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Faculty of Health Sciences
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Prevention of Fetal/Neonatal Alloimmune Thrombocytopenia (FNAIT) by prophylactic monoclonal antibodies

In vitro and preclinical evaluation of HPA-1a-specific antibodies

Trude Victoria Mørtberg

A dissertation for the degree of Philosophiae Doctor

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List of papers

I. **TV Mørtberg**, H Zhi, G Vidarsson, S Foss, S Lissenberg-Thunnissen, M Wuhrer, TE Michaelsen, B Skogen, TB Stuge, JT Andersen, PJ Newman, and MT Ahlen. *Prevention of Fetal/Neonatal Alloimmune Thrombocytopenia in Mice: Biochemical and Cell Biological Characterization of Isoforms of a Human Monoclonal Antibody.* ImmunoHorizons, 2022. 6(1): p. 90-103. doi: 10.4049/immunohorizons.2100097

II. **TV Mørtberg**, S Foss, LM van Ligtenberg, EL Bertelsen, O Ottersen, G Vidarsson, B Skogen, TB Stuge, JT Andersen, and MT Ahlen. *Preclinical evaluation of an HPA-1a-specific human mAb IgG1 modified for enhanced effector properties.* Unsubmitted manuscript.

III. **TV Mørtberg**, EL Bertelsen, H Tiller, B Skogen, TB Stuge, and MT Ahlen. *Exploring recombinant glycoproteins as reagents for anti-HPA-1a antibody subtype specificity in FNAIT.* Unsubmitted manuscript.

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Abbreviations

AAAG	L234A-L235A-N297A-P329G
ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
AMIS	Antibody-mediated immune suppression
APLDQ	T30A-S32P-Q33L-N39D-M470Q
CDC	Complement-dependent cytotoxicity
CMP	Complement receptor-mediated phagocytosis
DAT	Direct antiglobulin tests
EGF1	Epidermal growth factor 1
Fab	Fragment antigen binding
Fc	Fragment crystallizable
FcγR	IgG Fc receptors
FcRn	Neonatal Fc receptor
FNAIT	Fetal/neonatal alloimmune thrombocytopenia
GlcNAc	N-acetylglucosamine
HDFN	Hemolytic disease of the fetus and newborn
HG	High galactose
HLA	Human leukocyte antigen
HOD	Hen egg lysozyme-ovalbumin-Duffy
HPA	Human platelet antigen
ICH	Intracranial hemorrhage
IU	International units
IVIg	Intravenous Immunoglobulin
LF	Low fucose
mAb	Monoclonal antibody
MAHA	Mouse anti-human antibody
MAIPA	Monoclonal antibody-specific immobilization of platelet antigen
MHC	Major histocompatibility complex
NIPT	Non-invasive prenatal test
NNUPI	Norwegian National Unit for Platelet Immunology
OD	Optical density
PIFT	Platelet immune fluorescence test
PSI	Plexin-semaphorin-integrin
RBC	Red blood cells
REW	Q311R-M428E-N434W
RGD	Arginine-Glycine-Aspartic Acid
SNP	Single-nucleotide polymorphism
SRBC	Sheep red blood cells
WT	Wild-type

Thesis summary

Fetal/neonatal alloimmune thrombocytopenia (FNAIT) is a condition occurring in about 1:1000–2000 pregnancies, and in Whites, alloimmunization to human platelet antigen (HPA)-1a is most common. The maternal alloantibodies traverse the placenta, bind to, and eliminate fetal platelets from the circulation rendering the fetus thrombocytopenic. Clinical outcome ranges from non-symptomatic or mild bleedings to intracranial hemorrhage (ICH). To date, no prevention is available for HPA-1a alloimmunization, and screening programs to identify women at risk have not yet been implemented. Initial proof of concept for prevention of HPA-1a alloimmunization was previously demonstrated in a murine model, paving the way for the polyclonal or monoclonal HPA-1a-specific IgG preparations currently under development as a prophylaxis for FNAIT. As the polyclonal preparation is plasma-derived, it depends on plasma donations from alloimmunized individuals, and thus monoclonal antibodies offer an obvious preferred alternative. In this thesis, a human monoclonal antibody (mAb 26.4) specific for HPA-1a, as well as a panel of mAb 26.4 IgG1 isoforms with modified Fc regions, were tested as prophylactic candidates. They were characterized in vitro and in vivo, showing that the mAb 26.4 wild-type successfully mediated complete prevention of alloimmunization in a murine FNAIT model. Further, a novel isoform (mAb 26.4.REW), with longer plasma half-life and stronger effector functions, efficiently induced antibody-mediated immune suppression in low doses. Alloimmunization of women often occurs in connection with delivery, however a significant share of women is indeed immunized during pregnancy — even in their first pregnancy. In addition to fetal platelets as the source of antigen, placental cells or tissues also express the antigen and may be sources for immunization. It has been reported that maternal samples from FNAIT cases with ICH outcome contain HPA-1a-specific antibodies of anti-αVβ3 specificity, and that these sera disturb endothelial function, and induce endothelial cell apoptosis. In this thesis, samples from referred and confirmed FNAIT cases with ICH outcome in Norway during the last 20 years were examined for reactivity patterns using recombinant integrins. Most samples bound monomeric integrin β3, αVβ3 and αIIbβ3 on beads in a similar manner, in line with previous reports indicating that most FNAIT sera react with the HPA-1a epitope on β3, independent of the α-chain. However, upon absorbing antibodies binding to recombinant αIIbβ3, the remaining fraction was examined using αVβ3-beads and αVβ3-expressing cell lines, without significantly detectable remaining antibodies. This highlights the current challenge in detecting potential antibodies of the anti-αVβ3 subtype. Further characterization of such antibodies and their antigen conformation requirement is needed.

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1 Introduction

Upon encountering foreign antigens, an immune response is induced to eliminate the antigen. However, when these cells and tissues origin from same species individuals with population-based protein variants, alloimmunization can occur. The exposure to alloantigens typically arises in connection with blood transfusions and transplantation, but notably also in the pregnancy setting as the fetus express inherited paternal antigens.

In the settings of transfusion and pregnancy, the formation of antibodies to non-self antigens present in various blood group systems, e.g., ABO, Rh, Kell, Kidd and Duffy, are of clinical importance when undiagnosed and untreated, as they may lead to hemolysis of red blood cells (RBCs) [1]. Human leukocyte antigens (HLA) class I are widely expressed by almost all nucleated cells, and thus the risk of exposure is high in transfusion or pregnancy. Antibodies toward HLA class I may result in clinical complications related to transplantation, as they can induce graft rejection [2], and related to transfusion as they cause refractoriness to transfused platelets [3]. Whether maternal anti-HLA class I antibodies are associated with thrombocytopenia in the newborn is debated [4-6]. Notably, incompatibility to human platelet antigens (HPAs) during pregnancy may induce alloimmunization, causing fetal platelet removal that can lead to severe outcomes [7]. The focus in this thesis is pregnancy-related alloimmunization against HPA-1a and efforts to prevent it.

1.1 Platelets and platelet antigens

Platelets, or thrombocytes, are the smallest circulating cells with a diameter of 2–4 μm when in a resting state [8]. Platelets are abundant in the blood, with a platelet count of $150–450\times10^9$ cells per liter of blood in adults. Because platelets have a short lifespan of 7–10 days, they are continually produced from megakaryocytes in the bone marrow and released into circulation [9]. These discoid anucleate cells have important functions in hemostasis, where they are activated upon vessel damage and bind together to form a plug. Because of blood flow and the cell type composition of the blood, platelets appear with the highest concentration close to the vessel walls [10, 11]. Upon encountering disrupted endothelial walls or exposed extracellular matrix, surface receptors ensure platelet capture at the site of injury, which again triggers platelet activation. When activated, the platelet shape shifts from discoid to spherical, followed by the creation of filopodia and lamellipodia generated by inducing reorganization of the cytoskeleton [12]. In addition, platelets secrete pre-stored components from intracellular

granules — α-granules and dense granules — to communicate with surrounding cells and tissues [13].

Though platelets have been classically regarded as purely hemostatic agents, studies have shown the significance of platelets in aiding the immune system in response to infection and cancer (reviewed in [14-16]). Platelets express toll-like receptors able to recognize pathogen- and damage-associated molecular patterns produced by damaged or compromised cells, and they can interact directly with leukocytes for recruitment to the site of injury. In addition, they express receptors for direct detection of pathogens, and upon activation, they secrete their intracellular granules, which — among other factors — contain antimicrobial peptides to destroy foreign intruders [17].

1.1.1 Integrins

Platelets have various adhesion molecules and receptors on their cell surface — integrins being the most abundantly expressed — which they use to continually monitor the situation in the blood. Integrins are a large family of highly conserved heterodimeric, non-covalently associated glycoproteins important for adhesion between cells, and between cells and the extracellular matrix. However, they also have important roles in immunity, the development of the embryo, in cancer, and hemostasis [18]. These transmembrane receptors consist of two integrin subunits, α- and β-chain, and they have the ability of bi-directional signal transduction, inside-out and outside-in signaling, across the plasma membrane [19, 20]. To date, there are 18 α-subunits and 8 β-subunits described that, in different combinations, arrange to form 24 varied receptors with distinctive properties and functions (reviewed in ref. [19, 21]).

Integrins appear to occur in three conformational states that relate to the regulation of ligand affinity [22] (Figure 1). These rearrangements are important for diverse biological processes such as migration, development, and hemostasis. When in a bent state, integrins have low affinity for ligands because of the proximity of intracellular tails and a folded headpiece. An intermediate state exists where the integrin is extended but the headpiece is still closed, and therefore the integrin has an intermediate affinity for ligands. However, by further activation, there is a swing-out of the hybrid domain, and the lower legs are unclasped, leading to an extended-open conformation with high affinity for ligands.

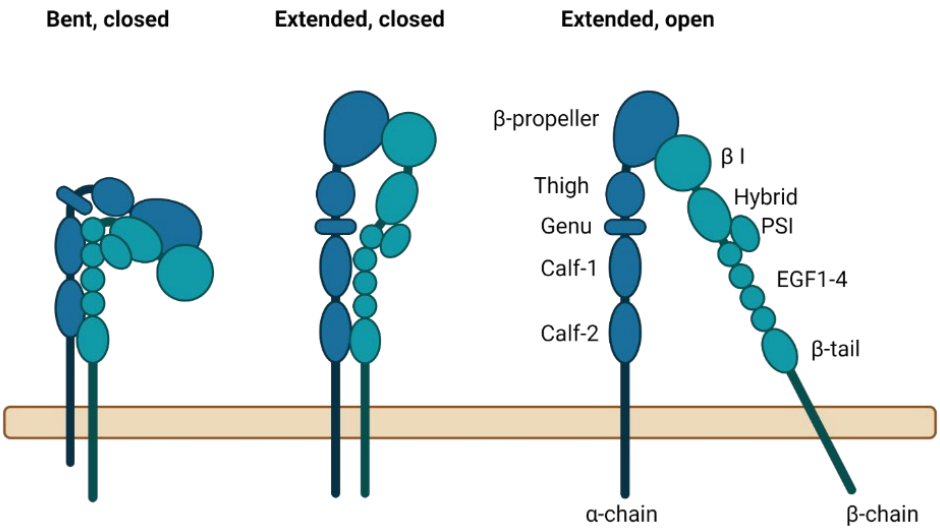

Figure 1: *Schematic illustration of integrin configurations related to ligand affinity. A bent conformation with a closed headpiece relates to inactive integrin, while rearrangements through the intermediate state (extended, closed) lead to an extended conformation with high ligand affinity. Created with BioRender.com.*

Integrin β3

Integrin β3 is a β-subunit that combines only with two closely related α-subunits, αIIb and αV, both of which are present on platelets [23-25]. These integrins are Arg-Gly-Asp (RGD) binding receptors; that is, they recognize ligands containing the RGD peptide motif [19, 24]. The binding site have also been confirmed by the efficient inhibition of ligand binding to platelets by RGD-containing peptides [26].

The integrin αIIbβ3 is a calcium-dependent heterodimer [27] also known as the fibrinogen receptor. However, it also binds to fibronectin, von Willebrand factor [19, 28], vitronectin [29], and multiple other ligands reviewed in ref. [21]. On platelets, αIIbβ3 is the most abundant glycoprotein complex, estimated to express approximately 80,000 molecules per platelet [30]. On resting, circulating cells, αIIbβ3 is present on the platelet surface in an inactive state, but upon platelet activation, it changes its conformation into a high-affinity state to induce platelet aggregation [25, 31]. In addition, upon activation, α-granules containing αIIbβ3 are secreted, increasing the number of receptors on the platelet surface to further support binding to additional platelets [32]. The integrin αIIbβ3 has also been reported to be expressed by mast

cells [33] and by placental trophoblasts early in gestation, suggesting that it is involved in implantation and early placental differentiation [34].

The β3-subunit also combines with αV to form integrin αVβ3. This receptor is defined as the vitronectin receptor, although it has several other ligands, i.e., osteopontin [35], thrombospondin [36], and more reviewed in ref. [21]. In contrast to αIIbβ3, αVβ3 is found only as a few hundred to a few thousand molecules on each platelet [36, 37], but it is also reported to be expressed on endothelial cells [38], on placental trophoblast cells [39], on osteoclasts [40], on fibroblasts [41] and on some cancer cells [42].

1.1.2 Human platelet antigens

Integrin β3 contains numerous single-nucleotide polymorphisms (SNPs) leading to single amino acid substitutions and therefore allovariants in the population. Depending on the position of the SNP, it might impact protein structure, function, and expression. The most common SNPs do not induce functional changes to the platelet proteins and, as such, damaging SNPs are normally removed by natural selection. Different allelic structures might be antigenic, leading to alloimmunization upon exposure by transfusion, transplantation, or pregnancy. Even though many of the glycoproteins are also expressed on other cells, these antigens are characterized as HPAs, consecutively upon documentation of their clinical importance in platelet alloimmunity by the Platelet Immunology Working Party of the ISBT. To date, the variants are divided into 35 HPA systems [43] (Figure 2).

The first HPA was found on integrin β3 [44], and the following HPA are numbered in the order they were officially assigned. Six of the systems — HPA-1, -2, -3, -4, -5, and -15 — are biallelic systems in which alloantibodies have been observed against both forms of the SNPs [43]. In each system, the allelic variant carried by most of the population is designated as “a” and the more infrequent variant designated as “b”. The different HPA systems are spread throughout six different glycoproteins: gpIIIa (β3), gpIIb (αIIb), gpIb (CD42b/c), gpIa (α2), gpIX (CD42a), and CD109. As outlined in Figure 2, most of the HPAs are present on the fibrinogen receptor αIIbβ3 (also defined as gpIIb/gpIIIa and CD41/CD61) [43].

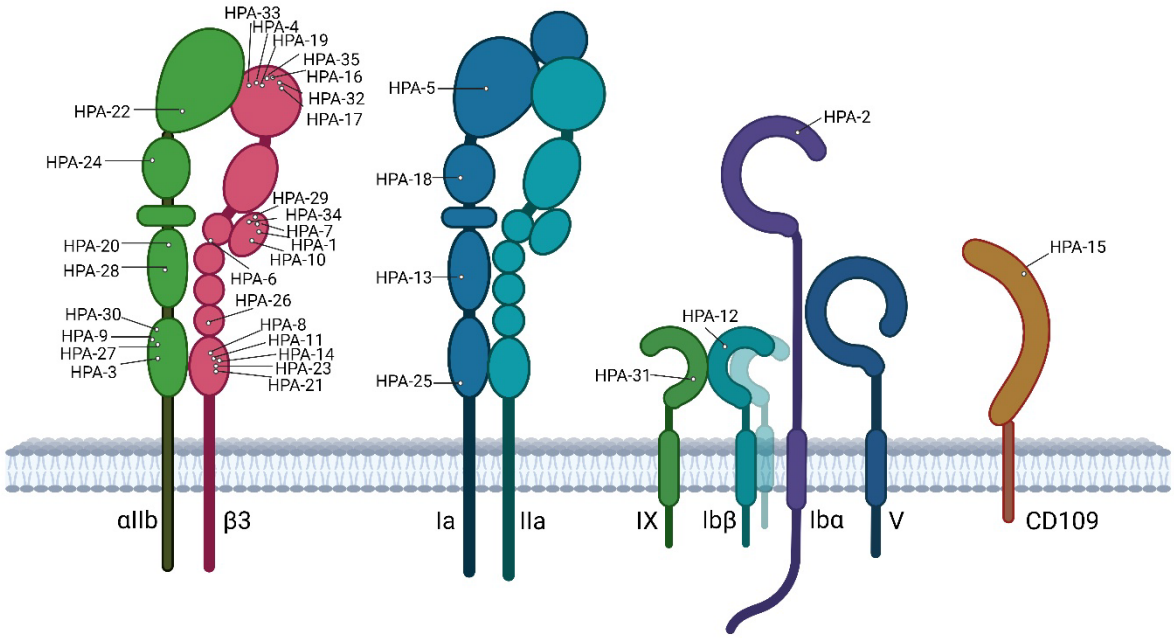

Figure 2: *Localization of HPA antigens on glycoprotein complexes on the surface of platelets. Created with BioRender.com, based on data from the Human Platelet Antigen database [43].*

1.2 Fetal/neonatal alloimmune thrombocytopenia

Maternal alloantibodies specific for paternally inherited HPAs may cause destruction of fetal platelets following transplacental transport during pregnancy. This condition is referred to as fetal/neonatal alloimmune thrombocytopenia (FNAIT) and is the most frequent cause of neonatal thrombocytopenia in otherwise healthy full term neonates [45]. This is a rare condition occurring in approximately 1 to 1000–2000 live births [46-49]. Thrombocytopenia is defined as a platelet count below 150×10^9/L with severe thrombocytopenia below 50×10^9/L. In Whites, the most frequent cause of severe FNAIT is alloimmunization against HPA-1a, accounting for approximately 80% of the cases [50, 51].

1.2.1 HPA-1a alloimmunization

To be at risk of HPA-1a alloimmunization, the mother must be homozygous for the HPA-1b antigen and be exposed to HPA-1a either by transfusion or by carrying a heterozygous (HPA-1ab) fetus. About 2.3% of Whites are homozygous for HPA-1b and therefore at risk of HPA-1a alloimmunization [52] (Figure 3).

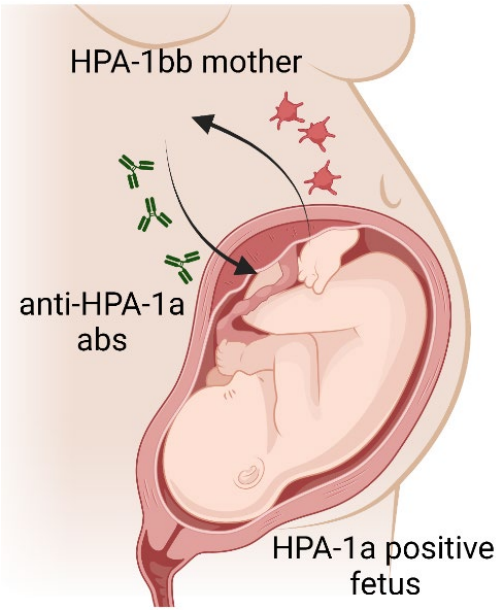

Figure 3: *Illustration of HPA-1a alloimmunization. Created with BioRender.com.*

HPA-1 (also defined as PI^A or Zw) is a polymorphism located on integrin β3 in amino acid position 33, where the majority of the population carries leucine (defined as HPA-1a), and the minority carries proline (defined as HPA-1b) [44]. Alloantibodies toward HPA-1b also occur, although they are less common than HPA-1a alloimmunization [53]. Individuals carrying a valine in this position have also been found, defined as HPA-1c, and some HPA-1a-specific antibodies have shown binding to recombinant β3-Val33 [54].

1.2.2 Other common HPA systems associated with FNAIT

Even though HPA-1a alloimmunization is the most common cause of FNAIT in Whites, other antigen incompatibilities also lead to alloantibody responses. Other common antigen incompatibilities are HPA-5 and -15; however, HPA-2 and -3 have also been observed [55]. Alloantibodies toward HPA-5b antigen present on glycoprotein GPIa have been regarded as the second most common cause, accounting for approximately 15% of FNAIT cases [56]. However, current studies call this into question, because the rate of HPA-5b alloimmunization is far more prevalent than previously reported and therefore might be an incidental finding in at least some FNAIT workups [57]. Still, anti-HPA-5b antibodies have been associated with severe FNAIT [58]. Anti-HPA-15b antibodies have been shown to induce FNAIT in 4% of cases [56], and severe cases have been reported [59]. HPA-15b-specific antibodies behave similarly to HPA-1a-specific antibodies, as they suppress angiogenesis and induce apoptosis in

endothelial cells in vitro [60]. FNAIT cases due to anti-GPIbα antibodies are less common; however, in a mouse model, preimmunized GPIbα$^{-/-}$ females bred with wild-type males led to miscarriage [61]. Because there were few live neonates in this murine model, this finding may indicate that the frequency of GPIbα-mediated FNAIT might also be underreported in humans. For early loss of pregnancy, platelet incompatibility might not even be considered in most cases. FNAIT caused by minor allele frequencies is also reported, although rarely [55, 62, 63].

In the Asian population, HPA-1b is an even lower frequency allelic variant (0.4%) [64]. The most frequent cause of FNAIT in this population is alloantibodies against HPA-4b (24.8%), HPA-5b (13.8%) and HPA-5a (4.3%), which are evidently less severe than in anti-HPA-1a-induced FNAIT [64]. Another cause of FNAIT in the Asian and African populations is immunization against CD36 (gpIV), where protein-deficient individuals produce isoantibodies against CD36 [64-66].

1.2.3 Clinical outcome

Most FNAIT cases are suspected only after the delivery of a newborn presenting with bleeding or thrombocytopenia, or because of FNAIT history in the immediate family. To date, there are no national screening programs to identify women at risk of HPA-1a alloimmunization.

Transplacental transport of platelet alloantibodies, the binding to and depletion of fetal platelets, renders the fetus or neonate at risk of thrombocytopenia, prone to bleeding, and in severe cases to intracranial hemorrhage (ICH) followed by lifelong disabilities or stillbirth [48, 56, 67]. Only 10% (7.9–12%) of HPA-1a-negative women generate anti-HPA-1a antibodies when exposed to an incompatible fetus; of these women, only 30% give birth to a severely thrombocytopenic neonate [48, 49, 67]. About 10–26% of severely thrombocytopenic neonates presents with ICH [47, 51, 68, 69]. Furthermore, the risk of severe FNAIT is increased when a sibling present with ICH [70]. A study showed that 54% of ICH cases occurred before 28 weeks of gestation and often in the first pregnancy [71].

Children with FNAIT-related ICH have a very high risk of perinatal death or long-term neurodevelopmental impairment [72]. The clinical effect on the fetus exerted by HPA-1a-specific antibodies is heterogenous as many are born healthy, while some present with skin manifestations such as petechiae, purpura or other bleeding complications. Although likely underreported, fetal internal organ hemorrhage other than ICH may also lead to severe

complications and deadly outcome [73]. A retrospective study reported a significant association between anti-HPA-1a antibody levels in the mother and reduced birthweight in newborn males [74]. In addition, a study conducted using data from the No IntraCranial Haemorrhage (NOICH) registry, reported that 65% of ICH cases were in male fetuses/neonates [71]. Together, these studies imply that neonatal gender might influence alloimmunization and potentially disease severity.

1.2.4 FNAIT-related associations and risk factors

Most HPA-1a-negative women are not alloimmunized, even though they carry an HPA-1ab fetus. In addition, not all fetuses or newborns of HPA-1a alloimmunized mothers present with FNAIT. This indicates that other unknown factors are important for alloimmunization, and for the pathophysiology of FNAIT.

Several studies have shown a strong association between HPA-1a alloimmunization and the expression of HLA-DRB3*01:01 (also defined as DR52a and DRw52) [75-78]. In Whites, approximately 28% carry the additional DRB variant DRB3*01:01 while approximately 90% of HPA-1a alloimmunized women carry this class II variant [67, 75-77]. Peptides with leucine in position 33 (Leu33, HPA-1a) but not peptides with proline (Pro33, HPA-1b), have been shown to bind stably to HLA-DRB3*01:01 [79, 80]. Leu33 has been shown to act as an anchor in the major histocompatibility complex (MHC) peptide-binding groove together with Trp25 and Asp28, generating a 1-4-9 motif of the peptide anchor residues [79, 81].

Laboratory investigations of HPA-1a-specific antibodies

Maternal anti-HPA-1a antibody levels during pregnancy are used in some countries as a risk stratification for FNAIT. A prospective study in Norway showed that levels of maternal HPA-1a alloantibodies above 3 international units (IU)/ml in week 22 and/or 34 were associated with the degree of thrombocytopenia in the newborn; antibody levels also correlated with neonatal platelet count [82]. The concentration and specificity of FNAIT-related antibodies are often measured by more than one method, including platelet glycoproteins with defined HPA composition.

A method for evaluating platelet-specific antibodies is by flow cytometry by indirect platelet immune fluorescence test (PIFT) where the maternal sample is cross-matched to a panel of platelets with known HPA status [83]. If paternal or neonatal platelets are available, they are

used as donor platelets, but this also requires a direct PIFT to disregard potentially prebound antibodies to the platelets.

Glycoprotein-specific antibody tests using intact platelet antigens, such as the monoclonal antibody-specific immobilization of platelet antigen (MAIPA) assay, is so far recognized as the gold standard method. Briefly, MAIPA is performed by incubating patient plasma with HPA-defined donor platelets prior to glycoprotein binding by murine monoclonal antibodies [84]. The platelets are solubilized, and glycoproteins are captured in a microplate by goat anti-mouse IgG. The glycoprotein-bound human antibodies are detected by enzyme-coupled anti-human IgG and its corresponding substrate and stop solution, measured as optical density (OD) by a microplate reader. The advantage of this method is that antibodies toward the different glycoproteins (gpIIb/IIIa, gpIb/IX, gpIa/IIa, gpV and CD109) are measured separately by using typed and selected donor platelets. In-house MAIPA assays are implemented in most reference laboratories that have donor platelets available; however, a commercial kit with typed platelets included is also available (apDia, Turnhout, Belgium).

For anti-HPA-1a IgG measurements, a quantitative MAIPA is performed by calibrating the patient sample to a defined standard (the international NIBSC standard; 03/152 anti-HPA-1a [85, 86]) and with read-out in IU [87, 88]. MAIPA may also be performed with paternal or neonatal platelets as an antigen source for direct detection of antibodies against fetal antigens. A downside to this assay is that some MAIPA-negative cases showed low-avidity HPA-1a-specific alloantibodies [89], which might be tentatively missed, because of extensive washing procedures. Notably, a new MAIPA using streptavidin-coated beads and biotinylated monoclonal antibodies for glycoprotein capture has shown enhanced sensitivity compared to the standard MAIPA protocol [90].

HPA-specific IgG detection is also performed using a commercial test — Pak Lx kit (Immucor, Georgia, USA) — which uses beads coupled with platelet-derived glycoproteins from a pool of donors to evaluate HPA specificity.

1.2.5 Treatment strategies

To date, there is no effective treatment for FNAIT available; however, several clinical guidelines recommend the off-label use of intravenous immunoglobulin (IVIg) to the pregnant women [91]. Typically, a previous sibling diagnosed with severe FNAIT leads to the treatment

of a pregnant HPA-1a alloimmunized woman with IVIg weekly from as early as 12 weeks of gestation to prevent FNAIT and ICH in the subsequent neonate. This treatment is costly: normal dosing is 1 g/kg/week [92]. IVIg treatment may have side effects such as headache and influenza-like symptoms and, with a weekly dosing frequency, the strain of this treatment strategy is high. Even though IVIg has not been tested in a placebo-controlled clinical trial, recommendations indicate that such a treatment is currently the best approach available because it has been used successfully for decades. It seems to be consensus for this treatment in a pregnancy where the previous child had FNAIT with ICH. Even though IVIg brings unwanted side effects to the mother, it is the first-line management, with or without corticosteroids [93-95].

A study investigating all FNAIT-related ICH cases from the NOICH registry from 2001 to 2010 reported reduced ICH risk from 79% to 11% in the group treated with IVIg in the subsequent pregnancy [71]. This was confirmed by another study that reported prevention of bleeding in the fetus or neonate in almost all cases when IVIg was used [96]. Although for low-risk pregnancies — defined as no previous sibling with FNAIT-related ICH — IVIg has not yet demonstrated significant beneficial effects [97]. However, in a future screening regimen, the implementation of IVIg as a treatment to low-risk pregnancies might cause overtreatment of many women.

In Norway, women with anti-HPA-1a levels ≥3 IU/mL are recommended for cesarean delivery around gestational week 38–39 [98]. According to the 2014 Norwegian National Clinical Guidelines for Obstetricians, the delivery should be performed at a hospital with a neonatal intensive care unit. At delivery, HPA-compatible platelets for transfusion to the newborn should be available if the platelet count is less than 35×10^9/L to prevent further bleeding. If compatible platelet concentrations are not available, random platelets should be used [91]. It is also recommended to perform ultrasound scan for ICH when the neonatal platelet count is less than 50×10^9/L [98].

Fetal blood sampling guided by ultrasound and weekly intrauterine platelet transfusions were procedures historically used for antenatal diagnosis and treatment, although these methods are no longer recommended because of the high risk of procedure-related fetal loss [91] — shown to be up to 8.3% per affected pregnancy [99]. These procedures also induce the risk of fetomaternal hemorrhage, leading to further sensitization of the mother [99].

Potential future therapeutic approaches

The expression of the neonatal Fc receptor (FcRn) in placental tissue increases throughout gestation and correlates with the transfer of IgG from the mother to the fetus [100]. Therefore, a treatment of interest is to block the FcRn molecule responsible for the transport of pathogenic antibodies. Inhibition of FcRn as a potential FNAIT treatment was demonstrated by Chen and colleagues in 2010 by treating pregnant, preimmunized mice by administering the anti-FcRn antibody (1G3). Improved platelet counts were demonstrated in pups compared to pups delivered from non-treated mice [101].

Clinical trials have demonstrated the safety and efficiency of blocking FcRn in autoimmune disorders and, as such, FcRn inhibition may be an alternative therapeutic approach for FNAIT [102]. M281 is a human anti-FcRn antibody [103] in the pipeline as a treatment for hemolytic disease of the fetus and newborn (HDFN) [104]. Furthermore, other FcRn inhibitors are also undergoing clinical trials for autoimmune diseases [105].

1.3 FNAIT-associated antibodies: structure and function

Antibodies are central to the defense against pathogens, but in auto- and allo-immunity, they may cause harm. Antibodies are potent and function through multiple biological systems. The performance of an antibody depends on the features of its Fab (fragment antigen binding) region, such as epitope specificity and antigen binding affinity, and the features of its Fc (fragment crystallizable) region, such as isotype, subclass, allotype, and glycosylation.

IgG antibodies consist of two identical heavy chains containing a variable domain followed by three constant domains (CH1-3), with a flexible hinge region between the two first constant domains, as well as two identical light chains containing a variable domain and a constant domain [106]. These chains are held together by interchain disulfide bonds; the CH2 and CH3 domains constitute the Fc region, while the light chain combined with the upper heavy chain constitute one Fab region.

In humans, IgG is the most abundant isotype, further divided into IgG1, IgG2, IgG3, and IgG4 subclasses. Depending on the nature of the antigen encountered, the immune system ensures class switching of the appropriate IgG subclass to induce targeted and improved defense [106]. Because most antibody-mediated effector functions are facilitated by binding of the antibody Fc region to IgG Fc-receptors (FcγRs) and/or complement, subclass variety in the constant

region and hinge region leads to the exertion of different effector functions. IgG1 and IgG3 are potent in their induction of effector functions through antibody-dependent mechanisms such as antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC) [107]. Although it varies between allotypes, IgG3 has a remarkably long hinge region — leading to increased flexibility of the antibody that corresponds to its augmented binding to effector molecules (Figure 4), which might be partially because of less blocking of the binding sites by the Fab arms [106, 108].

IgG antibody subclass compositions have also been investigated in FNAIT. No difference in isotype composition in mothers with or without FNAIT-affected newborns was reported by von dem Borne and colleagues in samples from HPA-1a alloimmunized individuals [109]. Proulx and colleagues later confirmed this finding through the analysis of 43 anti-HPA-1a sera [110]. In both studies, anti-HPA-1a IgG2 was not found, and IgG4 was rare. In addition, Mawas and colleagues reported higher levels of HPA-1a-specific IgG3 in mothers giving birth to severely thrombocytopenic newborns compared to less severe outcomes [111].

1.3.1 Antibody Fc glycosylation and its impact on effector functions

Located in conserved regions in the Fc region of IgG, there are N-linked glycans in position 297 (Figure 4). These biantennary glycans are positioned between the heavy chains in the region of FcγR and C1q binding [112] and affect the binding affinity of the receptors; they also impact the stability of the antibody [113]. However, it has been shown that they influence the conformation of the local FcγRIIIa binding site rather than the quaternary global structure of Fc regions [114].

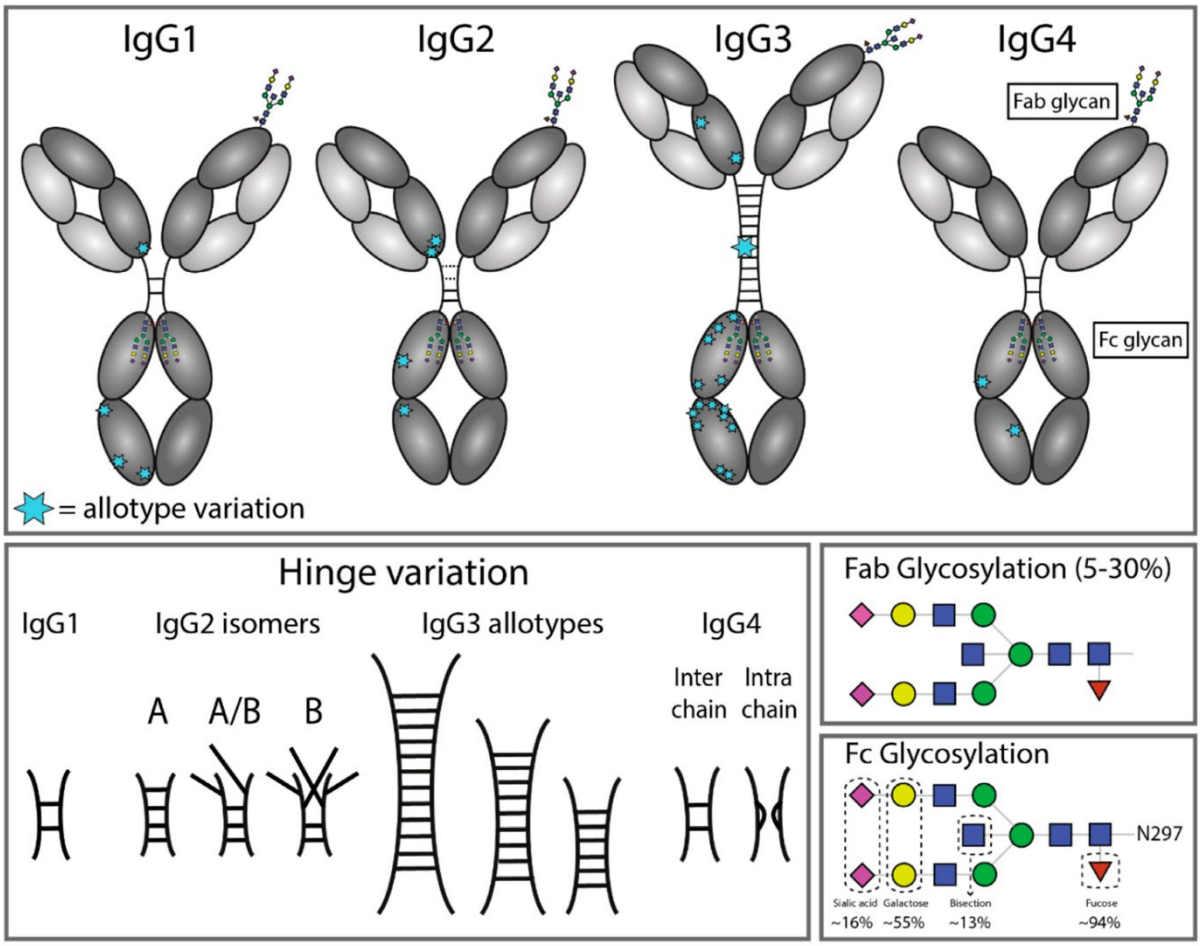

Figure 4: *Illustration of IgG subtypes, hinge variations, and glycan composition. From ref. [107] used under the Creative Commons CC-BY license.*

IgG Asp297-glycan consists of a core built up of N-acetylglucosamine (GlcNAc) and mannose (Figure 4). In addition, the glycan can exist with one or two galactose residues, one or two sialic acid residues, a core fucose, and a bisecting GlcNAc [115]. This generates the possibility of 36 unique glycans [116], of which over 30 have been demonstrated using mass spectrometry [117]. Total IgG in human serum contains glycans of which 94% comprise core fucose, 13% bisected GlcNAc, 55% galactose, and 16% sialic acid [107].

The glycan composition of IgG is formed in the golgi of each specific B cell, and because there are two N-glycans in the Fc region, the glycans can vary in composition on the same IgG. The glycan profile varies between individuals depending on age and sex [117, 118], and interestingly, increased levels of galactose and sialic acid have been seen during pregnancy [119, 120]. There has also been a difference in the profile between mothers and their newborns [121].

Glycosylation patterns in FNAIT are highly skewed toward reduced fucose levels

Reduced IgG-core fucosylation at levels down to 10% of anti-HPA-1a IgG1 has been reported for FNAIT cases, in contrast to normal levels of fucose found in total serum IgG1 [122, 123]. Interestingly, such reduced level of fucose was not found in anti-HLA antibodies from refractory thrombocytopenia cases — not even when detected in parallel with anti-HPA-1a in the same sample [123]. Therefore, this reduced fucosylation is not a pregnancy-related phenomenon but rather specific to the HPA-1a-specific alloantibodies. Reduced fucose levels also demonstrated a significant correlation between low neonatal platelet counts and more severe disease. Testing of core fucose levels might be a future diagnostic tool if feasible testing methods are in place for disease severity since samples connected to asymptomatic FNAIT patients had normal fucose levels. This trend was also reported in samples taken years after delivery [123]. The same trend was reported for anti-D antibodies, where core fucose levels correlated with HDFN disease severity [124]. Reduced core-fucosylation has later been reported to remain stable during pregnancy and in subsequent pregnancies [125]. Galactose levels of HPA-1a antibodies were also elevated in this study and remained high after delivery. Given that males hyperimmunized to RhD also showed decreased fucose levels in anti-D antibodies [126], this further confirms that skewed fucose levels are not caused by pregnancies itself but rather factors influencing alloimmunization.

1.3.2 HPA-specific antibodies interact with IgG receptors

FcRn and its role in antibody half-life and fetal transfer

To destroy fetal platelets and induce thrombocytopenia in the fetus, pathogenic HPA-1a-specific IgG depends on transportation into fetal circulation by the FcRn [127-129]. The FcRn receptor is a homolog to MHC class I molecules containing three extracellular domains (α1, α2, and α3) and is expressed together with β2-microglobulin, which it pairs with non-covalently [130]. Compared to classical MHC class I molecules that present peptides in a groove between the two first domains on top — α1 and α2 — FcRn does not have a groove in this position, but binds IgG and albumin on individual sites on the opposing side of the α1 and α2 domains [131]. FcRn binds IgG in a 2:1 manner, made possible by the presence of two CH2 domains — one on each heavy chain in the Fc region of IgG — and is notably not affected by Fc glycosylation [132].

Human FcRn is expressed by endothelial cells and many myeloid cells, where it serves to rescue monomeric IgG [133-135] and albumin [136] from degradation. Epithelial cells have been shown to be key for the transcytosis of monomeric IgG or IgG-bound immune complexes by FcRn from the apical side to the basolateral side of the cells [137, 138]. In addition, for antigen-presenting cells such as monocytes, macrophages, and dendritic cells, FcRn has also been shown to be crucial in the cross-presentation of IgG-bound immune complexes [139]. FcRn is also expressed by placental syncytiotrophoblasts, where it facilitates the transmission of IgG into the stroma [140] (Figure 5). No FNAIT symptoms were found in mouse pups from $\beta3^{-/-}$ $FcRn^{-/-}$ females when immunized with $\beta3^{+/+}$ $FcRn^{-/-}$ platelets, confirming the requirement of FcRn in the transplacental transport of harmful antibodies [101]. In addition, by using mice with different knockout combinations, it was demonstrated that only fetal, not maternal, FcRn is involved.

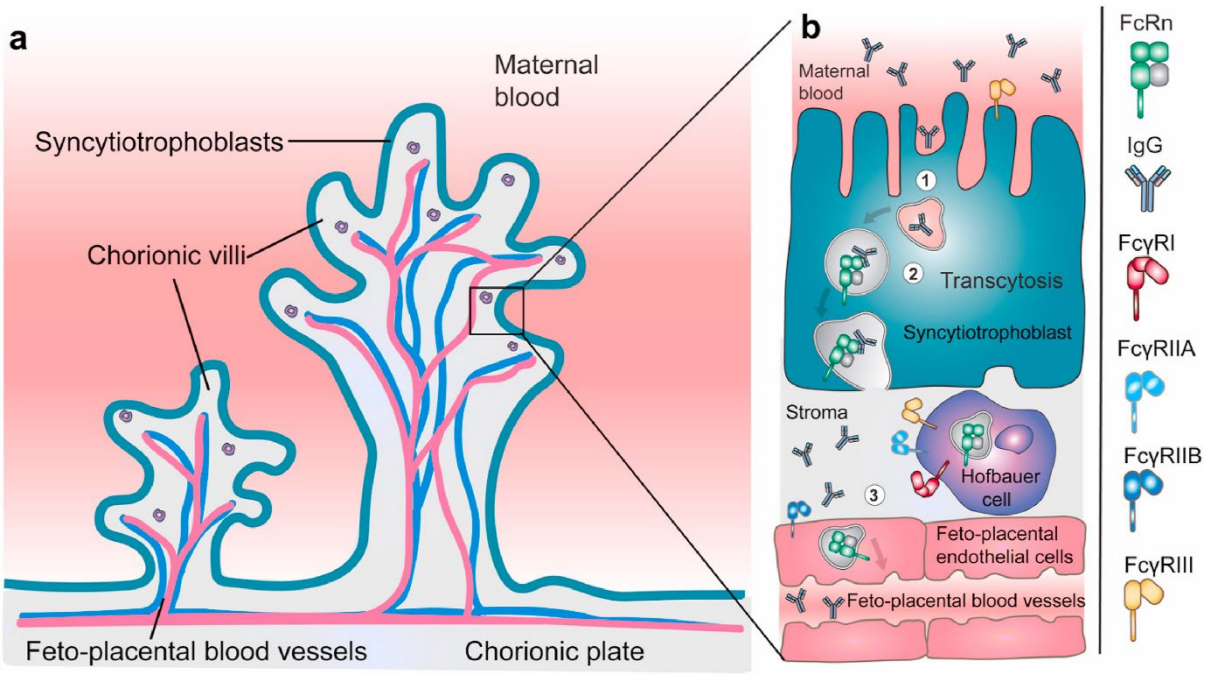

Figure 5: *Transplacental transport of IgG. From ref. [141], used under the Creative Commons CC-BY license.*

FcRn is present in the endosomal system and shows strict pH-dependent binding to IgG and albumin. There is no binding of ligands to FcRn at neutral pH; however, with the reduced pH (5–6) in endosomes, binding is facilitated. When bound, IgG is recycled back to the cell surface, where the pH increases, leading to antibody release. This is mostly because of the protonation of histidine residues on H310 and H435 in the Fc region of IgG at low pH [142]. Of the IgG

subclasses, IgG3 demonstrates a reduced plasma half-life (approximately 1 week) compared to the other subclasses (approximately 3 weeks). This was found to be because of an R435H, influencing FcRn binding and consequently antibody recycling [143] and transport of IgG into fetal circulation [144]. Indeed, an H435 allotype of IgG3 showed a comparable half-life to IgG1 [143]. Interestingly, this H435 IgG demonstrated enhanced effector functions in vivo by providing significantly better protection against pneumonia compared to IgG3 and IgG1 in mice.

In addition to Fc region variations, the antibody variable domains have also been shown to affect binding to FcRn through charge patches, causing modulation of FcRn binding and transport properties [145, 146]. FcRn may also be saturated and, as such, influence the level of IgG that is transferred to the fetus. The level of IgG in the fetus is between 5–10% at 17–22 weeks of gestation, although at term the levels even exceed maternal levels [147].

IgG accomplish effector functions through binding to FcγRs

Following the transport of maternal HPA-specific IgG alloantibodies into fetal circulation, they exert their functions through numerous mechanisms mediated by interactions with IgG receptors. There are multiple FcγR in humans: FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16) where I and II exist as three different receptors (FcγRIa, FcγRIb, and FcγRIc [148], and FcγRIIa, FcγRIIb, and FcγRIIc [149], respectively) and III as two receptors (FcγRIIIa and FcγRIIIb [150]). For FcγRI, only the "a" gene corresponds to the high-affinity receptor [151] and is the one discussed further. IgG1 and IgG3 are generally bound by all FcγRs [152]. Some of the receptors have specific polymorphisms, such as FcγRIIa (H131R) and FcγRIIIa (V158F), that might influence binding affinity and response to therapeutic antibodies [153, 154]. All FcγR binds IgG in a 1:1 pattern in the lower hinge and CH2 domain, but is also affected by the glycan in position 297 [112].

FcγRs have different immune cell expression patterns; FcγRI are typically expressed on monocytes, macrophages, dendritic cells, neutrophils, and mast cells [152, 155]; FcγRIIa on many myeloid cells, including monocytes, platelets, and neutrophils [154]; FcγRIIIa on NK cells, macrophages, and monocytes; and FcγRIIIb on granulocytes [155]. FcγRIIb is the only inhibitory receptor [156] and is typically expressed on B cells and macrophages [157].

Reduced core fucosylation in the N297-glycan of IgG results in increased binding affinity to FcγRIII by promoting carbohydrate-carbohydrate interactions between the Fc region glycans and FcγRIII glycans, improving binding and ADCC [158-161]. Complement activation is substantially increased by IgG having N297-glycans with high levels of galactose through increased binding to complement protein C1q [161]. This binding is induced by the hexamerization of IgG1, which increases binding to C1q (C1q:IgG binds 1:6) [162].

Potential antibody-dependent mechanisms for destruction of fetal platelets

Upon alloimmunization, the developed HPA-1a-specific antibodies differ in specificity, affinity, subclass, and glycosylation levels that influence their ability to induce effector functions. Potential antibody-dependent mechanisms leading to the depletion of fetal platelets by maternal HPA-1a-specific antibodies are presented in Figure 6.

When HPA-1a-specific alloantibodies bind to fetal platelets, one potential mechanism is the neutralization of platelet function without lowering the platelet count. A report showed that 9% of sera containing HPA-1a alloantibodies had the ability to bind HPA-1a on transfected CHO cells and block binding to immobilized fibrinogen [163]. Notably, sera containing antibodies with inhibitory properties were drawn from mothers of neonates that presented with severe bleeding.

IgG-opsonized platelets are removed from the fetal circulation by macrophages in the spleen and liver through ADCP [16]. Decreased fucosylation of HPA-1a-specific antibodies has been shown to enhance monocyte and neutrophil-mediated phagocytosis of HPA-1a-positive platelets because of increased affinity of IgG to FcγRIII [123]. Platelets may also be destroyed through ADCC performed by NK cells.

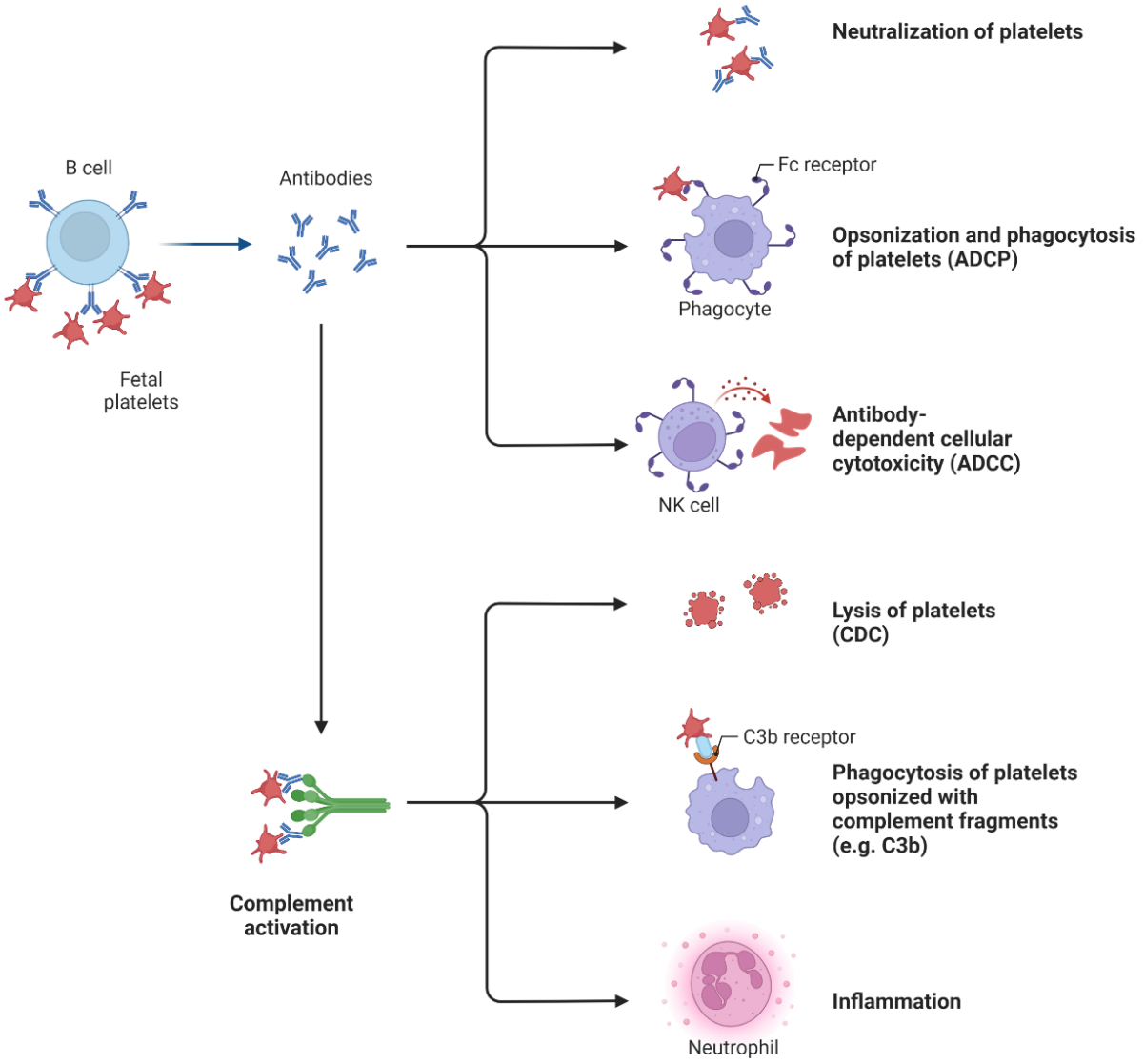

Figure 6: *FNAIT-related antibody effect on platelets. Created with BioRender.com*

The classical pathway of complement activation involves the binding of complement proteins to antibody-opsonized particles. This complement cascade involves the binding of C1q protein to antibody Fc regions, where the antigen-bound antibodies form a hexamer through Fc:Fc interaction, which again increase affinity for C1q [164]. At the end of the complement cascade, a membrane attack complex is deposited on the surface, leading to the lysis of the particle. As part of the complement cascade, membrane-bound C3b and C3d interact with complement receptors on various immune cells [165]. C3b interacts with complement receptor 1 on phagocytes, leading to complement receptor-mediated phagocytosis [166], while C3d leads to enhanced B cell functions through complement receptor 2 and, as such, links the innate and adaptive immune systems [167]. Contrary to this, van Osch and colleagues reported that neither

anti-HPA-1a nor anti-HLA antibodies induced complement deposition on platelets in their native form [168]. However, upon an increase of the galactose and sialic acid levels of these antibodies, complement deposition was demonstrated. In summary, complement activation might not be the main effector function for FNAIT-related thrombocytopenia.

1.4 Prevention of FNAIT alloimmunization by prophylactic treatment

HDFN may be regarded as the blood cell analogue to FNAIT and is today efficiently prevented by antibody prophylaxis that hinders the immunization against RhD. Routine postpartum administration of anti-D prophylaxis to RhD-negative women reduced the incidence of alloimmunization to 2%, and by the recent implementation of antenatal administration, the immunization rates were down to 0.17–0.28% [169]. This intervention has reduced the incidence to the degree that HDFN is considered a rare condition today, although only in the parts of the world where intervention is implemented [170].

A similar prophylactic approach has not been considered pertinent for FNAIT, because the prevailing view was that the immunization happens during gestation and not in connection with delivery. On the contrary, it was demonstrated that FNAIT resembles HDFN more than previously believed, as studies reported that a significant share of women at risk were alloimmunized in connection with or after delivery [48, 49, 82]. Consistent with these data, HPA-1a immunization may also be prevented in a similar manner by prophylactic anti-HPA-1a antibodies. Despite this, some women are immunized already during the first incompatible pregnancy and deliver severely affected neonates [171]. Evidently, both antenatal and postnatal prophylactic administrations should optimally be implemented to ensure a minimum number of alloimmunizations.

1.4.1 The mechanisms of prophylactic antibodies: AMIS

To implement a prophylactic regime, the effector functions of an antibody prophylaxis require careful evaluation, especially if implemented antenatally to avoid harm to the fetus. The knowledge that passively transfused antibodies may induce antibody-mediated immune suppression (AMIS) toward the corresponding antigen is mostly derived from RhD research on the use of anti-D prophylaxis. The main theories revolve around clearance of the cell or cell-derived particle from the circulation and inhibition of B cells through FcγRIIb, and although less likely, other mechanisms such as epitope masking and epitope modulation have been

proposed. The potential mechanisms have been reviewed previously [172-175], but they should be highlighted in our context.

Anti-D-coated RBCs are rapidly cleared from the circulation by phagocytosis, lysis, or adherence to macrophages in the spleen of D-negative individuals [176]. Since macrophages are poor antigen-presenting cells, they might not be able to induce a primary immune response. Therefore, by antigen deviation, the RBCs are cleared without mounting an immune response [174]. Interestingly, it has been shown that for larger fetomaternal hemorrhages in which the majority of cells are cleared 6 days post-delivery, anti-D prophylaxis induces effective AMIS even though there are remaining cells in the circulation [177]. This indicates that clearance is not the sole mechanism in effect and that suppression may be a vital part of the anti-D prophylactic effect. Another potential mechanism of RBC clearance is the activation of complement, leading to CDC or complement receptor-mediated phagocytosis (CMP). However, anti-D IgG does not activate complement [178]. The ability of IgG to induce AMIS have also been demonstrated to be independent of complement [179].

Since effective AMIS was induced by anti-D even when fetal cells were remaining in the maternal circulation, the effect is likely caused by inhibition of B cells by downregulating activation, proliferation, and antibody production. The FcγRIIb — being the only inhibitory FcγR — is present on the B cell surface and is believed to be crucial for AMIS. Immune suppression occurs by the binding of FcγRIIb on the B cell surface to the Fc region of an antigen-bound IgG and the simultaneous binding of the B cell receptor to an antigen on the same surface. This effect occurs only with immature B cells as suppression is not effective if the immune response is already established [175]. By downregulating the antigen-specific B cells, the remaining circulating antigen might not mediate antibody development.

AMIS has also been shown to function independently of epitope-specificity when it is present on the same particle i.e., Kell and RhD blood group antigens. This was demonstrated in D-negative K-negative individuals transfused with D-positive K-positive RBCs and simultaneously administered anti-K IgG, inducing rapid clearance of cells in the spleen [180]. Only 1 of 31 individuals developed anti-D, compared to 11 of 31 in the control group not subjected to anti-K IgG. This fits with the inhibitory theory as the B cell could bind antibodies bound to one antigen through its FcγRIIb while the B cell receptor binds another antigen at the same particle.

Another proposed mechanism is antigen modulation, demonstrated in mice by Maier and colleagues [181]. This response showed to be antigen-specific because modulation was detected for either KEL (k) antigen or for hen egg lysozyme-ovalbumin-Duffy (HOD) (when present simultaneously on murine RBCs) only when injected with anti-KEL or anti-HOD antibodies, respectively. In addition, T cells specific for sheep red blood cells (SRBC), even when opsonized with anti-SRBC, were found in AMIS-responsive mice [182, 183].

Notably, AMIS has been demonstrated in FcγRIIb-deficient mice, indicating that this inhibitory mechanism is not necessary for the induction of efficient AMIS [184, 185]. These studies also showed preventive effects of prophylaxis when using FcγR-deficient mice, eliminating both the clearance and inhibition theories. Notably, AMIS was also induced using $F(ab')_2$ fragments, implying that epitope masking was the effective mechanism in these murine studies. The theory of epitope masking — where anti-D antibodies block the antigen for binding to the B cell receptor — is not a likely mechanism of action in humans because AMIS occurs efficiently with only 20% antigen coverage by anti-D antibodies [172, 186]. Particularly, this theory is also unlikely as anti-K prophylaxis could prevent the development of anti-D antibodies in the study mentioned above [180].

1.4.2 AMIS was efficiently induced in a murine FNAIT model

Since the effector functions of an antibody prophylaxis are not entirely defined, testing new treatments directly on pregnant women does not come without safety concerns. Thus, a suitable mouse model to understand the pathogenesis and assess the potential of various drugs in FNAIT — treatments or prophylaxes — is highly desirable, and when choosing a murine model for FNAIT research, there is much to consider. Since the HPA-1 polymorphism is absent in murine integrin β3 [187], adaptions must be made to mimic the human system for HPA-1a alloimmunization. The first FNAIT mouse model was presented in 2006 by Ni and colleagues [188]. They introduced the use of integrin β3-deficient ($\beta3^{-/-}$) mice that, when challenged with platelets from wild-type mice, generated anti-β3 antibodies. Subsequent breeding of preimmunized females with wild-type males caused FNAIT in the corresponding pups. The pups presented with thrombocytopenia, ICH, and fetal death.

Using the β3-deficient mice, Tiller and colleagues demonstrated the successful use of antibodies as a prophylactic treatment [189]. Integrin β3-deficient mice were immunized by injection of wild-type platelets while AMIS was induced by pre-injection of anti-β3 sera. Crucially, they

also demonstrated AMIS by administering human anti-HPA-1a antibodies or the murine HPA-1a-specific monoclonal antibody (mAb) SZ21 prior to challenge with human HPA-1a^{+} platelets, thus showing the potential for prophylactic intervention to prevent FNAIT.

1.4.3 A monoclonal prophylactic antibody candidate: mAb 26.4

Based on these promising results demonstrating proof of concept for prophylactic treatment of FNAIT, the EU-funded PROFNAIT project collected plasma with anti-HPA-1a antibodies from immunized females for use as a prophylactic drug. Today, this drug (RLYB211, previously referred to as NAITgam in PROFNAIT) is tested in clinical trials as the first preparation to reduce the incidence of HPA-1a alloimmunization [190]. However, such a prophylactic approach would, in the long term, eliminate available plasma products; thus, monoclonal antibodies are attractive alternatives. Other advantages of shifting from polyclonal to monoclonal prophylaxis are that production is unlimited and that produced batches are more homogenous and safer than plasma collected from a number of individuals.

In 2015, a human HPA-1a-specific mAb, referred to as 26.4, was reported by Eksteen and colleagues — it was isolated from an HPA-1a alloimmunized woman that delivered a neonate with FNAIT [191]. From isolated immune cells, memory B cells were sorted and immortalized using Epstein-Barr virus, followed by the selection of HPA-1a-specific cells and the generation of hybridomas. Notably, by pre-incubating HPA-1aa platelets with mAb 26.4, binding of polyclonal HPA-1a-specific sera were inhibited, showing its potential as a therapeutic drug. Due to its human origin and its ability to bind both αIIbβ3 and αVβ3, this monoclonal is also of interest as a candidate for prophylactic treatment of FNAIT.

1.4.4 The generation of a new murine FNAIT model: APLDQ mice expressing HPA-1a

The previous FNAIT models were able to mimic the pathogenesis of the disease, but a more translational model allowing the testing of human HPA-1a-specific antibodies as therapeutic or prophylactic drugs is more ideal.

Reintroducing the HPA-1-defining Leucine 33 in the murine integrin β3 was insufficient for recreating the HPA-1a epitope, while additional humanization of the remaining amino acid residues in close proximity in the plexin-semaphorin-integrin (PSI) domain, differing between humans and mice (residues 30, 32 and 39, Figure 7), allowed efficient binding of mAb SZ21 to murine platelets [187]. To further understand the polyclonal immune response to HPA-1a,

Newman and colleagues generated two CRISPR/Cas modified transgenic mice strains with humanized integrin β3: the first harbor four amino acid substitutions in the PSI domain (C57BL/6N-APLD: T30A, S32P, Q33L, and N39D) while the second also harbors an additional substitution in the epidermal growth factor 1 (EGF1) domain (C57BL/6N-APLDQ: T30A, S32P, Q33L, N39D, and M470Q) [192]. mAb 26.4 was unreactive to platelets from APLD mice; however, efficient binding was achieved with platelets from APLDQ mice. Interestingly, another monoclonal HPA-1a-specific antibody, mAb B2G1, required even further substitutions in neighboring domain, and tentatively, other human monoclonals generated may require additional substitutions. Notably, the integrin β3 humanization will be systemic in the humanized APLDQ mice and, as such, all cells and tissues expressing integrin β3 should comprise the HPA-1a epitope required for 26.4 binding in these mice.

Because mAb 26.4 was non-reactive to wild-type murine platelets but efficiently bound APLDQ platelets expressing an HPA-1a-like epitope, the APLDQ mice (Figure 7) was the preferred model for our prophylactic studies presented further in this thesis.

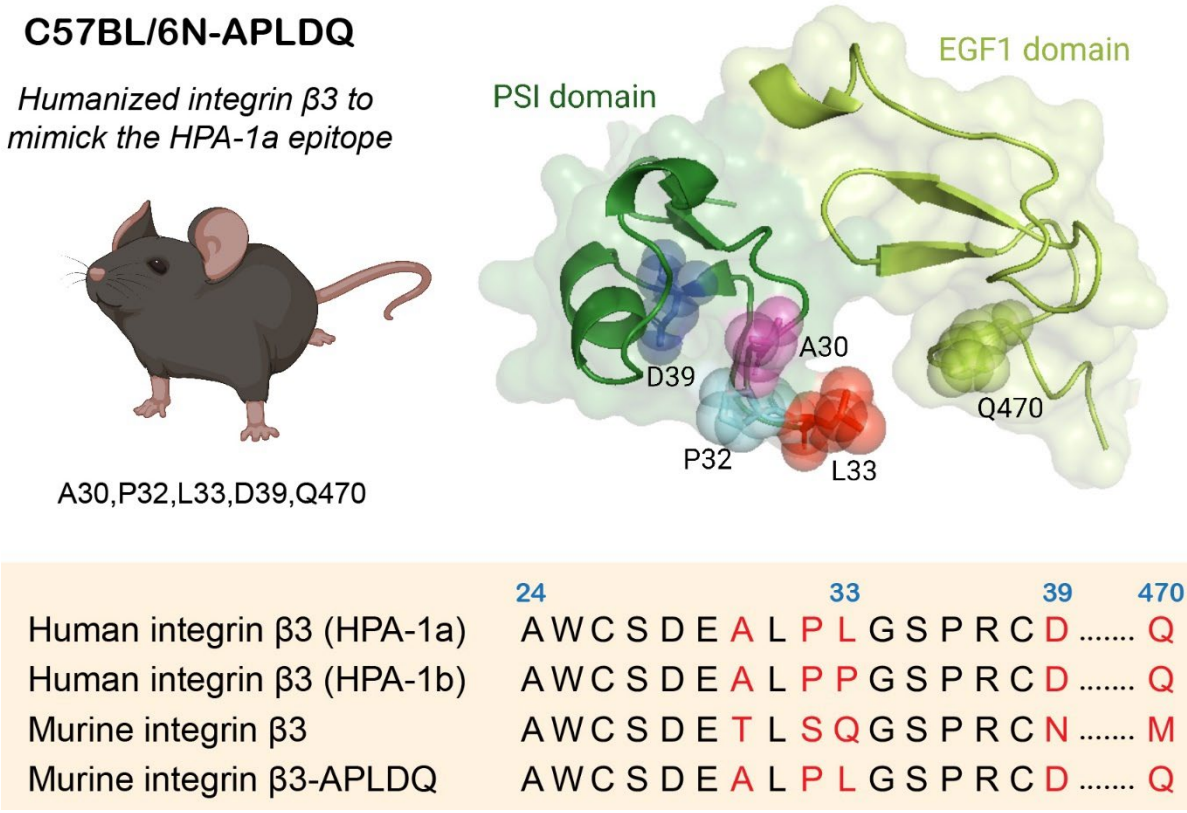

Figure 7: *Illustration of transgenic mice with humanized integrin β3 to express HPA-1a. Highlighted APLDQ residues in integrin β3 from both the PSI and EGF1 domains. PDB entry 3FCS [193], visualized by PyMOL v2.5.5. Mouse from BioRender.com.*

2 Aims of thesis

The main aim of this thesis was to characterize and evaluate the potential of the human mAb 26.4 as a prophylactic candidate for the prevention of HPA-1a alloimmunization related to FNAIT. By developing antibody isoforms designed to include functions believed to be important in antibody-mediated immune suppression, candidates were tested in vitro and in vivo in the APLDQ FNAIT mouse model.

Data have emerged suggesting that subtypes of anti-HPA-1a antibodies may exist — tentatively mounted against placenta-derived epitopes. It is yet unknown the extent to which mAb 26.4 also holds the potential to prevent such immunization; thus, comparative studies of such antibodies and mAb 26.4 would be valuable. An additional aim was therefore to characterize binding patterns of FNAIT-related antibodies for evaluation of the developed antibody response that, in some cases, leads to ICH.

More specific aims:

- Characterize mAb 26.4 isoforms in vitro by assessing their binding abilities to recombinant FcγRs and FcRn, and by evaluation of their effector functions.
- Evaluate antibody ability to clear platelets and induce AMIS in vivo in a murine FNAIT model and assess the outcome of delivered pups.
- Produce soluble recombinant integrin β3 proteins, both as monomers and dimers, using baculovirus and insect cells as a eukaryotic protein production platform.
- Analyze antibodies from women giving birth to FNAIT-affected neonates with respect to concentration and binding patterns using recombinant proteins.

3 Summary of papers

Paper I

Prevention of Fetal/Neonatal Alloimmune Thrombocytopenia in Mice: Biochemical and Cell Biological Characterization of Isoforms of a Human Monoclonal Antibody

TV Mørtberg, H Zhi, G Vidarsson, S Foss, S Lissenberg-Thunnissen, M Wuhrer, TE Michaelsen, B Skogen, TB Stuge, JT Andersen, PJ Newman, and MT Ahlen

Today, there are no prevention of FNAIT as there are no prophylactic drugs available on the market to prevent HPA-1a alloimmunization. The aim of this study was to test the potential of the fully human HPA-1a-specific mAb 26.4 as a prophylactic candidate to prevent alloimmunization. The specific mechanisms of AMIS are not entirely understood, and a panel of mAb 26.4 isoforms with modified Fc regions or altered Fc glycosylation was generated. The variants were evaluated for their binding ability to recombinant FcγRs and FcRn, and in vivo plasma half-lives investigated using mice with human FcRn.

A novel mouse strain expressing a humanized HPA-1a epitope on integrin β3 was used as an antigen source. Platelets were isolated and transfused to BALB/c mice to mimic FNAIT alloimmunization. Immunized mice developed substantial levels of antibodies, and by breeding with male mice expressing humanized HPA-1a, newborn pups showed signs of FNAIT. By administering mAb 26.4 wild-type and an effector-silent isoform to BALB/c mice prior to platelet challenge, induction of AMIS was demonstrated to be most efficient with the wild-type antibody isoform. This study generated proof of principle for FNAIT prevention using human HPA-1a-specific monoclonal antibodies.

Paper II

Preclinical evaluation of an HPA-1a-specific human mAb IgG1 modified for enhanced effector properties

TV Mørtberg, S Foss, LM van Ligtenberg, EL Bertelsen, O Ottersen, G Vidarsson, B Skogen, TB Stuge, JT Andersen, and MT Ahlen

In a prophylactic regimen, it would be advantageous to administer a drug with long half-life to reduce frequency of administration. Here we present a novel Fc-engineered isoform of mAb 26.4 (REW), modified to entail increased binding properties to FcRn and C1q, which leads to enhanced plasma half-life and increased on-target complement activation. Interestingly, this isoform also demonstrated higher binding affinity for FcγRs in ELISA assays, which could allow for better induction of AMIS. In addition, mAb 26.4.REW demonstrated efficient phagocytosis, and increased C3d deposition on beads containing integrin β3 compared to mAb 26.4.WT.

Based on a recent report demonstrating efficient prevention of alloimmunization at lower doses, we designed murine experiments testing our novel antibody candidate together with the wild-type antibody at 10 IU/ml and 2 IU/ml. mAb 26.4.REW was able to induce efficient suppression of alloimmunization at both doses, and normal platelet counts were displayed in the delivered pups. On the contrary, pups from immunized mice without antibody prophylaxis had thrombocytopenia, bleeding symptoms and ICH. In summary, this new modified antibody candidate shows promising results as a human prophylaxis, and with increased plasma half-life and effector functions, this candidate may also allow for reduced dosing and frequency of administration in a human prophylaxis regimen.

Paper III

Exploring recombinant glycoproteins as reagents for anti-HPA-1a antibody subtype specificity in FNAIT

TV Mørtberg, E Bertelsen, H Tiller, B Skogen, TB Stuge, and MT Ahlen

Today, there are no prophylactic drugs to prevent HPA-1a alloimmunization. Most women are immunized in connection with delivery, although studies show that a significant proportion of women are immunized early during their first HPA-1-incompatible pregnancy. It is unlikely that the number of platelets required to induce immunization originate from fetomaternal hemorrhage this early in the course; thus, other cells or tissues might contribute with integrin β3 causing HPA-1a alloimmunization.

Consistent with previous studies displaying HPA-1a-specific αVβ3-dependent antibodies in FNAIT samples with ICH outcomes, we aimed to evaluate anti-HPA-1a antibodies in maternal plasma samples from referral FNAIT cases with ICH outcome in Norway during the last decades, investigated at Norwegian National Unit for Platelet Immunology (NNUPI). Antibodies that bind independently of associated α-chain were preabsorbed from plasma samples using αIIbβ3-coated beads. The remaining supernatant was analyzed both in flow cytometric assays using recombinant integrin β3 proteins and in antigen capture assays using αVβ3-expressing cell lines. Prior to absorption, samples showed efficient binding to protein-coated beads and αVβ3-expressing cell lines. Interestingly, samples bound in similar patterns to all the beads (αIIbβ3, αVβ3 and monomeric β3), showing that most of the HPA-1a-specific antibodies might bind independently of associated α-chain. Notably, HPA-1a-specific antibodies of the anti-αVβ3 subtype were not significantly detected in preabsorbed samples from FNAIT cases with ICH. This highlights the challenge of detecting these potentially clinically harmful antibodies in maternal samples.

4 Methodological considerations

4.1 The quest for the optimal prophylaxis: Papers I and II

The main aim of this thesis was to evaluate the use of the human mAb 26.4 as a prophylaxis for FNAIT using murine models. Since the optimal antibody properties to be used as a prophylaxis are still under investigation, we wanted to test a panel of candidates.

4.1.1 Selecting antibody subclass

The first requirement of an antibody prophylaxis is its binding to the antigen of interest. Central to this thesis was the use of mAb 26.4, a human HPA-1a-specific antibody originating from a woman who gave birth to a child with severe FNAIT [191]. mAb 26.4 binds to HPA 1a-bearing integrin β3 independently of the associated α-chain, while completely ignoring HPA-1b. The characterized antibody was isolated from a single immortalized B cell and was of the IgG3 subclass. In principle, IgG1 or IgG3 antibodies because of their superior effector functions would be more suitable as effector functional drugs compared to IgG2 and IgG4. The longer hinge region found in IgG3 antibodies has been shown by Chu and colleagues to improve ADCP compared to antibodies of the IgG1 subclass, as demonstrated by modifying IgG1 antibodies by inducing longer hinge regions [108]. They also found that phagocytosis of serum-treated antibodies with elongated hinges was not affected, indicating that hinge length does not lead to decreased antibody stability [108]. This is contrary to another study showing that its long hinge makes it predisposed to proteolysis [194]. In addition, Wuhrer and colleagues reported a negative effect on ADCC with increased hinge length [195]. By producing all known allotypes of IgG, they showed that although IgG1, IgG2, and IgG4 allotypes performed similarly in ADCC assays, IgG3 allotypes demonstrated variations in their ADCC activity through variation in hinge length and in the CH2 domain [195]. In their study, IgG3 allotypes with short hinges — but also with IgG1 hinge — exerted stronger induction of ADCC even though the affinity for FcγRIIIa was similar.

Nevertheless, there has been a lack of interest in the IgG3 subclass in drug development because of its short half-life compared to the other IgG subclasses, and today most antibody drugs on the market are of the IgG1 subclass [196]. In addition, IgG3 holds many more polymorphisms than the other subclasses [106, 197]; some induce immense aggregation during production [198]. Considering this, we decided to produce the mAb 26.4 specificity with an IgG1 backbone, as introduced in Papers I and II.

4.1.2 Fc-region modifications alters effector functions

For prevention of alloimmunization toward RhD, prophylaxis is given by subcutaneous injection postnatally, but in the recent years it has also been given antenatally in gestational week ~28. Because the effect and dosing of 26.4 wild-type (WT; unmodified IgG1) as an antenatal prophylaxis are still undetermined, we expanded our candidate panel to include an effector-silent variant. This variant (26.4.AAAG) has the well-characterized PG-LALA mutations in the Fc region, although to completely abolish effector functions, an additional mutation was included in the conserved glycan-site N297 from an asparagine to an alanine.

In Paper II, we introduced a novel candidate, 26.4.REW, produced with the REW technology by introducing three mutations in the Fc region; Q311R, M428E, and N434W [199]. Harboring these three mutations generates an antibody with increased binding to FcRn and C1q; thus, the antibody shows an enhanced plasma half-life and an increased ability to activate complement. Our interest in this variant was to potentially lower the dosage and frequency of administration. In addition, the prophylactic effect of a candidate having enhanced complement activation has not been studied in the context of FNAIT prophylaxis.

The mechanism behind the success of the RhD antibody prophylaxis is still not definite, but likely combines mechanisms such as ADCC, ADCP and CDC, which influences the rapid clearance of antigen-containing particles, and the inhibition of B-cells through FcγRIIb binding. As the conserved glycan at N297 affects FcγR binding, important for most effector functions, we wanted to produce mAb 26.4 with altered glycans to accommodate the likely effects of a prophylaxis using techniques for glyco-engineering [200]. Because antibodies with low fucose levels increase binding to FcγRIIIa, followed by enhanced effector functions such as ADCC [159-161], it is believed to be important for AMIS and, as such, mAb 26.4.WT and 26.4.REW were produced as variants with low fucose levels (~20%). In addition, we produced mAb 26.4.WT and 26.4.REW with high galactose levels (~80%). The aim here was to explore the effect of higher C1q binding in our in vitro assay.

4.2 Production of recombinant integrin β3 allows for in vitro testing

To allow in vitro characterizations of our antibody panel, we made HPA-1a antigens for antibody immobilization to evaluate Fc region effects, and for direct binding assays and ADCP

assays (Papers I and II). In addition, to analyze binding patterns of antibodies from FNAIT-affected pregnancies, integrin dimer complexes were produced (Paper III).

To generate recombinant soluble proteins with eukaryotic post-translational modifications and protein folding, we used insect cell protein production. To ensure soluble proteins, the transmembrane and cytoplasmic domains were excluded. To produce dimer complexes, an α-helical coiled-coil was included to ensure self-dimerization. A purification tag was included on the α-chain while a 6×His-tag for binding to Dynabeads was located on the β-chain. Therefore, following purification, proteins captured through the His-tag on beads should be in a dimer complex.

In our current production, we also used the native sequences of integrin β3 for the monomeric form. Other studies have used monomeric chimeric integrin β3 where the βI domain is replaced with the αI domain from an αL integrin, generating an integrin β3 able to bind ligand [201, 202]. Even though it demonstrates that the overall structure resembles the integrin β3 structure, this is not guaranteed [201]. However, as the native integrin β3 were produced in satisfactory amounts and showed binding by mAb 26.4 and other HPA-1a-specific and integrin β3-specific antibodies, we used these monomeric antigens to capture antibodies by ELISA.

4.3 Murine studies: doses of platelets and prophylactic drug

In our prophylactic studies (Papers I and II), we wanted to mimic fetomaternal hemorrhage by introducing APLDQ platelets intravenously in the tail vein. Even though no adjuvant was included in this platelet challenge procedure, we chose to use the same number of injections (two administrations) and platelets (1×10^8) as Zhi and colleagues [203], which was also defined as “high platelet dose” by Tiller and colleagues in their prophylactic study [189]. Sufficient immunization levels are required in the control mice to allow evaluation of a prophylactic intervention in antibody-treated mice. When challenging BALB/c mice by transfusion of APLDQ platelets, the developed anti-APLDQ antibody response was acceptable but not as high as when supplying adjuvant. Upon successive breeding with APLDQ males, the delivered pups were thrombocytopenic (Papers I and II). For prophylactic intervention, we primarily decided (Paper I) to also use the highest dose (20 μg) as was tested using mAb SZ21 in the integrin β3-deficient mice study [189].

4.3.1 Limitations to bleeding assessments of pups

A limitation to the study in Paper II, is that the assessment of bleeds in pups and brain bleeds are evaluated by a single person, not specialized in examination of bleeds. The ideal setting would be to include a second trained person unaware of litter groups. Weight of the pups was not considered relevant as they are examined between 3-48 hours after delivery and the weight increase is rapid. Also, for the microscopy imaging, few brain sections are included here, and sections were from a few selected pups only.

4.4 Ethical consideration for murine studies

The use of animal models to test therapeutic or prophylactic drugs for FNAIT is essential as there are obvious ethical difficulties in performing research on pregnant women. The number of mice were reduced to a minimum, although at a level still achieving significant data. Sacrificed mice were used further in other in vitro studies to reduce the total number of research animals. To ensure animal welfare, the mice were housed in enriched environments containing houses, tunnels, and chewable material, with food and water always available. Because mice are social animals, they were housed together as much as possible, although pregnant mice were separated to link the moms with the corresponding pups.

The animal studies in Paper I were performed at Biological Resource Center at the Medical College of Wisconsin. All animal protocols were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee performed according to the human and animal experimentation guidelines of the U.S. Department of Health and Human Services and in adherence with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

The animal studies performed in Paper II were performed at UiT The Arctic University of Norway; all protocols were approved by the Norwegian Food Safety Authority (FOTS 23182).

5 Discussion

In order to reduce morbidity and mortality of FNAIT, efficient treatment or prevention of alloimmunization is required. Because there is still no prophylaxis available to date, the main aim of this thesis was to test the prophylactic potential of mAb 26.4 isoforms with altered Fc regions or modified Fc glycan structures. In conclusion, after in vitro characterization relevant for potential AMIS mechanisms, certain candidates were tested in vivo in a murine FNAIT model. Using prophylactic treatment, AMIS was successfully induced, and pups with normal platelet counts were delivered from incompatible pregnancies (Papers I and II).

5.1 Prophylactic studies using a murine FNAIT model

Using integrin β3-deficient mice, Tiller and colleagues demonstrated proof of concept for AMIS related to FNAIT by introducing anti-β3 sera prior to challenge by wild-type platelets, preventing endogenous production of anti-β3 antibodies [189]. This was also shown when using human HPA-1a+ platelets as antigen and human anti-HPA-1a antibodies or murine mAb SZ21 as prophylactic candidates. By transfusing murine wild-type platelets and anti-β3 sera to integrin β3-deficient female mice — followed by breeding with wild-type males — the resulting pups had higher platelet counts compared to pups from immunized mice. However, these platelet counts do not reach the same level as in pups from compatible pregnancies, and the authors speculate on the effect of remaining prophylaxis in the circulation upon breeding. Importantly, they also demonstrated a reduced incidence of ICH and miscarriage, and the number of live pups was at normal levels. A disadvantage of this study is that when using mAb SZ21 as a prophylactic treatment, it is not possible to distinguish the endogenous immune response toward human HPA-1a platelets from the administered SZ21. Furthermore, SZ21 is a pseudo-specific antibody binding HPA-1b at high concentrations, and the use of human platelets as an immunogen is not optimal as there are multiple glycoprotein differences between the platelets of these species.

5.1.1 APLDQ mice expressing HPA-1a used for prophylactic antibody testing

To allow testing of human anti-HPA-1a antibodies as therapies for FNAIT, Newman and colleagues produced mice with humanized integrin β3 to comprise the HPA-1a epitope, referred to as APLDQ mice [192]. The use of platelets from APLDQ mice as an antigen source to challenge wild-type mice for induction of an HPA-1a-like alloimmunization was successfully demonstrated by Zhi and colleagues [203]. In their immunization setup, they used 1×10^8

platelets mixed with adjuvant and injected intraperitoneally in BALB/c mice. After two injections at days 0 and 7, the mice showed efficient immunization against APLDQ platelets. By breeding with C57BL/6N-APLDQ males, these preimmunized females delivered thrombocytopenic pups. Bleeding in various tissues and dead pups were also reported using this model. Conversely, upon therapeutic intervention by IVIg at two timepoints within pregnancy, pups were delivered with higher platelet counts and without bleeding.

BALB/c mice are often used to study the mechanisms and effects surrounding antibody production while C57BL/6 mice seem to be beneficial when exploring cellular mechanisms. It could be discussed whether BALB/c mice as recipient mice in our studies are the best strain to use as they also differ in the MHC expression from the C57BL/6N-APLDQ mice. However, a study determining immune responsiveness by different mouse strains to allogenic platelets, demonstrated that BALB/c generated high levels of IgG to platelets from C57BL/6 mice, while the reversed setting mounted low levels of antibodies [204]. With respect to humans, MHC incompatibility during pregnancy is quite common. Nonetheless, in our studies, the level of anti-MHC antibodies toward C57BL/6N wild-type platelets was minimal compared to the levels of anti-APLDQ antibodies (Papers I and II).

The use of a new murine model, as in the current study, requires careful interpretation of measurable antibodies and clinical outcomes. In mice that have received both APLDQ platelets and prophylactic antibodies, sera from day 14 can contain both the human prophylactic IgG and potential in vivo-generated murine anti-APLDQ IgG as well as potential murine anti-human IgG (also referred to as anti-drug antibodies/MAHA mouse anti-human antibodies). During detection of developed endogenous murine anti-APLDQ, the human prophylactic mAb 26.4 may compete for the same epitope on APLDQ platelets, which could tentatively give a false-negative readout of the murine anti-APLDQ response even when using species-specific secondary detection antibodies. However, we did not see any signs of this in our results. On the other hand, this phenomenon is a circumstance limited to these kinds of preclinical studies as it occurs because of species differences. Therefore, this will not be a problem when using human mAb 26.4 in a human setting.

These transgenic APLDQ mice have normal platelets — as demonstrated by murine platelet counts, bleeding times, and the ability to aggregate [203]. Notably, just as for the previous murine FNAIT models, this model also has the disadvantage that it requires preimmunization, since mice are not immunized by breeding alone. Recently, it has also been demonstrated that

CD4+ T cells are required for the development of anti-APLDQ antibodies by B cells in BALB/c mice challenged with APLDQ platelets [205].

5.1.2 Induction of AMIS is dose-dependent in mice

Using the APLDQ mouse model, we successfully demonstrated AMIS, showing proof of concept using monoclonal HPA-1a-specific antibodies (Paper I). In the wake of our initial prophylactic study, Zhi and colleagues further optimized the prophylactic regimen by analyzing the lowest effective dose of both a monoclonal and a polyclonal batch [206]. Here they tested the prophylactic candidates RLYB211, the hyperimmune anti-HPA-1a IgG preparation (previously NAITgam), and RLYB212, originating from mAb 26.4. By intravenous injection of prophylactic antibodies 1 hour prior to transfusion of APLDQ platelets, Zhi and colleagues could prevent immunization with as low dose as 4 IU/ml for RLYB212 and between 1 and 4 IU/ml for RLYB211. Compared to our initial prophylactic study (20 µg/mouse), these doses are more than a hundredfold lower, but they still efficiently prevented immunization. In addition, the setup of prophylactic treatment differs between the two studies, because they treated the mice only 1 hour prior to platelet challenge. Different from their study, we injected our mice with the prophylactic dose 24 hours prior; therefore, the injected antibody dose is subjected to both biodistribution and degradation from the circulation. Measurement of antibody concentration directly before platelet transfusion was not feasible because of the volume restrictions for blood sampling in mice and because blood sampling prior to transfusion complicates the tail vein injection technique caused by lower blood volume in the mouse. Hence, the effective antibody concentration at the time of the platelet challenge could not be measured.

Another difference from our studies is that Zhi and colleagues challenged the mice with only one APLDQ platelet dose [206]; therefore, the developed anti-APLDQ antibody response was less than the generated response in our studies. However, they chose to rechallenge and reintroduce the mice with prophylaxis at day 21, and the treated mice still showed lasting protection. The control mice receiving only platelets generated an immense response toward APLDQ platelets, showing that three-week immunization by platelet challenge might be a more optimal test to generate a sufficient response compared to a rechallenge after only one week.

5.1.3 Panel of mAb 26.4 isoforms as prophylactic candidates

In addition to the successful use of mAb 26.4 wild-type to induce AMIS in BALB/c mice challenged with APLDQ platelets, other candidates were tested in vivo as prophylactic drugs and are discussed further here.

mAb 26.4.AAAG: antibody with abolished effector functions

The use of effector-silent antibodies as FNAIT treatments has been demonstrated previously. SZ21 — generated as an $F(ab')_2$ fragment to remove Fc-dependent effector functions — was able to block binding of HPA-1a-specific antibodies and, as such, prevent clearance of human HPA-1ab platelets in a NOD/SCID mouse model [207]. Also valuable for a FNAIT treatment, SZ21 $F(ab')_2$ was able to displace bound HPA-1a-specific antibodies. However, since transplacental transfer requires FcRn interaction with the Fc-region, SZ21 $F(ab')_2$ cannot inhibit platelet destruction in fetal circulation. Later, a deglycosylated variant of SZ21, which also abrogates Fc-related effector functions but retains FcRn transport, was tested as a treatment in the murine NOD/SCID model [208]. Transplacental transport and the ability to ameliorate platelet destruction by HPA-1a-specific antibodies were preserved even after deglycosylation. Because SZ21 is of murine origin, its use as a therapeutic drug in a human setting might induce the generation of anti-drug antibodies — eliminating them from circulation upon repeated administration. To overcome this problem, a study introduced the recombinant human antibody B2G1 modified with a nondestructive constant region, B2G1Δnab, as a therapeutic candidate [209]. The survival of HPA-1ab platelets sensitized with B2G1Δnab was similar to unsensitized platelets, while B2G1-sensitized platelets were rapidly cleared.

In our primary prophylactic study (Paper I), we partially induced AMIS when using mAb 26.4.AAAG (abolished of all effector functions) as a prophylactic candidate. This was demonstrated by a reduced anti-APLDQ response in wild-type mice administered with mAb 26.4.AAAG followed by transfusion of APLDQ platelets. Importantly, subsequent pups from breeding with APLDQ males had normal platelet counts. Furthermore, Zhi and colleagues later showed that 4 IU/ml of HPA-1a-specific antibody binds less than 10% integrin β3 receptors on APLDQ platelets [206]. Based on these results, we can estimate that our initial prophylactic dose might have been sufficient to saturate all epitopes of the transfused platelets, inducing AMIS by complete epitope masking. If we were to examine mAb 26.4.AAAG further as a candidate at doses as low as those analyzed in Paper II, it might not be sufficient to induce

AMIS. However, Zhi and colleagues tested mAb 26.4.AAAG as a therapeutic agent by administering the candidate during pregnancy to preimmunized females [203]. The platelet count in three of four deliveries was rescued, showing its potential as a treatment for FNAIT. Since the epitope in this murine model consists of five amino acid substitutions compared to one in humans, mAb 26.4 might not block all endogenous anti-APLDQ antibodies in this model, as seen by its inability to rescue platelet counts in one pregnancy. Nevertheless, this is unlikely in a human setting because mAb 26.4 has been shown to block binding of polyclonal HPA-1a-specific IgG to HPA-1aa platelets [191].

In summary, even though mAb 26.4.AAAG might not be able to induce AMIS in humans at the desired lowered doses, this does not eliminate the use of this candidate in a therapeutic setting.

mAb 26.4.REW: potent antibody by enhanced effector functions

In our subsequent prophylactic study, we introduced a new candidate, mAb 26.4.REW, with modified effector functions (Paper II). Based on the new knowledge generated by Zhi and colleagues showing that there is a dose-dependent prevention at reduced doses [206], we tested two different concentrations of the novel candidate together with mAb 26.4.WT. Using the same injection regimen as in our previous study, we demonstrated efficient induction of AMIS at lowered doses (2 IU/ml and 10 IU/ml) using the new candidate mAb 26.4.REW. Unexpectedly, mAb 26.4.WT did not efficiently prevent immunization in the 10 IU/ml group, but four of the five mice in the 2 IU/ml group did not generate anti-APLDQ antibodies. Because the 2 IU/ml doses were generated by direct dilution of the 10 IU/ml batch, a technical dilution error was unlikely. Interestingly, the mice in the 10 IU/ml treated group showed a higher level of MAHA toward mAb 26.4.WT than the other three groups, theoretically affecting the level of circulating antibody in these mice and leading to unsuccessful prevention of immunization in this experimental setup. In addition, because we introduced antibody prophylaxis 24 hours earlier, the effective dose is difficult to evaluate in these mice in the current transfusion regimen.

In conclusion, since mAb 26.4.REW efficiently induced AMIS even at 2 IU/ml, future studies exploring even lower doses may be performed to display the potency of the antibody.

mAb 26.4.LF: increased affinity for FcγRIII

In our initial antibody isoform panel, we included mAb 26.4 with either reduced fucose levels or increased galactose levels, although they were not tested in vivo (Paper I). Even though low fucose antibodies have increased binding to FcγRIIIa, Bruggeman and colleagues showed that hypofucosylated anti-D antibodies did not affect ADCP by macrophages [210], likely because of macrophages broader expression of other FcγRs compared to NK cells. This is consistent with what we saw in our phagocytosis assay using THP-1 cells, where our low fucose variant mAb 26.4.WT.LF showed no superior function compared to the other variants (Paper I, Figure 4). In addition, the mAb 26.4 low fucose variant was equally effective as the wild-type variant in clearing platelets in APLDQ mice (Paper I, Figure 5). Based on these results and the notion that the wild-type variant showed efficient in vitro effector functions without glycosylation modifications, the low fucose and high galactose variants were not tested in vivo in the murine model as prophylactic candidates.

Interestingly, Herter and colleagues demonstrated that afucosylated antibodies induced significantly higher levels of ADCP compared to wild-type antibodies in nonclassical monocytes ($CD16^{+}$) when assessed under physiological concentrations of human endogenous IgG [211]. In our in vitro experiments, monoclonal antibody candidates were analyzed in the absence of non-specific, endogenous IgG; tested candidates induced similar ADCP results (Paper I, Figure 4). Future ADCP studies should include endogenous IgG to simulate physiological conditions or the blocking of singular FcγRs. As mentioned above, we later learned that human HPA-1a-specific antibodies are functional in this mouse model at far lower doses [206]. Therefore, in hindsight, these mAb 26.4 candidates should be tested further in vivo as they might surpass the wild-type variant at lower doses. A study evaluating afucosylated anti-CD20 reported enhanced phagocytosis by neutrophils and increased expression of MHC class II [212] and, as such, afucosylated antibodies should be evaluated with care in a prophylactic setting as they might induce adaptive immune responses.

Importantly, anti-D preparations have demonstrated high galactosylation levels and lowered, although varied, levels of fucosylation between different preparations [126]. Decreased levels of IgG core fucose for HPA-1a-specific antibodies are also seen in samples from HPA-1a alloimmunized women [122, 123]. Hence, as FNAIT-related antibodies naturally demonstrate lowered fucose levels of Fc glycans, such antibodies might make important contributions to a monoclonal antibody prophylaxis for FNAIT.

5.1.4 Difference between mice and humans

In general, the immune systems of mice and humans are quite similar, and although there are some significant differences, mice represent a valuable surrogate in early studies of therapeutic substances [213]. When developing murine models for translational research, there are many factors to consider when it comes to immunization, such as antigen source and presentation.

Notably, when testing human antibodies in mice, the effector functions are carried out by murine FcγRs and murine effector cells. Compared to the three subfamilies of FcγR in humans, mice have a fourth receptor, FcγRIV (reviewed by ref. [214]). Importantly, mice do not express FcγRIIIb and FcγRIIc. FcγRs do not directly correlate between humans and mice, and their binding patterns and expression on cells are also different — which complicates evaluation of the effect of human antibodies.

For humans, the immune response to RhD when using antibody prophylaxis is either completely suppressed or not prevented at all, while in mice it has been shown to have a dose-dependent effect [184]. Prophylactic anti-D has been shown to have a long-term effect because there are both a reduced number of responders and reduced anti-D levels in treated individuals [215], while no long-term influence is demonstrated in mice.

Many different studies in murine models have been used to demonstrate which specific mechanisms are in effect for AMIS, although these models cannot entirely simulate the human setting. For humans, studies indicate that AMIS is induced by Fc-dependent mechanisms, while studies in mice show that AMIS could occur Fc-independently [184, 185]. Based on the knowledge gained, it is probable that multiple mechanisms are working simultaneously, and they might also functionally synergize with one another.

5.2 Translation to humans: prophylactic treatment of FNAIT

Prior to implementing a prophylactic regimen, routine screening programs are required to identify candidates. Furthermore, reflections regarding the origin of the antigen mounting the immune response to HPA-1a must be carefully evaluated prior to initiating an antenatal prophylaxis regime to avoid potential harm to the fetus.

5.2.1 Screening is required to identify individuals eligible for prophylactic treatment

Because there is no prophylaxis or effective treatment to date, national screening programs are not implemented, although it has been argued that screening would be cost effective [49, 216] and reduce mortality and morbidity [67]. Recently, large screening studies were performed in Norway [67], Poland [217], The Netherlands (unpublished HIP study), and also the ongoing international IPA2002 study (www.clinicaltrials.gov study NCT05345561, Rallybio IPA, LLC), to demonstrate the natural history of HPA-1a alloimmunization.

Since prophylactic treatments to preventing HPA-1a alloimmunization are under development, implementation of screening regimens is essential. The first approach in this setting would be to determine the HPA-1 status of pregnant women. As such, this implementation is feasible and may even be carried out in the same blood sample as for RhD screening. In addition, further determination of fetal HPA-1 status may be uncovered by a non-invasive prenatal test (NIPT) which involves typing of prenatal cell-free DNA in maternal plasma. Further, there are discussions regarding employing HLA-typing for the determination of HLA-DRB3*01:01 status, as this allele is highly associated with HPA-1a alloimmunization [75-78]. It has been estimated that women who carry the HLA-DRB3*01:01 allele have a 25-fold higher risk for alloimmunization compared to women lacking this HLA-allele [218]. Nevertheless, there are women who are negative for this allele that generates anti-HPA-1a antibodies, although they rarely delivers severely thrombocytopenic neonates [219]. Accordingly, the elimination of these women from prophylactic intervention should be carefully evaluated. Furthermore, after identifying maternal and neonatal HPA-1 status, anti-HPA-1a antibody detection is required to eliminate women that are already alloimmunized.

Importantly, the determination of antibody status in these women will allow closer follow-up in the pregnancy and direct intervention by treatment of high-risk pregnancies. It has been reported that in a non-screened population in Norway, only 14% of FNAIT cases were detected

by referrals compared to a screened population [220]. As such, screening will allow more women at risk to be closely monitored and followed up to avoid severe fetal/neonatal outcomes. However, to date, no international consensus on how to risk-stratify HPA-1a alloimmunized women has been determined. To implement screening, plans for both treatment and follow-up regimens for such women must be in place.

Importantly, screening for HPA-1 status will be required only in the first pregnancy, eliminating further typing in subsequent pregnancies for HPA-1a-positive women. On the other hand, prophylactically treated women will require further anti-HPA-1a antibody screening in future pregnancies, and if still non-alloimmunized, new rounds of prophylactic administrations are necessary. By limiting the screening to HPA-1 incompatibilities only, alloimmunization to less common HPAs that may result in severe disease is disregarded. Future screening of less recurring variants may be implemented as the immunization rate and subsequent disease outcome of HPA-1a alloimmunization are reduced. However, for other variants, the initial screening may not eliminate as many women as for the first step of HPA-1 screening that eliminates about 98 % of the women tested. As such, these screening regimens may be more troublesome. On the contrary, antibodies to CD36 are a frequent cause of FNAIT in Asian and African populations, and as demonstrated by Xu and colleagues, IVIg and deglycosylated anti-CD36 antibodies were able to rescue pups in a murine model [221]. As such, implementation of screening for women of risk of CD36 immunization should be considered.

In summary, prior to starting national prophylaxis programs, screening to identify women at risk of HPA-1a alloimmunization must be implemented. Such screening regimens also allows for the detection of high-risk pregnancies, and potential future therapies may be issued at an earlier stage.

5.2.2 What is the antigenic stimulus of HPA-1a alloimmunization?

For successful implementation of a FNAIT prophylaxis, administration must occur prior to the alloimmunization event. To date, the specific mechanisms of alloimmunization are unknown and, as such, there is a need to investigate the initial antigenic stimulus leading to the development of HPA-1a-specific antibodies further.

The first requirement for alloimmunization is obviously that HPA-1a-negative women are challenged with the HPA-1a antigen. In addition, a triggering of the immune system to the

antigen is required for antibody development. Antigen-presenting cells, upon encountering the HPA-1a antigen, process and present the antigen on HLA surface molecules to activate antigen-specific T cells. Such T cells are necessary to activate naïve B cells into antibody-producing B cells. Importantly, activation of antigen-presenting cells requires a secondary signal, and for HPA-1a alloimmunization, such a signal is apparently missing, or at least unknown for the time being. Since only ~ 10% of HPA-1bb woman develop HPA-1a-specific antibodies [48, 49, 67], more than the sole antigen challenge is required for the induction of alloimmunization.

Fetomaternal hemorrhage during pregnancy is quite common, with increasing volumes later in pregnancy — largely occurring during delivery. As the D antigen is restricted to RBCs and because the majority of women are immunized in connection with delivery, fetomaternal hemorrhage seems to be the priming stimulus for RhD immunization. On the contrary, FNAIT also occurs in the first incompatible pregnancy; antibodies have been detected as early as week 17 [48]. Since integrin β3 is not only present on platelets but also on other cells and tissues such as endothelial cells [38] and trophoblast cells [39], the site of origin for the stimulating antigen could be from any one of these cell types. αIIbβ3 is expressed by fetal platelets as early as 16 weeks of gestation [222], but has also been reported on early trophoblasts in placental sections of 8 and 15 weeks of gestation [34]; hence FNAIT-related alloimmunization may occur at a very early stage.

An alternative to fetomaternal hemorrhage as an antigen source and initial immunization trigger is the fact that the maternal immune system is in direct contact with trophoblast cells of fetal origin. Integrin β3 has also been visualized by immunolabeling on syncytiotrophoblasts as early as the first trimester [223]. In this interface of maternal and fetal cells, there is normally an immunosuppressive environment as extravillous trophoblasts uniquely express HLA-G, which has been connected to fetus-induced immune tolerance [224]. These cells are believed to serve as a barrier between the maternal effector cells and the placenta, protecting the fetus from rejection. Therefore, a break in tolerance at this interface may lead to the induction of HPA-1a alloimmunization. In addition, polymorphisms in HLA-G have been associated with placental complications [225]. Intriguingly, senescent syncytiotrophoblasts shed microparticles into maternal circulation throughout pregnancy, and as they also express HPA-1a, they could exert effects elsewhere. Notably, human blood monocytes can be activated in vitro by syncytiotrophoblast-derived microparticles [226]. However, if microparticles expressing HPA-

1a induce antibody development, we would believe that more women would be immunized, as the release of microparticles from placental tissue is part of normal turnover [227].

In summary, the initial trigger for HPA-1a alloimmunization is yet to be discovered. Importantly, this knowledge will also play a role in the implementation of an antibody prophylaxis. Notably, the murine FNAIT model lacks the ability to become immunized by breeding only, which is dissimilar from the human setting. Since this model requires preimmunization by APLDQ platelet transfusion, we test our prophylactic candidates for effector functions only in a fetomaternal hemorrhage-like situation. Prophylactic strategies based on preventing immunization against antigens of platelet origin may, or may not, be different from measures to avoid immunization against HPA-1a expressed by other cells or tissues.

5.2.3 Antibody effect on integrin β3 on endothelial cells and placental tissue

Until recent years, it was assumed that fetal ICH occurred by depletion of fetal platelets followed by a harmful event causing bleeding. The effect of HPA-1a-specific antibodies on endothelial cells was initially investigated by Radder and colleagues, without any significant damage measured [228]. With more sensitive methods, data have later emerged supporting the idea that, in addition to directly affecting platelet number and function, HPA-1a-specific antibodies also affect endothelial monolayer integrity and endothelial spreading [229]. Further, Yougbaré and colleagues reported that anti-β3 antibodies impair angiogenesis through binding to αVβ3 expressed by endothelial cells, eventually leading to apoptosis [230].

During pregnancy, placental trophoblast cells interact directly with maternal blood and cells. Since integrin αVβ3 is also expressed by placental trophoblast cells, antigen-specific antibodies may disturb placental function and thus affect fetal development [74, 231]. Associations between anti-HPA-1a levels and reduced birthweight for newborns — especially males — have been demonstrated, indicating that placental development and function may be affected [74]. Interestingly, a study by Althaus and colleagues looking at placentas from IVIg-treated and untreated FNAIT-related pregnancies demonstrated chronic villitis in the placentas from untreated pregnancies [232]. Dubruc and colleagues conducted a retrospective study demonstrating significant levels of chronic inflammatory lesions in placentas of FNAIT cases [233]. Notably, both studies involved pregnancies in which most of the women were alloimmunized against other HPAs than HPA-1a. Later, Nedberg and colleagues demonstrated

an association between anti-HPA-1a antibodies and chronic histiocytic intervillositis in placentas from HPA-1a alloimmunized women [234]. Notably, this study indicates that IVIg-treated women were not protected against chronic inflammation in the placenta, contrary to earlier findings by Althaus and colleagues [232].

Interestingly, de Vos and colleagues demonstrated that non-treated FNAIT placentas show increased deposition of complement protein C4d compared to IVIg-treated and control placentas [235]. These findings imply that untreated FNAIT pregnancies have a higher degree of classical pathway complement activation, which further influences placental function and fetal growth. Yougbaré and colleagues demonstrated that immune responses against integrin β3 lead to miscarriage and intrauterine growth restriction in a mouse model [236]. Importantly, they also reported that uterine NK cells were recruited and caused the apoptosis of trophoblasts in preimmunized pregnant mice. Therefore, NK cell-induced ADCC of placental trophoblasts might be a pathological mechanism in FNAIT. Interestingly, by depleting the NK cells or by inhibiting NK cell activating receptor, miscarriage was prevented.

In summary, placental damage related to FNAIT has been reported, and affected development and function may cause lowered neonatal birthweight and miscarriage. At the time being, it is unknown whether anti-HPA-1a antibodies induces placental inflammation or rather that placental inflammation triggers alloimmunization.

5.2.4 Prophylactic treatment: what about antenatal administration?

Following murine studies showing the potential of human HPA-1a-specific antibodies as prophylactic candidates [189, 206] and Paper I, the polyclonal batch RLYB211 has been further investigated for prophylactic use in humans [237]. By administering 1000 IU to HPA-1a-negative male study participants challenged with HPA-1ab platelets, a more rapid platelet clearance was successfully induced. Because platelet transfusion rarely causes the development anti-HPA-1a IgG [238, 239], plasma-derived hyperimmune IgG isolated from pregnant women will not be available for long if the polyclonal prophylaxis is successful. Hence, the need for monoclonal antibodies as a prophylactic treatment for FNAIT is even more in demand than for RhD. One major difference from RhD is that for FNAIT, the epitope is generated by a single SNP while for RhD, a polyclonal immune response is developed towards numerous epitopes on the protein. This might be part of the reason that the development of monoclonal antibodies has not been successful for RhD (reviewed in ref. [240]).

It is well established that the implementation of antenatal administration of anti-D prophylaxis has been successful, with no reports of harmful effects to the fetus [169]. Compared to the D-antigen, which is present only on RBCs, integrin β3 and its combination with both αV and αIIb are expressed by multiple cells and tissues. Hence, with the introduction of antenatal administration of prophylactic antibodies, there is more to consider regarding unintentional harmful effects to the fetus or placental tissues.

When optimizing an antenatal antibody prophylaxis, one ideal situation would be to prevent transplacental transport and thus limit the antibodies to maternal circulation. However, by eliminating antibody binding to FcRn by substituting H435A (mAb 26.4.H435A), the antibody plasma half-life was decreased to less than a day in transgenic mice for human FcRn (Paper I, Figure 3). It has been observed that glycosylated Fab regions could reduce FcRn-mediated transplacental transport [241]. However, this approach also reduces antibody half-life as both transport and antibody recycling is facilitated by FcRn. Another approach would be to extend the half-life of mAb 26.4 but to limit transplacental transport. For instance, albumin also interacts with FcRn but is not transported to the fetus to the same extent as IgG [131]. By conjugating the Fab fragment of mAb 26.4 to albumin, the drug might be mainly retained in the maternal circulation. However, by removing the Fc region of the antibody, effector functions are abolished — with epitope masking as the only remaining functional mechanism. In addition, this might also limit potential harmful effects on the placental tissue; however, it calls the preventive efficiency of such a drug variant into question. IgG2 has reduced transplacental transport while still demonstrating a similar half-life as IgG1 [242]. Hence, generating mAb 26.4 with an IgG2 backbone may hold prophylactic potential, although IgG2 are not as potent antibodies as IgG1 and IgG3 — demonstrating by lowered binding to most FcγRs [106].

For an antenatal prophylaxis regimen, longer injection intervals are beneficial not only to cause minimal strain to the mothers but also from a cost perspective. Enhanced antibody plasma half-life may limit prophylactic dosage, and as presented in Paper II, mAb 26.4.REW was designed for this purpose. In addition to its enhanced plasma half-life and increased ability to activate complement by design, the isoform also demonstrated increased binding to FcγRIIa, FcγRIIb, and FcγRIIIa (Paper II, Figure 1). This candidate demonstrated effective induction of AMIS even at 2 IU/ml, and in line with our results, this candidate displays promising results as a postpartum prophylaxis. However, because this candidate entails enhanced effector functions in addition to its increased plasma half-life, its use as an antenatal prophylaxis should be

carefully evaluated. The strong binding to FcRn may induce more efficient transport to the fetus and its uprated effector functions might harm the fetus.

Using our effector-silent prophylactic variant (mAb 26.4.AAAG), we were able to partially induce AMIS in vivo using a murine FNAIT model (Paper I, Figure 6). This antibody has epitope-blocking abilities but does not induce effector functions to undesired tissues such as HPA-1a-expressing placental tissue. However, as discussed previously, this effect is likely dependent on dose and the animal model used.

Furthermore, it has been argued that at the calculated prophylactic dose of 1000 IU, antenatal administration of prophylaxis (RLYB211) would not exceed the safety threshold of 3 IU/ml and, thus not cause severe harm to the fetus [237]. For anti-D prophylaxis, positive direct antiglobulin tests (DAT) are often reported [243], even though they do not generally cause severe outcome. mAb 26.4 isoforms are excellent prophylactic candidates as they demonstrate successful induction of AMIS in the murine FNAIT model. If proven successful at preventing HPA-1a alloimmunization in humans at doses that do not generate harm to the fetus, the inclusion of antenatal administration of prophylaxis may further reduce alloimmunization rates compared to postpartum prophylaxis alone. Nevertheless, there are reports of FNAIT cases with severe neonatal outcomes even at very low levels of anti-HPA-1a antibodies detected in the mother. Importantly, low avidity alloantibodies toward HPA-1a have been detected by SPR [89]. Here, two severe FNAIT cases that were false-negative in MAIPA were found to contain clinically relevant antibodies with low avidity. Reports have shown that such low avidity HPA-1a-specific antibodies can destroy HPA-1a-expressing platelets in a mouse model, confirming their clinical relevance [244, 245].

In conclusion, the generation of a monoclonal prophylactic antibody variant with an AMIS effect and enhanced plasma half-life while simultaneously retaining the drug in the maternal circulation is complicated as both antibody recycling and transplacental transport are connected to the same receptor.

5.2.5 HPA-1a-specific antibodies depend on conformational structures

The notion that anti-HPA-1a antibodies bind integrin β3 in different aspects was demonstrated by Valentin in 1995 [246]. Although some antibodies bound to the HPA-1a epitope without other requirements than surrounding amino acids in the PSI domain (type I antibodies), others

depend on distinct but conformationally close amino acids in the EGF1 domain for binding (type II antibodies).

Later discoveries by Santoso and colleagues uncovered that HPA-1a-specific antibodies have different binding requirements depending on the dimerization of integrin β3 [247]. Three binding subtypes were suggested: antibodies binding integrin β3 independent of dimerized α-chain (anti-β3 subtype), antibodies binding only when expressed as αVβ3 (anti-αVβ3 subtype), and antibodies binding when combined with αIIbβ3 (anti-αIIbβ3 subtypes). They demonstrated HPA-1a-specific antibodies of the anti-αVβ3 subtype in samples from FNAIT cases with ICH as an outcome. Notably, non-ICH cases did not comprise antibodies of this subtype. Furthermore, sera containing such antibodies were able to induce apoptosis in endothelial cells and disturb the formation of endothelial cell tubes and endothelial adhesion to vitronectin in vitro. In conclusion, maternal production of HPA-1a-specific antibodies of the anti-αVβ3 subtype could be responsible for the induction of ICH.

Recent evidence suggests that a third category of binding abilities exists — it has been demonstrated that HPA-1a-specific antibodies bind the epitope dependent on either open or bent conformation of the integrin [201]. By employing HEK cells transiently transfected with αVβ3, αIIbβ3, chimeric β3, or mutated variants stabilizing β3 in either open or closed conformations, Thinn and colleagues displayed sera that bound both complex-dependent and conformation-dependent.

Studies involving mAb 26.4, have reported few requirements for the antibody regarding binding to HPA-1a. mAb 26.4 binds integrin β3 apparently independently of complex [191], and our studies have also demonstrated binding to monomeric β3 (Papers I, II, III). Zhi and colleagues additionally showed that mAb 26.4 is a type II antibody because it shows dependency on certain amino acids in the EGF1 domain, and is unreactive to platelets from APLD mice [192]. In addition, Bayat and colleagues later reported that mAb 26.4 binds to integrin β3 in a conformation-independent manner, binding both open and bent forms of integrin β3 tested [202]. The binding characteristics of a prophylactic candidate are also properties that also must be considered in a preventive perspective. Since mAb 26.4 binds to both αIIbβ3 and αVβ3, and also binds independent of conformation (both open and bent forms of the integrin), this antibody may have the potential to clear all integrin β3-expressing particles — platelets and microparticles from both platelets and placental tissue.

Intriguingly, the antigen source for HPA-1a alloimmunization might steer the generation of antibody specificity and dependency in a certain direction. Suggested by Stam and colleagues, HPA-1a-specific antibodies of the αVβ3 subtype might develop by immunization against αVβ3 originating from trophoblasts [248]. Notably, if anti-αVβ3 subtype antibodies discovered by Santoso and colleagues are responsible for the development of ICH, detection of such antibodies may be used as a risk stratification tool. Their important results also prompt further investigation of similar FNAIT cohorts to confirm this association and further develop methods to allow diagnostic testing. Thus, in Paper III, we wanted to investigate the binding specificity of antibodies from FNAIT cases with ICH as an outcome investigated at NNUPI. By employing a similar strategy as Santoso and colleagues [202, 247] we preabsorbed samples using αIIbβ3-coated beads prior to analyzing the remaining supernatant in antigen capture assays. However, when performing antibody detection using αVβ3-coated beads or αVβ3-expressing cell lines, antibodies of the αVβ3 binding character were not significantly detected (Paper III, Figure 4 and 6).

The explanations for our shortcoming in detecting such dimer-dependent antibodies as previously reported are unknown. Differences in material, buffers, or other methodological variations for absorption and detection might influence our results. Our techniques likely encompass integrin β3 proteins of different conformation than in previous studies. We chose to use αIIbβ3-coated beads for the absorption of antibodies of anti-β3 and anti-αIIbβ3 subtypes. After testing with conformation-dependent mAbs (319.4 and 370.2), our data indicate that αIIbβ3 on beads forms an open conformation (Paper III, Figure 8). If HPA-1a-specific antibodies of the anti-αVβ3 subtype implicated in ICH also have a conformational-dependent binding for the extended form of integrin β3, we might not apply the correct proteins for absorption or detection. However, this highlights the need for more detailed characterizations and standardizations for antigens used for absorption, in order to measure and isolate, and further study these subtypes of anti-HPA-1a antibodies.

In conclusion, additional studies should be performed to further characterize pathogenic antibodies from FNAIT-affected pregnancies. Through increased understanding concerning antibody binding patterns and disease outcome, this might bring further clarity about the initial events leading to HPA-1a alloimmunization.

6 Future aspects

In the current work to evaluate the use of mAb 26.4 as a prophylaxis for FNAIT, we only tested a few antibody candidates in the murine FNAIT model. However, isoforms with different subclasses and modified glycosylation are of great interest. For instance, the combination of high galactosylation and low fucosylation has demonstrated further enhancement of ADCC than low fucosylation alone [161]. Thus, mAb 26.4.WT with this combination (LF and HG) could be an interesting prophylactic candidate for future testing. Also, as tested in Paper II, the mAb 26.4.REW isoform successfully induced AMIS at both 10 IU/ml and 2 IU/ml and, as such, this candidate would be interesting to further compare with the mAb 26.4.WT at even lower drug concentrations — to evaluate at what concentration this antibody is still effective. In addition, the inclusion of an additional blood sampling prior to platelet transfusion, would allow evaluation of the actual plasma concentration of the antibody at the time of platelet challenge. Further, more extensive assessments of mouse pup brains from Paper II, by microscopy and tentatively antibodies specific for other structures and cells, may be performed to gain more insight into the outcome of pups from immunized mice.

To investigate any potential harmful effects of antenatal prophylactic treatment, the prophylactic candidates may be further evaluated in the murine model by APLDQ incompatible pregnancies. By injection of different concentrations of antibody preparations — including doses in the same range as those inducing efficient AMIS — to non-immunized mice bred with APLDQ males, any harm to pups or placenta could potentially be monitored. It would also be interesting to perform murine follow-up studies to investigate in what organs of the mice antibody sensitized platelets are depleted, and kinetics thereof. This could potentially be assessed by transfusion of the prophylactic antibody candidates and stained platelets, followed by PET scans to determine site of elimination. It could also be worth considering using APPDQ mice (expressing proline instead of leucine) as recipient mice for APLDQ platelet challenge. Here, the model would only carry one amino acid difference, and thus mimic the setting of human alloimmunization even more closely.

Since FNAIT-related antibodies demonstrate such diversity in their binding patterns to HPA-1a, more knowledge regarding the potential epitope variations is essential. By generating various recombinant β3 integrins associated with both αV and αIIb — that also include mutations or modifications to force the integrins in either a bent or extended conformation — may allow for more extensive absorption and detection studies to evaluate antibody reactivity

of relevant FNAIT samples. Also, the development of such assays and thus further knowledge about the various subtypes of HPA-1a-specific antibodies, may hold potential for future risk stratification for FNAIT outcome.

7 Concluding remarks

By employing a human HPA-1a-specific antibody, we successfully demonstrated prevention of alloimmunization in a murine FNAIT model, demonstrating proof of principle for use as prophylactic drugs. In a follow-up study, we showed effective induction of antibody-mediated immune suppression by implementing far lower doses of an isoform having enhanced binding to FcRn and increased ability to induce complement activation. To date, this murine FNAIT model is the closest representation of the disease progress in humans. In this model, platelets are used as the immunizing agent as pregnancy alone is not sufficient to induce immunization and antibody development. As such, if other fetal cells or tissues — in addition to platelets — provoke alloimmunization in humans, the translational value is only partial. However, even if initial immunization events might differ, the model allows for exploration of potential therapeutic or preventive drugs.

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Paper I

ImmunoHorizons

RESEARCH ARTICLE

Clinical and Translational Immunology

Prevention of Fetal/Neonatal Alloimmune Thrombocytopenia in Mice: Biochemical and Cell Biological Characterization of Isoforms of a Human Monoclonal Antibody

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ABSTRACT

Maternal alloantibodies toward paternally inherited Ags on fetal platelets can cause thrombocytopenia and bleeding complications in the fetus or neonate, referred to as fetal and neonatal alloimmune thrombocytopenia (FNAIT). This is most commonly caused by Abs against the human platelet Ag (HPA)-1a in Caucasians, and a prophylactic regimen to reduce the risk for alloimmunization to women at risk would be beneficial. We therefore aimed to examine the prophylactic potential of a fully human anti–HPA-1a IgG1 (mAb 26.4) with modified Fc region or altered N-glycan structures. The mAb 26.4 wild-type (WT) variants all showed efficient platelet clearance capacity and ability to mediate phagocytosis independent of their N-glycan structure, compared with an effector silent variant (26.4.AAAG), although the modified N-glycan variants showed differential binding to FcγRs measured in vitro. In an in vivo model, female mice were transfused with platelets from transgenic mice harboring an engineered integrin β3 containing the HPA-1a epitope. When these preimmunized mice were bred with transgenic males, Abs against the introduced epitope induced thrombocytopenia in the offspring, mimicking FNAIT. Prophylactic administration of the mAb 26.4.WT, and to some extent the mAb 26.4.AAAG, prior to platelet transfusion resulted in reduced alloimmunization in challenged mice and normal platelet counts in neonates. The notion that the effector silent variant hampered alloimmunization demonstrates that rapid platelet clearance, as seen with mAb 26.4.WT, is not the sole mechanism in action. Our data thus successfully demonstrate efficient Ab-mediated immunosuppression and prevention of FNAIT by anti–HPA-1a monoclonal variants, providing support for potential use in humans. *ImmunoHorizons*, 2022, 6: 90–103.

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Abbreviations used in this article: AMIS, Ab-mediated immune suppression; AP, alkaline phosphatase; EIA, enzyme immunoassay; FNAIT, fetal and neonatal alloimmune thrombocytopenia; HG, high galactose; HPA, human platelet Ag; iMFI, integrated mean fluorescence intensity; LF, low fucose; MAHA, mouse anti-human Ab; MFI, median fluorescence intensity; RT, room temperature; WT, wild-type.

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INTRODUCTION

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a clinical condition caused by generation of maternal IgG Abs directed toward alloantigens on fetal platelets. The human platelet Ag (HPA) most commonly associated with FNAIT in Caucasians is HPA-1a (1). Women lacking this Ag may become alloimmunized when encountering it during pregnancy or in connection with delivery. The maternal IgG Abs are then transferred over the placenta and enter the fetal circulation. At this point, the IgG Abs bind HPA-1a followed by induction of platelet depletion, which renders the fetus prone to bleeding, with the risk for intracranial hemorrhage and lifelong disabilities or, in the worst case, death (2). Most identified cases are recognized after delivery because of bleeding symptoms in the neonate, and the condition is thus underdiagnosed (3). Unfortunately, no preventive or effective treatment for FNAIT is available, but the off-label use of IVIg to treat immunized women is recommended in several clinical guidelines (4).

Alloimmunization may occur during pregnancy; however, data from a prospective screening study in Norway showed that up to 75% of the women were immunized in connection with delivery (5). This opens the possibility for prophylactic treatment to prevent HPA-1a alloimmunization, in line with administration of anti-D IgG prophylaxis that has safely and efficiently prevented alloimmunization to RhD Ag, which has reduced the incidence of hemolytic disease of the fetus and newborn in the past 50 y (6). This notion motivated the development of the hyperimmune human plasma-derived anti–HPA-1a IgG preparation named NAITgam (also referred to as RLYB211), which is currently undergoing clinical trials to evaluate its ability to reduce the incidence of HPA-1a immunizations in HPA-1a–negative pregnant women (7). An even more attractive strategy is the use of a monoclonal IgG Ab specific for HPA-1a as a prophylaxis, because this will allow streamlined manufacturing of a product with a well-defined mechanism of action without the need for plasma supply from immunized individuals.

To develop prophylactic treatment for FNAIT, it may be worthwhile to look into experiences gained on anti-D prophylaxis for hemolytic disease of the fetus and newborn. However, despite decades of research and clinical studies, recombinant monoclonal anti-D prophylactic products have not yet proven to be effective substitutes for the polyclonal products on the market (8). The reason for this is not known, although it could be related to the size of the D-Ag, which could harbor multiple potential epitopes. In contrast, HPA-1a is defined by a single amino acid difference between mother and child (1, 9). Thus, a single HPA-1a–specific Ab will in principle sterically block the entire alloantigenic epitope when bound. The use of prophylactic Abs to prevent alloimmunization is based on the concept of Ab-mediated immune suppression (AMIS) (10), but optimal design for mAbs directed against HPA-1a has not yet been defined.

In this study, we made use of the human anti–HPA-1a, mAb 26.4, derived from a woman who gave birth to a child with severe FNAIT (11). This mAb has been shown to be highly specific to the HPA-1a epitope on integrin β3. Here, we explored it as a prophylactic candidate against HPA-1a immunization by a combination of in vitro and in vivo tests. To do so, we engineered a panel of recombinant IgG1 variants of mAb 26.4 where we introduced specific Fc-region amino acid substitutions or altered the composition of attached N-glycan structures. The panel of isoforms was studied for their ability to interact with recombinant effector molecules, including the plasma half-life regulator FcRn, combined with cellular studies addressing phagocytosis and platelet clearance.

For in vivo studies, we used a recently developed humanized FNAIT preclinical mouse model (12), where five amino acid substitutions (APLDQ) have been engineered into the murine integrin β3, which mimics the human HPA-1a epitope and thus allowing for mAb 26.4 binding. We demonstrate that the mAb 26.4 wild-type (WT) is able to induce rapid platelet clearance and prevent alloimmunization and subsequent FNAIT symptoms in offspring. Notably, an engineered mAb 26.4 variant lacking effector functions also showed dampened immunization, although it was not capable of inducing platelet clearance. These results support further preclinical and clinical studies of mAb 26.4 as a prophylactic drug to prevent HPA-1a alloimmunization in humans.

MATERIALS AND METHODS

Ab production and verification

Vectors encoding recombinant mAb 26.4 isoforms were produced as previously described (13, 14). Briefly, cDNA fragments encoding the 26.4 variable H and L chain regions were codon optimized for mammalian expression and subcloned in-frame with the cDNA sequence of the human IgG1 constant H and L chain regions in pcDNA3.1 expression vectors. In addition to the mAb 26.4.WT, the effector silent variant 26.4.AAAG (L234A, L235A, N297A, P329G) and 26.4.H435A with reduced FcRn binding (15, 16) were produced. mAb 26.4.WT was also produced containing altered N-glycans, low fucose (LF; 26.4.WT.LF), and high galactose (HG; 26.4.WT.HG). The mAb variants were produced using FreeStyle 293-F cells (Thermo Fisher Scientific, Waltham, MA). Variants with modified N297-linked N-glycans were produced by supplying a fucose decoy, 2-deoxy-2-fluoro-L-fucose, posttransfection to decrease fucosylation, or by adding 5 mM D-galactose (Sigma-Aldrich, Burlington, MA) and expression vector encoding β-1,4-galactosyltransferase 1 to the transfection mix to increase galactosylation. The mAb variants were purified using a Protein A HiTrap HP affinity column (GE Healthcare, Chicago, IL), and their integrity was verified by SDS-PAGE. Fc glycosylation, including galactosylation and fucosylation, was assessed by mass spectrometry.

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Ag production and verification

To verify Ab characteristics, we produced recombinant soluble integrin β3 proteins using the baculovirus system (17, 18). Originally designed constructs for the extracellular domain murine integrin β3 (synthesized GeneArt; Thermo Fisher Scientific) were codon optimized for *Spodoptera frugiperda* with XhoI and NheI restriction sites flanking the insert for cloning. Because some construct changes were necessary for this study, tags and epitopes were changed using the GeneArt Site-Directed Mutagenesis System (Thermo Fisher Scientific). Optimized constructs contained a kozak sequence at the N terminus, and a 6xHis and a Twin-Strep-tag (WSHPQFEK-GGGSGGGSGGS-SAWSHPQFEK), followed by two stop codons, at the C terminus. The constructs were first moved into the acceptor vector pFL (gift from EMBL) followed by transformation into EMBacY (19) expressing *Escherichia coli* cells (gift from EMBL) through Tn7 transposition (18). Cells with correctly inserted pFL vector were chosen through blue/white selection. Bacmids were purified by isopropanol precipitation from subcultured EMBacY-expressing *E. coli* cells and used for transfection of Sf9 insect cells (Thermo Fisher Scientific) for baculovirus production. Proteins were expressed in H5 insect cells (Thermo Fisher Scientific) using titered viral stocks with daily monitoring of the coproduced YFP protein by flow cytometry.

After dialysis and concentration of the supernatant using the polyethersulfone VivaFlow 200 system, 30,000 molecular weight cutoff (Sartorius, Göttingen, Germany), the proteins were purified by gravity flow using columns with Strep-Tactin XT Superflow resin (IBA Lifesciences, Göttingen, Germany). The presence and purity of recombinant proteins were verified by nonreducing SDS-PAGE. Proteins were coupled to Dynabeads His-Tag Isolation & Pulldown (Thermo Fisher Scientific) according to protocol and analyzed using anti–integrin β3 Abs (mAb 26.4 and AP-3, both conjugated to Alexa Fluor 488) by flow cytometry.

ELISA

Binding of mAb 26.4 variants to recombinant mouse integrin β3-APLDQ. Ninety-six-well enzyme immunoassay (EIA)/RIA plates (CorningCostar) were coated overnight (4°C) with Ag (1 μg/ml in PBS) and blocked with PBS/4% skimmed milk powder (S) (Acumedia)/0.05% Tween 20 (T) (Sigma-Aldrich) (PBS/S/T). Titration series of the mAb 26.4 variants (1.0 to 0.008 μg/ml) in PBS/S/T were added for 1 h at room temperature (RT). Bound Abs were detected using an alkaline phosphatase (AP)-conjugated goat anti-human κ L chain Ab (1:5000; Sigma-Aldrich) and visualized by addition of phosphatase substrate (10 μg/ml in diethanolamine) (Sigma-Aldrich) before absorbance values (405 nm) were recorded using a Sunrise plate reader (TECAN). PBS/T was used as washing buffer in between each step.

Binding of mAb 26.4 variants to recombinant human FcγR. Ninety-six-well EIA/RIA plates were coated with mouse integrin β3-APLDQ (1 μg/ml PBS) and blocked as described earlier, prior to incubation with titration series of the mAb 26.4 variants (2.0 to 0.016 or 1.0 to 0.00005 μg/ml, PBS/S/T) for 1 h at RT. Then, soluble recombinant GST-tagged FcγRI (0.25 μg/ml), FcγRIIa-R131 (2 μg/ml), FcγRIIb (2 μg/ml), or FcγRIIIa-V158 (2 μg/ml) (20) was added and incubated for 1 h at RT before bound receptors were detected using an HRP-conjugated goat anti-GST Ab (1:5000, PBS/S/T; Rockland Immunochemicals, Limerick, PA). Binding was visualized by addition of TMB solution (Calbiochem), and the enzymatic reaction was stopped with 50 μl 1 M HCl before absorption values (450 nm) were recorded as described earlier. PBS/T was used as washing buffer in between each step.

Binding of mAb 26.4 variants to recombinant mouse and human FcRn. Ninety-six-well EIA/RIA plates were coated with mouse integrin β3-APLDQ (1 μg/ml PBS) and blocked as described earlier, prior to incubation with titration series of mAb 26.4 variants (1.0 to 0.008 μg/ml, PBS/S/T) for 1 h at RT. All remaining steps were performed using phosphate buffer (pH 6.0; 67 mM phosphate, 0.1 M NaCl, 0.05% S/T) or PBS/S/T (pH 7.4) as dilution and wash buffers. Recombinant soluble mouse or human GST-tagged FcRn (21, 22) (1 μg/ml) was added and incubated for 1 h at RT. Bound receptors were detected using an HRP-conjugated anti-GST Ab as described earlier, before absorption values (450 nm) were recorded.

In vivo plasma half-life

Hemizygous FcRn [B6.Cg-*Fcgrt*^{tm1Dcr} Tg(FCGRT)32Dcr/DcrJ] mice (JAX stock no. 014565), which are knockout for mouse FcRn HC (*Fcgrt*^{tm1Dcr}) and express the genomic transgene of the human FcRn HC (FCGRT) under the control of the human FcRn promoter (Tg32), were used to determine plasma half-life. Male mice aged 7–9 wk, weighing between 17 and 30 g, received 1 mg/kg mAb 26.4 variants by i.v. administration. Blood samples (25 μl) were drawn from the retro-orbital sinus at days 1, 2, 3, 5, 7, 10, 12, 16, and 19 postadministration and were mixed with 1 μl 1% K3-EDTA to prevent coagulation followed by centrifugation at 17,000 × *g* for 5 min at 4°C. Plasma was isolated and diluted 1:10 in 50% glycerol/PBS solution and stored at −20°C. Half-life data were plotted as percent of Ab remaining compared with the first concentration measured. Data points from the β-phase were used to calculate half-life using the following formula:

$$t\frac{1}{2} = \frac{\log(0.5)}{\log\left(\frac{A_c}{A_o}\right)} \times t,$$

where $t_{1/2}$ is the half-life of the Ab, A_c is the amount of Ab remaining, A_o is the original amount of Ab at day 1, and t is the elapsed time (23). The experiment was performed by JAX Services at The Jackson Laboratory, and samples were shipped for analyses.

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Quantification of 26.4 mAb variants in plasma

26.4 mAb variants in plasma were captured on a mouse anti-human IgG Fc mAb (1 μg/ml) (Southern Biotechnology, Birmingham, AL) coated in 96-well EIA/RIA plates (CorningCostar) detected by an AP-conjugated polyclonal goat anti-human κ L chain Ab (Southern Biotechnology) (1:5000), which was visualized by adding the AP substrate (10 μg/ml in diethanolamine) (Sigma-Aldrich). Absorbance was measured at 405 nm and recorded as described earlier. PBS/T was used as washing buffer in between each step.

Phagocytosis assay using THP-1 cells

FluoSpheres Carboxylate-Modified Microspheres (1.0 μm yellow-green fluorescent; Thermo Fisher Scientific) were coupled to recombinant murine integrin β3-APLDQ protein according to protocol. Briefly, proteins and microspheres were incubated overnight at RT in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide at pH 6.5. After removal of unbound protein, beads were blocked with BlockAid solution (Thermo Fisher Scientific) and stored at 4°C. THP-1 (ATCC TIB-202) cells were cultured at 37°C and 5% CO_2 in ATCC-modified RPMI 1640 medium (Thermo Fisher Scientific), 0.05 mM 2-ME, and 10% FBS.

The phagocytosis assay protocol was optimized from Ackerman et al. (24). In a 96-well U-bottom plate, 10 μl Fluospheres at ∼3.6 × 10^8/ml were incubated with 10 μl 26.4 Ab (20 μg/ml) for 2 h at 37°C. After washing, beads were resuspended by supplying 200 μl THP-1 cells at 2.5 × 10^5/ml. The plate was incubated overnight at 37°C and 5% CO_2. A total of 100 μl supernatant was replaced with 100 μl 4% paraformaldehyde before analyzing the cells using high-throughput screening on BD LSRFortessa (BD Biosciences, San Jose, CA). The gate was set around live THP-1 cells, and 10,000 events were collected. Integrated mean fluorescence intensity (iMFI) was calculated by multiplying the percentage of bead-positive cells with the mean fluorescence intensity of bead-positive cells (24). 26.4.WT and 26.4.AAAG were incubated with APLDQ-coated FluoSpheres as described earlier, followed by incubation with THP-1 cells for 1 h. The cells were analyzed by Amnis ImageStreamX Mk II (Luminex, Austin, TX) using 488 laser 0.3 mW and ×60 focus. The data were further analyzed using the IDEAS software and Internalization Wizard.

Mice

BALB/c WT female mice (6–10 wk) and C57BL/6N mice (both sexes; 8 wk) were purchased from Charles River. C57BL/6N-APLDQ mice (in-house) were mainly used for blood platelet isolation (both sexes; >8 wk of age). Mice were maintained in the Biological Resource Center at the Medical College of Wisconsin.

All animal protocols were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee and performed according to the human and animal experimentation guidelines of the U.S. Department of Health and Human Services and in adherence to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Blood sampling

Blood from adult mice was sampled using EDTA-coated capillary tubes from the submandibular vein. Platelet count was measured using a ScilVet ABC (scil Animal Care Company, Gurnee, IL). Samples were diluted 1:3 in PBS with 0.3% EDTA and collected after centrifugation at 700 × *g* for 15 min. Blood from pups was collected within 48 h of delivery from the submandibular vein using heparinized microhematocrit capillary tubes (Thermo Fisher Scientific) and transferred to tubes with anticoagulant acid-citrate-dextrose. All pups were euthanized directly after blood collection. Samples were diluted 1:3 in PBS with 0.3% EDTA and collected after centrifugation at 700 × *g* for 15 min. All samples were stored at −70°C until use.

Platelet isolation, immunization, and prophylaxis

APLDQ mice were anesthetized using a ketamine mix (20 mg/ml ketamine and 2 mg/ml xylazine) 0.5 ml/20 g body weight injected i.p. When deep anesthesia was reached, the mouse was strained and opened, exposing the posterior vena cava. Blood was drawn slowly into syringes prefilled with anticoagulant acid-citrate-dextrose. Death was assured by cervical dislocation. Blood collected from the APLDQ mice was pooled, diluted 1:2 in PBS, and layered on top of Fico/Lite-LM (Atlanta Biologicals, Flowery Branch, GA). The tubes were centrifuged at 350 × *g* for 15 min without brakes. Platelet-rich plasma was recovered, and the platelet count was measured as described earlier. Washed platelets were resuspended to 5 × 10^8/ml in PBS. Platelets (1 × 10^8/dose) were injected i.v. into female BALB/c mice to be immunized (days 2 and 9). 26.4 Abs were diluted in PBS, 20 μg Ab/200 μl, and injected i.v. 24 h prior to APLDQ platelet transfusion for prophylactic experiments (days 1 and 8).

Breeding

At least 3 d after the last blood collection, BALB/c females from the prophylaxis experiment were placed in separate cages and introduced to APLDQ males. Within 48 h after delivery, pups were weighed, and blood samples were drawn as described earlier. Both blood platelet count and the presence of anti-APLDQ Abs in plasma were analyzed.

Plasma analysis

Abs against mouse platelets. To analyze Abs in plasma from immunized mice or pups, 96-well U-bottom plates were blocked with 1% PBSA for 1 h. 1 × 10^6 platelets from APLDQ mice or control mice and plasma samples (end dilution 1:6 to test for mouse anti-APLDQ Abs and 1:12 to test for 26.4 Abs), were incubated for 30 min at RT. FITC-conjugated anti-mouse IgG or anti-human IgG (Jackson ImmunoResearch, Philadelphia, PA) diluted 1:200 was incubated for 30 min at RT, and Ab

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binding was measured using BD Accuri C6 (BD Biosciences). A total of 10,000 events were collected from the platelet gate.

Mouse anti-human Abs. 26.4.WT Abs were coupled to Dynabeads M-270 Epoxy (Thermo Fisher Scientific) following Ab-coupling protocol. Briefly, washed beads were incubated with Ab on a roller at 37°C overnight. Washed beads were stored at 4°C until use.

Ab-coated beads (500,000 beads/well) were incubated with murine plasma (end dilution 1:200) by shaking for 20 min. Mouse anti-human Abs (MAHAs) in plasma were detected using anti-mouse IgG conjugated with Alexa Fluor 647 (1:500; Jackson Immunoresearch), and beads were analyzed using high-throughput screening on BD LSRFortessa (BD Biosciences).

Statistical analysis

ELISA data are plotted as mean ± SD. Flow cytometric data are plotted with median fluorescence intensity (MFI) and presented as mean ± SD. Statistical analyses were done using GraphPad Prism 9.2.0 software by ordinary one-way ANOVA and Tukey multiple comparison test (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$).

RESULTS

IgG1 mAb 26.4 variants engineered for distinct effector molecule binding properties

The preferred effector function properties of a prophylactic mAb to prevent HPA-1a immunization should be tailored prior to clinical testing in humans, and the requirement may vary depending on the treatment regimen. Thus, to study the effect of modified Fc-mediated effector functions, we engineered a panel of IgG1 isoforms based on mAb 26.4 (Fig. 1A). To completely abolish the ability of WT IgG1 mAb 26.4 (26.4.WT) to engage all classical human FcγRs and the complement factor C1q, we introduced four amino acid substitutions in the CH2 domain of the H chain, namely, L234A, L235A, N297A, and P329G (AAAG) (25). Notably, N297 is the only N-glycan site in the Fc, and distinct biantennary glycan structures may modulate binding to individual effector molecules (26). As such, we

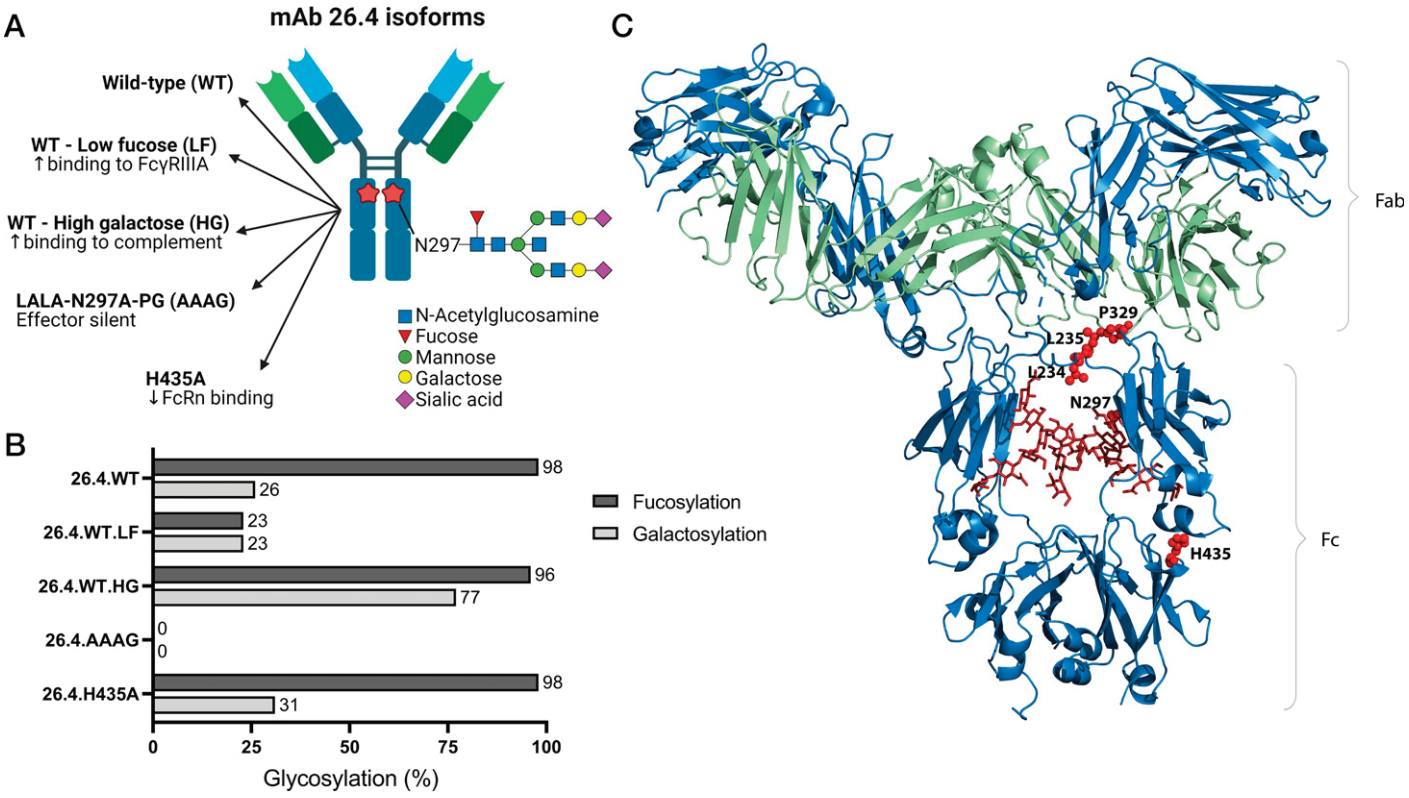

FIGURE 1. Design of anti–HPA-1a mAb 26.4 isoforms with different Fc regions.

(**A**) Schematic illustration of a monoclonal anti-HPA-1a IgG1 26.4 Ab with different modifications highlighted (created with BioRender.com). (**B**) The N-glycan structures attached to N297 of recombinantly produced 26.4 variants were analyzed by mass spectrometry to map the content of fucose and galactose. (**C**) Illustration showing the crystal structure of a human IgG1 molecule. The heavy chains are shown in blue, light chains in green, and N297-linked N-glycans as red sticks. The amino acid residues L234, L235, P329, N297, and H435 are shown as red spheres. The figure was made in PyMOL using the crystallographic data from PDB: 1HZH. AAAG, LALA-N297A-PG.

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produced recombinant 26.4.WT variants with LF (23% fucose versus 98% in WT) and HG (77% galactose versus 26% in WT) content (Fig. 1B), which enhances binding to FcγRIIIa on NK cells (27) and binding to C1q, respectively (28).

The designed mAbs were produced by FreeStyle 293-F cells, and secreted Abs were purified with expected molecular size as determined by SDS-PAGE analysis (Supplemental Fig. 1A).

To confirm functional binding to human integrin β3, we produced recombinant soluble proteins. Size and purity were confirmed by SDS-PAGE analysis (Supplemental Fig. 2A), and appropriate epitope conformation was confirmed by reactivity with anti–integrin β3 mAbs (AP-3 and 26.4) in flow cytometry (Supplemental Fig. 2B).

After coating of the recombinant integrin β3 in ELISA wells, we added serial titrations of the purified mAb 26.4 variants followed by detection, which revealed that they bound the HPA-1a epitope equally well (Supplemental Fig. 1B, 1C). This was also the case when tested for binding to a recombinant version of the murine integrin β3 with the five amino acid substitutions (APLDQ), which introduce the human HPA-1a epitope (Fig. 2B). Thus, neither the AAAG substitutions nor alteration of posttranslational modification of the attached N-glycan structures affected Ag–Ab interactions.

Next, this ELISA setup was used to compare mAb binding with recombinant soluble versions of human FcγRs (Fig. 2A), which revealed that the AAAG substitutions completely eliminated binding to the high-affinity FcγRI (Fig. 2C), as well as the low-affinity FcγRIIa (Fig. 2D), FcγRIIb (Fig. 2E), and FcγRIIIa (Fig. 2F). In addition, reduced fucosylation of the N-glycans attached to N297 dramatically enhanced binding to FcγRIIIa (Fig. 2F), as expected. In terms of potential FcγR-mediated suppression of B cell activation (29, 30), 26.4.WT.LF showed increased binding to FcγRIIb compared with 26.4.WT (Fig. 2E), but also to FcγRIIa (Fig. 2D). The presence of galactose slightly enhanced binding to the low-affinity receptors compared with 26.4.WT (Fig. 2D–F).

To verify the FcRn binding properties of the designed mAb 26.4 variants, we used the ELISA setup as described earlier, followed by adding recombinant forms of human and mouse FcRn (Fig. 3A), relevant for both the potential future in vivo use in humans and the current mouse model. In addition, a variant of 26.4 with the H435A substitution (26.4.H435A) was produced

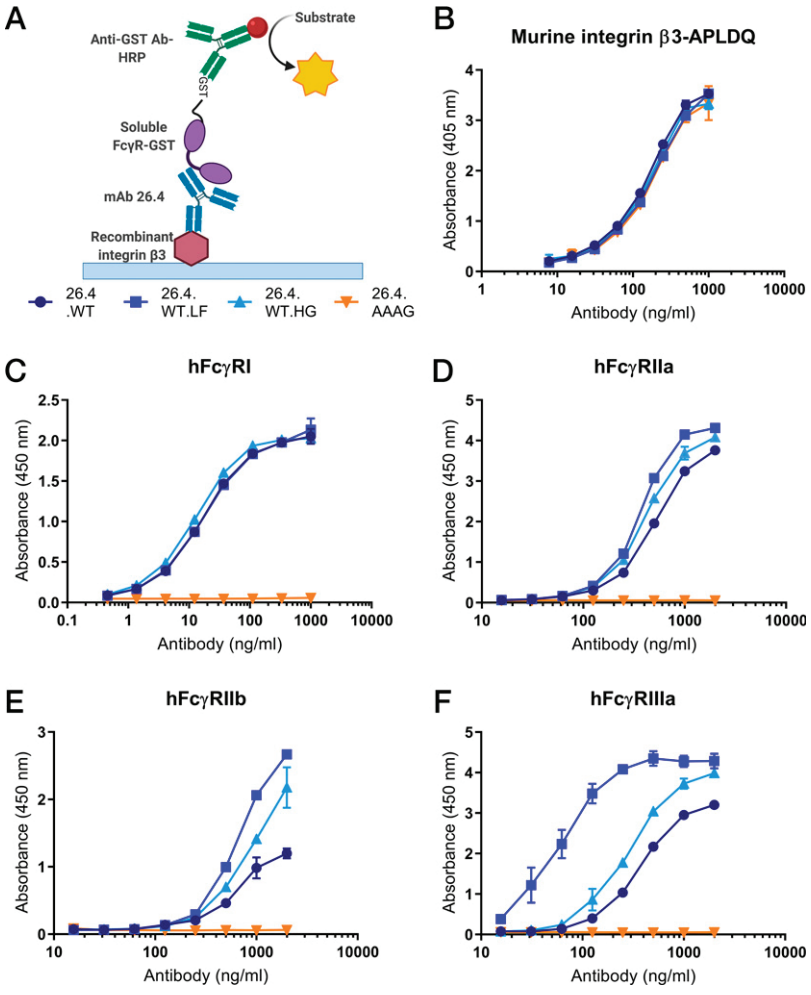

FIGURE 2. mAb 26.4 variants show distinct FcγR binding properties.

(**A**) Schematic illustration of ELISA protocol for FcγR binding (created with BioRender.com). (**B**) Binding of titrated amounts of mAb 26.4 variants to recombinant integrin β3, a murine backbone, engineered with the HPA-1a epitope (APLDQ), coated in wells, and detected with an anti-human Fc Ab by ELISA. Each point is a mean of duplicates from one representative experiment out of two, and error bars indicate SD. (**C**–**F**) Binding of GST-tagged soluble recombinant human FcγRI (C), FcγRIIa-R131 (D), FcγRIIb (E), and FcγRIIIa-V158 (F) to titrated amounts of the mAb 26.4 variants captured on the integrin β3-APLDQ and detected with an HRP-conjugated anti-GST Ab. Each point is a mean of duplicates from one experiment, and error bars indicate SD. AAAG, LALA-N297A-PG.

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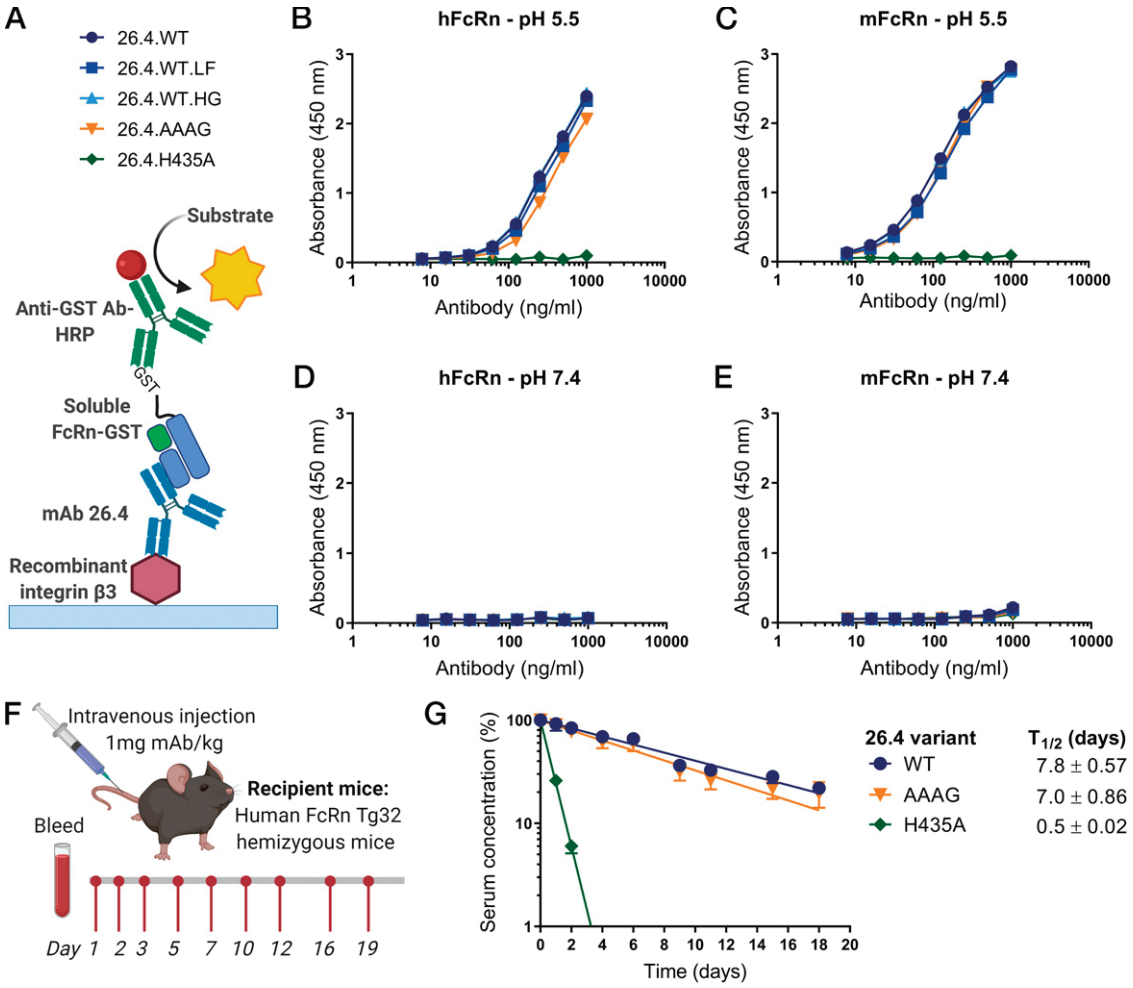

FIGURE 3. FcRn binding properties of the designed mAb 26.4 variants.

(**A**) Schematic illustration of ELISA protocol for FcRn binding (created with BioRender.com). (**B–E**) Binding of recombinant GST-tagged soluble human (B and D) and mouse (C and E) FcRn to titrated amounts of the mAb 26.4 variants captured on recombinant integrin β3, a murine backbone, engineered with the HPA-1a epitope (APLDQ), at pH 5.5 (B and C) and pH 7.4 (D and E) measured by ELISA. Each point is a mean of duplicates from one representative experiment out of three, and error bars indicate SD. (**F**) Schematic illustration (created with BioRender.com) of the in vivo setup to measure the plasma half-lives of three mAb 26.4 variants injected (1 mg/kg) into human FcRn Tg32 hemizygous mice. Blood samples were drawn at days 1, 2, 3, 5, 7, 10, 12, 16, and 19 postadministration. (**G**) The presence of the mAb 26.4 variants in plasma derived from the Tg32 mice (n = 5 per Ab) measured by ELISA and analyzed using nonlinear regression analysis (GraphPad Prism 7) followed by plotting as percentage compared with day 1 (100%). Each point is a mean of duplicates, and error bars indicate SD. $t_{1/2}$ is presented as days ± SD. AAAG, LALA-N297A-PG.

as a control Ab for reduced FcRn binding (15, 16). The results demonstrated equal binding of the 26.4 variants to the two receptor species at pH 5.5, except for 26.4.H435A (Fig. 3B, 3C), while none of them bound at pH 7.4 (Fig. 3D, 3E). Hence this is in line with the fact that the AAAG substitutions are structurally distal from the principal FcRn binding site (Fig. 1C) and, as such, are not expected to affect the plasma half-life. To address the latter, we used mice transgenic for human FcRn, which are the state-of-the-art model for plasma half-life determination of human IgG1 candidates (23). After i.v. administration of 26.4.WT and the AAAG variant, blood samples were collected over time, and the presence of the Abs in isolated plasma was quantified by ELISA (Fig. 3F). The results revealed a plasma half-life of 7–8 d for the mAbs; however, as a control for FcRn engagement, injection of 26.4.H435A gave rise to ∼14-fold shorter plasma half-life (Fig. 3G).

mAb 26.4 variants induce in vitro phagocytosis and in vivo platelet clearance

For in vitro functional testing of the capacity of the Ab variants to induce phagocytosis, we measured uptake by THP-1 cells in a flow cytometric assay after binding to fluorescent beads with recombinant APLDQ Ag. Ab-dependent phagocytosis was efficient, independent of N-glycan difference, for all three mAb 26.4 WT variants (26.4.WT, 26.4.WT.LF, 26.4.WT.HG; Fig. 4A), in contrast with the binding differences in FcγRIIa (Fig. 2D)

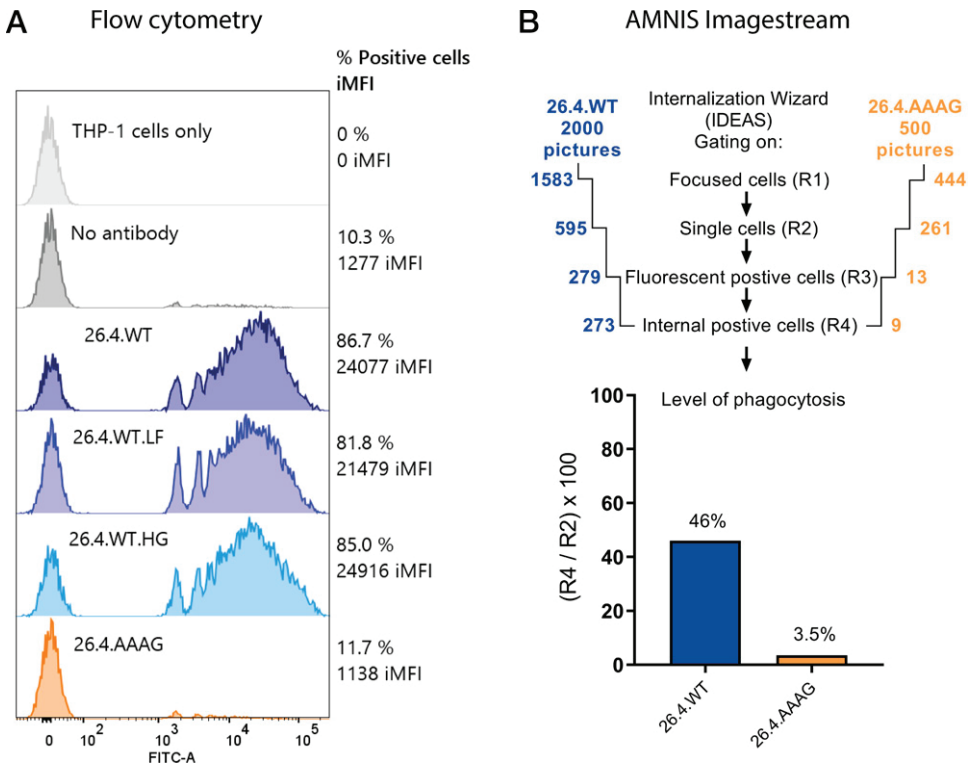

FIGURE 4. FcγR-mediated phagocytosis requires a fully functional Fc region of mAb 26.4.

(**A**) Yellow-Green FluoSpheres coupled with recombinant integrin β3, a murine backbone, engineered with the HPA-1a epitope (APLDQ), coated with 10 μg/ml mAb 26.4 variants. Ab-dependent phagocytosis was measured in THP-1 cells after overnight incubation and measurement of 10,000 events in cell gate by flow cytometry. Data represent one experiment of multiple experiments with different time points and concentrations showing the same trend. THP-1 cells that were yellow-green fluorescent (intensity $> 10^3$) are denoted with percentage, and iMFI was calculated with the formula: iMFI = percentage THP-1^+ events × mean fluorescence intensity of positive events. (**B**) To confirm engulfment and not sticking of beads, 26.4.WT Ab (2000 pictures analyzed) and 26.4.AAAG Ab (500 pictures analyzed) were examined in a 1-h phagocytosis assay, measured by Amnis ImageStreamX Mk II (laser 488 0.3 mW and focus ×60; Luminex), and analyzed using the guide Internalization Wizard in the software IDEAS. Briefly, internalization was analyzed through multiple steps by gating on focused cells (R1), single cells (R2), fluorescent-positive cells (R3), and internal positive cells (R4). AAAG, LALA-N297A-PG.

and FcγRIIIa (Fig. 2F) binding in ELISA. However, because phagocytosis is facilitated through multiple FcγRs on THP-1 cells (24, 31), the low FcγRIIIa expression in nondifferentiated monocyte-like THP-1 cells (32) may explain the poor correlation between phagocytic activity and FcγR binding efficacy for the Ab variants. To further support that the read-out in flow cytometry represents internalization of beads rather than mere surface binding, we also demonstrated internalization with 26.4.WT by imaging flow cytometry (Fig. 4B).

Because the prophylactic effect may depend on platelet clearance, 26.4.WT glycosylation variants (LF and HG) and 26.4.AAAG were injected into humanized APLDQ-expressing mice (Fig. 5A). 26.4.WT and glycosylation variants induced efficient platelet clearance in vivo, measured at 5 and 24 h after injection (Fig. 5B), with normalized platelet counts after 7 d. In contrast, 26.4.AAAG did not cause platelet clearance, although it was demonstrated to be bound on platelets at 5 and 24 h, but not detected after 7 d (Fig. 5C). To further investigate how the differential properties identified by in vitro studies translated into immunosuppression in the preclinical model, we included 26.4.WT and 26.4.AAAG in subsequent experiments.

Administration of mAb 26.4 prevents immunization against HPA-1a in vivo

By the use of an FNAIT mouse model where murine APLDQ platelets lead to the generation of anti-APLDQ Abs when injected into BALB/c mice, the prophylactic potential of the two mAb 26.4 variants was studied by injecting 20 μg mAb 24 h prior to platelet transfusion (Fig. 6A). Efficient endogenous anti-APLDQ Ab responses were seen at days 14 and 21 in mice challenged with platelets only, while prophylactic treatment with 26.4.WT completely prevented this immune response (Fig. 6B). Mice injected with 26.4.AAAG showed detectable anti-APLDQ Abs at day 14, but the mean level was significantly lower than in the immunization control group at day 21 ($p = 0.0008$). The administered Abs were both detectable in plasma at day 7 (Fig. 6D), while only 26.4.AAAG was present in plasma at days

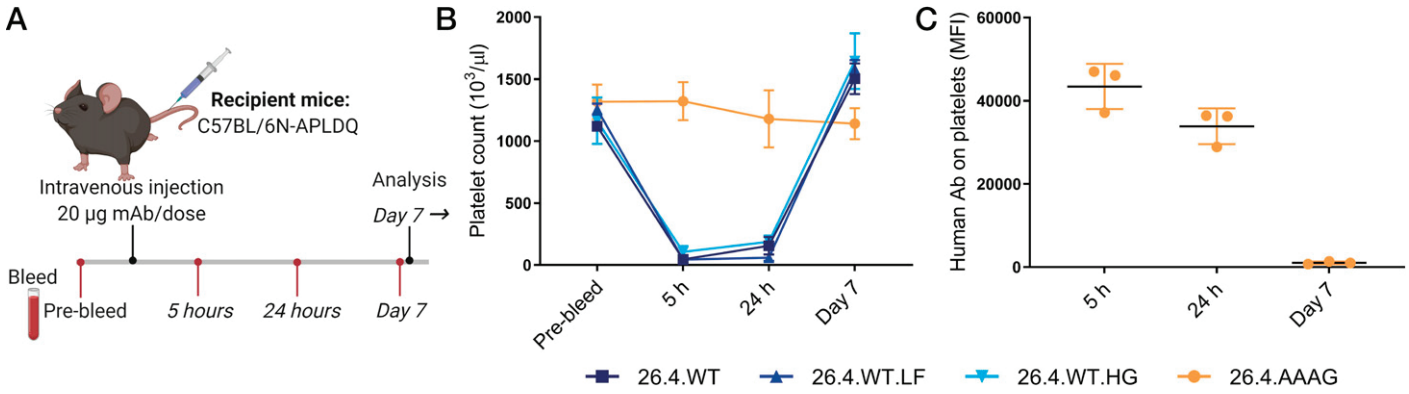

FIGURE 5. APLDQ mice injected with mAb 26.4 eliminate platelets from circulation.

(**A**) Schematic illustration (created with BioRender.com) of C57BL/6N-APLDQ mice receiving mAb 26.4 injections i.v. following a prebleed. Blood samples were taken at 5 h, at 24 h, and after 1 wk. (**B**) Platelet count of APLDQ mice injected with 20 µg mAb 26.4 isoforms. (**C**) Platelets from APLDQ mice injected with 26.4.AAAG were isolated from blood samples and incubated with FITC-conjugated anti-human Ab to detect Abs on the platelet surface by flow cytometry, gated on platelets. MFI is plotted ($n = 3$) with mean and SD. AAAG, LALA-N297A-PG.

14 and 21. Notably, generation of MAHAs was observed in plasma from 26.4.WT-injected mice at day 14, but interestingly, not against 26.4.AAAG (Fig. 6C). All plasma samples showed no or very low Ab reactivity against platelets from BALB/c and C57BL/6N mice (Supplemental Fig. 3).

To assess the effect on clinical outcome, we bred the preimmunized female mice from the prophylaxis experiment with APLDQ males. No remaining 26.4.AAAG Abs were detected in plasma from pups or moms in the 26.4.AAAG group (Fig. 6E). Pups from immunized mice showed significantly reduced platelet counts ($p < 0.0001$), whereas pups from Ab-treated females all presented normal platelet counts (Fig. 6F), even though detectable levels of anti-APLDQ Abs were seen at day 14 in 26.4.AAAG-treated mice. When measuring the anti-APLDQ Ab levels in plasma of both mothers and pups, only pups from immunized mothers showed the presence of anti-APLDQ Abs (Fig. 6G). Taken together, these data provide the proof of principle that mAb 26.4 has potential as a specific drug for FNAIT prophylaxis by hindering immunization in BALB/c mice against transfused APLDQ platelets carrying the HPA-1a epitope. Prophylactic administration of the mAb 26.4 WT, and to some extent the mAb 26.4 effector silent variant, prior to platelet transfusion resulted in reduced alloimmunization in challenged mice and normal platelet counts in neonates.

DISCUSSION

The purpose of the study was to explore the prophylactic potential of a panel of recombinant isoforms of mAb 26.4 to prevent HPA-1a immunization in a recently developed preclinical FNAIT mouse model (12). The HPA-1a homologous APLDQ epitope has been shown to be a target for several polyclonal alloanti–HPA-1a sera, and several, but not all, monoclonal anti–HPA-1a Abs tested (33). Importantly, mAb 26.4 binds efficiently to the APLDQ epitope while being nonreactive with WT murine platelets, making this preclinical model highly suitable for in vivo therapeutic (12) and prophylactic evaluation. Indeed, mAb 26.4 showed great potential as an FNAIT prophylaxis by inducing AMIS in BALB/c mice transfused with APLDQ platelets, with no sign of thrombocytopenia in subsequent neonates.

A requirement for a prophylactic mAb is that it exclusively binds HPA-1a, thereby eliminating any harmful interaction with maternal HPA-1bb platelets. However, the optimal design of a prophylactic recombinant mAb for prevention of HPA-1a alloimmunization in humans may also depend on the planned timing of administration. For a tentative postpartum treatment strategy, the prophylactic effect may well be immediate and without the need for a long plasma half-life. In contrast, antenatal prophylactic mAb administration requires attention to the risk of potential pathogenic effect on placenta and fetal platelets. With this in mind, we designed mAb 26.4 variants as prophylactic candidates, with and without effector functions.

Although the plasma half-life of IgG1 is 3 wk on average in humans, clinical studies have shown that the half-lives of IgG1 mAbs vary greatly, between 6 and 32 d, despite the fact that they all contain the same Fc (34). One reason for this is that the composition of the variable regions may affect cellular handling, and subsequently pharmacokinetics. Such effects may be dependent on the interaction with the half-life regulator FcRn (35, 36), which is a cellular receptor that interacts in a strictly pH-dependent manner at the CH2-CH3 elbow region of the IgG Fc (37–39). Specifically, this mode of binding is regulated by protonation of histidine residues (H310 and H435) (Fig. 1C), allowing strong binding at acidic pH (5.0–6.0) and no binding or release at neutral pH. Importantly, this pH-dependent binding is also required for FcRn-mediated mother-to-fetus transport across the placenta (40–44). The plasma half-lives of 26.4.WT and 26.4.AAAG were determined to be 7–8 d in the human FcRn in vivo

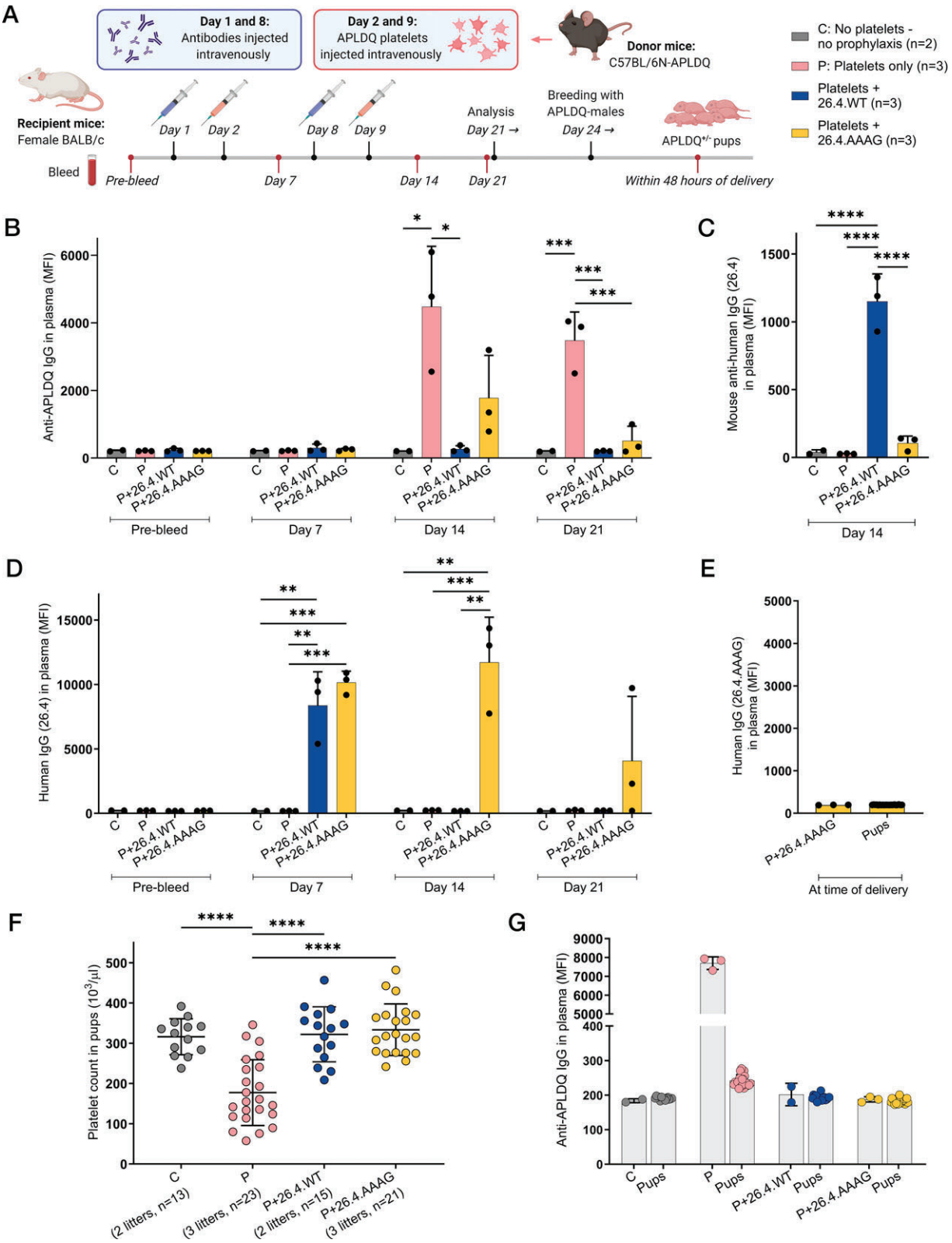

FIGURE 6. mAb 26.4 isoforms induce AMIS in FNAIT mouse model.

(**A**) Schematic illustration (created with BioRender.com) of the FNAIT mouse model where BALB/c mice receive Ab injections (20 µg/dose) at days 1 and 8, and APLDQ platelets (10^8/dose) isolated from C57BL/6N-APLDQ mice on days 2 and 9. Mice were bled 1 week prior to experiment start and at days 7, 14, and 21. After plasma analysis for anti-APLDQ mouse IgG Abs (**B**), MAHAs (**C**), and human IgG Abs (**D**), the mice ***(Continued)***

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mouse model. This is a relatively long plasma half-life in this mouse model, which predicts that the pharmacokinetics in humans should be favorable.

The mechanisms that likely contribute to AMIS have been reviewed for anti-D Abs against RhD immunization (29, 30). Briefly, by the Ag clearance or destruction theory, the particles containing Ags are rapidly removed through Fc-mediated mechanisms, such as Ab-dependent cellular phagocytosis by splenic macrophages or Ab-dependent cellular cytotoxicity. In the B cell inhibition theory, the BCR binds the Ag concurrently with the inhibitory FcγRIIb on the B cell binding to the Fc region of Abs bound to the same particle (coinhibition), thus hindering further proliferation, maturation, and Ab secretion of the B cell. These two theories are the most likely mechanisms behind AMIS, but also epitope masking and Ag modulations may contribute. Accordingly, Ag clearance in this model is represented as platelet clearance, which was efficiently induced by mAb 26.4.WT variants when injected into APLDQ mice, in line with recent data on this model (12). In contrast, the effector silent 26.4.AAAG did not induce in vivo platelet clearance, although present on the platelet surface.

In the immunization experiment with prophylactic administration of mAb 26.4, the generation of murine anti-APLDQ Abs was prevented by 26.4.WT but also significantly reduced with 26.4.AAAG. Because this engineered isoform lacks effector functions, the less efficient prevention by 26.4.AAAG is likely due to epitope masking only, as seen in the platelet clearance experiment, and less efficient removal of either Ab or bound transfused platelets. Thus, the prophylactic effect of 26.4.AAAG demonstrates that rapid platelet clearance is not the sole mechanism of action at the relatively high dose (20 μg Abs/mouse) used in this study. For RBC alloimmunization, there are indications that AMIS most often confers Ag-specific prevention (45), but on high-density Ag expression also non–epitope-specific prevention to neighboring Ags, tentatively by both epitope masking and steric hindrance (46). However, because no or very low Ab responses were detected to other alloantigens on C57BL/6N platelets in all groups, our results could not indicate whether the protection was solely epitope specific (Supplemental Fig. 3).

In subsequent breeding experiments, litters from mice that received Ab prophylaxis had normal platelet counts, including those in the 26.4.AAAG group that had only partially prevented immunization. Circulating 26.4.AAAG Abs in females during pregnancy could potentially be protective against fetal platelet clearance by pathogenic maternal anti-APLDQ Abs. However, the remaining low levels of 26.4.AAAG Abs at the time of breeding and the notion that no 26.4.AAAG Abs were detected in plasma from either pups or mothers at time of delivery makes it unlikely that these Abs have masked epitopes on fetal platelets to the level of protection.

Plasma from mice given both APLDQ platelets and prophylactic Abs, may eventually contain prophylactic IgG in addition to endogenous murine anti-APLDQ IgG. Even with use of species-specific secondary reagents for IgG detection, the human prophylactic Ab and the murine anti-APLDQ Abs may compete for the same epitope on the APLDQ test platelets and thus might impact the read-out of Ab detection. However, this is not likely impacting our data because both anti-APLDQ Abs and 26.4.AAAG Abs plasma levels were reduced from day 14 to 21 in the 26.4.AAAG mice. Further, it is known that human Abs can trigger generation of MAHA (anti-drug Abs) in preclinical models. Interestingly, the 26.4.AAAG variant raised little MAHA response, which otherwise could negatively affect its protective effect.

The proof of concept for AMIS, relevant for FNAIT, was previously demonstrated as dampened immune response by injecting murine anti-integrin β3 Abs into β3-knockout mice after transfusion of WT platelets (47). In addition, human polyclonal anti–HPA-1a Abs from alloimmunized women or murine mAb SZ21 also induced AMIS in the same model when transfusing human HPA-1a$^+$ platelets (47). However, AMIS was also induced in knockout mouse models for activating or inhibitory FcγR using intact Ab, but also with the use of Fab fragments, indicating that AMIS can occur through Fc- and FcγR-independent pathways (48). Although convincingly demonstrating AMIS in the earlier models, their intrinsic drawback is the lack of ability to include the specific epitope relevant to test human Ab candidates on otherwise normal murine platelets.

In our model, APLDQ immunization mimicking HPA-1a alloimmunization can be achieved by either i.p. injections with adjuvant or i.v. by tail-vein injections. In the literature, the administration protocols of prophylaxis in AMIS/clearance experiments varies from protocols where injection occurs prior to administering a dose of immunizing agent, to ex vivo incubation of Ag and Ab prior to injection, to simultaneous administration of Ag and Ab, or to Ab administration shortly after Ag

(n = 2–3) were bred with APLDQ males and newborn pups measured for the presence of 26.4.AAAG in plasma (**E**), platelet count (**F**), and anti-APLDQ Ab levels (**G**). (B and C) APLDQ platelets were isolated and incubated with plasma samples followed by detection using FITC-conjugated anti-mouse IgG Ab (B) or anti-human IgG Ab (D) measured by flow cytometry gated on platelets. (C) MAHAs were detected from plasma on day 14 using Dynabeads coated with mAb 26.4 and detection using Alexa Fluor 647–conjugated goat anti-mouse Ab measured by flow cytometry gated on a single bead population. (E–G) Blood samples were collected from newborn pups within 48 h of delivery, and presence of 26.4.AAAG Abs was measured in plasma from pups and moms in the 26.4.AAAG group (E), platelet count (F) was measured using ScilVet ABC (scil animal care company), and anti-APLDQ Abs (G) measured in both pups and moms. MFI is plotted with mean and SD. Statistical data were analyzed with one-way ANOVA and Tukey multiple comparison test of means against mean of immunized mice (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$). Nonsignificant comparisons were not shown. AAAG, LALA-N297A-PG.

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administration (47, 49–51). Here, we administered the prophylactic Abs 24 h prior to platelet transfusion, to ensure presence of prophylactic Abs in circulation. By using tail-vein administration without adjuvant, we mimic Ag exposure through fetomaternal hemorrhage during pregnancy or in connection with delivery. Notably, by using this i.v. immunization protocol, the Ab responses did not reach levels achieved with i.p. injections with adjuvant, and accordingly, FNAIT was less severe in affected pups (12). We currently have no data on the fate of the transfused APLDQ platelets with regard to the persistence in circulation and anatomical location for destruction in vivo after administration of the different prophylactic Ab variants. Future in vivo studies on this may give further insight into the preventive mechanism. The mAb 26.4 has previously been reported not to induce aggregation of HPA-1aa platelets, in line with other anti–HPA-1a Abs (11, 52). A similar impact on murine APLDQ platelets was not investigated in this study because the transfused platelets served mainly as Ag in our model. However, given that a change in integrin β3 because of mAb 26.4 binding likely depends on the Ab–epitope interaction, all Ab isoforms should exert the same effect. We did not find any indications that the effector silent mAb 26.4.AAAG caused platelet aggregation in vivo in APLDQ mice, because opsonized platelets were still circulating after 24 h.

From a translational perspective, the human HPA-1a epitope on αIIbβ3 is in addition to being abundantly expressed on platelets, also present on the αvβ3 on placental syncytiotrophoblasts (53) and endothelial cells (54). Importantly, maternal anti–HPA-1a Ab selectively reactive with αVβ3 complexes on endothelial cells has been reported to be more strongly associated with FNAIT-associated intracranial hemorrhage than those selectively reactive with αIIbβ3 (54). Relevant to this, mAb 26.4 binds independently of the α subunit (11) and also to the β3 subunit alone (Supplemental Fig. 1B). For postpartum prophylactic administration of mAb 26.4, this may be appropriate, while for antenatal prophylactic use, an effector silent isoform therefore could be preferential to avoid harmful effects on placenta, endothelial cells, or platelets of fetal origin. However, future dosage experiments may conceivably balance the dose required for prevention and undesirable interaction with fetal Ag for antenatal use. In theory, a variant with abolished binding to FcRn and thus limited transport into fetal circulation would limit the risk of pathogenic effect in the fetus. However, because the IgG1 half-life in circulation and transplacental transport are both dependent on FcRn, such a prophylactic variant would require a design that could allow dissection of these two parameters.

In conclusion, we have successfully shown that variants of the mAb 26.4 can prophylactically prevent FNAIT in the APLDQ preclinical model, supporting further studies toward use in humans.

DISCLOSURES

P.J.N. is a consultant for RallyBio Inc. in the field of platelet immunology. B.S. belongs to a group of founders and owners of Prophylix AS, a Norwegian biotech company that produced the hyperimmune anti–HPA-1a IgG for the prevention of HPA-1a immunization and FNAIT now under further development by RallyBio Inc. The other authors have no financial conflicts of interest.

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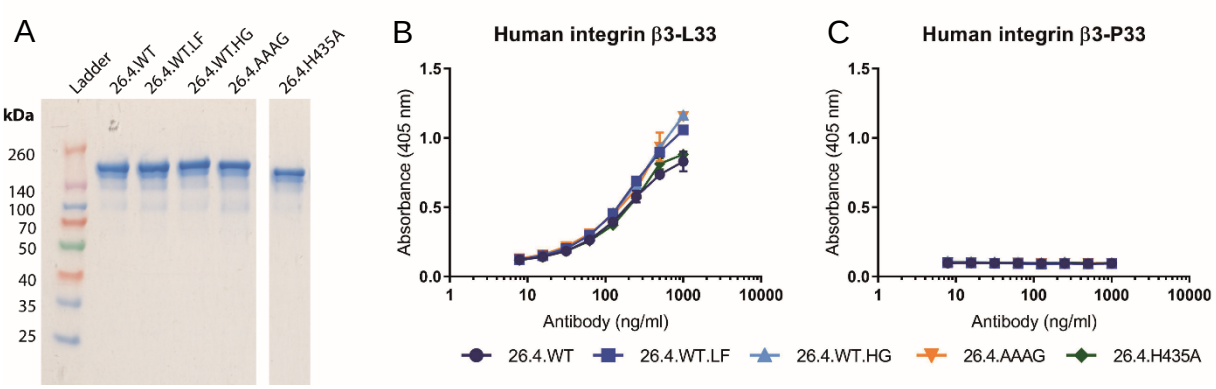
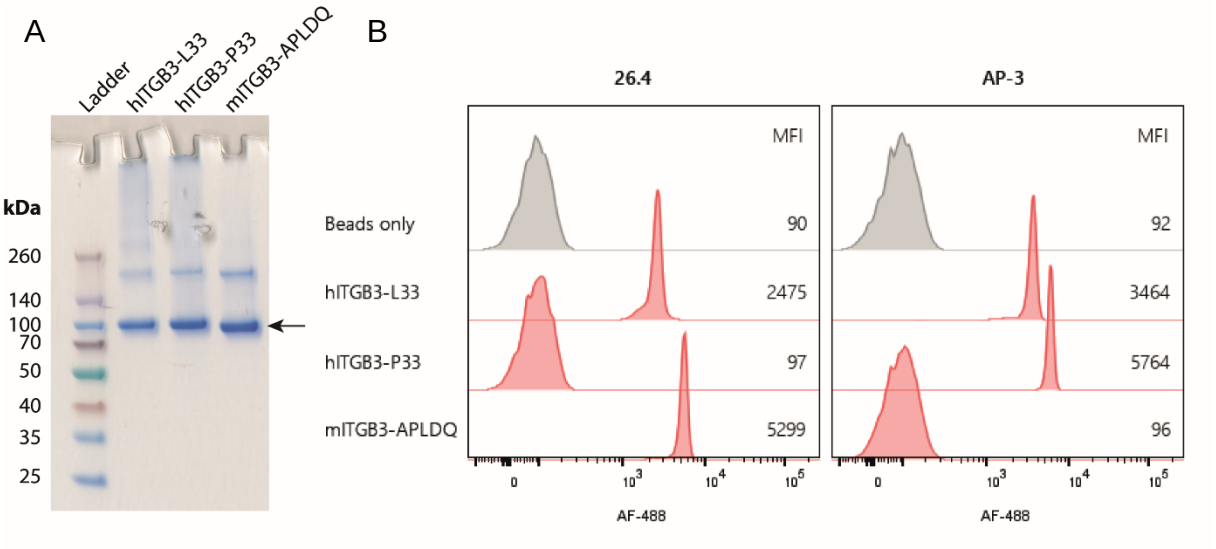
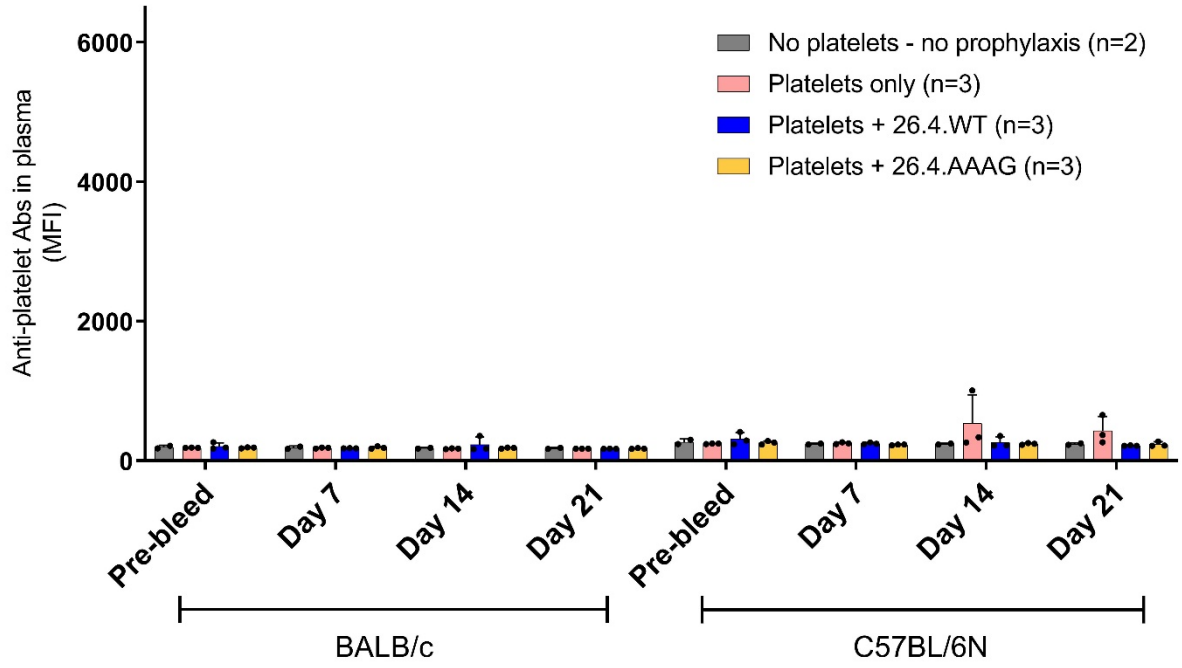
Supplementary materials



SUPPLEMENTARY FIGURE S1. Recombinant Fc region variants of mAb 26.4 bind specifically to HPA-1a. *Anti-HPA-1a 26.4 IgG1 isoforms produced recombinantly with modifications. (**A**) Purified mAb 26.4 variants were run on a non-reducing SDS-PAGE, and all migrated with expected molecular weights (150 kDa) and purity. (**B-C**) Binding of titrated amounts of mAb 26.4 variants to recombinant soluble β3 integrins variants L33 (**B**) and P33 (**C**) coated in ELISA wells (absorbance 405nm). Each point is a mean of duplicates from one representative experiment out of two, and error bars indicate SD.*



SUPPLEMENTARY FIGURE S2. mAb 26.4 bind soluble recombinant integrin β3, a murine backbone, engineered with the HPA-1a epitope (APLDQ). *The extracellular domains of ITGB3 with a 6xhis-tag and Twin-Strep-tag were produced as soluble proteins in insect cells using the baculovirus system. Constructs for human integrin β3-L33 (hITGB3-L33), human integrin β3-P33 (hITGB3-P33) and murine integrin β3 engineered with the HPA-1a epitope (mITGB3-APLDQ) were produced. (**A**) Recominant integrin β3 proteins were run on a non-reducing SDS-PAGE showing the presence of proteins around 100 kDa (arrow) similar to a teoretical size of approximately 85 kDa. (**B**) Binding of recombinant integrin β3 proteins, captured on Dynabeads™ His-Tag Isolation and Pulldown, to anti-integrin β3 Abs detected by flow cytometry. Expectedly, AP-3 (AF-488), a mouse anti-human Integrin β3 Ab, bound both human integrins-L33 and -P33 (hITGB3-L33/P33), and 26.4 (AF-488) bound recombinant proteins comprising the HPA-1a epitope (L33 and APLDQ variants: hITGB3-L33 and mITGB3-APLDQ). 10000 events were collected. MFI=median fluorescence intensity.*



SUPPLEMENTARY FIGURE S3. No or negligible binding of mAb 26.4 variants to control murine platelets from AMIS experiments. *Platelets isolated from BALB/c and C57BL/6N mice were isolated and incubated with plasma samples followed by detection using FITC-conjugated anti-mouse IgG Ab and analyzed flow cytometry. Median fluorescence intensity (MFI) is plotted with mean and SD.*

Paper II

Paper III

Supplementary review paper

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Murine models for studying treatment, prevention and pathogenesis of FNAIT

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ABSTRACT

Maternal alloimmunization to paternally inherited antigens on fetal/neonatal platelets can cause fetal/neonatal alloimmune thrombocytopenia (FNAIT) after antibody-mediated removal of platelets from the fetal circulation. The complications vary from mild bleeding symptoms to severe intracranial hemorrhage and subsequent neurological impairment or death. Studies on *in vivo* mechanisms are challenging to measure directly in pregnant women, rendering murine models as valuable and attractive alternatives, despite some critical differences between mice and men affecting the translational value. Here we present and discuss, the different murine models that substantially have increased our knowledge and understanding of FNAIT pathogenesis – as well as preclinical evaluation of therapeutic and preventive strategies.

1. Introduction

Fetal/neonatal alloimmune thrombocytopenia (FNAIT) is a condition caused by maternal alloimmunization against paternally inherited platelet antigens on fetal/neonatal platelets, and subsequent antibody-mediated removal of platelets from the circulation of the fetus or newborn.

Alloantigens are formed due to allelic variations of glycoprotein complexes important for hemostasis, by their function as receptors for fibrinogen, fibronectin, vitronectin, collagen and von Willebrand factor (vWf). The human platelet antigens (HPA) that may cause alloimmune complications have been designated into 35 systems (HPA-1 to -35) by the International Platelet Immunology Nomenclature Committee of the International Society of Blood Transfusion (ISBT). An updated overview is available in the HPA database at http://www.versiti.org/HPA.

The GPIIb/IIIa (αIIbβ3 or CD41/CD61) complex is a heterodimeric αβ integrin (encoded by *ITGA2B* and *ITGB3*) that form a receptor for fibrinogen, fibronectin, vitronectin and vWf, and most of the HPA systems are present on this complex. Alloimmunization to HPA-1a cause the vast majority of FNAIT (~80 %) in Caucasians [1] whereas antibodies to HPA-4 antigens are more frequently seen in the Asian population. Other HPA determinants that cause FNAIT are located on the GPIb-IX-V complex, the GPIa/IIa complex and the CD109 protein on the platelet surface. In addition, isoantibodies to CD36 in CD36-deficient individuals are also a cause of FNAIT [2,3].

In humans, maternal IgG antibodies are transported across the placental membranes and into the fetal circulation by Fc receptor-mediated mechanisms that provides the fetus with humoral protection during gestation and the first 3–6 months after birth while the endogenous immune system is developing and maturing. This transport of antibodies has been studied in detail the last decades, and has been shown to be effectuated by the neonatal Fc receptor (FcRn). This receptor is not exclusively expressed on human syncytiotrophoblasts in placental tissue, but importantly also on endothelial cells and in epithelial tissue. FcRn is also crucial for the significantly longer half-life of IgG compared to the other immunoglobulin classes by pH-dependent recycling [4]. Even though FNAIT is defined as thrombocytopenia with or without bleeding in the fetus/newborn, the complications due to potential harmful effect of the antibodies towards antigen-epitopes expressed on placental tissue is in the spotlight after observations of low birthweight in babies of HPA-1a alloimmunized mothers [5]. Today, there are no licensed drugs for neither prevention nor safe and effective treatment of FNAIT. However, there is an increasing trend for off-label use of human intravenous IgG (IVIG) treatment of women with anti-HPA-1a antibodies, and many clinical guidelines recommend IVIG treatment of women with a known obstetric history of severe FNAIT with intracranial hemorrhage (ICH) [6].

Knowledge about genetic and molecular basis, pathogenesis and prevalence of FNAIT has greatly improved the last 30 years, both due to the improved repertoire of laboratory analyses, and a number of

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retrospective and prospective human studies. However, studies on *in vivo* mechanisms are challenging to measure directly in pregnant women and fetuses, rendering murine models as valuable and attractive alternatives. To date, several different models have been used for studying FNAIT. Here, we present and discuss the insights previous and current models have given.

2. Similarities and differences - man *versus* mice relevant for FNAIT

In order to translate results from murine experiments to the human setting, the differences between several facets of FNAIT biology should be carefully acknowledged. These factors include differences in hematology, pregnancy-related anatomy, and immunization: antigen source, antigen presentation and immune cell interactions, but also antibody-related factors as transplacental transfer, platelet opsonization and removal as well as effect on endothelial cells in the vascular system and bleedings.

2.1. Human versus murine platelets and platelet integrins

The murine platelets are smaller and their life-span of 3–4 days is markedly shorter than the 8–12 days in humans [7]. Platelet counts in inbred mice, show variations between strains, age and gender, with reports of normal counts in the range of $600-1500 \times 10^3/\mu L$, with most commonly reported platelet count in the range of $900-1200 \times 10^3/\mu L$ [8–12]. The platelet count in pups is roughly doubling from day of delivery ($\sim 400-600 \times 10^3/\mu L$) during the first two weeks of life, to adult levels within 8 weeks [9]. Thus, the baseline platelet count should be determined carefully for every model, keeping in mind that there are also variations in the observed platelet counts due to differences in sampling techniques (sampling point, sampling method, platelet activation and clotting) and quantitation methods (manual counting, automatic analyzers or flow cytometry) [10,11]. Severity of thrombocytopenia in or between FNAIT models based on platelet counts alone should be compared with caution.

Platelet integrin complexes function as receptors for hemostatic factors important for platelet function and hemostasis in mice as in humans. Amino acid sequence identity between human and murine platelet integrins are generally more than 80 % for the mature proteins (not including the signal peptide, based on NCBI reference sequences): 0.91 for GPIIIa (*ITGB3*), 0.80 for GPIIb (*ITGA2B*), 0.93 for GPIbα (*ITGB1*) and 0.83 for GPIa (*ITGA2*) in line with original characterizations [13,14]. High amino acid sequence identity score may however, not necessarily represent epitope homology – depending on the conformational nature of the given epitope. Since there are no reports of alloantigenic platelet integrin variants between the inbred strains, immunogenicity is created either by knockout of murine glycoprotein genes or by using human platelets. Human anti-HPA-1a antisera do not bind the murine wild-type GPIIIa, which otherwise would have precluded all use with such antibodies with the mouse as a host [15]. Thus, for studies of antibodies reactive with the human GPIIIa (their reactivity patterns, *in vivo* effect and treatment potential), chimeric models, recombinant proteins, or murine platelets, with reconstructed human epitopes are required.

2.2. Human versus murine placenta and placental transport

The placenta is of high evolutionary diversity among species, and for some research areas (placental disease/toxicology studies), the murine placenta has been demonstrated to have less translational value due to its differences in major key factors for placentation and placental function, reviewed by Schmidt et al. [16].

In mice, the pups are growing in multiple embryonic sacs in each of the two uterine branches, where each embryonic sac contains a single pup and its own placenta. Importantly, for models of alloimmunization, both the human and mouse have discoidal, hemochorial placentas where maternal blood are in direct contact with the trophoblast cells (of fetal origin). Maternal immune cells may interact with fetal antigens expressed on the outer cell layer in the intravillous space in humans, which correspond to the structurally different placental labyrinth in mice [16]. In addition to the chorioallantoic placenta, the mouse also has a secondary placental structure - the inverted yolk sac - that surrounds the fetus throughout gestation, and is the structure that allows transfer of maternal antibodies to the fetus by transcytosis by the FcRn in the inner layer of the inverted yolk sac structure [17]. Thus, transplacental transport of antibodies is mediated by anatomically different structures in humans and mice, while facilitated by the same receptor.

3. Mouse models in FNAIT – Insights from different models

Different mice strains demonstrate diverse immune responses due to variations in genetic background [18,19]. Alloantigens, that in most cases differ from self by a single amino acid residue substitution, have a limited number of antigenic peptides to be presented in MHC class II, compared to other foreign substances as microbes, parasites and viruses. Therefore, differences in antigen-presenting capacity due to different MHC haplotypes may affect immunization in this setting. In addition, it should be kept in mind that the uniformity within the mouse colony due to genetic identity within an inbred mice strain, dramatically changes whenever an antigen-specific immune response is mounted by the adaptive immune cells, due to its nature of random sequence generation – affecting the potency, titer and polyclonality of antibody repertoire.

The choice of mouse strain or design of knockout models may significantly influence the results of the study, as their intrinsic phenotype may affect their overall health, relevant hematology, immune responses and breeding.

3.1. Murine model designs

In 2006, Ni and colleagues presented a model of GPIIIa knockout ($\beta3^{-/-}$) mice backcrossed onto a BALB/c background [20]. The original β3-deficient mouse strain was designed by Richard O. Hynes and his group to study Glanzmann thrombasthenia [21], and due to its suitability to study platelet conditions it was utilized to generate the first murine model of FNAIT [20]. Use of a corresponding GPIbα-knockout model, originally made to study Bernard-Soulier syndrome [22], was also reported by Li and colleagues [23]. In these two models, alloimmunization (or rather isoimmunization) is achieved by repeated injection of wild-type platelets, and pre-immunized females are bred with wild-type males to assess the effect of the antibodies on pregnancy and pups. Platelet glycoprotein-deficient mice are however phenotypically affected: GPIIIa-deficient mice have higher fetal/neonatal mortality rate due to placental defects, anemia and hemorrhages, and by design impaired platelet function [21], whereas GPIbα-deficient mice have reduced platelet count and reduced platelet function [22]. A chimeric model expressing human GPIIIa (originally used to study Glanzmann thrombasthenia [24]) allows studies on human anti-GPIIIa antibodies in a murine system [25].

For other studies, non-obese diabetic/severe combined immunodeficient (NOD/SCID) or SCID mice has been used. These mice harbor the SCID mutations leading to mice without functional B and T cells and the NOD mutations leading to mice without 1) functional natural killer (NK) cells, 2) functional antigen-presenting cells and 3) circulating complement; the mice will be affected in many ways [26]. In this model, the mice functions as a host, injected with human material (antigen and antibodies), but may nevertheless depend on immune-related effects like Fcγ receptor-mediated functions. The knowledge acquired from all these models, with respect to treatment, prevention and pathogenesis is discussed below.

3.2. Studies on therapeutic strategies

3.2.1. Therapeutic administration of IVIG to treat FNAIT

In the GPIIIa knockout model, repeated injections of wild-type platelets into $\beta3^{-/-}$ mice gave robust and potent generation of β3 integrin-specific antibodies, which subsequently caused thrombocytopenia when injected into wild-type mice [20]. The titer of maternal antibody against β3 integrin correlated with the severity of FNAIT in the pups, in pre-immunized females bred with wild-type males. High antibody titer caused severe FNAIT with signs of bleeding, ICH, and in the most severe cases miscarriage and increased mortality rate [20]. This model, resembling human anti-GPIIIa-mediated FNAIT conditions, allows studies of the pathogenic mechanisms as well as studies attempting to interfere with pathogenesis. IVIG is licensed for use to treat a number of immune-deficient conditions, but is also used to treat autoimmune diseases. Human IVIG has also been used in mouse models of immune thrombocytopenia (ITP) [27]. By applying this strategy to the above FNAIT model, it was shown that administration of IVIG in pre-immunized $\beta3^{-/-}$ mice (bred with male wild-type mice) during pregnancy, increased platelet counts of the newborn pups, and more importantly, bleeding symptoms, pup mortality and number of miscarriages were diminished [20]. The therapeutic effect of IVIG was further confirmed in a number of other, subsequent studies mentioned below.

3.2.2. FcRn – a potential therapeutic target

The transplacental transport of maternal antibodies is a central part of human FNAIT pathogenesis. In 2010, Ni and colleagues reported another mouse model, generated through the combination of β3-deficient mice and FcRn-deficient mice, creating $\beta3^{-/-}FcRn^{-/-}$ mice [28]. By pre-immunizing and breeding deficient and wild-type β3/FcRn mice in different combinations, they showed that also in this murine model, fetal rather than maternal FcRn, is crucial for transportation of antibodies across the placenta and thus, critical for the development of FNAIT. When pre-immunized $\beta3^{-/-}FcRn^{-/-}$ females were bred with $\beta3^{-/-}FcRn^{-/-}$ males, no FNAIT occurred in the pups even though anti-β3 antibodies were present in the mother. However, by treating these pregnant mice with IVIG, there was a decrease in antibody titer in the mothers, showing that IVIG also may function through FcRn-independent pathways [28]. In the light these results, they targeted this receptor to reduce harmful effect of anti-β3 antibodies, as had previously been done in animal models of autoimmune conditions [29]. Indeed, they were able to show that by injecting an anti-FcRn antibody into pregnant pre-immunized $\beta3^{+/+}FcRn^{+/+}$ (bred with wild-type males; $\beta3^{+/+}FcRn^{+/+}$) the newborn pups had normal platelet counts and showed no abnormalities. Less severe thrombocytopenia was also seen in pups when treating pregnant pre-immunized $\beta3^{-/-}FcRn^{-/-}$ mice (bred with wild-type males) with anti-FcRn antibody [28]. As the authors pointed out, the monoclonal anti-FcRn antibody could be administrated with good effect in lower doses than IVIG. Thus, an anti-FcRn antibody could be an attractive alternative to IVIG which is a plasma-derived product of limited source, has batch-to-batch variations, and has adverse effects when administered in high dose.

Noteworthy, when comparing FcRn between species, human FcRn shows immense restrictions when binding to IgG from different species with no or very low binding to murine IgG, while murine FcRn binds human IgG better than murine IgG [30]. Andersen and colleagues also confirmed this in an *in vitro* experiment using soluble recombinant murine and human FcRn binding to IgG1 [31]. These cross-species differences may affect the experimental results of half-life and transplacental transfer analyses as well as potential mechanistic artifacts in the model [32]. Several anti-FcRn antibodies are in the drug development pipeline for autoimmune and alloimmune conditions. Of utmost relevance to FNAIT, the M281, a human anti-FcRn antibody has been demonstrated to efficiently reduce transplacental transport of IgG in a human *ex vivo* placental perfusion model [33]. This molecule is currently in clinical phase trials as a potential treatment for the alloimmune complication hemolytic disease of fetus and newborn [34,35].

3.2.3. FNAIT treatment by prevention of fetal platelet destruction

A chimeric mouse model expressing a hybrid complex of murine αIIb/human β3 [24] was used by Ghevaert and colleagues to examine the ability of the recombinant IgG1 antibody B2G1 (HPA-1a-specific mAb) to prevent platelet destruction [25]. Using bone marrow from β3-deficient mice [21], they transduced it with a lentivirus vector containing cDNA of the human *ITGB3* comprising either HPA-1a or 1b, and next, the bone marrow was transplanted into lethally irradiated β3-deficient mice [24,25]. Interestingly, $F(ab')_2$ B2G1 antibody fragments was used as a proof of principle for the effect of non-FcγR-binding reagents to inhibit and prevent platelet destruction by intact B2G1 antibody or polyclonal anti-HPA-1a sera in the chimeric mouse model [25]. Consequently, a modified B2G1 variant without FcγR-binding can have platelet protective effects and thus potentially be a candidate for FNAIT treatment, given proper FcRn-binding for transport to the fetus.

Another mouse model was presented in 2009 by Bakchoul and colleagues, in an FNAIT study focusing on platelet clearance in NOD/SCID mice (JAX stock #001303) [36]. This immunodeficient mouse model does not have functional lymphoid cells and have no or low levels of immunoglobulin making the model ideal for graft studies [26] but also antibody-mediated platelet clearance studies [37]. The injection of maternally derived human anti-HPA-1a IgG after supplying NOD/SCID mice with resting human platelets from HPA-1ab donors, mediated clearance of these platelets *in vivo* [36]. By introducing a monoclonal murine antibody (mAb SZ21, $F(ab')_2$) directly after platelet transfusion, the platelet clearance by human anti-HPA-1a antibodies was efficiently inhibited. Importantly, this study confirmed the potential to treat FNAIT using agents that block the binding of the maternally derived antibodies to the HPA-1a epitope [36]. The SZ21 antibody was later deglycosylated by Bakchoul and colleagues and injected into pregnant BALB/c mice, where it was transferred to the fetus to the same degree as native SZ21, and still blocked the binding of maternal antibodies to the HPA-1a epitope [38]. As deglycosylation of IgG antibodies is well known to terminate Fc receptor-binding and thus Fc-related effector functions [39], it could make the antibody suitable for treatment [38]. Thus, this study supports that deglycosylated monoclonal antibodies could be a potential FNAIT treatment in the future.

3.2.4. Potential therapeutic targeting of T cells by peptides

There has also been interest in targeting the T cell response in FNAIT, as the peptide harboring the HPA-1a epitope (the Leu33 residue) is efficiently presented to HPA-1a-specific T cells by antigen-presenting cells expressing the DRA/DRB3*01:01 molecule [40–46]. Jackson and colleagues made attempts to dampen already established immune responses by tolerization with peptide treatment [47]. In a SCID mice model depleted of NK cells and macrophages, PBMC from *HLA-DRB3*01:01*-positive HPA-1a alloimmunized women were intraperitoneally injected followed by administration of HPA-1a-derived peptides. After 2 and 3 weeks, $HPA\text{-}1a^{+}$ platelets were injected to boost any present response against the antigen. However, in this system, no consistent effect of this regimen was seen, measured by anti-HPA-1a antibody responses *in vivo* [47]. This kind of therapeutic strategy still holds potential, but may require additional factors targeting the antigen-presenting cells, other than peptide alone, to induce an effective tolerogenic effect on T cells [48].

3.3. Potential prevention of alloimmuniztion

While many studies are designed to study treatments of FNAIT, the notion that immunization often takes place in connection with delivery [49], opened the possibility to employ the prophylactic strategy that has successfully reduced the incidence of RhD alloimmunizations. The

administration of human plasma-derived anti-D antibody treatment is based on the concept of antibody-mediated immune suppression (AMIS). Still it is unclear exactly how passively injected antibodies suppress the immune system, but different mechanisms are proposed, as discussed by Kumpel and Elson [50]. Briefly, this includes inhibition of B cells through crosslinking of the B-cell receptor and the inhibitory receptor FcγRIIB on B cells with the antibody-antigen complex, or clearance of antigens, where the antigen is removed from the circulation before the antigen is seen and processed by the immune system [51]. However, Bernardo and colleagues could induce AMIS in knockout mice for either activating or inhibitory Fcγ receptors [52]. AMIS was similarly induced in these models when using antibodies lacking their Fc region indicating that AMIS also works through Fc and Fcγ receptor-independent pathways [52]. In addition, antibody masking of the epitope that blocks the recognition of antigen by B-cell receptors has been suggested as another possibility. However, based on stoichiometric calculations only a small portion of the RhD epitopes would be masked [50].

In investigation of the potential prophylactic approach, Tiller and colleagues managed to induce AMIS by injection of mouse anti-β3 antisera subsequent to transfusion of murine wild-type platelets into $\beta3^{-/-}$ mice [53]. Importantly, AMIS was also seen using human anti-HPA-1a antibodies or the monoclonal murine antibody SZ21 after transfusion of human HPA-$1a^{+}$ platelets [53]. Thus, the findings from this study supported the concept that also FNAIT may be prevented through a prophylactic regimen.

3.4. Insights into pathogenesis from murine models

3.4.1. Anti-GPIbα-antibodies may cause miscarriages through reduced placental function

Although the majority of FNAIT complications are due to αIIbβ3 incompatibilities, there are also cases reported due to alloantibodies against GPIbα [1]. By making a GPIbα knockout mouse model similar to the GPIIIa knockout, Li and colleagues showed that pre-immunized GPIbα-deficient female mice have an very high miscarriage rate when bred with wild-type mice compared to the β3-deficient mouse model [23]. The miscarriages were accompanied by reduced placental function and development, while treatment with either anti-FcRn antibodies or IVIG successfully increased the number of live pups. This study suggests that there might be many unreported FNAIT incidents due to frequent miscarriages caused by antibodies against glycoprotein GPIbα, which then masks the severity of this condition. Nevertheless, this study also supports the use of IVIG or antibodies against FcRn as FNAIT treatment [23].

In a follow-up study, it was shown that immunogenicity of GPIbα is lower than for GPIIIa in these knockout models, as more wild-type platelets were required for potent antibody responses in immunization experiments [54]. Mounting an efficient antibody response to foreign structures generally depends on activation of dendritic cells and induction of a pro-inflammatory environment, by binding of pattern recognition receptors to microbe-specific structures and/or endogenous stress signals. Allogenic proteins, such as blood type antigens and platelet glycoproteins will most likely not activate the innate immune cells by their sole presence in circulation. It was previously reported that ongoing authentic infection, or the mimicking of inflammation with Poly I:C can induce or enhance antibody responses in RBC-alloimmunization models [55]. Accordingly, injection of LPS or Poly I:C (mimicking bacterial or viral infections, respectively) both enhanced antibody production and subsequent miscarriages in the knockout mice [54]. This is adding a piece to the puzzle that factors like inflammation may influence immunization and the quality and quantity of the alloreactive response. Whether the timing of the inflammation signals are critical for immunization to platelet antigens, as was indicated in a mouse model where the timing of Toll-like receptor 3-induced inflammation and exposure to RBC antigens affects the degree of alloimmunization [56], is not yet known.

3.4.2. Intracranial hemorrhage might be caused by impaired angiogenesis

The last decades, the focus of FNAIT treatment has been on improving the platelet count of the fetus or neonate, but in a recent study by Yougbaré and colleagues, they highlight that β3 integrin has an important role in angiogenesis in relation to development of the fetus [57]. Here, they showed that it might not be thrombocytopenia that causes ICH, but rather the maternal anti-β3 antibodies that impair angiogenesis leading to ICH in the fetus. In the β3-deficient mouse model, anti-β3 antibodies impaired the vessel density in the brain and retina of the delivered pups, also accompanied by increased endothelial cell apoptosis and inhibited angiogenic signaling. Importantly, anti-HPA-1a antibodies of anti-αvβ3 specificity in development of FNAIT-associated ICH, mediated through binding to endothelial cells, have also been reported [58]. As shown previously [20,23,28], IVIG can be used to ameliorate FNAIT, but in this study they also saw improvement of vascular density and less apoptosis [57]. These findings indicate that treatment of FNAIT preferentially should be focused on eliminating the maternal anti-β3 antibodies rather than increasing the platelet count of the fetus. Another study using the β3-deficient mouse model recently found that activated NK cells causes apoptosis of β3 integrin-positive trophoblasts in the placenta, which subsequently leads to placental dysfunction and miscarriages [59]. By depleting the NK cells or through functional blockage of the NK receptors FcγRIIIa or NKp46, both placental growth restriction and miscarriage were reduced [59]. This study introduces another alternative approach to treat FNAIT, but in addition, it also sheds light on the pathological mechanisms in the development of FNAIT.

3.4.3. Clinical significance of low-avidity anti-platelet antibodies

Bakchoul and colleagues used the NOD/SCID model to show that low-avidity anti-HPA-1a antibodies from a series of FNAIT cases cleared human platelets from HPA-1ab donors *in vivo* - even though these antibodies may not be detected in standard serological and solid-phase glycoprotein-specific assays [60]. Such antibodies can be detected by surface plasmon resonance (SPR) measurements which allows increased sensitivity in part by eliminating the washing steps used in conventional methods [60].

Peterson and colleagues later confirmed (using the NOD/SCID mice model) that low-avidity antibodies can cause FNAIT and that many cases go undetected [61]. In this study, most women that had low-avidity antibodies only, were *HLA-DRB3*01:01*-negative suggesting that these women have a predisposition to generate such antibodies [61]. Tentatively, this could be due to a less efficient underlying T cell response supporting potent antibody responses, as several reports support the role of *HLA-DRB3*01:01* in HPA-1a antigen presentation [40–45] and clinical outcome [46,62].

3.4.4. Using the murine system to study the antigenic epitopes for antibodies

Comparative analyses to study antibody reactivity have used human and murine recombinant proteins. Based on amino acid sequence comparisons, residues important for anti-HPA-1a antibody binding was determined two decades ago [15]. Since then, detailed characterizations of the glycoprotein complex and conformation important for antibody binding have been resolved by crystal structures and experiments with site-directed mutagenesis variants [63–65]. However, due to recent CRISPR/Cas9 technology for site-directed mutagenesis in the murine genome, mouse platelets are now valuable again: designed, humanized murine platelets to map the epitope for different anti-HPA-1a monoclonal antibodies [66].

4. Conclusions and future perspectives

Important insights into the pathogenesis, treatment and prevention of FNAIT have been given by the elegant studies on knockout mice models. An advantage with knockout models is that injections with wild-type antigen causes immunization against the entire native

protein, likely representing a more general strategy, not restricting the translational value to a single alloantigen system. However, from a molecular interaction point of view, the glycoprotein knockout models unfortunately lack the essential fine-tuned balance of reactivity of self and non-self, both regarding conformational epitopes for antibody-specificity and for peptide-antigen presentation and recognition by antigen-specific T cells. Most antibodies causing FNAIT are thought to be specific to a single epitope, at least caused by a single amino acid substitution. Thus, the study of preventive or therapeutic treatments using monoclonal antibodies could be more challenging to examine in knockout mice. Models that take these factors into account, would better mimic the epitope-specific responses in future studies, as improved strategies are needed for diagnosis and treatment of women with a potential high risk of FNAIT in their newborns.

Declaration of Competing Interest

None.

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