Inhibition of HPA-1a alloantibody-mediated platelet destruction by a deglycosylated anti-HPA-1a monoclonal antibody: towards targeted treatment of fetal-alloimmune thrombocytopenia

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Key points:

1) The study describes a potential novel treatment for fetal alloimmune thrombocytopenia by dissecting the effector activities of an epitope-specific IgG antibody.

2) Neither the *in vivo* transplacental transport nor the inhibiting properties of the blocking antibody are impaired by the N-glycan modification.
Abstract

Fetal/neonatal alloimmune thrombocytopenia (FNAIT) is often caused by maternal alloantibodies against the human platelet antigen (HPA)-1a, which opsonize fetal platelets. Subsequent platelet destruction is mediated via the Fc-part of the alloantibodies. The monoclonal antibody SZ21 binds to the HPA-1a-epitope and inhibits binding of maternal alloantibodies. However, it also promotes complement activation and phagocytosis. Deglycosylation of antibodies abrogates the Fc-related effector functions. We modified the N-glycan of SZ21 by Endoglycosidase F. The in vivo transplacental transport of N-glycan modified (NGM)-SZ21 was not impaired. When injected into pregnant mice, both native SZ21 and NGM-SZ21 were transported equally into fetal circulation (8.9% vs. 8.7%, respectively, p=0.58). Neither the binding properties of NGM-SZ21 to HPA-1a in surface plasmon resonance, nor inhibition of anti-HPA-1a-induced platelet phagocytosis were affected by N-glycan modification. NGM-SZ21 prevented platelet destruction induced by maternal anti-HPA-1a antibodies in vivo in a NOD/SCID mouse model (platelet clearance after 5h; 18% vs. 62%, in the presence or absence of NGM-SZ21, respectively, p=0.013). Deglycosylation of SZ21 abrogates Fc effector functions, without interfering with placental transport or the ability to block anti-HPA-1a binding. Humanized, deglycosylated anti-HPA-1a monoclonal antibodies may represent a novel treatment strategy to prevent anti-HPA-1a-mediated platelet destruction in FNAIT.
Introduction

Fetal/neonatal alloimmune thrombocytopenia (FNAIT) results from maternal immunization against fetal-specific human platelet antigens (HPAs).¹⁻³ Analogous to Rhesus D incompatibility, maternal antibodies to alloantigens (blood groups) on fetal platelets can result in destruction of fetal platelets after transplacental transport from the maternal to the fetal circulation. Alloantibodies against the epitope HPA-1a on glycoprotein (GP) IIb-IIIa are responsible for most of the severe cases of FNAIT.⁴⁻⁷ The incidence of HPA-1a-mediated FNAIT in the Caucasian population is about 1 in 1,500 live birth, based on a large population study,⁷ with no prophylactic measures to prevent maternal immunization.⁷ The most devastating risk of FNAIT is intracranial hemorrhage, which may lead to death or persistent neurological sequel in 10% of the clinically symptomatic cases.⁴,⁸ After delivery, FNAIT can be treated by platelet transfusion.⁹,¹⁰ However, in almost 50% of affected cases, intracranial hemorrhage occurs before delivery, sometimes as early as in the 20th week of gestation.⁸,¹¹,¹² This makes antenatal treatment essential to avoid deleterious consequences.⁵ Ideally, treatment should be initiated from about the 20th week of gestation, as from then on the placenta transports maternal IgG to the fetus, and fetal platelets already express the HPAs.¹³⁻¹⁵

Currently, antenatal treatment options include intrauterine platelet transfusion (IUT) to the fetus, or treatment of the pregnant mother with intravenous immunoglobulin (IVIG) with or without additional steroids.¹,³ All three treatment options have limitations. IUT is associated with the risk of severe procedure-related complications causing iatrogenic fetal death ¹⁶, high dose steroids for 12-20 weeks during gestation increase the risk for gestational diabetes and put the mother at an increased risk for infection, and little is known about the long term effects of immuno-modulation of the mother during pregnancy. In addition, these treatments have limited efficacy. About 20% of the newborns remain severely thrombocytopenic despite treatment of the mother with IVIG and steroids.¹²,¹⁷
As fetal platelet destruction is initiated after binding of maternal allo-antibodies to the fetal platelet surface, an attractive treatment option would be to block binding of these maternal alloantibodies to the respective alloantigens on fetal platelets. Recently, we demonstrated the protective effect of F(ab)\(^2\) fragments of the monoclonal antibody (mAb) SZ21 on platelet clearance induced by maternal anti-HPA-1a allo-antibodies.\(^{18}\) This mAb binds to the HPA-1a epitope and competes with the human alloantibodies. As the mother lacks the antigen to which it binds, one could safely inject the mAb SZ21 into the mother, taking advantage of the materno-fetal transport of antibodies. However, this concept has two major practical obstacles. Monoclonal antibodies with an intact Fc-moiety are as effective as maternal alloantibodies in inducing platelet destruction \textit{in vivo} via Fc-receptors, while F(\(ab\)\(^2\))-fragments are not efficiently transported across the placenta to the fetus.

IgG is transported from the maternal circulation to the fetus by binding to the neonatal Fc receptor of (FcRn) that is expressed in the placental villous syncytiotrophoblast.\(^\text{19,20}\) FcRn-mediated IgG transport does not require carbohydrate moieties on the Fc-portion of the antibody for binding or transplacental transport.\(^\text{15,21}\) Thus, removal of the N-glycan should not affect placental transport. In contrast deglycosylation of the N-glycan attached to Asn297 inhibits recognition by Fc-receptors on macrophages (FcRI, FcRIIa, FcRIIIa), as well as its ability to activate complement factor C1q.\(^\text{22,23}\)

In this study, we demonstrate that deglycosylation of the anti-HPA-1a mAb, SZ21, neither affects its efficient transplacental transport, nor its ability to bind with high affinity to the HPA-1a epitope on platelets, thereby blocking binding of maternal HPA-1a alloantibodies and subsequent destruction of HPA-1a platelets \textit{in vivo}. These studies may indicate a new approach for a minimally invasive treatment strategy for prevention of fetal platelet destruction by maternal anti-HPA-1a alloantibodies.
Material and Methods

Antibodies

mAb SZ21, which binds the epitope HPA-1a on GPIIIa, was obtained from Beckman Coulter (Krefeld, Germany). Isotype matched mouse IgG1 was obtained from Beckman Coulter. mAb AP2, which recognizes a complex-dependent epitope on GPIIb-IIIa, but does not interfere with the HPA-1a epitope, was kindly provided by Dr. Robert R. Montgomery (Blood Research Institute, Milwaukee, WI). The deglycosylated variants of these two antibodies are named NGM-SZ21 and NGM-AP2, respectively. Human anti-HPA-1a alloantibodies were obtained from sera of women who developed these antibodies during pregnancies that were complicated with severe FNAIT, and purified by Melon Kit methodology (Thermo Fisher Scientific, Bonn, Germany). The animal experiments were approved by the local animal authorities in Hessen/Germany and Milwaukee/USA.

N-glycan modification of mAbs

To deglycosylate the mAbs, the N-linked glycan attached to Asn297 of the IgG heavy chain was enzymatically removed under native conditions using Endo F (Native Protein Deglycosylation Kit, Sigma-Aldrich, Munich, Germany). In brief, 200 µg SZ21 or iso-matched mAb (AP2) were incubated with 2 µl of Endo F in PBS at 37°C for 2 hours and then purified using the melon IgG purification kit (Thermo Fisher Scientific).

Degradation of antibodies by Endo F-treatment was evaluated by Coomassie Blue stained SDS-PAGE. Efficiency of antibody deglycosylation was investigated using lectin blotting. A total of 1 µg of SZ21 or NGM-SZ21 was resolved by 10-20% precast gradient SDS-PAGE (BioRad, Munich, Germany) under reducing conditions and transferred to polyvinylidene fluoride membrane. Biotinylated lens cullinaris agglutinin (LCA) (Sigma-Aldrich, Munich, Germany) was
added to a final concentration of 50 µg/ml for 45 min at room temperature (RT) and the membrane was washed 10 times (0.05% Tween/Tris buffered saline). Subsequently, peroxidase-conjugated streptavidin (Sigma Aldrich) was added in a final concentration of 1 µg/ml for 30 min at RT and bound LCA was visualized by ECL detection kit (GE Healthcare, Munich, Germany).

To further analyze the specificity of antibody deglycosylation, SZ21 and NGM-SZ21 were separated on SDS PAGE as described above. Gel matrix containing IgG heavy chain was extracted, digested and analyzed by MALDI-TOF mass spectrometry (Voyager DE Biospectrometry workstation, Applied BioSystems, Foster City, CA).

Assessment of transplacental materno-fetal transport of NGM-SZ21

For maternal antibody transfer, age matched pregnant female BALB/c mice at 17 days of gestation were injected i.v. with a total of 40 µg of SZ21, NGM-SZ21, or isotype-matched mouse IgG 1 (Beckman Coulter). After delivery, i.e. 3-4 days after antibody injection into the pregnant mother, blood was collected from the 1-8 hours-old pups by carotid bleeding. After pooling blood samples from all pups of each pregnancy, sera were obtained to assess the amount of free anti-HPA-1a alloantibodies in the neonatal mouse blood using GPIIIa surface plasmon resonance (SPR) as described (see below). This experiment was done in triplicate.

Antibody-binding characterization using surface plasmon resonance (SPR)

The binding kinetic of NGM-SZ21 was analyzed by SPR technology using ProteOn XPR36 (ProteOn XPR36, Bio-Rad, Munich, Germany) as described. In brief, GPIIb-IIIa from human platelets was isolated using affinity chromatography and immobilized onto flow cells of a GLM-sensor chip (25 µg in 250 µl ProteOn acetate buffer, flow rate 30 µl/min until saturation). Bovine serum albumin (BSA) was immobilized onto the control flow cell.
To quantify the mAbs transported across the placenta into the fetal circulation, we used both variants of SZ21 (native and NGM) as standards, as previously described. The mAbs were initially diluted in reaction buffer to a concentration of 20 ng/ml. A standard curve was constructed with a non-linear four-parameter logistic regression using GraphPad Prism 5, by plotting the response units (RUs, means of duplicates) of fetal or maternal blood samples against six dilutions of the reference sample. The RU values were then read against this standard curve.

To determine the equilibrium dissociation constant (KD) of the mAbs, 6 different concentrations with 2-fold dilution of the maximum concentration 10 nM (1,500, 750, 375, 188, 94 and 47 ng/ml) of SZ21 or NGM-SZ21 were injected over the coated sensor ships at flow rate of 30 µl/min. Antibody binding was evaluated during an association phase (antibody injection, 0-350 sec), which was followed by a dissociation phase (injection of buffer only, 350-700 sec). The association phase, where the antibody is flowed across the coated sensorship and binding is measured, allows the determination of the rate of formation of the antibody-antigen complex over the time which is reflected by an increase in the RUs. The kinetic of the increase in RU determines the association constant (Ka). In the dissociation phase the antibody is removed from the flow (concentration in buffer is zero) and the rate of complex dissociation follows exponential decay kinetics. This kinetic determines the dissociation constant (Kd). Data were double referenced by subtraction of control flow cell and data from interspots, as recommended by the manufacturers. Antibody affinity to GPIIb-IIIa and rate constants of the antibody-antigen interactions were determined by global analysis using a simple 1:1 Langmuir binding model provided by the Proteon X36 software (Bio-Rad). Further details are explained in the respective result section.
**Antibody-mediated platelet phagocytosis assay**

To test the ability of NGM-SZ21 to prevent maternal anti-HPA-1a-induced platelet phagocytosis in vitro, a platelet phagocytosis assay was performed.

Monocytes were isolated from peripheral blood samples of healthy donors (HPA-1ab, blood group O) by anti-CD14-microbeads using AutoMACSTM technology according to the manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated monocytes were adjusted to 1x10^4/µl in 10% FCS-RPMI (PAA Laboratories, Pasching Austria).

Platelets were isolated by centrifugation from 10 ml citrated whole blood of the monocyte donor (72 g for 25 min at RT). 100 µl of platelet rich plasma (PRP) (1x10^5/µl) were labeled with 2 µl of 1 mM CMFDA (an intracellular dye, CellTracker™ Green CMFDA, Molecular Probes Invitrogen, Karlsruhe, Germany) for 45 min at RT. Excessive CMFDA was then removed and platelet concentration was adjusted to 1x10^5/µl.

Aliquots of 100 µl labeled platelets were pre-incubated with NGM-SZ21 or NGM-AP2 (5, 15, 30, and 60 µg/ml) for 15 min at RT. 50 µl IgG-fraction of human sera containing anti-HPA-1a antibodies were added to the suspension for further 30 min. Opsonized platelets were then incubated with monocytes (ratio 1:10) for 2 hours at 37°C. Thereafter, monocytes were labeled with 5 µl PE-labeled anti-CD14 monoclonal antibody (Becton Dickinson GmbH, Heidelberg, Germany) and analyzed by flow cytometry (BD FACS Canto™, company BD Biosciences, San José, California, USA). Monocytes (PE-positive events) were gated and the percentage of FITC-positive monocytes, i.e. those who ingested labeled platelets, was identified as the phagocytic activity. Human anti-HPA-1a alloantibodies were tested in triplicate with monocytes and platelets of three different donors.

**Analysis of maternal alloantibody-mediated HPA-1a platelet destruction in vivo**

The survival of human platelets was investigated in the NOD/SCID mouse model for alloimmune thrombocytopenia as described.¹⁸,²⁶ Thirty µg of NGM-SZ21 or NGM-AP2 were added to 200 µl
resting platelets ($2 \times 10^9 / \mu l$) from healthy donors in autologous plasma prior to injection into the retro-orbital plexus of NOD/SCID mice (The Jackson Laboratory, Bar Harbor, Maine, USA). After 30 minutes, a blood sample was collected to define the baseline of circulating human platelets (100%), and 800 µg of the human anti-HPA-1a IgG-fraction were intraperitoneally (IP) injected. Survival of human platelets in the mouse circulation was estimated by analyzing blood samples obtained at 60, 180, and 300 minutes after antibody injection by flow cytometry using FITC-labeled Gi5 and PE-labeled MWreg30 (Becton Dickinson GmbH) specific for human and mouse GPIIb-IIIa, respectively.

**Statistical analyzes**

Statistical analyzes were performed using Prism, Version 5.0 (GraphPad, La Jolla, CA, USA). Comparisons between two groups were calculated using t-test and between 3 and more groups using One-way ANOVA test and Mann-Whitney-test. P-values < 0.05 were considered statistically significant.
Results

*N-glycan modification of mAb SZ21 using Endo F*

To modify the N-glycan of the mAbs, deglycosylation was performed under native conditions using Endo F and degradation of antibodies was evaluated by Coomassie Blue stained SDS-PAGE. Untreated SZ21 showed two bands in SDS-PAGE under reducing conditions with apparent molecular weights of 50 kDa (heavy chain) and 25 kDa (light chain) (figure 1). Treatment with Endo F resulted in a reduction of ~3 kDa of the apparent weight of the heavy chain, but not of the light chain of NGM-SZ21. No additional bands were detected.

The efficiency of antibody deglycosylation was investigated using lectin blotting. By lectin blotting, LCA bound to the heavy chain of untreated SZ21 but not of NGM-SZ21 (Figure 1), indicating efficient removal of the IgG-glycan. Removal of N-linked glycan at Asn297 residue under native conditions was also documented by MALDI-TOF mass spectroscopy. The mass chromatogram (figure 1) revealed that the major glycan present in SZ21 (fragment 1506) was absent in the heavy chain of NGM-SZ21. No additional cleavage was observed in the heavy chain of the NGM-SZ21. These results indicate that Endo F treatment only removed the N-glycan attached to Asn-297 of the antibody heavy chain without altering the protein structures.

*N-glycan modification does not impair the transplacental transport of SZ21*

To investigate transplacental transport, three pregnant BALBc mice were i.v. injected with 40 µg of SZ21, NGM-SZ21 or isotype-matched control IgG antibody at gestation day 17. Maternal blood samples (100 µl) were collected from pregnant mice 5 minutes after injection and within 8 hour after delivery. Blood samples from newborn pups were collected within 8 hours after birth. Because of the small collected volume (10-25 µl), neonatal blood samples of pups of one
pregnancy were pooled together and the respective IgG-fractions were isolated from 50 µl of pooled sera for SPR analysis.

By SPR, we found SZ21 and NGM-SZ21 in the blood of the pups (Figure 2A). As these antibodies had been i.v. injected into pregnant mother mice, they must have been transported via the placenta to the pups. At the end of the association phase ($B_{350}$): 44 response units (RU) ± 4 (SD) and 38 RU ± 5, and at the end of the dissociation phase ($B_{700}$): 32 RU ± 3 and 25 RU ± 4, respectively were found for SZ21 and NGM-SZ21. These data demonstrate that the deglycosylated antibody is transported with the same efficacy through the placenta as is the glycosylated antibody. No relevant binding was detected with the IgG-fraction of the control pups (Figure 2A).

Quantification of circulating SZ21 and NGM-SZ21 in the maternal and neonatal circulation was performed against the corresponding standard curves. In maternal IgG-fractions, concentrations of $2,190 \pm 15$ ng/ml and $1,888 \pm 123$ ng/ml of SZ21 and NGM-SZ21, respectively, were measured 5 minutes after antibody injection, of which $1,613 \pm 21$ ng/ml (71%) and $1,393 \pm 114$ ng/ml (72%) were still detectable 8h after delivery (gestation day 21 ± 1 day), figure 2B. Eight hours after delivery, concentrations of $145 \pm 18$ ng/ml of SZ21 and $115 \pm 16$ ng/ml of NGM-SZ21 were determined in the pubs, indicating that approximately 9% of SZ21 and NGM-SZ21 ($p = 0.58$) had been transported from the maternal into the fetal circulation (figure 2B). To compare antibody quantification by SPR with solid phase ELISA, purified GPIIb/IIIa-complex was coated on the microtiter plate, and antibody binding was measured by the use of enzyme labeled secondary antibodies. Comparable standard curves between ELISA and SPR were obtained (see supplementary Figure 1).

**NGM-SZ21 displays binding properties similar to unmodified SZ21**

To investigate the impact of N-glycan removal on the binding affinity of SZ21 for the HPA-1 epitope on GPIIb-IIIa, we compared untreated mAb SZ21 and NGM-SZ21 using SPR. SZ21 and
NGM-SZ21 were injected over the coated sensor ships in six different concentrations (range 1,500-47 ng/ml). Antibody binding was analyzed during an association phase and a dissociation phase. For SZ21, a Ka of 4.7x10^{-5} M and a Kd of 5.5x10^{-4} M were observed, giving an estimated KD of 1.17x10^{-9} M, which compares to NGM-SZ21, for which a Ka of 8.6x10^{-4} M and a Kd of 3.76x10^{-4} M were determined, resulting in an estimated KD of 4.36x10^{-9} M. When analyzed using GPIIb-IIIa from HPA-1bb homozygous platelets low affinity binding was documented. Both antibodies showed similar high dissociation rates, and consequently higher Kd values, 8.36x10^{-8} M and 1.78x10^{-8} M, respectively. Taken together, these data confirm that N-glycan removal did not impair the binding affinity of SZ21 to the HPA-1a epitope.

**NGM-SZ21 inhibits platelet phagocytosis induced by maternal HPA-1a alloantibodies**

To test the impact of N-glycan removal on the phagocytic activity of SZ21, a platelet phagocytosis assay was used. While SZ21 induced a high platelet phagocytic activity, opsonization with NGM-SZ21 did not cause significant phagocytosis (Figure 3).

To investigate the ability of NGM-SZ21 to inhibit phagocytosis of platelets induced by maternal anti-HPA-1a alloantibodies, HPA-1a-positive platelets were treated with NGM-SZ21 or NGM-AP2 before adding human anti-HPA-1a antibodies (n=4). A marked reduction of anti-HPA-1a-mediated platelet-phagocytosis was observed after pre-incubation with NGM-SZ21, but not with NGM-AP2 (Figure 3).

**NGM-SZ21 prevents maternal HPA-1a alloantibody-mediated platelet clearance in vivo**

The ability of NGM-SZ21 to prevent anti-HPA-1a-mediated clearance of human platelets *in vivo* was investigated by injecting NGM-SZ21 (HPA-1a-epitope specific) prior to the injection with anti-HPA-1a alloantibodies (n=4). NGM-AP2 (HPA-1a-epitope non-specific) was used as control. Consistently with the results of the *in vitro* experiments described above, platelet clearance was significantly inhibited by the administration of NGM-SZ21 but not by NGM-AP2 (median of
human platelet clearance (CL5h): 18%, range 10-30% vs. 62% range 60-84, p=0.013) (Figure 4A).

To simulate the situation of advanced pregnancy during which maternal anti-HPA-1a alloantibodies are already present in the fetal circulation, we injected maternal anti-HPA-1a antibodies into mice 30 minutes prior to giving NGM-SZ21 or NGM-AP2. NGM-SZ21 but not NGM-AP2 was able to ameliorate platelet destruction induced by circulation maternal anti-HPA-1a antibodies (median CL5: 38%, range 28-48% vs. 64%, range 58-80%, p=0.023) (Figure 4B).

Taken together, these data suggest that NGM-SZ21 prevents anti-HPA-1a antibody-mediated platelet destruction by competing with the binding of maternal alloantibodies, even when injected at a time point during which the maternal alloantibodies were already present in the circulation.
Discussion

This study provides the basis for a novel strategy to prevent fetal platelet destruction in FNAIT. We show that an engineered HPA-1a-specific mAb, deglycosylated at amino acid Asn297 of its heavy chain, is transported through the placenta as efficiently as is its native, fully glycosylated counterpart. The deglycosylated antibody binds via its Fab-domain to the HPA-1a epitope on GPIIb-IIIa. However, it lacks key effector functions that promote undesirable Fc-receptor dependent phagocytosis and complement activation and effectively blocks platelet phagocytosis and destruction by native human anti-HPA-1a alloantibodies \textit{in vitro} as well as \textit{in vivo}. As the human anti-HPA-1a alloantibodies were obtained from women in whom these antibodies induced severe FNAIT during pregnancy, it is very likely that the engineered monoclonal antibody will also be protective in human FNAIT.

As a proof of principle, we took advantage of the well characterized mAb SZ21, which specifically binds to the HPA-1a epitope and is well known to block binding of human anti-HPA-1a antibodies.\textsuperscript{18,27} We enzymatically removed the N-glycan attached to Asn-297 of the antibody heavy chain. This enzymatic digestion was highly effective and specific as shown by mass spectrometry, and seemed not to alter the protein structure of the antibody, as shown by lack of protein degradation products, and – most importantly – preserved binding specificity and affinity of the antibody to its antigen, as shown by SPR.

In our \textit{in vivo} mouse model the transplacental transport of the deglycosylated antibody NGM-SZ21 was as effective as the transplacental transport of its native form SZ21. In the pup circulation we recovered about 9% of the concentrations determined in the maternal blood of both forms of the antibody (NGM-SZ21 and native-SZ21), which is in accordance with the relative levels of other epitope-specific antibodies recovered in newborns in humans as well as in mice.\textsuperscript{26,29} The amount of blood we could obtain from the newborn mice was very limited. We, therefore, had to pool the blood of several of the newborn pups from one pregnancy for analysis. Theoretically, it would be possible that the amount of antibody transferred from the maternal to
the fetal circulation varied across pups. Due to this limitation, and due to the small number of animals used in our study, we can only assume that the antibodies had been transported to all pups in similar concentrations.

Most importantly NGM-SZ21 did prevent platelet phagocytosis induced by different maternal anti-HPA-1a alloantibodies \textit{in vitro} and also protected against destruction of HPA-1a-positive platelets by human anti-HPA-1a alloantibodies \textit{in vivo} in a NOD/SCID mouse model.\textsuperscript{18,26} Anti-HPA-1a antibody-mediated platelet destruction was also inhibited by NGM-SZ21 when we injected the human anti-HPA-1a alloantibodies prior to the administration of NGM-SZ21 to better simulate the situation of FNAIT in pregnancy. These results indicate that NGM-SZ21 is capable of preventing anti-HPA-1a-mediated platelet destruction \textit{in vivo} even when maternal anti-HPA-1a alloantibodies are already present in the fetal circulation. Likely this is mediated by competing with maternal HPA-1a alloantibody binding to fetal platelets or even by displacing the maternal alloantibodies as previously described for F(ab)'\textsubscript{2}-fragment of SZ21.\textsuperscript{18,26}

Unfortunately no mouse model for fetomaternal incompatibility in human platelet alloantigens currently exists. Due to this limitation, we cannot directly assess the capability of deglycosylated epitope-specific monoclonal antibodies to protect fetal mice from platelet destruction by maternal allo-antibodies. The existing mouse model of feto-maternal platelet incompatibility employs GPIIb-IIIa knock-out mice. These mice develop a broad isoimmune response to multiple epitopes on the large GPIIb-IIIa complex when immunized with platelets of wild type mice.\textsuperscript{28,28} In this model multiple antibodies with different binding sites on GPIIb-IIIa are transported to the pups. Their binding cannot be blocked by one epitope-specific monoclonal antibody. The development of a novel mouse model for fetomaternal incompatibility using humanized mice carrying the correct human MHC class II that can be challenged by human \(\beta3\) integrin with HPA-1a and -1b would help overcoming this limitation.
Due to anatomical and functional differences between human and murine placentas, the final proof of our concept can only be provided by a clinical study in humans. This will require a humanized form of the mAb SZ21 (or another HPA-1a-specific antibody), a technology well established for other therapeutic antibodies. For human use, however, there are important issues that cannot be assessed in our study and need to be taken into consideration. It is known that the beta-subunit of αVβ3 integrin on endothelium and placenta bears the HPA-1a-epitope(s). This fact may create some problems for the application of the engineered humanized mab SZ21. First, it is not clear whether endothelial cells expressing HPA-1a epitope can mop up the antibody. Since the αVβ3 complex on endothelial cells is expressed on the basal, rather than apical cell surface, it is unlikely that humanized mab SZ21 would be absorbed by maternal endothelial cells. Second, this antibody may react with the fetally-derived trophoblasts, which are most probably heterozygous for the HPA-1 polymorphism. However, maternal anti-HPA-1a alloantibodies get past them quite well on their way into the fetal circulation and induce platelet destruction in FNAIT. Therefore, this problem likely can be overcome by adjusting the dose of SZ21 to ensure delivery of a therapeutically effective dose.

The general concept of “dissociating” the effector activities of an IgG antibody from its transplacental transport potentially may allow development of preventive treatments of other antibody mediated feto-maternal incompatibilities and may even provide some perspectives for treating other fetal disorders resulting from transplacental antibody transfer.

In conclusion, removal of N-glycan of amino acid Asn-97 of the heavy chain of a mAb specific for the HPA-1a epitope on GPIIb-IIIa modifies the effector functions of the antibody, but still allows its efficient materno-fetal transport. This modified antibody prevents in vivo destruction of HPA-1a-positive platelets by maternal anti-HPA-1a alloantibodies. This provides the basis to develop a new approach to prevent severe thrombocytopenia in FNAIT.
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Authorship

Author’s Contribution: T. B. designed and performed the experiments, analysed the data, and wrote the paper; A.G. analysed the data, provided constructive criticism and wrote the paper; A. K., and H. H. performed in vitro and in vivo experiments, collected and analyzed the data. U. J. H., H. R. and G. B. provided novel reagents and important suggestions. P. J. N., and S. S. designed and supervised the study, analysed the data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References


Figure legends:

**Figure 1: N-glycan modification of the anti-HPA-1a monoclonal antibody SZ21**

Coomassie Blue staining (CBS) was used to analyze general structure modifications of SZ21 after its treatment with Endo F. Two bands with apparent molecular weights (MW) of <50 kDa (~47 kDa) and 25 kDa representing the heavy and light chains of mAb SZ21 and NGM-SZ21 were visible under reducing conditions (upper left panel). N-glycan was then detected using biotinylated lens culinaris agglutinin (LCA) (upper right panel). A strong signal was solely detected at a MW of ~50 kDa of the lane loaded with SZ21 (lane 1). No signal was detected by testing Endo F treated NGM-SZ21 (lane 2) indicating removal of the N-glycan.

The specificity of deglycosylation was analyzed by MALDI-TOF mass spectroscopy. As shown in the lower panels, the only difference between the heavy chain of mAb SZ21 (left panel) and the heavy chain of NGM-SZ21 (right panel) was in the fragment 1506 corresponding to the N-glycan that is linked to the asparagine at the position 297. No additional modifications were observed in the heavy chain of the NGM-SZ21.

**Figure 2: The impact of the N-glycan on the in vivo transport of monoclonal antibodies through the placenta**

To investigate the effect of N-glycan on IgG-transport, a total of 40 µg of SZ21, NGM-SZ21 or isotype-matched (non-platelet binding) IgG were injected in age matched pregnant female BALB/c mice at day 17 of gestation. Blood samples from newborn pups were collected within 8 hours after birth.

A) Binding of transported antibodies: In SPR, similar antibody binding to GPIIb-IIIa from HPA-1aa platelets was detected in the blood of pups whose mothers were injected with either SZ21 or NGM-SZ21. No relevant antibody binding was detected using the blood of the control pups.
B) Quantification of circulating antibodies: Equal amount of SZ21 and NGM-SZ21 (8.9% and 8.7%, respectively, \(p=0.58\)) was transported from maternal (black bars) into neonatal (white bars) circulation at the end of the pregnancy. This figure shows data from three different experiments.

**Figure 3: Ability of NGM-SZ21 to prevent anti-HPA-1a-mediated phagocytosis of HPA-1a platelets**

The effect of antibody deglycosylation on the phagocytic activity was investigated by opsonising CMFDA (FITC)-labelled platelets with SZ21 or NGM-SZ21 before adding the monocytes. Monocytes were then gated and the percentage of FITC-positive monocytes, i.e. those who ingested labeled platelets, was identified as the phagocytic activity (%). Note that the SZ21 but not NGM-SZ21 was capable of inducing platelet phagocytosis (first and second bars, respectively).

To assess the protection property, labeled platelets were incubated with NGM-SZ21 (black bars) or with NGM-AP2 as an isotype-matched control (grey bars) before adding maternal anti-HPA1a IgG. Then monocytes were added and phagocytosis of the opsonized platelets was assessed and expressed as phagocytic activity. White bars represent the native phagocytic activity of the maternal anti-HPA-1a antibodies. Grey bars show that the control antibody NGM-AP2 did not inhibit phagocytosis, while the black bars show inhibition of the phagocytic activity by NGM-SZ21 (median of inhibition 47% vs. 0%, respectively, \(p=0.008\)).

**Figure 4: NGM-SZ21 prevents anti-HPA-1a antibody-mediated platelet destruction in a NOD/SCID mouse model of alloimmune thrombocytopenia**

Resting human platelets (HPA-1ab) were injected retro-orbitally into NOD/SCID mice and the survival of platelets was analyzed. Results are shown as a median and range of experiments that were performed in duplicate with anti-HPA-1a antibodies from four FNAIT cases.
A) Epitope-Blockade: When NGM-SZ21 was injected prior to maternal anti-HPA-1a antibodies, elimination of platelets was largely inhibited (black symbols), while the control antibody NGM-AP2 did not prevent rapid elimination by human anti-HPA-1a antibodies (grey symbols). NGM-SZ21 alone did not induce relevant platelet destruction (white symbols).

B) Antibody competing: To simulate the situation of advanced pregnancy maternal anti-HPA-1a antibodies were injected 30 minutes prior to giving NGM-SZ21 or NGM-AP2. The injection of maternal anti-HPA-1a alone resulted in a rapid destruction of circulating human HPA-1a platelets (white symbols). Note that NGM-SZ21 (black symbols), but not NGM-AP2 (grey symbols) was able to ameliorate platelet destruction induced by circulation maternal anti-HPA-1a antibodies.
Figure 2B

Anti-HPA-1a (ng/ml)

Maternal level  Fetal level Maternal level  Fetal level

\[ p=0.58 \]
Figure 4A

- NGM-SZ21 + maternal anti-HPA-1a
- NGM-AP2 + maternal anti-HPA-1a
- NGM-SZ21 alone

Platelet survival (%) vs. Time (min)
Figure 4B

- Maternal anti-HPA-1a + NGM-SZ21
- Maternal anti-HPA-1a + NGM-AP2
- Maternal anti-HPA-1a alone

Platelet survival (%) vs. Time (min)

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