Differentiating pathogenic from bystander autoantibodies in immune thrombocytopenia using intact glycoprotein-deficient megakaryocytes

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Received: July 29, 2025.
Accepted: December 5, 2025.
Early view: December 11, 2025.

https://doi.org/10.3324/haematol.2025.288832

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Abstract

Autoantibodies targeting platelet surface glycoproteins (GP) are the primary cause of immune thrombocytopenia (ITP), however testing for these autoantibodies is not routinely employed mainly because current diagnostic tests lack sufficient sensitivity. To overcome this and other diagnostic limitations, we generated HLA class I-negative, blood group O, induced pluripotent stem cell lines, and gene-edited them to produce a novel panel of GP-deficient, *in vitro*-derived megakaryocytes (MK). Using GPIIb-, GPIbα- and GPIX-deficient MK allowed sensitive and specific identification and characterization of both GPIIb-IIIa- and GPIb/IX-specific plasma autoantibodies, as well as rare, previously undescribed patient autoantibodies targeting GPIX. The availability of frozen bioengineered MK expressing select platelet GP antigens on their surface simplifies the detection of anti-platelet autoantibodies involved in disease progression, while avoiding detection of non-pathogenic bystander autoantibodies that are sometimes generated by secondary exposure to "cryptic epitopes", and that can otherwise confound diagnosis and treatment. Use of intact MK selectively deficient in the GP that comprise the major targets of platelet autoantibodies has the potential to significantly improve clinical diagnosis and treatment of ITP.

Introduction

Immune thrombocytopenia (ITP) is an autoimmune disorder primarily characterized by a low platelet count. Many patients have few or only mild bleeding symptoms, but severe and life-threatening bleeding may occur and sometimes requires extensive, long-term care that can compromise the health-related quality of life.[1] Even today, ITP remains a diagnosis of exclusion, due to the lack of reliable standard tests or biomarkers to confirm diagnosis. Nearly one in seven patients with primary ITP are misdiagnosed,[2] with significant implications for ITP management, and may result in exposer of patients to unnecessary treatment. Platelet autoantibodies are the primary drivers of ITP, although other mechanisms, such as cytotoxic T cells and natural killer cells, may also contribute to ITP pathogenesis. Despite their central role, platelet autoantibody tests are not routinely employed for diagnosing ITP mainly due to the low sensitivity of existing methods. Thus, while a positive autoantibody test is useful for confirming ITP, a negative test does not rule it out.[3] Therefore, there is a critical need for alternative, practical methods that can detect anti-platelet autoantibodies with high specificity and sensitivity to provide a positive laboratory confirmation for the diagnosis of ITP.

Platelet surface glycoproteins (GP), primarily GPIIb-IIIa and GPIb/IX, are the major targets of anti-platelet autoantibodies causing ITP.[4-6] Subsequent clearance of opsonized platelets is largely mediated through Fcγ receptor-dependent phagocytosis, or via platelet desialylation mechanisms.[7] Uncertainty regarding the relationship between autoantibody specificity, the mechanism of platelet destruction and the response to certain treatments, however, has limited the usefulness of diagnostic testing for ITP, and is therefore often not even prescribed.[8-17] Currently, antigen capture assays (ACA) like enzyme-linked immunosorbent assay (ELISA),[18] monoclonal antibody-specific immobilization of platelet antigens (MAIPA)[19] and platelet antibody bead array[20] are favored in clinical diagnostic laboratories for their GP specificity. However, they are labor-intensive and

employ detergents to solubilize platelet GP, which may destroy labile epitopes. Monoclonal antibodies used in these assays may also compete with the binding of anti-platelet autoantibodies, resulting in false negative outcomes. Additionally, these tests are often conducted only in specialized laboratories, each setting their own threshold for positivity. Even minor differences in these threshold values can affect the detection of weak- or low-affinity autoantibodies, potentially causing false-negative or false-positive results. Regardless of the assay used, platelet autoantibodies can be derived from two sources: antibodies bound to the patient's platelets, and free-circulating antibodies present in the patient's plasma. Diagnostic laboratories typically prefer, when possible, to obtain platelet-associated antibodies by eluting them from patient platelets, as this approach is more sensitive due to the enrichment of autoantibodies on the cell surface. However, this method requires a substantial blood volume and an adequate platelet count, which may not be feasible in patients with severe thrombocytopenia. In such cases, detection of circulating plasma antibodies, though less sensitive, is often the only viable option. Analyzing plasma autoantibodies also holds significant value in research, as plasma samples from ITP patients serve as important resources for studying disease pathogenesis, prognosis, and treatment response. Unlike platelet-associated antibodies that target only extracellular domains of platelet GP, plasma may contain antibodies against intracellular epitopes.[21,22] While these intracellular-targeting antibodies are not thought to be pathogenic, they may arise as a secondary immune response in the context of platelet destruction, and have the potential to confound both diagnosis and treatment.

Assays based on the use of intact platelets were developed long ago to detect platelet-specific antibodies. By preserving intact, native surface antigens, these assays offer high sensitivity. However, they were ultimately abandoned for ITP autoantibody detection due to poor specificity, stemming from the complex array of antigens expressed on the platelet surface. Reliable detection of ITP autoantibodies using intact platelets has only been achieved in rare cases - typically when GP-deficient donor platelets from individuals with Glanzmann's thrombasthenia or Bernard-Soulier syndrome (BSS) served as negative controls.[4,23] In addition to platelet-specific antigens, platelets also express ABO blood group antigens and HLA class I molecules, which further complicate the interpretation of antibody specificity. In the current study, we aimed to revive the whole-cell assay for detecting ITP autoantibodies in patient plasma by replacing donor-derived platelets with bioengineered megakaryocytes (MK), achieving high sensitivity and exceptional specificity. By creating GP-knockout (KO) induced pluripotent stem cell (iPSC)-derived MK, we were able to sensitively detect and accurately characterize autoantibodies against major platelet surface GP complexes, namely GPIIb-IIIa and GPIb/IX, including those previously undetectable by standard ACA. Moreover, the use of GPIbα KO and GPIX KO MK enabled detection of a previously undescribed anti-GPIX autoantibody, demonstrating enhanced resolution for autoantibody characterization. Importantly, the use of intact MK avoids detecting non-pathogenic 'innocent bystander' autoantibodies in patient sera that are detectable using standard ACA. The ready availability of frozen bioengineered MK should streamline the detection of anti-platelet autoantibodies, thus significantly enhancing the research and the clinical diagnosis and treatment of ITP.

Methods

Study design

The use of deidentified ITP patient plasma samples was approved by the Medical College of Wisconsin/Froedtert Hospital Institutional Review Board (IRB) (PRO00036374). Patient consent was waived in accordance with the approved IRB protocol. Patient plasma samples were obtained from two clinical diagnostic laboratories: the Platelet and Neutrophil Immunology Laboratory at Versiti, Wisconsin, USA (Site 1), and the Institute for Clinical Immunology and Transfusion Medicine at the University Hospital of Tübingen, Tübingen, Germany (Site 2). To ensure the inclusion of true ITP cases, only samples from patients previously confirmed to have platelet-associated or circulating antibodies were selected by the diagnostic laboratories. Twenty-eight ITP plasma samples from Site 1 had been previously tested using diagnostic PABA[20] or ELISA,[18] while 20 samples from Site 2 had been tested using diagnostic MAIPA.[19] All ITP patient samples were blinded and coded by the diagnostic laboratories before being transferred to the research laboratory for testing using intact MK. As negative quality control, twenty deidentified normal human plasma samples obtained from Versiti were pooled and included in each test. Additionally, Site 1 provided three coded normal plasmas randomized among the ITP samples to ensure assay accuracy. Diagnostic test results were released subsequently by the laboratories for comparative analysis and are summarized in *Online Supplementary Table S1*.

Flow cytometric analysis

To analyze MK surface glycoprotein expression, 2×10^5 cells were incubated with the following fluorescently conjugated antibodies at room temperature for 20 minutes (min): phycoerythrin (PE)/Cyanine7-conjugated anti-GPIIb, FITC-conjugated anti-GPIIIa, APC-conjugated anti-GPIbα (BioLegend), PE-conjugated anti-αVβ3, clone LM609 (Sigma), AF647-conjuated anti-GPVI (11A12), FITC-conjugated anti-GPIX (BD Biosciences), Alexa Fluor 488-conjugated anti-IL-4Rα (R&D systems). Fluorescently labeled isotype controls corresponding to each monoclonal antibody were included as background staining. GPV surface expression was monitored by incubating the MK with sheep anti-GPV

primary antibody (R&D systems) followed by Alexa Fluor 488-conjugated donkey anti-sheep immunoglobulin (Ig)G (Jackson ImmunoResearch Laboratories). For autoantibody detection (*Online Supplementary Figure S1*), $5x10^5$ iPSC-derived MK were incubated with 10-25 μL of human plasma for 30 min at room temperature. After washing, the cells were incubated with PE-conjugated donkey anti-human IgG (Jackson ImmunoResearch Laboratories), PE/Cyanine7-conjugated anti-GPIIb, and either APC-conjugated anti-GPIbα or AF647-conjuated anti-GPVI at room temperature for 20 min. Cells not exposed to human plasma, but stained with secondary and gating antibodies, served as negative controls. Anti-HPA-1a (26.4) alloantibody, a kind gift from Dr. Maria Therese Ahlen (University Hospital of North Norway), was used as a positive control. Flow cytometry was performed using a BD FACSCelesta or FACSymphony A5 SE cell analyzer. Data were analyzed using FlowJo software (Tree Star Inc.). Flow cytometry data were visualized on a biexponential scale. Due to digital background subtraction, some fluorescence intensity values for unstained populations were negative; these values were considered background noise.

Statistical analysis

Statistical tests were performed using GraphPad Prism 9 (GraphPad Software). Analysis between multiple groups was performed using one-way ANOVA with Dunnett's test for comparison between individual groups. $P<0.05$ was considered statistically significant.

Results

Generation and characterization of GPIIb-IIIa- and GPIb/IX-deficient induced pluripotent stem cell lines

A blood group O, HLA class I-negative iPSC line[24] was used as the founder line for all gene-editing procedures because its differentiated MK exhibited low background binding to plasma from 21 randomly selected healthy donors (*Online Supplementary Figure S2*). iPSC clones in which the gene encoding platelet membrane GPIIb was deleted were then generated using CRISPR/Cas9 technology (*Online Supplementary Figure S3A*). Disruption of the *ITGA2B* gene did not affect differentiation of iPSC into MK, and the size of GPIIb KO MK was comparable to that of WT MK (*Online Supplementary Figure S4*). Flow cytometric analysis confirmed the complete loss of GPIIb surface expression (Figure 1A). As expected, GPIIb-deficiency also resulted in a significant reduction in MK surface expression of GPIIIa; the remaining low level of GPIIIa is due to rescue of its expression by an endogenous integrin αV subunit and formation of αVβ3 (Figure 1A). Disruption of GPIIb did not affect the expression of other major GP, such as GPIbα and GPVI, on the MK surface (Figure 1A).

The GPIb/IX/V complex is comprised of a large GPIbα subunit disulfide linked to GPIbβ and non-covalently associated with GPIX and GPV.[25] We generated GPIbα, GPIX and GPV single KO iPSC clones (*Online Supplementary Figure S3B-D*), and also introduced a transgene encoding a chimeric IL4Rα-GPIbα[26] protein into the AAVS1 locus of the GPIbα KO iPSC clone (*Online Supplementary Figure S5*). This fusion protein, depicted on the left side of Figure 1B, replaces almost the entire extracellular domain of human GPIbα with the extracellular domain of human interleukin-4 receptor α (IL4Rα). IL4Rα-GPIbα has previously been shown to associate with GPIbβ in a manner mimicking the intact GPIbα subunit, and fully supports assembly of the GPIb/IX complex.[26] WT iPSC clones were then differentiated into MK, and found to express high levels of GPIbα, GPIX and GPV on the cell surface (Figure 1B). In contrast, GPIbα-deficient MK completely lost GPIbα expression, and also exhibited significantly reduced surface GPIX and GPV expression (Figure 1B), as expected. Similarly, GPIX-deficient MK showed significantly reduced levels of GPIbα and GPV (Figure 1B). In contrast, GPV-deficient MK expressed normal levels of GPIbα and GPIX (Figure 1B), consistent with previous findings that GPV biosynthesis and surface expression depend on the presence of GPIb/IX complex, but GPIb/IX surface expression doesn't require GPV.[27,28] Expression of IL4Rα-GPIbα restored GPIX surface expression in GPIbα-deficient MK, as expected, but only partially restored expression of GPV (Figure 1B), suggesting that interaction with the extracellular domain of GPIbα may play an important role in trafficking GPV to the cell surface. Finally, targeted disruption of GPIb/IX/V did not significantly affect the expression of other major GP, such as GPIIb and GPVI (Figure 1B).

Identification of glycoprotein-specific autoantibodies from immune thrombocytopenia plasmas using bioengineered megakaryocytes

As shown in Figure 2A, ITP patient plasmas 1-4, previously typed using diagnostic ACA (*Online Supplementary Table S1*), showed strong binding to both WT MK and GPIbα KO MK. Patients 1 and 2 completely lost binding to GPIIb KO MK, suggesting that the autoantibodies target either GPIIb or elsewhere on the GPIIb-IIIa complex, while plasmas from patients 3 and 4 showed significantly decreased, but not negative, binding to GPIIb KO MK, suggesting the autoantibodies are highly likely targeting the GPIIIa (β3) subunit, which remains expressed in αVβ3 complex on GPIIb KO MK. Supporting this notion, a GPIIIa-specific alloantibody (anti-HPA-1a)[29] demonstrated a similarly low levels of binding to GPIIb KO MK. Notably, the use of whole intact MK also allowed detection of ITP patient antibodies from the plasmas previously undetectable using standard diagnostic ACA methods. For example, plasma samples from ITP patients 5-8, which tested negative in the ACA (*Online Supplementary Table S1*), exhibited various degrees of binding to both WT and GPIbα KO MK, but did not react with GPIIb KO MK (Figure 2B), consistent with the notion that autoantibod-

ies in these patients target so-called "labile epitopes" that become disrupted during detergent solubilization procedures commonly used in ACA. Additionally, blocking FcγRIIa receptors with anti-FcγRIIa antibody IV.3 did not affect ITP autoantibody binding to MK, demonstrating that non-specific antibody binding via FcγRIIa receptor did not play a role in this autoantibody detection system (*Online Supplementary Figure S6*).

ITP patient plasmas containing confirmed anti-GPIb/IX autoantibodies were also examined for their ability to react with iPSC-derived MK. As shown in Figure 3A, plasma from patients 9, 11, and 12 exhibited varying degrees of binding to both WT and GPIIb KO MK, but lost reactivity with GPIbα KO and GPIX KO MK, indicating the presence of antibodies targeting the GPIb/IX complex. Patient 10 showed strong binding to WT, GPIbα KO, and GPV KO MK, but markedly reduced binding to GPIIb KO and GPIX KO MK, suggesting the co-existence of anti-GPIIb-IIIa and anti-GPIX autoantibodies (Figure 3A). Notably, this case highlights the enhanced resolution provided by the MK panel, enabling the identification of a previously unrecognized anti-GPIX autoantibody in patient 10. Although anti-GPIIb-IIIa autoantibodies were also detected in patients 11 and 12 by ACA, their presence could not be confirmed using the whole MK panel.

Similar to the findings with GPIIb-IIIa-specific autoantibodies, the MK panel also detected plasma autoantibodies against the GPIb/IX complex that were not identified by ACA. Plasma from patients 13 to 15 exhibited strong binding to WT MK, markedly reduced binding to GPIIb KO MK, and moderately reduced binding to GPIbα KO and GPIX KO MK (Figure 3B), suggesting the co-existence of a strong anti-GPIIb-IIIa antibody and a weaker anti-GPIb/IX antibody in these samples. Patient 16 demonstrated strong antibody binding to WT MK, with significantly reduced binding to GPIIb KO, GPIbα KO, and GPIX KO MK, indicating the presence of both strong anti-GPIIb-IIIa and anti-GPIb/IX autoantibodies

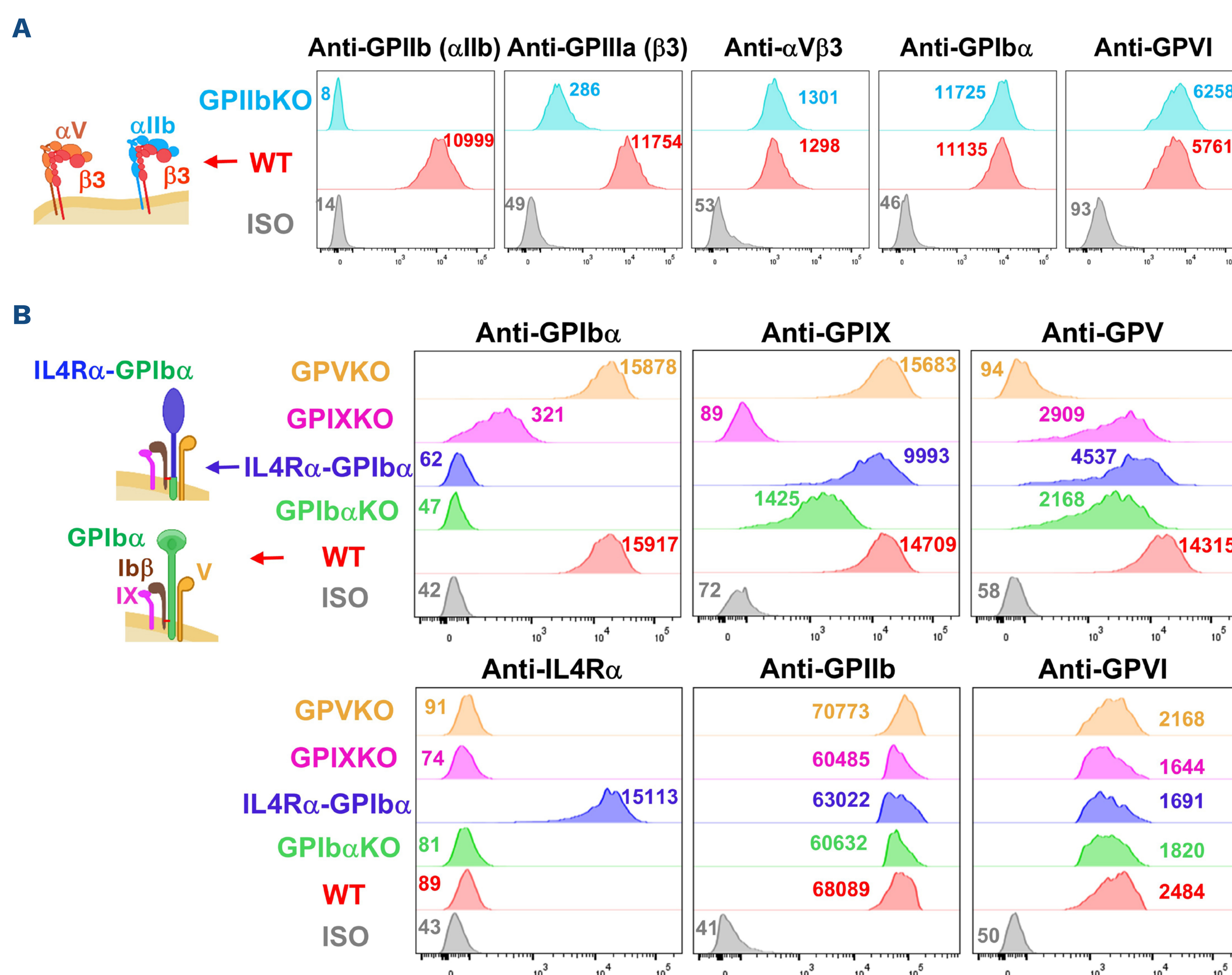

Figure 1. Characterization of surface antigen levels in glycoprotein-deficient megakaryocytes. (A) Flow cytometric analysis demonstrating the loss of surface expression of GPIIb-IIIa, but not αVβ3 and other major platelet surface glycoproteins (GP), in GPIIb knockout (KO) megakaryocytes (MK). The schematic illustration on the left shows integrin β3 (GPIIIa) pairs with either αIIb (GPIIb) or αV to form complexes on the MK surface. αVβ3 expression is detected with a complex-specific monoclonal antibody LM609. (B) Flow cytometric analysis of the surface expression of individual component in GPIb/IX/V complex in each GP KO MK. The schematic illustration on the left shows that the chimeric IL4Rα-GPIbα replaces most of the GPIbα extracellular domain (residues 1-472) with the interleukin-4 receptor α chain (IL4Rα) extracellular domain (residues 1-198). The red line represents the disulfide bond formed between GPIbα and GPIbβ. Color-coded numbers indicate the median fluorescence intensity of the corresponding peaks. ISO: isotype control for the staining antibody; WT: wild-type.

(Figure 3B). Collectively, these results provide compelling evidence for the ability of iPSC-derived, GP-deficient MK to both detect and characterize ITP antibodies with increased sensitivity and resolution - qualities that could be of future importance in directing therapy (see Discussion below). As expected, this whole-cell assay is also applicable for detecting anti-platelet isoantibodies, as demonstrated by the successful identification of a previously missed anti-GPIb/IX antibody in a BSS patient with suspected anti-platelet antibodies following blood transfusion (*Online Supplementary Figure S7*).

Discrimination of non-membrane-reactive autoantibodies from pathogenic antibodies causing platelet destruction

As shown in *Online Supplementary Table S1*, a total of 48 ITP patient plasma samples were tested with whole MK (WMK) in this study - 28 from Site 1 and 20 from Site 2. Although different ACA methods were used by the two diagnostic laboratories, the results could be categorized into three groups, as illustrated in Figure 4A. WMK confirmed the diagnostic ACA findings in 46.4% and 44.4% of cases from Site 1 and Site 2, respectively. Notably, WMK identified previously undetectable autoantibodies in 21.4% of samples from Site 1 and 22.2% from Site 2, underscoring the advantage of preserving labile native antigens in these cases. Importantly, autoantibodies in 32.1% (Site 1) and 33.3% (Site 2) of samples were not detected by WMK, raising concerns that some laboratory ACA results may be misleading and highlighting the need for further evaluation and cautious clinical interpretation.

Previous studies have shown the existence in the plasmas of ITP patients of antibodies that bind the cytoplasmic domains of platelet membrane GP,[21,22] and although detected by some ACA-type assays, have little to do with the pathology of ITP because they do not bind to or mediate the clearance of intact cells. In this regard, the use of bioengineered MK provides an advantage by only reporting the presence of those autoantibodies capable of actually binding to and removing platelet from circulation. As a case in point is demonstrated in Figure 4, in which ITP plasma from patient 17 was found to react strongly with GPIb/IX in

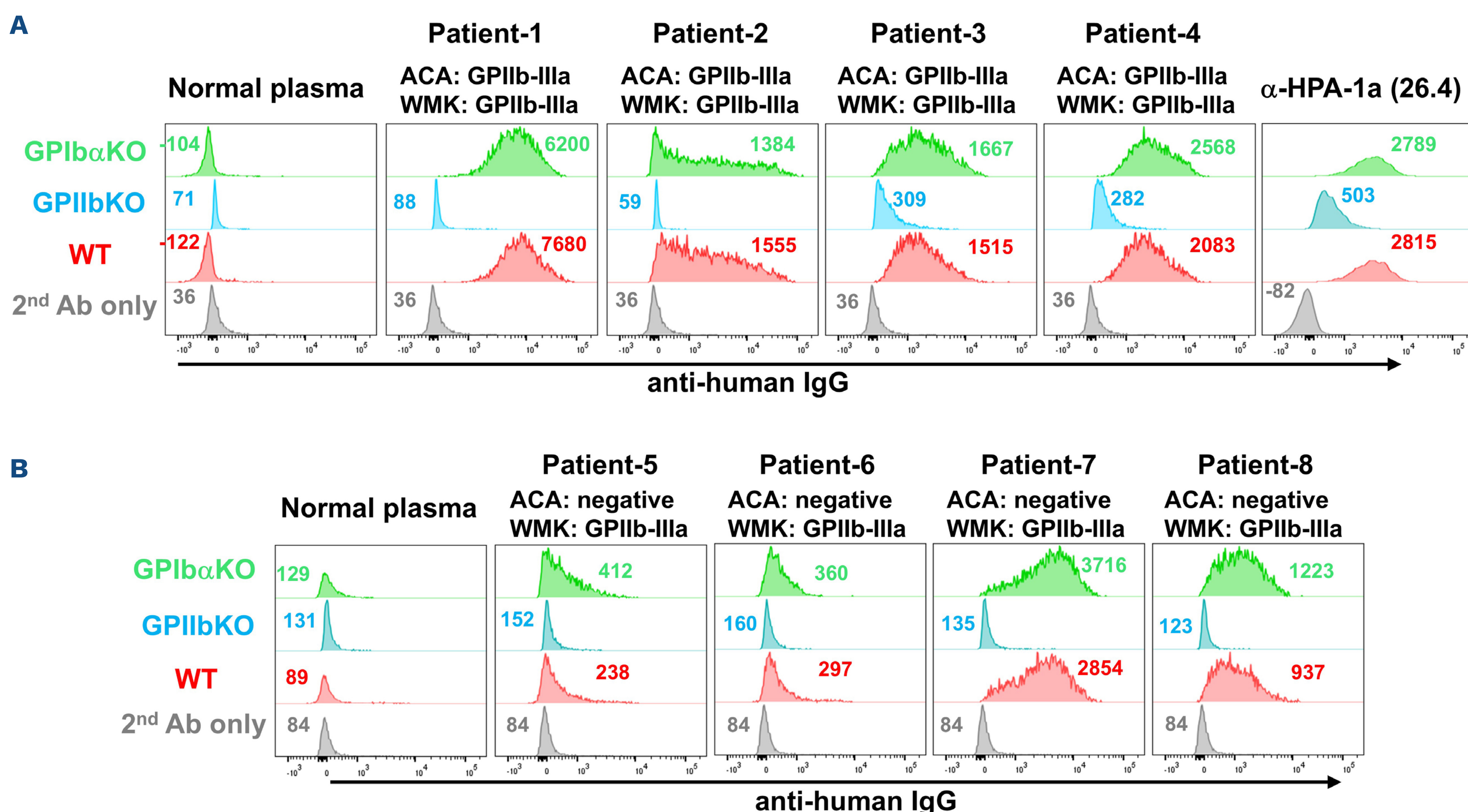

Figure 2. Detection and characterization of anti-GPIIb-IIIa autoantibodies from immune thrombocytopenia patient plasmas using whole megakaryocytes. The wild-type (WT) and glycoprotein (GP)-deficient megakaryocytes (MK) were incubated with plasma from normal healthy controls or immune thrombocytopenia (ITP) patients. The MK-bound antibodies were detected with phycoerythrin (PE)-conjugated donkey anti-human immunoglobulin (Ig)G using flow cytometry. Color-coded numbers indicate the median fluorescence intensity of the corresponding peaks. Autoantibodies detected by diagnostic antigen capture assay (ACA) and whole MK (WMK) in each patient's plasma are shown above the corresponding flow cytometry plots for comparison. (A) WMK detected anti-GPIIb-IIIa antibodies from ITP patient plasmas that had been previously confirmed with ACA in clinical diagnostic laboratory. Normal plasma is shown as a representative for negative controls. Anti-HPA-1a (26.4) antibody is a GPIIIa-specific human alloantibody. (B) WMK detected anti-GPIIb-IIIa antibodies from ITP patient plasmas that were previously undetectable with diagnostic ACA. Ab: antibody.

diagnostic ACA (Figure 4B), while showing little reactivity with intact iPSC-derived MK (Figure 4C) and intact human platelets (*Online Supplementary Figure S8*), suggesting that the detected antibody is non-pathogenic and likely represents a bystander in the patient's plasma. Such ACA results may be misleading, particularly when platelet-associated antibody testing cannot be performed due to severe thrombocytopenia, as was the case for this patient (*Online Supplementary Table S1*). To further investigate the nature of this antibody and localize its epitope, we performed a modified ACA using WT MK or IL4Rα-GPIbα MK expressing a chimeric protein lacking the GPIbα extracellular domain (Figure 4D). The antibody bound to the WT GPIb/IX complex but failed to bind the chimeric protein complex, indicating that its epitope is located on or associated with a cryptic region of the GPIbα extracellular domain. While the mechanisms by which such cryptic epitopes are exposed in ACA-type assays remain unclear, these findings underscore the value of using intact bioengineered, GP-deficient iPSC-derived MK as reliable tools for identifying pathogenic autoantibodies in ITP.

ITP is a heterogeneous disorder. Although the presence of bystander antibodies detected by ACA may explain some cases that were negative in WMK but positive in ACA (Figure 4A), this alone may not account for all instances, and other explanations are possible. First, ACA may in some cases be more sensitive than flow cytometry-based whole-cell assays, as they present a higher density of target antigens on beads or microplate surfaces. This may be particularly relevant for autoantibodies that recognize linear peptide

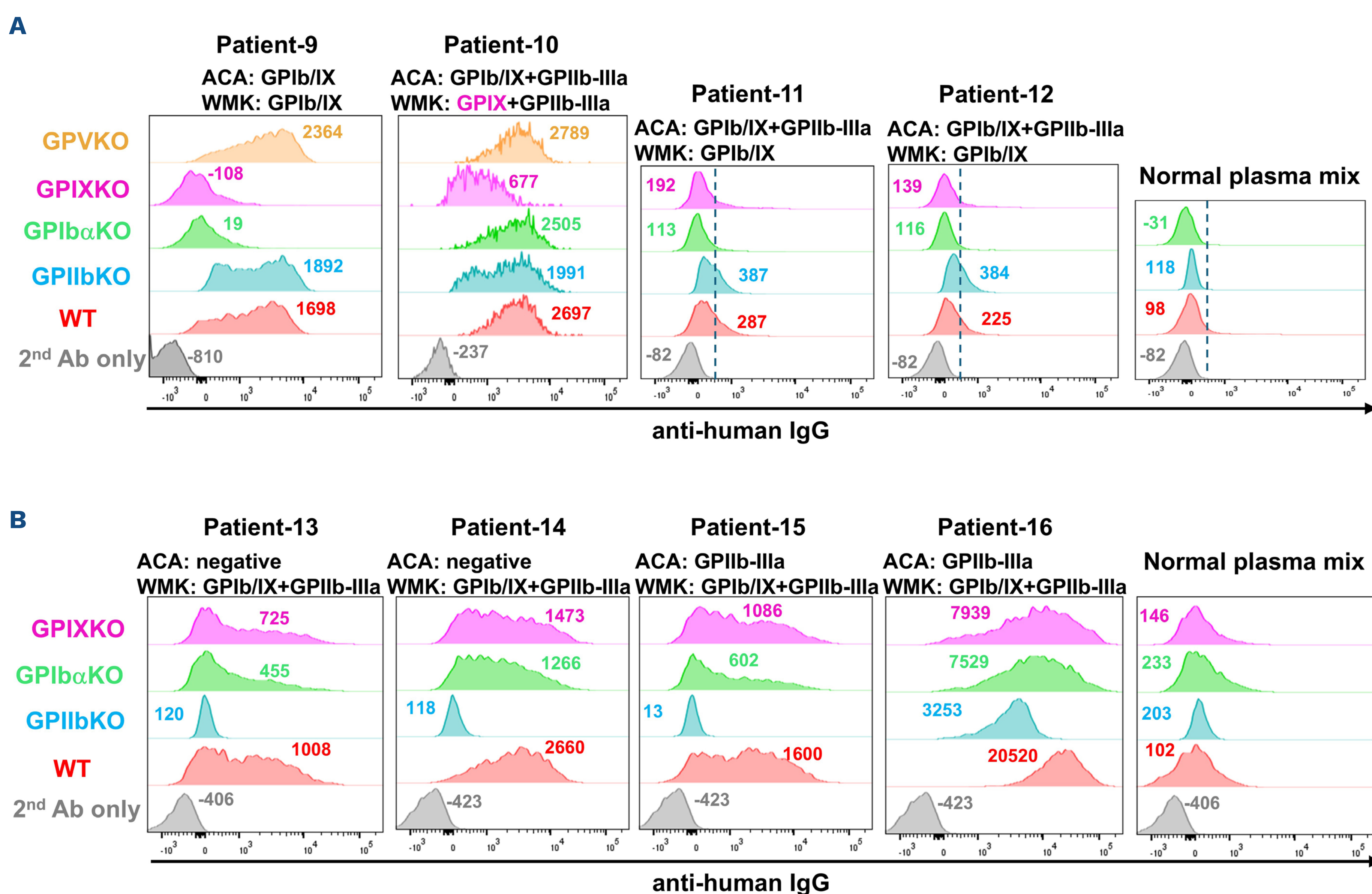

Figure 3. Detection and characterization of anti-GPIb/IX autoantibodies from immune thrombocytopenia patient plasmas using whole megakaryocytes. The wild-type (WT) and glycoprotein (GP)-deficient megakaryocytes (MK) were incubated with immune thrombocytopenia (ITP) patient plasma or a mixture of normal human plasma from 20 healthy donors. The MK-bound antibodies were detected by phycoerythrin (PE)-conjugated donkey anti-human immunoglobulin (Ig)G with flow cytometry. Color-coded numbers indicate the median fluorescence intensity of the corresponding peaks. Autoantibodies detected by antigen capture assay (ACA) and whole MK (WMK) in each patient's plasma are shown above the corresponding flow cytometry plots for comparison. (A) WMK detected anti-GPIb/IX autoantibodies from ITP patient plasmas that had been confirmed with ACA in clinical diagnostic laboratory. WMK detected a strong anti-GPIb/IX antibody in patient 9, weak anti-GPIb/IX antibodies in patients 11 and 12, and a unique strong anti-GPIX antibody coexisting with a relatively weak anti-GPIIb-IIIa antibody in patient 10. (B) WMK detected anti-GPIb/IX autoantibodies from ITP patient plasmas that were previously undetectable with clinical ACA. WMK detected weak anti-GPIb/IX antibodies co-existing with strong anti-GPIIb-IIIa antibodies in patients 13 to 15, and a strong anti-GPIb/IX antibody co-existing with a strong anti-GPIIb-IIIa antibody in patient 16. Ab: antibody.

epitopes or conformational epitopes that remain stable during platelet solubilization. Second, we cannot fully exclude the possibility that certain disease-relevant epitopes are absent or underrepresented in iPSC-derived MK, although these cells demonstrated robust binding to multiple well-characterized monoclonal antibodies against diverse epitopes on GPIIb-IIIa and GPIbα.

Discussion

Identification of antiplatelet autoantibodies that are clinically relevant to the pathology of ITP has historically been constrained by the relatively low sensitivity and specificity of the diagnostic tests commonly used to detect them. The major contribution of the present investigation is the development and use of intact iPSC-derived megakaryocyte cell lines lacking selected platelet membrane GP autoantibody targets (*Online Supplementary Figure S1*). Combined with flow cytometric detection, these offer a number of unique advantages over existing ELISA-based methods.

Firstly, the use of intact cells expressing antigens in their native form on the cell surface improves detection of antibodies directed against labile antigens that become disrupted following platelet solubilization. Labile antigens have been reported for some hard-to-detect anti-platelet alloantibodies, including those specific for the human platelet alloantigens (HPA)-3a, 3b, and 9b.[24,30-33] Detecting autoantibodies can be even more challenging than detecting alloantibodies due to often low abundance and affinity of autoantibodies present in ITP patient plasma. Surprisingly, we found that many autoantibodies, undetectable in standard ELISA-based tests, showed strong binding to intact megakaryocytes (Figures 2B and 3B), providing additional

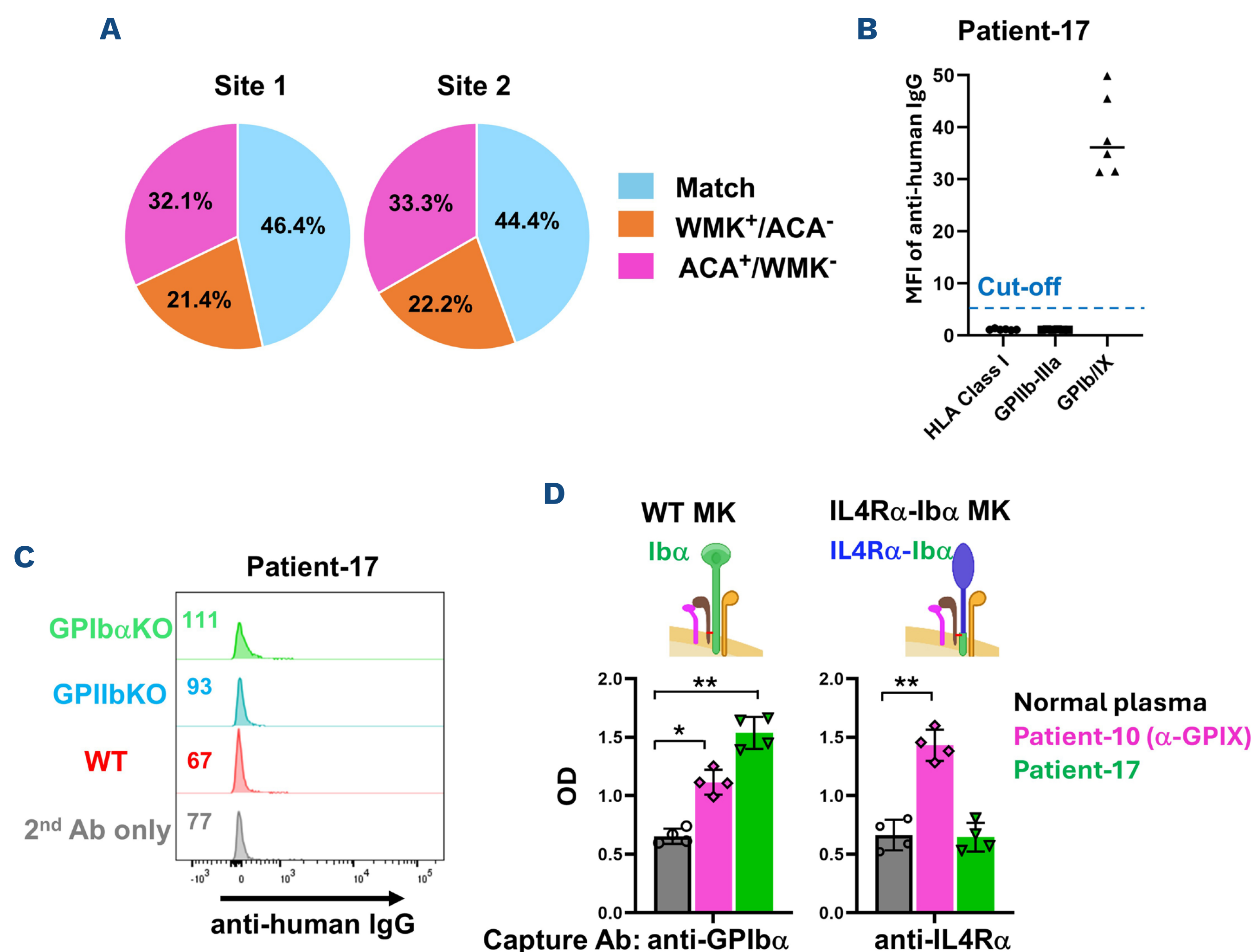

Figure 4. Discrimination of non-membrane-reactive autoantibodies using whole megakaryocytes. (A) Comparison of whole megakaryocytes (WMK) and clinical antigen capture assay (ACA) in detecting antiplatelet autoantibodies from immune thrombocytopenia (ITP) plasmas. As shown in *Online Supplementary Table S1*, blue indicates the cases that the WMK result matches the patient's laboratory ACA result. Orange indicates the cases that the patient's plasma autoantibody is detectable only with WMK, but not ACA. Pink indicates cases where at least 1 of the patient's plasma autoantibodies identified by ACA is not detected by WMK. (B) Clinical platelet antibody bead array (PABA) test of plasma from patient-17 showed strong reactivity to the GPIb/IX complex. Platelets from 6 individual donors were used in the testing panel. Data show the median fluorescence intensity (MFI) of anti-human immunoglobulin (Ig)G detected from beads conjugated with MBC143.1 (anti-HLA class I), AP2 (anti-GPIIb/IIIa) and MBC142.17 (anti-GPIb/IX) antibodies. (C) WMK detected no platelet-reactive antibodies in the plasma from patient-17. (D) ACA using induced pluripotent stem cell (iPSC)-derived MK demonstrates that the epitope recognized by patient-17's antibody is located on or associated with the extracellular domain of GPIbα. The GPIb/IX complex was captured from wild-type (WT) MK by anti-GPIbα monoclonal antibody MBC142.17. The IL4Rα-GPIb/IX complex was captured from IL4Rα-GPIbα MK by anti-IL4Rα monoclonal antibody clone 25463. Autoantibody binding to the captured antigens was detected by horseradish peroxidase-conjugated anti-human IgG. Values represent the means ± standard deviation from 2 independent experiments. *$P<0.05$, **$P<0.01$ by one-way ANOVA with Dunnett's test compared with normal plasma binding. Ab: antibody; OD: optical density.

evidence for ITP autoantibodies that target labile conformational antigens that become disrupted upon detergent solubilization.

Second, GP-deficient MK serve as their own internal controls, providing both enhanced specificity and increased resolution for autoantibody epitope characterization. For example, GPIbα is the only member of the GPIb/IX complex previously found to harbor autoreactive epitopes.[34] Using intact iPSC-derived MK cell lines lacking carefully selected platelet membrane GP, however, we were able to identify an autoantibody with specificity for the GPIX component of this complex (Figure 3A) - a critical first step in the design of GP-specific chimeric autoantibody receptor (CAAR) T cells aimed at reducing the titer of circulating autoantibodies (*confer* reference[35] and discussion below).

Third, the use of intact cells restricts identification to only those autoantibody subpopulations able to react with cell surface epitopes, ignoring so-called bystander antibodies that can arise downstream of antibody-mediate platelet destruction. Such cryptic epitopes, often on the intracellular regions of platelet surface GP[21,22] or cytoplasmic proteins,[36,37] are generated as a secondary immune response, and have been found in both ITP as well as other platelet-destructive conditions like posttransfusion purpura and drug-induced ITP.[38] Cryptic epitopes may also be located within the extracellular domains of platelet surface glycoproteins, and are exposed under conditions such as platelet aging, activation, or damage.[39-42] Naturally occurring autoantibodies to such epitopes are thought to help clear aged or damaged platelets from circulation.

Fourth, as platelet precursors, MK present a comprehensive array of platelet surface glycoprotein targets, thereby offering the potential to detect autoantibodies against rare antigens that are not included in ELISA-based testing panels. Autoantibodies targeting GPVI have been reported in some cases of ITP.[43-46] Such cases are likely under-recognized since GPVI is not offered as a target in the current ITP testing panels. Although we have not generated GPVI-deficient MK for antibody specificity characterization, we expect to detect currently unknown antibodies binding to the WT MK in initial screens. Further studies can then be conducted to address autoantigen specificity. Such an approach will reduce the possibility of missing potentially disease-causing autoantibodies.

The anti-CD20 monoclonal antibody, rituximab, is often used as second-line therapy to eliminate B cells, independent of their specificity, for ITP treatment. Though initially developed for hematologic cancers, chimeric antigen receptor (CAR)-bearing T cells directed against CD19 and CD20 are currently undergoing clinical trials for a number of autoimmune disorders.[47] Beginning in 2016, CAR T cells were repurposed to express chimeric autoantigens on their surface, facilitating specific elimination of pathogenic autoreactive B cells, while sparing B cells uninvolved in the autoimmune disease.[48-50] In the ITP field, GPIbα-CAAR T cells have been developed to eliminate GPIba-specific B cells.[35] Future advancements may involve the development of a wide range of GP-specific CAR T cells, further highlighting the need for sensitive assays such as the one described here that precisely identify targets of anti-platelet autoantibodies, crucial for selecting appropriate CAR T cells for ITP treatment.

ITP is a heterogeneous disorder comprising distinct groups of patients with varied clinical and serological profiles. Identifying pathogenic antiplatelet autoantibodies is critical for advancing our understanding of the disease and holds significant promise for improving ITP research, diagnosis, and treatment. As demonstrated in the current study, the whole MK assay enables sensitive detection and characterization of pathogenic autoantibodies from ITP plasma. This approach could also be applied to analyze platelet-associated autoantibodies, provided they are eluted from patient platelets (*Online Supplementary Figure S9*). However, we were only able to conduct a pilot study to demonstrate feasibility, as additional patient platelets and eluates were not available.

Currently, ITP diagnosis relies on exclusion of other causes, which carries a risk of misdiagnosis. To mitigate this risk when working with deidentified plasma samples lacking full clinical histories, we included only patients previously confirmed to harbor platelet-associated or circulating autoantibodies. Future clinical studies incorporating both antibody-positive and antibody-negative ITP cases, together with complete patient medical histories, will be required to fully establish the clinical utility of the WMK assay. We anticipate that WMK may uncover missed antibodies in serologically unconfirmed ITP cases, analogous to our detection of a previously undetectable anti-GPIb/IX isoantibody in a patient with BSS (*Online Supplementary Figure S7*), thereby enhancing the sensitivity and accuracy of ITP diagnosis.

The current GP-deficient MK panel could be expanded to include additional GP targets, such as GPIa/IIa and GPVI. However, validation of these targets is currently limited by the lack of relevant ITP plasma samples from our diagnostic laboratory. A major challenge for clinical application of this novel strategy lies in producing sufficient quantities of MK from established iPSC lines, as most diagnostic laboratories are not equipped to perform MK differentiation. Future efforts to immortalize bioengineered iPSC-derived MK may overcome this barrier and facilitate broader clinical implementation.

Disclosures

BRC is a consultant for Rallybio in the field of platelet alloimmunity. The remaining authors have no conflicts of interest to disclose.

Contributions

NZ conducted experiments and analyzed data. GU and TB provided MAIPA-tested ITP patient plasma and helped

interpret data. BRC provided PABA- and ELISA-tested ITP patient plasma and helped interpret data. NZ and PJN designed experiments and wrote the manuscript.

Acknowledgment

We thank Dr. Taisuke Kanaji at the Scripps Research Institute for providing the pCDNAzeo-hGPIbα pro-hIL4Rα-GPIbα plasmid, and Dr. Maria Therese Ahlen at the University Hospital of North Norway for providing anti-HPA-1a (26.4) alloantibody.

Funding

National Heart Lung and Blood Institute of the National Institutes of Health grant R35 HL139937 (to PJN)

Data-sharing statement

For original data, please contact the corresponding author.

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