Received: 1 December 2023 | Revised: 26 January 2024 | Accepted: 27 January 2024

DOI: 10.1111/trf.17758

IMMUNOHEMATOLOGY

TRANSFUSION

Generation of human antibodies targeting human platelet antigen (HPA)-1a

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Funding information
Landsteiner Foundation for Blood Transfusion Research, Grant/Award Number: 1908

Abstract

Background: Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a condition during pregnancy, which can lead to thrombocytopenia and a bleeding tendency with intracranial hemorrhage (ICH) being the most concerning complication in the fetus or neonate. An incompatibility between human platelet antigen (HPA)-1a accounts for the majority of FNAIT cases. Binding of HPA-1a-specific alloantibodies to their target on fetal platelets and endothelial cells can induce apoptosis of megakaryocytes, disrupt platelet function, and impair angiogenesis. Currently, there is no screening program to identify pregnancies at risk for severe disease. A better understanding of HPA-1a-specific antibody heterogeneity in FNAIT could aid in identifying pathogenic antibody properties linked to severe disease.

Study Design and Methods: This study aimed to isolate HPA-1a-specific B-cells from an HPA-1a-alloimmunized pregnant woman. Using fluorescently labeled HPA-1a-positive platelets, single B-cells were sorted and cultured for 10 days to stimulate antibody production. Subsequently, supernatants were tested for the presence of antibodies by enzyme-linked immunosorbent assay and their reactivity towards HPA-1a-positive platelets. Amplification and

Abbreviations: FcRn, neonatal Fc receptor; FNAIT, fetal and neonatal alloimmune thrombocytopenia; HPA, human platelet antigen; I-EGF-1, integrin epidermal growth factor 1; Mabs, monoclonal antibody; MAIPA, monoclonal antibody immobilization of platelet antigens.

sequencing of variable regions allowed the generation of monoclonal antibodies using a HEK-Freestyle-based expression system.

Results: Three platelet-specific B-cells were obtained and cloned of which two were specific for HPA-1a, named D- and M-204, while the third was specific for HLA class I, which was named L-204.

Discussion: This study outlined an effective method for the isolation of HPA-1a-specific B-cells and the generation of monoclonal antibodies. Further characterization of these antibodies holds promise for better understanding the pathogenic nature of alloantibodies in FNAIT.

KEYWORDS

alloantibodies, antibody heterogeneity, bleeding, FNAIT, HPA-1a, integrins

1 | INTRODUCTION

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) can cause severe intracranial hemorrhage (ICH) and organ bleeding during pregnancy and shortly after birth.[1,2] Among the white population, most FNAIT cases are caused due to an incompatibility between human platelet antigen-1a (HPA-1a) leading to the formation of maternal alloantibodies.[3] Antibodies of the IgG subtype are transported to the fetus via the neonatal Fc-receptor (FcRn), which is expressed on syncytiotrophoblasts cells of the placenta, and binds to target cells including fetal platelets and endothelial cells.[4] Since no routine screening on antibodies is in place and FNAIT is often diagnosed after birth, effective antenatal treatment can only be given in subsequent pregnancies.[5,6]

The clinical presentation of FNAIT varies, encompassing a wide spectrum of symptoms ranging from no bleeding to severe ICH and intrauterine fetal death.[7] Over the last years, evidence has accumulated that the specificity and functional characteristics of HPA-1a-specific antibodies are heterogeneous[8–14] and might be linked to disease outcomes.[15,16] Although all antibodies target the HPA-1a epitope, formed by a single amino acid substitution (Leu33Pro) on integrin β3, their specific footprint varies and differentially affects target cells. Integrin β3 can dimerize with αIIb to form the fibrinogen receptor which is abundantly expressed on the platelet membrane.[17] Binding of HPA-1a-specific antibodies can induce platelet phagocytosis by splenic macrophages thereby mediating thrombocytopenia.[14,18,19] Moreover, inhibition of megakaryopoiesis[20] and decreased fibrinogen binding have been reported for some.[21,22] Aside from its expression on platelets, β3 is also expressed on endothelial cells and syncytiotrophoblasts cells where it is dimerized with αv forming the vitronectin receptor.[23,24] Upon engagement of HPA-1a-specific antibodies to this receptor, some antibodies may disrupt integrin function leading to endothelial cell apoptosis, inhibition of adhesion, impaired barrier function, and reduced placental development.[10–12,15] It is likely that the combined effect of thrombocytopenia and impaired angiogenesis in the fetus or neonate contributes to the bleeding tendency in FNAIT.

Currently, there is no screening program for the identification of pregnancies at risk for severe FNAIT in part due to the lack of clear understanding of which factors correlate with disease severity.[25] A better understanding of the antibody heterogeneity in FNAIT holds potential for the identification of specific antibody properties that play a role in the development of severe disease. However, until now, only one HPA-1a-specific monoclonal antibody has been cloned from a patient[26] highlighting our limited understanding of the potential functional heterogeneity in this response. Here, we describe a fast method for the generation of recombinant platelet-specific monoclonal antibodies and describe the cloning of two new HPA-1a-specific monoclonals derived from a pregnant woman diagnosed with severe FNAIT.

2 | MATERIALS AND METHODS

2.1 | Donor material

Peripheral blood was donated by a woman (G10P2) with a history of FNAIT in previous pregnancies. Informed consent was in accordance with de Declaration of Helsinki and the medical ethics committee of the Amsterdam University Medical Center approved the protocol for the use of peripheral blood samples to isolate antibody sequences. Written informed consent was obtained from the participants in this study. Obstetric history included a healthy child and six miscarriages in

the past without any abnormalities detected. Perinatal death due to ICH at weeks 19 and 16 occurred at 6 and 2 years before the current pregnancy, respectively, and were related to HPA-1a-specific antibodies. In the current pregnancy, several treatments were applied including weekly dosage of intravenous immunoglobulin (IVIg, 1 g/kg) and prednisone from 10 weeks gestational age onwards. Fetal ICH was suspected at 27 weeks gestational age leading to weekly administration of five intrauterine platelet transfusions (IUPT, fetal platelet count nadir 11×10^9/L). Blood was donated 8 days before delivery, preceding the fourth IUPT. At 32 weeks gestational age, a cesarean section was performed and a healthy HPA-1a positive child was born with no overt bleeding symptoms, post-hemorrhagic abnormalities or complications on neonatal MRI scan of the brain. Neonatal platelet count was 148×10^9/L.

2.2 | Isolation of peripheral blood mononuclear cells, B-cells, and platelets

Peripheral blood mononuclear cells (PBMCs) were isolated using standard Ficoll-Paque density gradient centrifugation. $CD19^+$ B-cells were purified by positive selection with Dynabeads CD19 Pan B followed by detachment using DETACHaBead CD19 (Invitrogen, Carlsbad, USA). Platelets were isolated from ethylenediamine tetraacetic acid (EDTA) blood obtained from an HPA-1a-positve healthy donor. Blood was centrifuged at 200 g for 10 min at room temperature and platelet-poor plasma was saved. The platelet-rich plasma was washed with sequestrine buffer (17.5 mM Na_2HPO_4, 8.9 mM Na_2EDTA, 154 mM NaCl, pH 6.9, containing 0.5% bovine serum albumin) and centrifuged at 800 g for 10 min. Platelets were counted and diluted to a final concentration of 50×10^6 platelets/mL.

2.3 | Selection and culture of HPA-1a-specific B-cells

HPA-1a-positive platelets were labeled with either 5 μM CellTrace™ Violet (Invitrogen) or 1 μM CellTrace™ Far Red (Invitrogen) for 45 min at 37°C. Labels were quenched by addition of platelet-poor plasma and platelets were diluted to a final concentration of 2×10^6 platelets/mL in sequestrine buffer. Isolated PBMCs or B-cells were stained with FITC-conjugated anti-CD19 (BD Biosciences, San Jose, USA), PE-Cy7-conjugated anti-CD27 (BD Biosciences) and PE-conjugated anti-CD38 (BD Biosciences) for 30 min at 4°C. PBMCs or B-cells were then incubated with a mix of labeled platelets in a 1:10 ratio for 45 min at 37°C. After washing, cells were resuspended in sequestrine buffer for FACS analysis and cell sorting (FACSAria™ III, BD Biosciences). B-cells bound to HPA-1a-positive platelets were single-cell sorted in 96-well flat-bottom plates, pre-seeded with EL4.B5 feeder cells (50 Gy irradiated) to provide CD40L stimulation, as described previously,[27–29] and cultured for 10 days at 37°C and 5% CO_2.

2.4 | Supernatant analysis

The presence of IgM or IgG in B-cell supernatant was assayed through enzyme-linked immunosorbent assay (ELISA). ELISA plates (Nunc MaxiSorp flat-bottom, Thermo Fisher Scientific) were coated overnight with mouse anti-human IgG-Fc (Southern Biotech) or mouse anti-human IgM (Peliclass, M1267) diluted in phosphate-buffered saline (PBS). Plates were washed with 0.05% PBS-Tween 20 and blocked with 2% milk powder for 1 h at room temperature (Campina, Amersfoort, Netherlands). Afterward, 1:2 diluted supernatants from cultured B-cells were incubated for 1 h at room temperature. Antibody binding was detected with biotinylated mouse anti-human IgG-Fc (Southern Biotech, Birmingham, USA) or biotinylated mouse anti-human IgM-Fc (Southern Biotech) and streptavidin poly-HRP (Peliclass, M2032). Plates were developed with 0.1 mg/mL tetramethylbenzidine solution with 0.11 M NaAc and 0.003% H_2O_2 and terminated with 2 M H_2SO_4. Supernatants of antibody-producing cells were then incubated with HPA-1a-positive or -negative platelets and assessed by staining with a FITC-conjugated anti-IgG (clone JDC-10, BD Biosciences) or an APC-conjugated anti-IgM (clone G20-127, BD Biosciences) for antibody specificity towards HPA-1a. Results were acquired by flow cytometry (LSR III, BD Biosciences).

2.5 | Amplification and sequencing of Ig V region genes

Feeder cell plates were centrifuged and pellets of wells that were shown to be reactive towards HPA-1a-positive platelets were lysed in buffer RLT (Qiagen, Venlo, Netherlands) supplemented with 40 mM dithiothreitol (DTT) before RNA isolation was performed using the RNeasy Plus Micro kit (Qiagen) according to manufacturer's instructions. RNA was subsequently converted to cDNA using the SuperScript™ III CellsDirect kit by manufacturer's instructions. The resulting cDNA was used as a template for first and nested polymerase chain reaction (PCR, 98°C for 20 s, 59°C for 30 s, 72°C for 1 min;

35 cycles) to amplify IgG variable heavy (VH) and variable light (VL) with Phusion High-Fidelity polymerase (Thermo Scientific, Waltham, USA). Several primer mixes specifically recognizing variable domains or constant regions of heavy (CH), and kappa light (CL) chain were used, as described before[28,30] (Table 1), and PCR products were analyzed on 1.2% agarose gel. Nested PCR products were subsequently sequenced with the appropriate nested PCR primers using Big Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems).

2.6 | Production of recombinant IgG

Monoclonal antibodies were cloned and produced as described before.[16,31,32] In short, sequences were ordered at GeneArt (Thermo Scientific) and VH was cloned in pcDNA3.1 with HindIII and NheI (FastDigest, Thermo Scientific) in front of IgG constant heavy (IGHG1*03). VL was ordered in pcDNA3.1 harboring the kappa constant domain with no further cloning needed. The sequences of already existing HPA-1a-specific antibodies B2G1 and 26.4 had been described previously.[26,33] HEK-293F cells, cultured in Freestyle™ Expression Medium (Thermo Scientific) at 37°C and 5% CO2 while shaking at 125 rpm, were cotransfected with heavy chain vector, light chain vector and pSVLT/p21/p27-mix using polyethylenimine (PEI) MAX (Polysciences, Warrington, USA) as described by Vink et al.[34] Approximately 4 h post-transfection, 0.9% w/v Peptone Primatone® RL (Sigma-Aldrich), 100 units/mL penicillin, and 100 μg/mL streptomycin (Thermo Scientific) were added to the culture. Culture supernatants were harvested 6 days after transfection and purified on a 5-mL protein A column (GE Healthcare). The eluate was dialyzed against sodium acetate buffer (5 mM, [pH 4.5]) and samples were stored at −20°C.

2.7 | Generation of HLA class I-deficient HEK-293T cells expressing αIIbβ3 integrin

As the β3 integrin can also dimerize with endogenous αv, and serum of pregnant women often contains HLA class I-specific antibodies, these proteins were knocked out via CRISPR-Cas9 for the generation of a clean model system in HEK-293T cells to study binding of antibodies towards αIIbβ3. Forward and reverse RNA guide sequences targeting exon 12 of the integrin αv subunit-encoding gene, *ITGAV*, were designed using CRISPOR by selecting RNA sequences with the highest specificity score (*ITGAV*-g-fw: GCACCTCTCTTCATGGATCGTGG, *ITGAV*-g-rv: ATCCATTCAGCTTTGTCGTCTGG) and purchased from Integrated DNA technologies (IDT). Equimolar amounts of crRNA and tracrRNA were annealed by heating at 95°C for 5 min in a thermocycler, under RNase-free conditions, and cooled down at room temperature (RT) to allow for sgRNA formation. Ribonucleoparticle (RNP) complexes were prepared by combining 12 μg sgRNAs with 30 μg *Streptococcus pyogenes* Cas9 (spCas9) for 10 min at RT.[35] A total of 1×10^6 HEK-293F cells were spun down, resuspended in nucleofection solution (Amaxa SF cell line kit, Lonza, Basel, Switzerland) and gently mixed with RNPs. Cells were transferred to a nucleofector cuvette and electroporated using program CM-130 of a 4D-Nucleofector X unit (Lonza). After nucleofection, cells were resuspended in fresh medium and cultured for 7 days. Cell-surface expression of αv was analyzed by flow cytometry and the αv negative population was bulk-sorted and expanded. For generation of a polyclonal cell line in which HLA class I was ablated, a single RNA guide sequence was used against a conserved region present in HLA-A, B, and C genes, as described previously (*HLA class I*-g: CGGCTACTACAACCAGAGCG).[36] Formation of sgRNAs and nucleofection was performed as described above. HLA class I negative bulk cells were sorted and expanded. pEF1-αIIb and pcDNA3.1-β3, containing integrin cDNA sequences, were obtained from Addgene (a kind gift of Timothy Springer, plasmids #27288 and #27289 respectively).[37] To produce β3 with the HPA-1b epitope, referred to as HPA-1a-negative, a single amino acid alteration was made at position 33 (Leu33Pro) using Benchling Software. The new gene fragment was synthesized by IDT and cloned into pcDNA3.1-β3. HEK-293T cells (ATCC) were cultured in DMEM (25 mM HEPES, Gibco) supplemented with 10% v/v FCS, 4 mM L-Glutamine (Thermo Scientific), 100 U/mL penicillin and 100 μg/mL streptomycin (Thermo Scientific) in Nunclon Delta Surface 80-cm^2 flasks (Thermo Scientific) and passaged with 0.05% m/v Trypsin with 0.02% EDTA (Thermo Scientific) when 80% confluency was reached. For transient transfection, cells were seeded in a six-well plate (Corning) until 50%–70% confluency was reached followed by cotransfection using 1 μg of αIIb and β3 expression vectors and PEI (Polysciences). Receptor surface expression was monitored by flow cytometry and cells were used 48 h after transfection.

2.8 | Determination of monoclonal antibody specificity

Aliquots of 5×10^4 HLA class I-deficient HEK-293T cells expressing integrin αIIbβ3 were incubated with 50 μL of 10 μg/mL monoclonal antibodies for 30 min at 4C°. After washing, cells were incubated with a FITC-conjugated

TABLE 1 Overview of first and nested polymerase chain reaction (PCR) primers.

First PCR reaction primers	
IgG heavy chain forward primers	
5′ L-VH1	ACAGGTGCCCACTCCCAGGTGCAG
5′ L-VH2	CTGCAACCGGTGTACATTGTGCCATC
5′ L-VH3	AAGGTGTCCAGTGTGARGTGCAG
5′ L-VH4/6	CCCAGATGGGTCCTGTCCCAGGTGCAG
5′ L-VH5	CAAGGAGTCTGTTCCGAGGTGCAG
IgG heavy chain reverse primer	
3′ Cγ CH1	GGAAGGTGTGCACGCCGCTGGTC
Kappa light chain forward primers	
5′ L VK1/2	ATGAGGSTCCCYGCTCAGCTGCTGG
5′ L VK3	CTCTTCCTCCTGCTACTCTGGCTCCCAG
5′ L VK4	ATTTCTCTGTTGCTCTGGATCTCTG
Kappa light chain reverse primer	
3′ Ck1	CCAGATTTCAACTGCTCATCAGA
Nested PCR reaction primers	
IgG heavy chain forward primers	
5′ AgeI VH1	CTGCA*ACCGG*TGTACATTCCCAGGTGCAGCTGGTGCAG
5′ AgeI VH1/5	CTGCA*ACCGG*TGTACATTCCGAGGTGCAGCTGGTGCAG
5′ AgeI VH3	CTGCA*ACCGG*TGTACATTCTGAGGTGCAGCTGGTGGAG
5′ AgeI VH3-23	CTGCA*ACCGG*TGTACATTCTGAGGTGCAGCTGTTGGAG
5′ AgeI VH4	CTGCA*ACCGG*TGTACATTCCCAGGTGCAGCTGCAGGAG
5′ AgeI VH4-34	CTGCA*ACCGG*TGTACATTCCCAGGTGCAGCTACAGCAGTG
5′ AgeI VH1-18	CTGCA*ACCGG*TGTACATTCCCAGGTTCAGCTGGTGCAG
5′ AgeI VH1-24	CTGCA*ACCGG*TGTACATTCCCAGGTCCAGCTGGTACAG
5′ AgeI VH3-33	CTGCA*ACCGG*TGTACATTCTCAGGTGCAGCTGGTGGAG
5′ AgeI VH3-9	CTGCA*ACCGG*TGTACATTCTGAAGTGCAGCTGGTGGAG
5′ AgeI VH4-39	CTGCA*ACCGG*TGTACATTCCCAGCTGCAGCTGCAGGAG
5′ AgeI VH6-1	CTGCA*ACCGG*TGTACATTCCCAGGTACAGCTGCAGCAG
IgG heavy chain reverse primer	
3′ IgG	GTTCGGGGAAGTAGTCCTTGAC
Kappa light chain forward primers	
5′ AgeI VK 1–5	CTGCA*ACCGG*TGTACATTCTGACATCCAGATGACCCAGTC
5′ AgeI VK1-9	TTGTGCTGCA*ACCGG*TGTACATTCAGACATCCAGTTGACCCAGTCT
5′ AgeI VK1D-43	CTGCA*ACCGG*TGTACATTGTGCCATCCGGATGACCCAGTC
5′ AgeI VK2-24	CTGCA*ACCGG*TGTACATGGGGATATTGTGATGACCCAGAC
5′ AgeI VK2-28	CTGCA*ACCGG*TGTACATGGGGATATTGTGATGACTCAGTC
5′ AgeI VK2-30	CTGCA*ACCGG*TGTACATGGGGATGTTGTGATGACTCAGTC
5′ Age VK3-11	TTGTGCTGCA*ACCGG*TGTACATTCAGAAATTGTGTTGACACAGTC
5′ Age VK3-15	CTGCA*ACCGG*TGTACATTCAGAAATAGTGATGACGCAGTC
5′ Age VK3-20	TTGTGCTGCA*ACCGG*TGTACATTCAGAAATTGTGTTGACGCAGTCT
5′ Age VK4-1	CTGCA*ACCGG*TGTACATTCGGACATCGTGATGACCCAGTC
Kappa light chain reverse primer	
3′ BsiWI Jκ 3	GCCAC*CGTACG*TTTGATATCCACTTTGGTC
3′ BsiWI Jk1/2/4	GCCAC*CGTACG*TTTGATYTCCACCTTGGTC
3′ BsiWI Jκ 5	GCCAC*CGTACG*TTTAATCTCCAGTCGTGTC

anti-IgG (clone JDC-10, BD Biosciences) and analyzed by flow cytometry (FACSympony, BD Biosciences). HLA specificity was further characterized by Luminex assay (Lifecodes LSA, Immucor GTI Diagnostic, USA) according to manufacturer's instructions. Specificities with a raw MFI value above 750 were considered positive.

2.9 | Data analysis

Flow cytometry data were analyzed using FlowJo 10.8.1 Software (BD Biosciences). Antibody sequences were analyzed with IgBlast[38] to view matches to the germline VDJ genes and multiple sequence alignment was performed with Clustal Omega (1.2.4).[39]

3 | RESULTS

3.1 | Detection and single-cell sorting of HPA-1a-specific B-cells

Peripheral blood was obtained from an alloimmunized pregnant woman at week 31 of gestation, with a history of FNAIT in previous pregnancies. HPA-1a-specific antibodies in the current pregnancy were quantified by monoclonal antibody immobilization of platelet antigens assay (MAIPA, 135 IU/mL) around week 8 of gestation, with very strong MAIPA signals remaining during pregnancy. Within a day after blood drawing, PBMCs were isolated followed by enrichment of $CD19^+$ B-cells (Figure 1). In order to select for HPA-1a-specific B-cells, PBMCs and the B-cell fraction were incubated with HPA-1a-positive platelets labeled separately with two different dyes in order to increase specificity.[29] Sequential gating was performed involving either $CD27^+CD38^+$ to select for plasmablasts from PBMCs or $CD19^+$ to exclude debris from purified B-cells followed by gating on cells positive for both platelet dyes (Figure 2). Notably, the percentage of double positive cells from PBMCs was increased when compared to PBMCs isolated from a healthy control (1.34% vs. 0.13% respectively) indicating specific binding. Around 377 cells were then single-cell sorted by FACS and expanded onto feeder cells to promote B-cell differentiation and antibody production. Production of IgG or IgM was assessed after 10 days of culture by ELISA. A total of 33 clones produced antibodies (approximately 10% of total) of which 10 clones produced only IgM, 21 clones produced only IgG and one cloned produced both.

3.2 | Supernatant of several clones are highly specific for HPA-1a-positive platelets

To determine the reactivity of cultured B-cells, the supernatants that were tested positive by IgG/IgM ELISA were also screened by antigen-specific flow cytometry using HPA-1a-positive and -negative platelets. Four supernatants (~12% of supernatants containing IgG/IgM) were found to be of

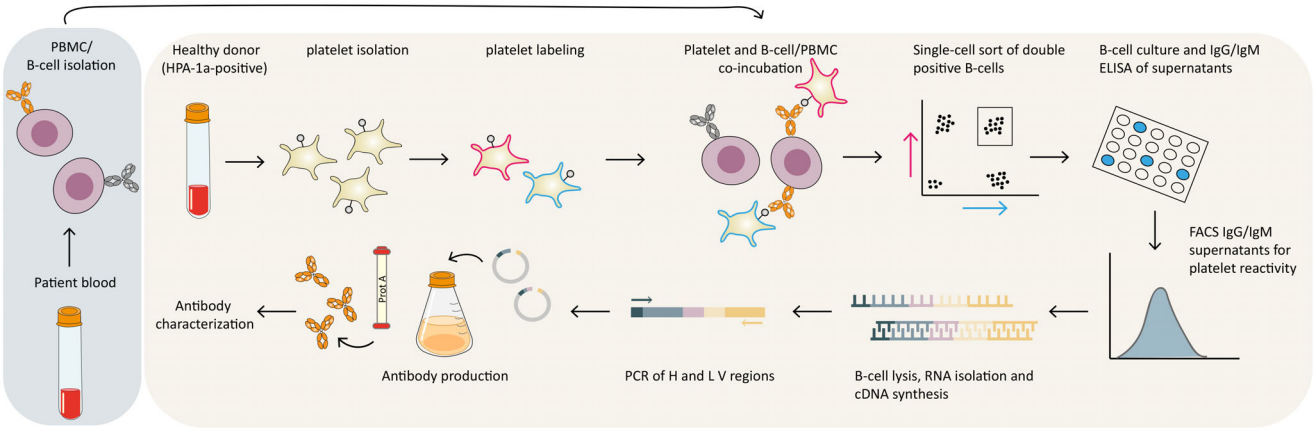

FIGURE 1 Graphical overview of human platelet antigen (HPA)-1a-specific B-cell isolation and monoclonal antibody production. Fresh blood from a hyperimmunized pregnant woman was obtained which was used for peripheral blood mononuclear cell (PBMC) isolation and B-cell enrichment. In parallel HPA-1a-positive platelets were isolated from a healthy donor and labeled with two fluorescent dyes. PBMCs/B-cells were incubated with labeled platelets and gating was performed on cells double positive for the platelet dye which was used as indication for HPA-1a specificity. Cells were single-cell sorted and cultured onto feeder cells for 10 days to promote differentiation and antibody production. Supernatants were tested by IgG/IgM enzyme-linked immunosorbent assay (ELISA) for the presence of antibodies and positive wells were incubated with HPA-1a-positive platelets to test for specificity by flow cytometry. The mRNA was subsequently isolated followed by cDNA synthesis. VH and light transcripts were amplified by nested polymerase chain reaction (PCR) and the identified V genes were cloned into pcDNA3.1 for antibody production using a HEK-293F based expression system. [Color figure can be viewed at wileyonlinelibrary.com]

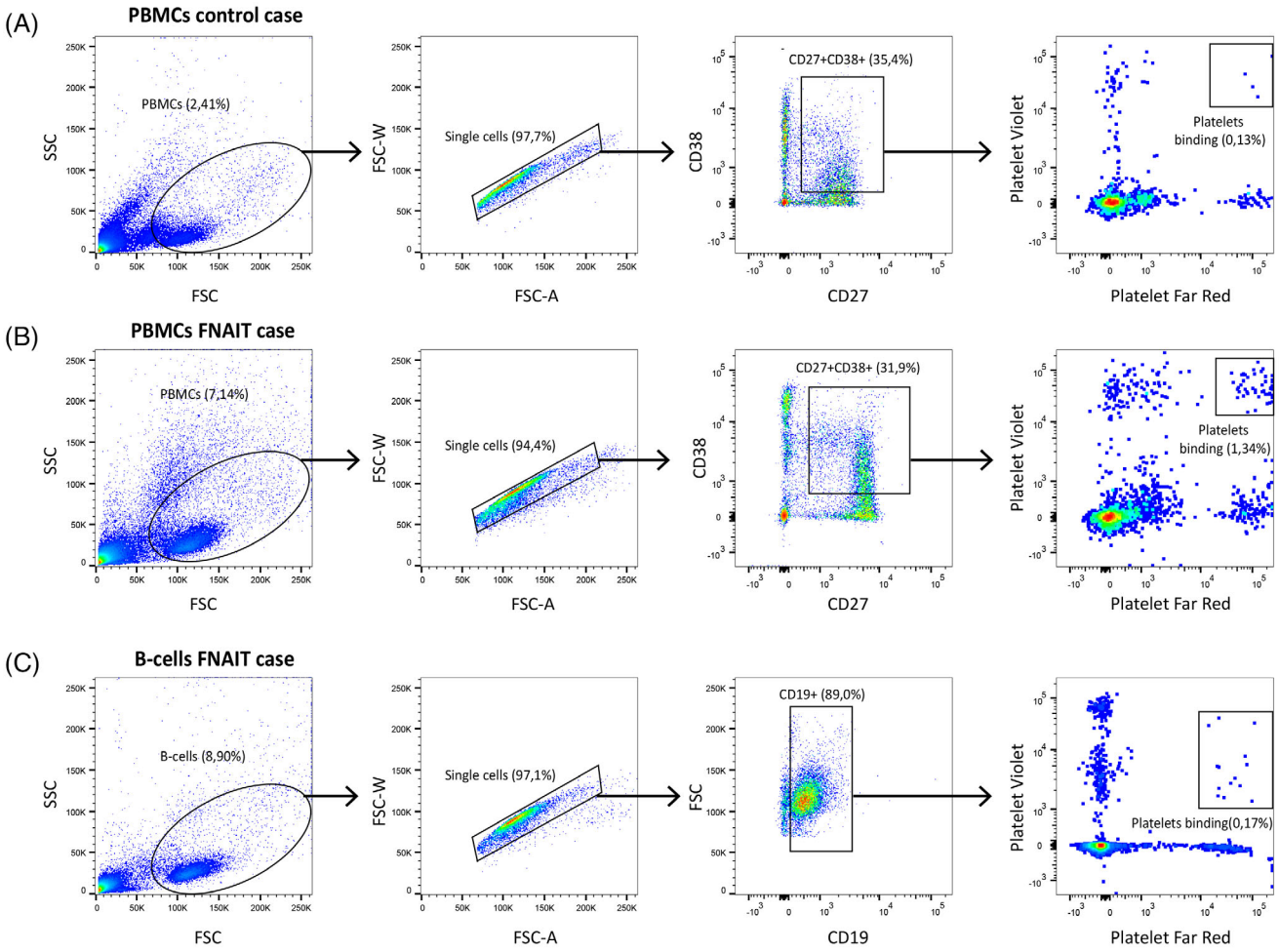

FIGURE 2 Gating strategy for selection of human platelet antigen (HPA)-1a-specific B-cells. Peripheral blood mononuclear cells (PBMCs) from (A) control case, (B) fetal and neonatal alloimmune thrombocytopenia (FNAIT) case, and (C) isolated B-cells from FNAIT case were first gated by FSC-A versus SSC-a, extending the normal lymphocyte gate beyond standard limits in order to compensate for the increased granularity and size of plasmablasts. Then, single cells were gated based on FSC-A versus FSC-W followed by selection for plasmablasts based on (A, B) CD27 and CD38 markers or (C) CD19 for selection of B-cells and exclusion of debris. Final gating identified B-cells binding to both fluorescently labeled platelet populations which were single-cell sorted. [Color figure can be viewed at wileyonlinelibrary.com]

the IgG isotype, while none of the IgM-positive supernatants were specific, with strong binding to HPA-1a-positive platelets and no reactivity towards its negative counterpart. The reactive clones were named D-, L-, M-, and N-204 (Figure 3).

3.3 | Analysis of V gene sequences

We then purified RNA, generated cDNA from the corresponding B-cells and amplified it by PCR. The products were assessed by size and purity on agarose gel (Figure 4A). Sizes of all amplified products matched the theoretical size (450-500 bp) except no band was amplified for the VH chain of clone N-204. The VH and light sequences were obtained by Sanger sequencing and aligned against two already existing HPA-1a-specific monoclonal antibodies (Mabs), B2G1 and 26.4, of which the latter was also obtained in connection with pregnancy (Figure 4B,C). Variations were mainly observed in the complementarity-determining regions (CDRs) in terms of amino acid composition and length. Mab 26.4 showed the longest CDR H3 while L- and M-204 contained the longest CDR L1. The closest germline genes used for V, D, and J gene segments were also identified (Table 2).

3.4 | Mabs D- and M-204 are highly specific for the HPA-1a epitope

Vectors were assembled and full antibodies were produced as described previously.[31,32] The purified Mabs were tested for reactivity towards HEK-293T cells, which

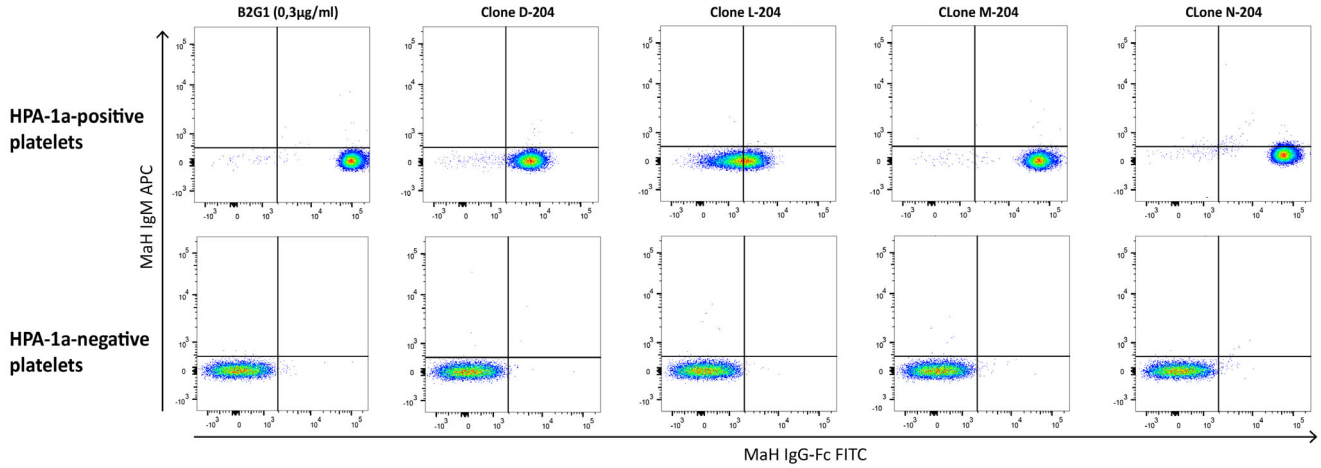

FIGURE 3 Platelet reactive B-cell supernatants are of the IgG isotype and only react to human platelet antigen (HPA)-1a-positive but not -negative platelets. Supernatants containing IgG or IgM, as observed by enzyme-linked immunosorbent assay, were tested for platelet reactivity by FACS. Dot plots are only shown for supernatants that showed reactivity to HPA-1a-positive platelets which were all IgG. Clone D-204 was obtained from peripheral blood mononuclear cells (plasmablasts) while L- M- and N-204 were generated from purified B-cells. B2G1 (IgG) was used as positive control. [Color figure can be viewed at wileyonlinelibrary.com]

were made deficient of HLA class I using CRISPR/Cas9 and transiently transfected with αIIbβ3 with and without the HPA-1a epitope. A clear HPA-1a reactivity was found for D- and M-204 clones, but not L-204 (Figure 5). As immune responses against HLA class I are quite common in pregnancy, which would also register as platelet reactivity,[40] we, therefore, tested the remaining L-204 clone for its reactivity to HLA class I. Additional testing by Luminex showed clone L-204 to be HLA class I-specific (A*02:01-03, A*02:05, A*68:01-02, A*69:01), while clone D- and M-204 tested negative in the same assay (see Figure S1), and was further characterized.[41]

4 | DISCUSSION

In this study, a fast method was described for the isolation of platelet-specific B-cells from an HPA-1a alloimmunized pregnant woman. Subsequently, we successfully generated monoclonal antibodies from these isolated B-cells which led to the discovery of two novel clones named D- and M-204. These clones strongly bound to HPA-1a-positive platelets and HEK-293T cells expressing integrin αIIbβ3 (HPA-1a-positive) but not towards their respective negative counterparts, HPA-1a-negative platelets or HEK-293T cells (HPA-1a-negative), highlighting their specificity. Given the patient's history of severe FNAIT, further characterization of these monoclonals may contribute to our comprehension of pathogenic antibodies involved in FNAIT.

The correlation between antibody titer and severity of FNAIT has been extensively investigated with no absolute correlation being established, yet remains a subject of debate.[1,7,22,42–45] Currently, it is increasingly recognized that not only the quantity but also the quality and specificity of HPA-1a-specific antibodies matter and are related to disease severity. Antibody quality can be controlled, among others, by the composition of a conserved N-linked glycan in the Fc tail of IgG.[46] Although plasma IgG-Fc glycans are highly fucosylated (∼94%), Fc fucosylation of HPA-1a-specific IgG has been found to vary significantly and decreased fucosylation of these antibodies was shown to correlate with disease severity.[14,16] This is explained by the fact that afucosylated IgG exhibits increased affinity for FcγRIII translating into enhanced phagocytosis of fetal platelets.[14,47] In addition, HPA-1a-specific antibodies have been suggested to distinguish between the HPA-1a epitope expressed on platelets, expressing integrin αIIbβ3, and endothelial cells, expressing integrin αvβ3.[15,48] Antibodies specifically recognizing αvβ3 were found in FNAIT sera with ICH and not in cases with mild disease suggesting endothelial damage to be an important factor in ICH occurrence.[15] Also, the specific footprint on β3 varies with some antibodies requiring distinct amino acids in the integrin epidermal growth factor 1 (I-EGF1) domain, further supporting the notion that the local conformation surrounding the HPA-1a epitope influences alloantibody binding.[49] Finally, integrin function is heavily regulated by transitions in different conformational states including the bent state, which has low affinity for ligand, and the extended state, with high affinity for ligand.[50] Antibodies preferably binding to bent or extended integrins have been described but its

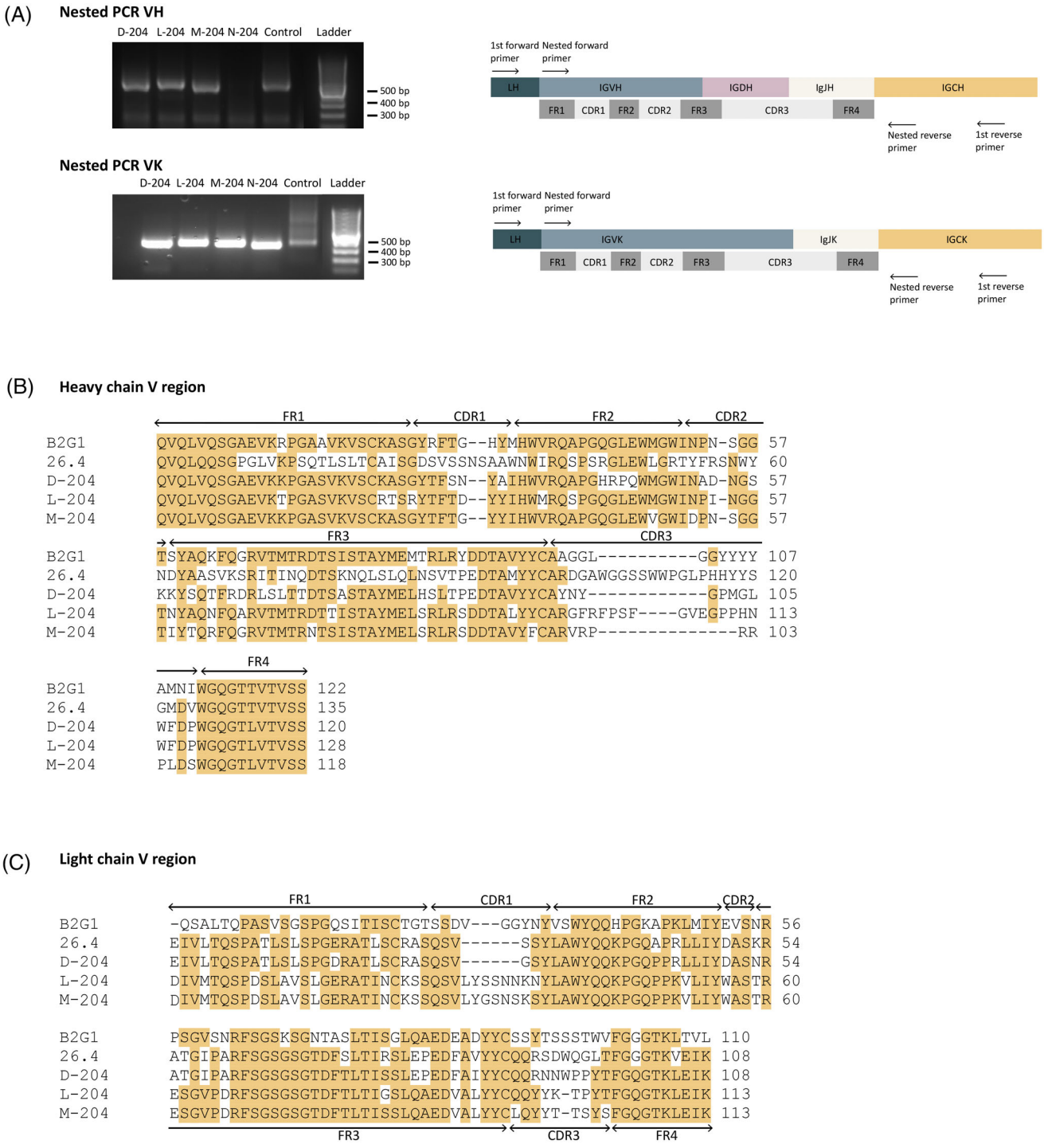

FIGURE 4 Cloning of the variable region of platelet-specific antibodies and amino acid sequence analysis. (A) The Ig variable heavy (VH) and light domain (kappa, VK) sequences were amplified from cDNA using primers designed as depicted on the right, for 1st and nested polymerase chain reaction (PCR). Aliquots of the nested PCR products were analyzed by agarose gel electrophoresis for the presence of the correct products at 450-500 bp. The cDNA of a B-cell clone targeting rhesus D was taken along as positive control. (B, C) Amino acid sequence of two already existing human platelet antigen-1a-specific antibodies B2G1 and 26.4 compared to D-, L-, and M-204. Framework and CDR regions were annotated with NCBI's IgBlast[37] and sequence alignment was performed with Clustal Omega multiple sequence alignment.[38] VH, variable heavy. [Color figure can be viewed at wileyonlinelibrary.com]

relation with disease severity remains unstudied.[8] Thus, the interplay between antibody titer, quality, and specificity in relation to FNAIT disease severity is complex and emphasizes the multifaceted factors that shape the functional effects of HPA-1a-specific antibodies. The extent of D- and M-204 in contributing to disease

TABLE 2 Analysis of closest germ line genes of human platelet antigen-1a-specific antibodies.

	V	D	J	Total identity V gene (%)	CDR-H3 length
Variable heavy					
26.4	V6-1*01	D6-13*01	J6*02	95.4	25
B2G1	V1-2*02	D1-26*01/D3-10*01/D3-10*02	J6*02	93.5	15
D-204	V1-3*01	D5-18*01/D5-5*01	J5*02	88.3	13
M-204	V1-2*02	D-24*01 (In ORF2)	J4*02	94.9	11
Variable light					
26.4	V3-11*01		J4*01	97.2	10
B2G1[a]	-		-	-	-
D-204	V3-11*01		J2*01	97.2	10
M-204	V4-1*01/V4-1*03		J2*03	95	9

[a]A non-immune variable light was recombined with a variable heavy, the latter obtained from an alloimmunized individual and selected using phage display, for generation of B2G1.

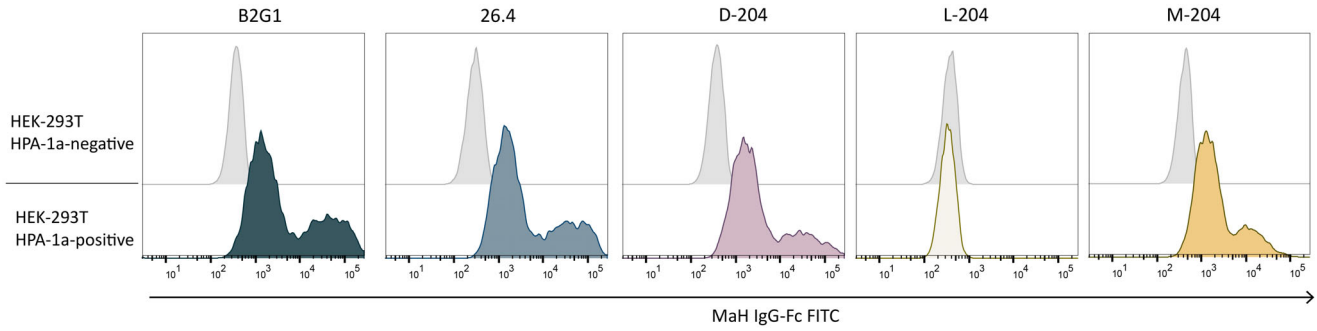

FIGURE 5 Characterization of antibodies using HEK-293T cells expressing human platelet antigen (HPA)-1a shows D- and M-204 to be HPA-1a-specific. Recombinant Mabs were produced and reactivity towards the HPA-1a epitope was tested using HEK-293T cells overexpressing the αIIbβ3 integrin and negative for HLA class I. Histograms of a representative experiment are shown comparing the binding of two already existing HPA-1a-specific Mabs; B2G1 and 26.4, and newly made Mabs; D-, L- and M-204, towards HPA-1a-negative (upper panel) and HPA-1a-positive (lower panel) HEK-293T cells. Two peaks are observed in the histograms of reactive antibodies, which is consistently observed when using transiently transfected HEK-293T cells. [Color figure can be viewed at wileyonlinelibrary.com]

severity is unclear, especially as the contribution of peripheral antigen-specific memory B-cells to the circulating antibody pool has not yet been firmly established. In the end, mapping the characteristics of pathogenic antibodies could provide a rationale for screening pregnant women for antibodies with such traits, aiding in the identification of those who might need treatment intervention.

Several monoclonal antibodies against human HPA-1a have been generated including SZ21,[51] isolated by hybridoma technology using mice immunized with human platelets, ML1[52] and B2G1,[33] which were derived by phage display, and 26.4.[26] The latter clone is the only one which was isolated from an HPA-1a immunized pregnant woman with a comparable approach as used in the current study, containing naturally paired heavy and light chains. No other HPA-1a-specific monoclonals, that recognize specific integrin conformations or integrin complexes, have been generated. The ability to efficiently isolate antigen-specific B-cells from maternal serum is among others dependent on the antigen bait used. Here, we used fluorescently labeled HPA-1a-positive platelets which have a high abundance of αIIbβ3 on the membrane. Instead of platelets, baits such as integrin αIIbβ3 or αvβ3 in either bent or extended conformation could be presented on transfected cells or vesicles to select for certain B-cells. Interestingly, one of our clones, L-204, appeared to be specific for HLA class I which was shown by Luminex testing. An incompatibility between HLA class I allotype in HPA-1a-positive and -negative platelets could explain the fact that L-204 did only react with the former. In addition, no reactivity was observed against HPA-1a-positive HEK-293T cells which are deficient for HLA class I. Such HLA class I-specific antibodies are frequently detected in pregnant women[40] and underline the use of our method to select

for B-cells that target different epitopes aside from HPA-1a. Current evidence suggests that these HLA class I-specific antibodies do not generally play a major role in FNAIT disease severity.[53]

In conclusion, we have developed an efficient method to isolate HPA-1a-specific B-cells from maternal serum and generated two HPA-1a-specific monoclonal antibodies. In future studies, their detailed specificity and functional effects will be addressed. The availability of this method will pave the way towards more efficient generation of HPA-1a-specific monoclonals which is needed to dissect the heterogenous antibody repertoire in FNAIT.

ACKNOWLEDGMENTS

The authors wish to thank Han Verhagen and Roos Krimpenfort for their substantial support regarding the generation of knockouts. In addition, we are most grateful to our patients who agreed to donate blood for this study.

FUNDING INFORMATION

JJO was supported by Landsteiner Foundation for Blood Transfusion Research (LSBR) grant 1908 to GV.

CONFLICT OF INTEREST STATEMENT

The authors have disclosed no conflicts of interest.

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How to cite this article: Oosterhoff JJ, Linty F, Visser R, de Vos T, Hofstede-van Egmond S, van de Weerd M, et al. Generation of human antibodies targeting human platelet antigen (HPA)-1a. Transfusion. 2024;64(5):893–905. https://doi.org/10.1111/trf.17758