interaction by homogeneous time-resolved fluorescence. Purified scFv were analyzed for binding to FcγRIIIA by ELISA and Octet; minimal cross-reactivity with the other human receptors and inhibition of hIgG-FcγRIIIA interaction (ie, FcγRIIIA blockers) were part of the selection process. The final antibody fragment, 17C02-scFv, was selected from 10 candidates based on these assessments as well as sequencing analysis to screen for glycosylation, oxidation, aggregation, deamidation/isomerization, and proteolytic sites to exclude scFv molecules with low biochemical stability.

Selection of the optimal scFv (17CO2) to express as an albumin fusion protein, a normal bivalent mouse antibody, and a 1-armed antibody

The 10 clones previously selected were purified for further analysis, with 2 clones (10E07 and 17A09) excluded due to low protein recovery after the purification process. The clone (19H02) was included as a "weak" control, considering it did not meet the selection criteria, but it did inhibit roughly 28% of binding of hIgG to FcγRIIIA. The clones were then analyzed for binding to FcγRI, FcγRII, FcγRIIIA, FcγRIIIB, and cynoFcγRIIIA by ELISA. Three scFv (17E03, 17F07, and 17C02) were selected (supplemental Figure 3), considering good binding to FcγRIIIA and cynoFcγRIIIA, limited perceived cross-reactivity with FcγRIIIB, and nonbinding to FcγRI and FcγRII. Clones 15B07 and 17C02 had a higher percentage of inhibition than 3G8-scFv-albumin, whereas 17F07 and 17E03 had a similar or lower percentage of inhibition than 3G8-scFV-albumin (supplemental Figure 4). Accordingly, the biomolecular interaction between 17C02 and FcγRIIIA evaluated using ForteBio Octet Red96e, showed this scFv had a better association and more stable interaction with the receptor than 3G8-scFv (supplemental Figure 5A). In fact, 17C02 was the best candidate when its association-dissociation curve was compared with curves from the other scFvs (supplemental Figure 5B). In addition, the sequence 17C02 possessed the lowest number of undesirable

sites (deamidation/isomerization, proteolytic cleavage, oxidation, aggregation, and glycosylation) of all scFv analyzed. The amino acid sequence of 17C02 was then used to obtain 3 different constructs (Figure 2).

17C02-based molecules bind to FcγRIIIA$^+$ cells

The binding of 17C02-based molecules to FcγRIIIA was evaluated using THP-1 transgenic cells expressing the CD16A molecule (THP-1-CD16A cells)[9] and peripheral blood mononuclear cells (PBMC) from human donors. The 3 molecules 17C02-albumin, 17C02-IgG2a (deglycosylated), and 17C02-IgG1$_{OA}$ bound to both THP-1-CD16A and NK cells in a dose-dependent manner (Figure 3A-B,D-E,G-H). 17C02-albumin and 17C02-IgG2a had greater binding capacities to FcγRIIIA-expressing cells than 3G8-albumin and 3G8, respectively (Figure 3A-B,D-E). As expected, 17C02-IgG1$_{OA}$ showed binding to THP-1-CD16A cells with no binding to wild-type THP-1 cells, which lack FcγRIIIA (Figure 3G). In addition, 17C02- and 3G8-based molecules demonstrated roughly equal binding with neutrophils (Figure 3C,F,I), suggesting interaction of 17C02 with FcγRIIIB.

17C02-based molecules inhibit the phagocytosis of human platelets sensitized with serum from patients with ITP

Human platelets sensitized with sera from patients with ITP were used to perform phagocytosis assays using THP-1-CD16A macrophages. Sensitization of platelets with ITP sera caused platelet phagocytosis (Figure 4A). The treatment of cells with FcγR-blocking antibodies showed that FcγRI and FcγRIIIA contribute to phagocytosis (Figure 4B). In fact, the blockade of either of these receptors inhibited ~70% of phagocytosis of serum-opsonized platelets. Contrarily, ~12% of phagocytosis was inhibited with an FcγRIIA/B/C-blocking antibody, suggesting that the activity of this receptor is not required under these in vitro conditions. In accordance with these results, blocking FcγRIIIA with 17C02-based molecules led to a significant inhibition of platelet phagocytosis (Figure 4B). Inhibition was obtained by all 17C02-based constructs.

17C02-albumin successfully ameliorates thrombocytopenia in a passive mouse model of ITP without causing major adverse events

FcγR-humanized mice were used to evaluate the capacity of 17C02-albumin and 17C02-IgG2a to ameliorate ITP in comparison with 3G8 (Figure 5). Animals were treated with 540 μM per mouse of either deglycosylated 17C02-IgG2a, 17C02-albumin, or deglycosylated 3G8 to block FcγRIIIA. Changes in body temperature were assessed after treatment as a potential adverse effect commonly associated with FcγRIIIA blocking antibodies.[8,14] As expected, deglycosylated 3G8 provoked a decrease in body temperature in accordance with previous studies[14] (Figure 5A). Similar adverse reactions were observed for deglycosylated 17C02-IgG2a. In contrast, the monovalent construct (17C02-albumin) did not cause a measurable change in body temperature (Figure 5A).

Analysis of select immune cell populations revealed that mice administered with 17C02-IgG2a exhibited a reduction in neutrophil counts both in the blood and spleen, along with a decrease in NK cell numbers in the blood (supplemental Figure 6). Conversely, mice treated with 17C02-albumin maintained immune cell levels comparable with those observed in untreated mice (supplemental Figure 6).

Two hours after the administration of anti-FcγRIIIA molecules, mice were injected with a rabbit antimouse platelet serum to induce thrombocytopenia. Animals that received antiplatelet serum alone (Nil) developed thrombocytopenia compared with untreated animals (Figure 5B). Treatment with 17C02-albumin successfully ameliorated ITP compared with animals in the Nil group. Interestingly, despite the ability for 17C02-IgG2a and 3G8-based molecules to inhibit phagocytosis, these compounds did not ameliorate thrombocytopenia (Figure 5B).

Considering the lack of ameliorative capacity observed with 3G8 and 17C02-IgG2a, platelet counts were assessed after the administration of these antibodies alone, before the induction of ITP. Treatment with either deglycosylated 3G8 or deglycosylated 17C02-IgG2a alone caused a significant decrease in platelet count compared with their respective isotype controls ($P < .05$; Figure 5C). In contrast, 17C02-albumin did not affect the platelet counts (Figure 5C).

17C02-IgG1$_{OA}$ ameliorates thrombocytopenia in murine ITP

FcγR-humanized mice were treated with 540 μM per mouse of 17C02-IgG1$_{OA}$, the same equimolar amount previously used for the other 17C02-based molecules. To evaluate potential adverse events associated with this treatment, changes in body temperature and induction of thrombocytopenia on its own were assessed

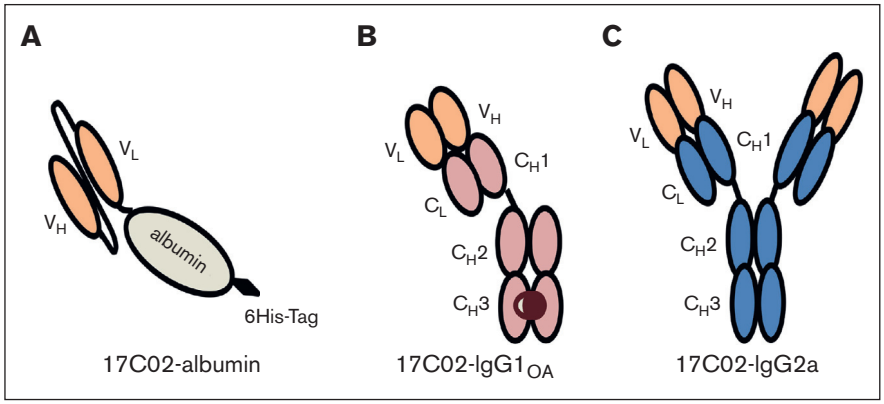

Figure 2. Schematic representation of the 17C02-based molecules. (A) The gene that encodes for the 17C02-scFv with a linker (connecting the VH with the VL chains) as well as an additional RGGGGSGGGGS were used to connect the scFv to the N-terminal sequence of human albumin and a 6xHis tag at the C-terminal end of albumin. (B) The genes encoding the V_L and V_H of 17C02 were used to express a human IgG1 1-armed antibody with the LALA mutation to impair Fc-FcγR interactions, using the "knob-into-hole" strategy. (C) The genes that encode for the V_L and V_H of 17C02 were expressed as a full-length mouse IgG2a antibody.

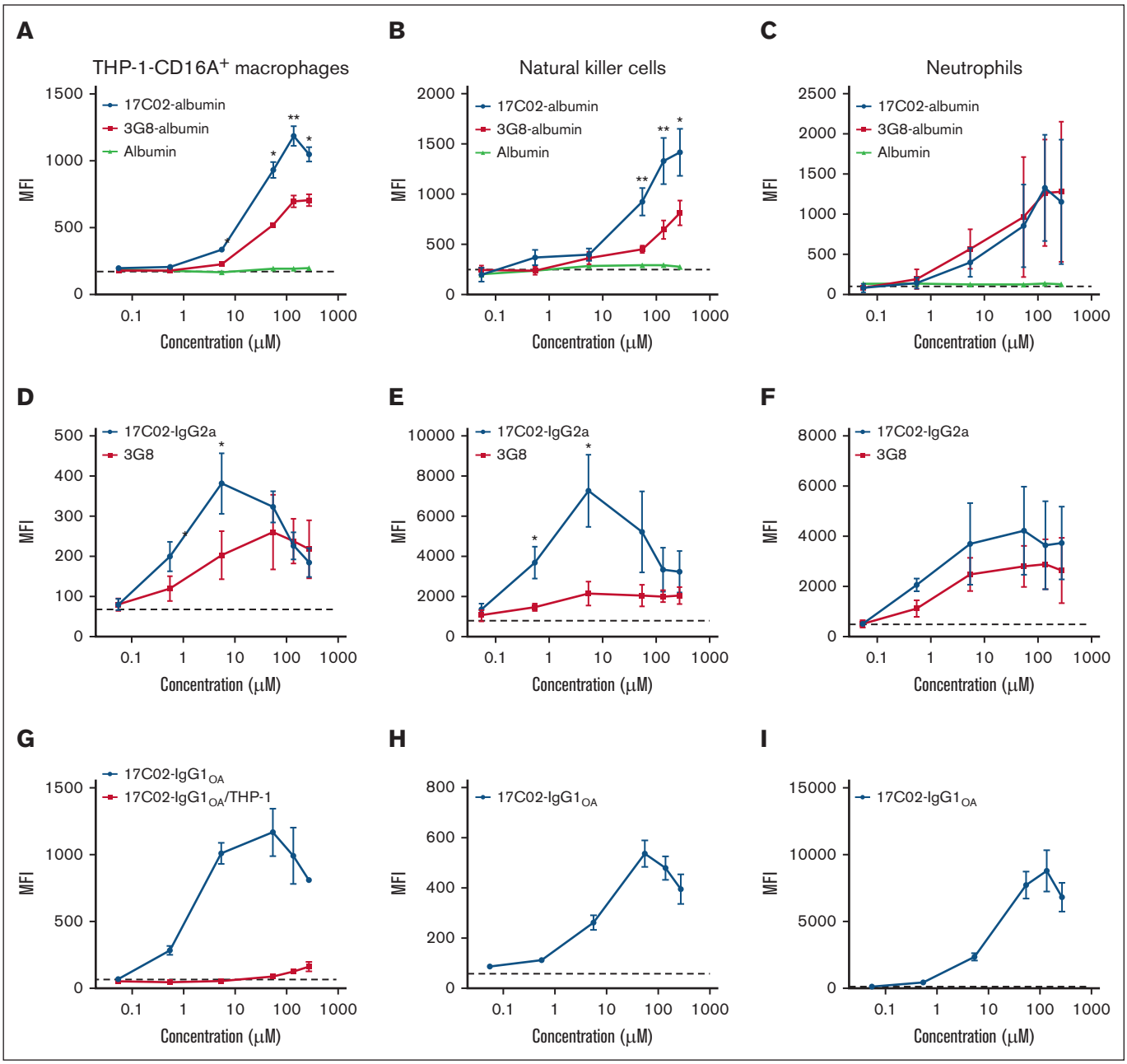

Figure 3. Binding of 17C02-based molecules to human FcγRIIIA expressed on THP-1-CD16A cells, THP-1 cells, as well as primary cells. Cells were incubated with the indicated concentration of either 17C02-based molecules, 3G8-based molecules, or albumin alone (negative control). The following were the cell types used for each panel: (A,D) THP-1-CD16A cells; (B,E,H) NK cells from healthy human donors; and (C,F,I) neutrophils from healthy human donors; as well as (G) a comparison between THP-1-CD16A and THP-1 cells. Binding of albumin fusion proteins was detected using a FITC-labeled monoclonal antihuman albumin antibody, whereas binding of deglycosylated full-length antibodies or the 1-armed antibody was detected using an APC-labeled goat $F(ab')_2$ antimouse IgG (Fc-specific) or an AF647-labeled donkey antihuman IgG (H + L), respectively. Stained cells were washed and analyzed by flow cytometry using the BD LSRFortessa X-20. Data analysis was performed using FlowJo v10. Data are presented as mean ± standard deviation from 3 to 5 independent experiments. The dashed line represents the mean fluorescent intensity (MFI; arbitrary units) value for the corresponding secondary antibody alone at 5 μg/mL. Statistical analysis was performed using a 2-way analysis of variance (ANOVA) and Sidak multiple comparisons test, comparing the MFI values of the 3 molecules at each antibody concentration (*$P < .05$; **$P < .01$).

after administration. Unfortunately, despite the molecule being monovalent, it provoked a drop in body temperature, compared with untreated mice (Figure 6A). The 17C02-IgG1$_{OA}$ also induced a minimal but statistically significant decrease in platelet counts when given alone (Figure 6B). To evaluate its ability to increase platelet counts in ITP, thrombocytopenia was then induced in the animals by administering rabbit antimouse platelet serum, and 2 hours after this injection, platelet counts were recorded again.

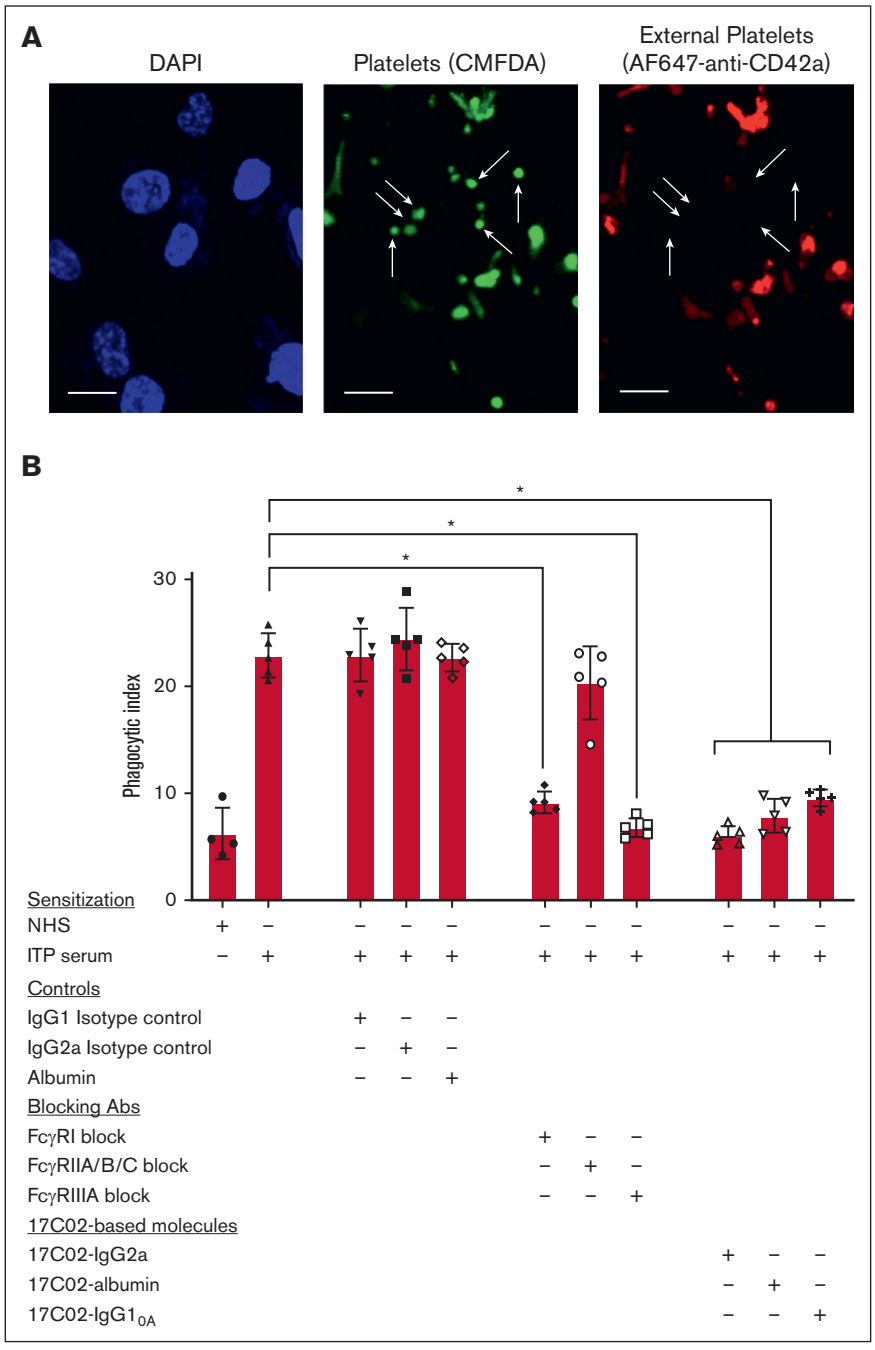

Figure 4. Blocking capacity of 17C02-based molecules and FcγR utilization by THP-1-CD16A cells in the phagocytosis of IgG-opsonized human platelets. (A) Images of platelets sensitized with ITP serum and later incubated with THP-1-CD16A macrophages. Images were taken at the center of each well with Z-stacking. Phagocytosis was quantified using Imaris v9.6.0. The white arrows denote examples of phagocytosis of platelets; scale bar, 10 µm. (B) PI from 4 independent experiment are shown. Sensitization: "+" indicates platelets were incubated with normal human serum vs serum from patients with ITP. The PI was calculated as the number of platelets engulfed per 100 macrophages. The contribution of FcγRI, II, and III to phagocytosis was evaluated using Fc region deglycosylated blocking antibodies (final concentration of 10 µg/mL; 0.07 µM each): anti-FcγRI (clone 10.1), anti-FcγRIIA/B/C (clone AT10), or anti-FcγRIIIA (clone 3G8). The deglycosylated mouse IgG1 (clone MOPC-21), the deglycosylated mouse IgG2a (clone N/A-CP150), and human albumin were used as controls (final concentration of 0.07 µM). The blocking capacity of 17C02-based molecules was evaluated (17C02-albumin, 17C02-IgG1$_{OA}$, and deglycosylated 17C02-IgG2a) using the same comparative final molar concentration. Data are presented as the mean ± the standard deviation (n = 4-5). The statistical analysis was performed using Kruskal-Wallis and Dunn multiple comparison test (*$P < .05$).

Animals that received no treatment (Nil) developed thrombocytopenia, compared with untreated animals (Figure 6C). However, treatment with 17C02-IgG1$_{OA}$ successfully ameliorated ITP, increasing platelet counts by ~55%, compared with animals in the Nil group (Figure 6C).

Discussion

FcγRs serve as vital components of the immune system, mediating an array of functions, from phagocytosis to cytokine release.[15] These receptors play key roles in both innate and adaptive immunity and have been implicated in various pathological conditions, including autoimmune disorders and infectious diseases.[16] Therapeutically targeting FcγRs has therefore emerged as a significant area of research.

FcγRs on immune cells, such as macrophages, dendritic cells, and neutrophils, can both activate and inhibit immune responses.[17] The balance between activating receptors such as FcγRIIA and inhibitory ones such as FcγRIIB is considered to be essential for immune homeostasis.[18,19] Therefore, indiscriminate Fc receptor blockade can result in unforeseen consequences, such as dampened immune responses to pathogens or uncontrolled activation leading to autoimmunity.[20]

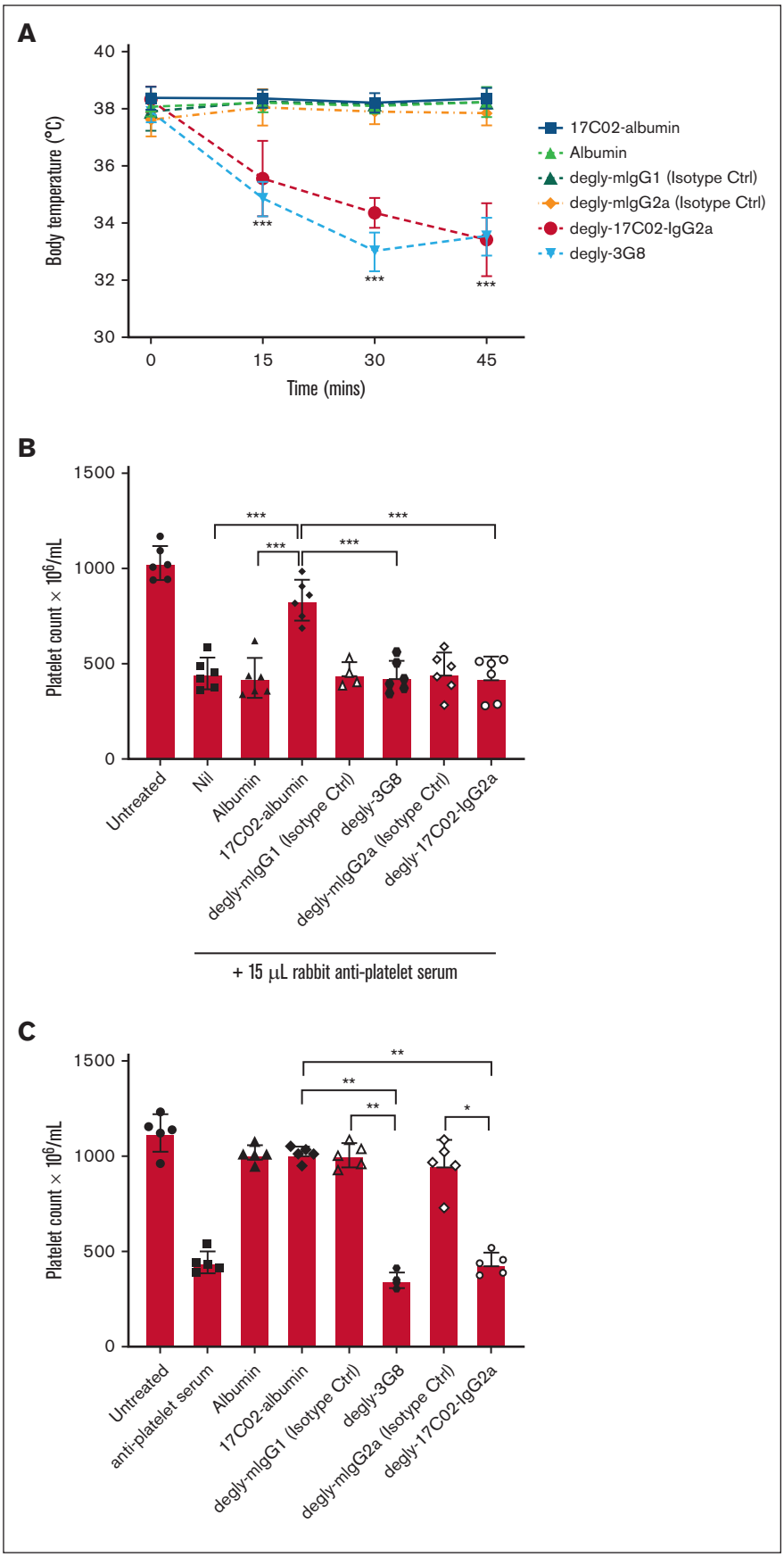

Figure 5. Ameliorative effects and adverse events caused by 17C02-based molecules and 3G8 in an antibody-mediated model of ITP. FcγR-humanized mice were treated with an IV administration of either deglycosylated full-length 17C02 or deglycosylated full-length 3G8 (81 µg/mouse; 540 µM/mouse), 17C02-albumin (50 µg/mouse; 540 µM/mouse), deglycosylated full-length IgG1 and IgG2 isotype controls (81 µg/mouse; 540 µM/mouse), or albumin alone (35.1 µg/mouse; 540 µM/mouse). (A) Decreases in rectal temperature were evaluated as an indicator of an inflammatory adverse event comparing 0-minute (pretreatment) with 15-, 30-, and 45-minute posttreatment conditions. Data are presented as mean ± standard deviation (n = 5-7). The statistical analysis was performed by a 2-way ANOVA and Sidak multiple comparisons test (***$P < .001$). (B) ITP was then induced in mice with 15 µL of a rabbit antiplatelet serum 2 hours after the anti-FcγRIIIA therapeutic intervention. Additional mice were either left untreated (Untreated) or treated with the antiplatelet serum alone (Nil) as comparative controls. Data are presented as mean ± standard deviation (n = 6). The statistical analysis was performed by a 1-way ANOVA and Tukey multiple comparisons test (***$P < .001$). (C) The ability of FcγRIIIA-blocking reagents and controls to directly induce thrombocytopenia (as an adverse event) was evaluated 2 hours after treatment. The antiplatelet serum alone (15 µL/mouse) was used as a positive control. Deglycosylated mouse IgG1 (degly-mIgG1) and IgG2a (degly-mIgG2a) isotype controls were used as negative controls for amelioration. Data are presented as mean ± standard deviation (n = 5). The statistical analysis was performed using Kruskal-Wallis and Dunn multiple comparison test (*$P < .05$; **$P < .01$).

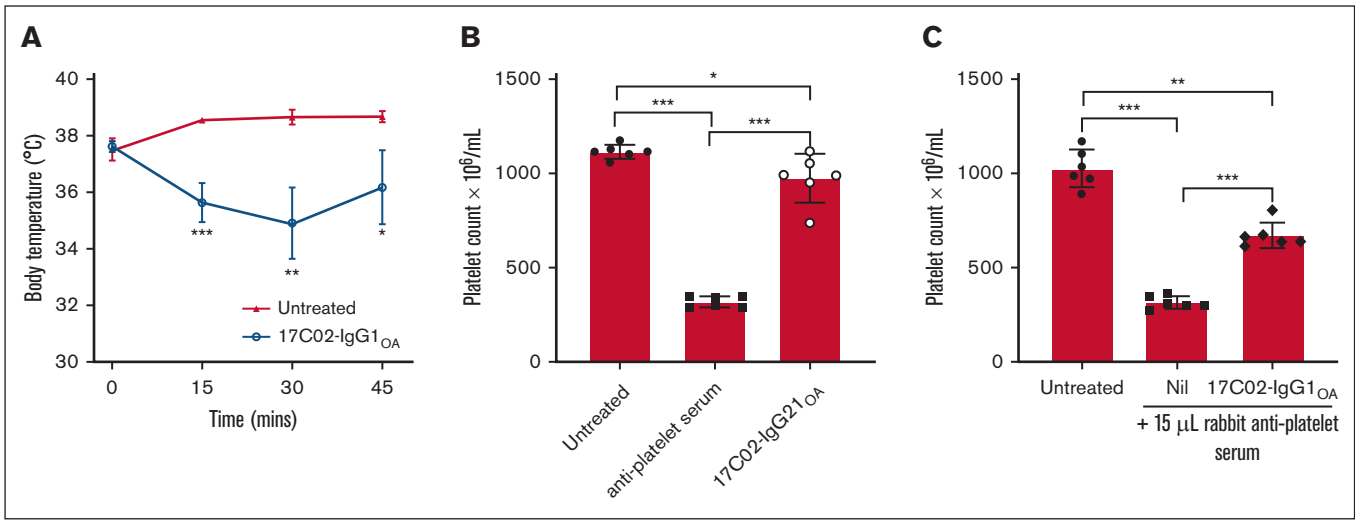

Figure 6. Ameliorative effects and adverse events caused by 17C02-IgG1$_{OA}$ in an antibody-mediated model of ITP. (A) FcγR-humanized mice were IV injected with 540 µM of 17C02-IgG1$_{OA}$, and body temperatures of mice were assessed for 45 minutes after treatment to investigate the inflammatory nature of the molecule (time "0" indicates before treatment). Data are presented as mean ± standard deviation (n = 6). The statistical analysis was performed by a 2-way ANOVA and Sidak multiple comparisons test (*P < .05; **P < .01; ***P < .001). (B) Mice were bled and platelet counts assessed 2 hours after 17C02-IgG1$_{OA}$ treatment to determine the ability of the antibody itself to cause thrombocytopenia. Rabbit antiplatelet serum alone (15 µL/mouse) was used as a positive control. Data are presented as mean ± standard deviation from 3 independent experiments (n = 6). The statistical analysis was performed by a 1-way ANOVA and Tukey multiple comparisons test (*P < .05; ***P < .001). (C) Mice were IV injected with 15 µL of rabbit antiplatelet serum 2 hours after 17C02-IgG1$_{OA}$ treatment to induce thrombocytopenia. Two hours after injection with the antiplatelet serum, mice were bled for enumeration of platelet counts to assess the ability of 17C02-IgG1$_{OA}$to ameliorate thrombocytopenia. Data are presented as mean ± standard deviation (n = 6 mice). The statistical analysis was performed by a 1-way ANOVA and Tukey multiple comparisons test (**P < .01; ***P < .001).

Various therapeutic approaches have been explored to target FcγRs. IV immunoglobulin and anti-D are both FDA-approved treatments for ITP and can be considered to exert their effects via FcγR blockade,[11,21] although the precise mechanism of both treatments is not fully understood.

Another strategy to target the FcγR pathway has been the use of fostamatinib, a small molecule ATP analog that binds and inhibits spleen tyrosine kinase, a key enzyme required for FcγR-dependent phagocytosis.[22] The initial double-blind phase 3 trial demonstrated therapeutic effectiveness in 18% of patients with highly treatment-refractory ITP. In later studies that used fostamatinib as a second-line treatment (with patients less likely being highly treatment-refractory), the response rate was as high as 78%.[23] Thus, it is evident that FcγR targeting is a strong candidate for future therapeutics.

Other approaches targeting FcγRs include obinutuzumab, a type 2 anti-CD20 monoclonal antibody with enhanced FcγRIIIA-dependent cellular cytotoxicity,[24] and MGD010, a bispecific antibody targeting CD32B and CD79B, to selectively suppress B-cell activity.[25] In addition, hexameric Fc-fusion proteins have been studied,[26] as well as some small molecule drugs that inhibit phagocytosis.[21,27]

The road to effective blockade possesses a fair share of challenges. First, the redundancy of FcγRs makes it difficult to achieve precise modulation.[28] Second, the high degree of interindividual variability in FcγR expression adds another layer of complexity to design effective therapeutics.[29,30] Third, the off-target effects and possible unintended immune suppression or activation continue to challenge the field. Although monovalent FcγR blockade partially addresses these concerns, it remains to be definitively established whether such targeted blockade is effective in patients. Importantly, selective blockade may still permit other FcγRs to carry out their protective roles in innate immunity.

As previously mentioned, the monoclonal anti-FcγRIIIA antibody 3G8 has been studied as an ITP treatment.[5] Notably, more than half of the patients who were unresponsive to alternative treatments exhibited significantly elevated platelet counts after 3G8 therapy.[31-33] However, the continued use of this treatment was hindered due to adverse effects including vomiting, nausea, and fever.[6,7]

Unfortunately, the monovalent 3G8-albumin fusion protein had insufficient efficacy in the humanized murine ITP model, possibly due to its rapid dissociation from FcγRIIIA (supplemental Figure 5A). We, therefore, created the monovalent 17C02 construct to overcome this limitation. The 17C02 scFv constructs exhibited superior binding affinity compared with 3G8. Although the albumin-stabilized scFv-FcγRIIIA provided excellent therapeutic results, the monovalent 1-armed 17C02 with an inactivated Fc region retained some undesired inflammatory activity, as indicated by the induction of body temperature changes and mild thrombocytopenia. The underlying cause for these adverse effects remains unclear but may stem from either incomplete Fc inactivation or the minuscule presence of contaminating full-length bivalent antibodies from the preparation process. Importantly, despite these drawbacks, the 1-armed antibody demonstrated efficacy in ameliorating ITP, and the adverse effects were less severe than those triggered by the bivalent 17C02-IgG2a.

In the context of incomplete Fc inactivation, the Fc domain of the 1-armed antibody was engineered with LALA mutations to impair

its affinity to FcγRs.[34] Although LALA mutations substantially reduce FcγR binding, they do not eliminate it entirely. Thus, it remains theoretically plausible that residual low-level binding could be sufficient to incite adverse events.

Concerning the trace amounts of contaminating full-length antibody, although we used the Knobs-into-holes technology[35] to engineer a monovalent IgG, forcing a full-length heavy chain to pair with a truncated heavy chain through complementary mutations in both chains, a minor fraction of full-length bivalent antibodies was still detected via western blot (~2.3% of total protein as determined by densitometry) (supplemental Figure 7). This trace presence of bivalent antibodies could also potentially account for the observed adverse events.

Given the role of FcγRs in various pathological conditions, the potential applications for Fc receptor blockade are vast. There is considerable interest in expanding the therapeutic applications beyond ITP to include other autoimmune diseases and inflammatory states in which IgG and FcγRs are implicated in pathophysiology.

Subsequent investigations focusing on any monovalent formulation should delve into understanding pharmacokinetics, pharmacodynamics, and biodistribution in animal models. In-depth toxicological studies will be crucial for a thorough evaluation of monovalent FcγR blockade safety. Ultimately, transitioning toward clinical trials to rigorously assess safety and efficacy of a monovalent FcγRIIIA blocker in human participants stands as a pivotal step.

In our study using a mouse model of ITP, we have used rabbit IgG as an inducer of thrombocytopenia. Although this choice was made for practical reasons (ie, rabbit IgG binds well to human FcγRIIIA),[36,37] it is important to acknowledge that rabbit IgG has limitations in mirroring human IgG binding to human FcγRs.

In summary, FcγRIIIA blockade with 17C02-albumin may offer an effective potential treatment strategy for patients with ITP and perhaps other disease status in which IgG and FcγRs play a role in the disease pathophysiology.

Acknowledgments

The authors thank Donna Lyons from The Research Vivarium of St. Michael's Hospital for her exceptional dedication to breeding and nurturing the FcγR-humanized mice. Additionally, the authors extend their appreciation to Monika Lodyga from Keenan Research Centre of Biomedical Science Core Facilities at St. Michael's Hospital for her invaluable technical guidance and expertise in the field of flow cytometry.

The study was supported by an intramural grant from the Canadian Blood Services (A.H.L.).

Authorship

Contribution: L.G.G. and A.H.L. conceived the study; L.G.G., A.H.L., K.D.W., Z.T., and E.C. designed experiments; U.J.S., A.M., M.S., and W.P.S. provided critical reagents; L.G.G., K.D.W., Z.T., E.C., Y.C.L., Y.T.C., P.A.A.N., Y.S., V.B., J.L., I.S., B.S.M., L.C.P., A.P.O., P.B., and P.C. performed experiments; L.G.G., K.D.W., E.C., Z.T., J.L., I.S., B.S.-M., L.S.-P., and A.H.L. analyzed and interpreted data; L.G.G. and A.H.L. wrote the manuscript; and A.H.L., K.D.W., and L.G.G. edited the manuscript.

Conflict-of-interest disclosure: Patent applications describing the 17C02-based molecules are assigned to the Canadian Blood Services with the participation of adMare BioInnovations and Unity Health/St. Michael's Hospital. A.H.L. has had research funding from CSL and has other patents on IV immunoglobulin alternatives. The remaining authors declare no competing financial interests.

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