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ORIGINAL ARTICLE

An HPA-1a–positive platelet–depleting agent for prevention of fetal and neonatal alloimmune thrombocytopenia: a randomized, single-blind, placebo–controlled, single-center, phase 1/2 proof-of-concept study

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Abstract

Background: Fetal/neonatal alloimmune thrombocytopenia (FNAIT) is a rare and potentially life-threatening bleeding disorder of the fetus/newborn. Antibodies against human platelet antigen 1a (HPA-1a) are associated with the most frequent FNAIT cases. There are no approved therapies for FNAIT prevention or treatment. RLYB211 is a polyclonal HPA-1a hyperimmune IgG being developed to prevent FNAIT.

Objectives: To investigate whether a single dose of anti–HPA-1a (1000 IU) could markedly accelerate the elimination of HPA-1ab platelets transfused into healthy, HPA-1a–negative participants as compared with placebo.

Methods: This randomized, single-blind, placebo–controlled, single-center, phase 1/2 proof-of-concept study (EudraCT: 2019-003459-12) included HPA-1a– and HLA-A2–negative healthy men. Cohort 1 received intravenous RLYB211 or placebo 1 hour after transfusion of HPA-1ab platelets. Cohort 1B received RLYB211 or placebo, followed by platelet transfusion 1 week later. Primary endpoint was the half-life of transfused platelets in circulation after administration of RLYB211 or placebo, determined by flow cytometry. Proof of concept was ≥90% reduction of half-life relative to placebo.

Results: Twelve participants were allocated to cohort 1 or 1B and randomized to receive RLYB211 (n = 9) or placebo (n = 3). RLYB211 markedly accelerated the elimination of HPA-1ab platelets in all participants vs placebo. In cohort 1B, this effect was

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observed 7 days after RLYB211 administration. Two treatment–emergent adverse events were possibly related to treatment, both in RLYB211–treated participants. No participants developed HPA-1a antibodies at 12 or 24 weeks.

Conclusion: These data support the hypothesis that anti–HPA-1a could be used as prophylaxis in women at risk of having an FNAIT–affected pregnancy.

KEYWORDS
alloantibodies, human platelet antigen, intracranial hemorrhage, neonatal alloimmune thrombocytopenia, prophylaxis

1 | INTRODUCTION

Fetal/neonatal alloimmune thrombocytopenia (FNAIT) is a rare and potentially life-threatening disorder that can cause uncontrolled bleeding in fetuses and newborns [1]. It is estimated to occur in approximately 1 in 1000 pregnancies with potentially severe consequences, including fetal and neonatal intracranial hemorrhage (ICH) that may result in irreversible brain damage or death [1–3]. FNAIT is caused by maternal alloantibodies directed against paternally inherited antigens on fetal platelets [4,5]. Antihuman platelet antigen 1a (HPA-1a) is the most frequently implicated antibody in FNAIT, accounting for 75% to 80% of severe FNAIT cases in White people [6–8]. Maternal alloantibodies of the immunoglobulin G (IgG) class cross the placenta and opsonize the fetal platelets, which are then removed by the mononuclear phagocyte system of the fetus, resulting in fetal thrombocytopenia [9,10]. Furthermore, HPA-1a alloantibodies may have a direct effect on fetal vessel wall integrity via binding of the β3 integrin on the endothelial cell surface [11]. If the endothelial lining is damaged, hemostasis will be compromised due to the low platelet count, which may result in bleeding, spanning a continuum from petechiae to ICH [4,12]. ICH due to anti–HPA-1a occurs in approximately 1 in 10,000 pregnancies [2], which translates into approximately 1000 yearly cases of ICH in Europe and North America. The propensity to develop HPA-1a alloantibodies is closely linked to a certain human leukocyte antigen (HLA) type [13]. Thus, the risk for women who are HPA-1a negative to become HPA-1a immunized is approximately 25 times higher in those who are positive for the *HLA-DRB3*01:01* allele than in women who do not carry this allele [14].

An estimated 25% of all HPA-1a immunization cases occur during gestation of the first pregnancy [15], and alloantibodies to HPA-1a are detectable as early as gestational week 17 [16]. Current guidelines recommend that HPA-1a–immunized pregnant women be treated with intravenous (i.v.) immunoglobulin, with or without corticosteroids [17]. However, i.v. immunoglobulin does not prevent alloimmunization, nor does it consistently improve fetal platelet count in all individuals [18]. Furthermore, this treatment is costly and is often associated with adverse events (AEs) [19–22].

At present, there are no approved therapies for preventing alloimmunization or treatments for an HPA-1a–negative pregnant woman carrying an HPA-1a–positive fetus. Previous proof-of-concept studies in a murine model of FNAIT using β3 integrin (GPIIIa)–deficient mice demonstrated that administration of platelet antibodies inhibited the maternal alloantibody response, increased platelet counts in pups, and decreased the number of pregnancies with miscarriages and dead pups compared with control mice that did not receive platelet antibodies [23,24]. These results have been confirmed in recent studies applying a novel biallelic murine model of FNAIT, in which the human HPA-1a allogeneic epitope was expressed on the murine GPIIIa backbone [25,26]. Administration of anti–HPA-1a, either as a hyperimmune plasma-derived anti–HPA-1a IgG (RLYB211) [27] or as a human monoclonal antibody (RLYB212) [28], followed by administration of HPA-1a–positive platelets, led to rapid elimination of HPA-1a–positive platelets from circulation and prevented the development of HPA-1a alloantibodies [26,29]. Additionally, HPA-1a–negative female mice treated prophylactically with RLYB211 before exposure to HPA-1a–positive platelets, gave birth to HPA-1a–positive pups with improved platelet counts and no bleeding symptoms [26].

These nonclinical data support the hypothesis that administration of anti–HPA-1a to HPA-1a–negative women may prevent HPA-1a alloimmunization and FNAIT, an approach resembling anti–rhesus D (RhD) prophylaxis that has been successfully used in RhD–negative women for almost 50 years for the prevention of hemolytic disease of the fetus and newborn (HDFN) [30]. The purpose of this clinical proof-of-concept study was to determine whether a dose of 1000 IU of anti–HPA-1a accelerates the elimination of HPA-1ab platelets transfused into healthy, HPA-1a–negative participants.

Essentials

- Fetal/neonatal alloimmune thrombocytopenia (FNAIT) can cause uncontrolled bleeding in fetuses and newborns.
- RLYB211, a plasma-derived polyclonal anti–HPA-1a IgG, is being developed to prevent FNAIT.
- RLYB211 showed rapid and complete HPA-1a platelet elimination in HPA-1a–negative individuals.
- Proof-of-concept was established for RLYB211, supporting potential use in FNAIT prophylaxis.

2 | METHODS

2.1 | Study design

This was a randomized, single-blind, placebo–controlled, single-center phase 1/2 proof-of-concept study (EudraCT Number: 2019-003459-12) investigating the safety and efficacy of a single dose of i.v. RLYB211 for eliminating HPA-1a–positive platelets transfused into HPA-1a–negative and HLA-A2–negative healthy male participants. RLYB211 is a human anti–HPA-1a immune globulin produced using source plasma from HPA-1a immunized women in North America, collected and tested in compliance with the US Food and Drug Administration and the European Medicines Agency standards and guidelines [27]. Both RLYB211 and placebo were formulated as clear, colorless solutions. The study, conducted at the Fraunhofer Institute for Translational Medicine and Pharmacology in Frankfurt am Main, Germany, was initiated in September 2020. Here, we report safety and proof-of-concept efficacy data for 12 treated participants, based on an interim data cutoff of January 2022.

2.2 | Study participants

Following approval by the Paul Ehrlich Institut, Germany, and the Ethics Committee at Frankfurt University Hospital (Case number 20-628-AMG), written informed consent was obtained from 12 healthy men aged 18 to 65 years. Only study participants who were both HPA-1a negative and HLA-A2 negative were included because donor/recipient discrepancy with regard to HLA-A2 was used for the identification of transfused platelets. Women were not included to avoid the very low risk of alloimmunization after transfusion of such a small number of incompatible platelets. Participants were excluded if they had a body mass index (BMI) $\geq$35 kg/m^2, had a history of hypersensitivity to platelet concentrates or human plasma proteins, had IgA levels < 0.06 g/L, received a blood transfusion within 3 weeks of screening, had platelet counts < 150 $\times$ 10^9/L or >450 $\times$ 10^9/L, had any platelet function disorder, received a nonsteroidal anti–inflammatory drug or selective serotonin reuptake inhibitor within 7 days of screening or had any chronic or active infectious disease requiring systemic treatment.

2.3 | Randomization and masking

This study included 2 cohorts, cohort 1 (n = 8) and cohort 1B (n = 4). Upon completion of the safety review of cohort 1, new participants were enrolled in cohort 1B. Eligible participants were assigned to cohort 1 or cohort 1B using a unique randomization number in ascending numerical order. For the first 4 participants in cohort 1, randomization was arranged to include 1 placebo participant as number 1 or 2 and 1 placebo participant as number 3 or 4, whereas the remaining participants received RLYB211 (Figure 1). Participants enrolled in cohort 1B (n = 4) were randomized 3:1 in a blinded manner to receive either RLYB211 (n = 3) or placebo (n = 1) (Figure 1). The randomization schedule was generated prior to the study by the Statistics Department at Larix. Participants were not informed of their assigned randomization group and were dispensed blinded study drugs labeled with their unique randomization numbers.

2.4 | Procedures

Cohort 1 (n = 8) received a transfusion of 10 $\times$ 10^9 HPA-1ab platelets from an HLA-A2–positive donor (where 10 $\times$ 10^9 HPA-1ab platelets are equivalent to the number of platelets in 30 mL of fetal blood), followed 60 minutes later by either i.v. RLYB211 (1000 IU of anti–HPA-1a; n = 6) or placebo (n = 2), with a follow-up period of 24 weeks (Figure 2). The dose of platelets for transfusion was based on the paradigm for RhD prophylaxis, according to which anti–D is dosed to neutralize the red blood cells (RBCs) from up to 30 mL of fetal blood, where 30 mL represents a large fetal-maternal hemorrhage, and volumes above this are typically associated with fetal mortality [31].

Cohort 1B received either i.v. RLYB211 (1000 IU of anti–HPA-1a; n = 3) or placebo (n = 1), followed 7 days later by transfusion of 10 $\times$ 10^9 HPA-1ab platelets from an HLA-A2–positive donor (day 1), with a 24-week follow-up period (Figure 2).

There were no premedications administered with the i.v. fusions, and i.v. administration time was approximately 1 to 2 minutes. Cohort 1 was designed to assess the ability of RLYB211 to eliminate HPA-1ab platelets immediately upon its administration. Cohort 1B, in contrast, would inform on how the reduction in anti–HPA-1a that occurs during the 7-day period from drug administration until platelet transfusion would affect the speed of platelet elimination. Thus, cohort 1B was designed to simulate prophylactic administration of RLYB211 prior to a fetal-maternal bleed.

It was estimated that an i.v. dose of up to 2000 IU human anti–HPA-1a IgG would be sufficient for opsonizing HPA-1ab platelets for phagocytosis. This calculation was based on: 1) knowledge about the physiological changes that occur during pregnancy and immediately after delivery; 2) knowledge about the pharmacokinetics of IgG; 3) experience from RhD prophylaxis; 4) previously published studies of antibody-mediated elimination of platelets; 5) clinical experience; and 6) *in vitro* experiments. As the calculation of dose was based on a number of assumptions, it was decided to apply a conservative approach and use 1000 IU as a starting dose for the current study.

2.5 | Platelet and plasma preparation

Platelets to be transfused were obtained by plateletpheresis from existing ABO-compatible platelet donors at the German Red Cross Blood Donor Service Baden-Württemberg-Hessen, Frankfurt am Main, Germany. Plateletpheresis were completed 20 to 24 hours prior to transfusion. All platelet donors were HPA-1ab heterozygous and HLA-A2 homozygous. None of the platelet donors had HLA antibodies.

Platelet-rich plasma prepared from whole blood collected from the recipients was used to determine the proportion of HLA-A2–positive platelets. Platelet-rich plasma was prepared from the

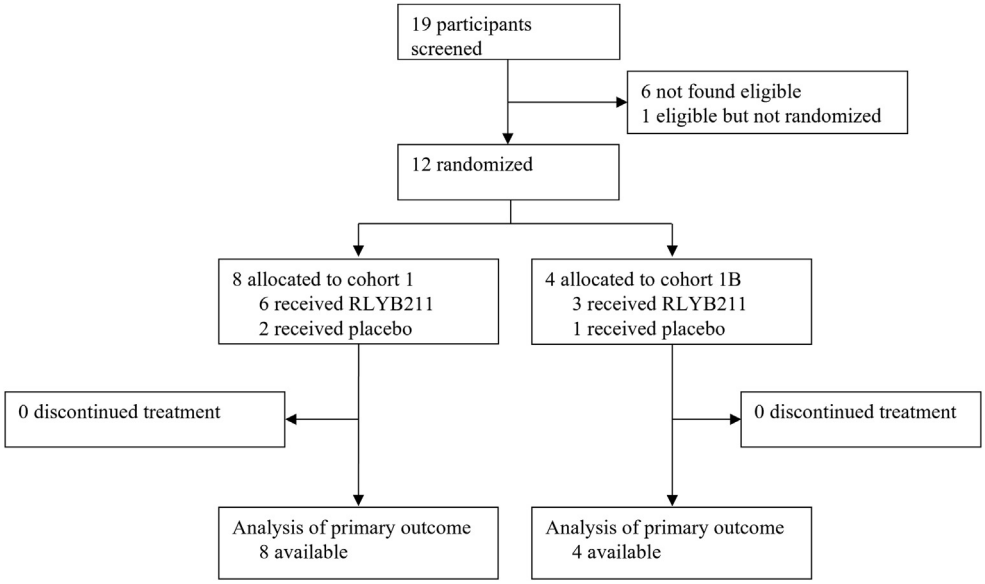

FIGURE 1 Trial profile.

anticoagulant citrate dextrose (ACD-A) plasma collected from the study participants, and platelets were immediately preserved with ThromboFix Platelet Stabilizer (Beckman Coulter) according to the manufacturer's instructions.

Both endogenous and transfused platelets in cohort 1 were analyzed 15 minutes before platelet transfusion; 15 minutes before study drug administration (ie, 1 hour later); 10, 20, 30, 40, and 50 minutes and 1, 2, 3, 4, and 24 hours after study drug administration; on day 3; and on day 7 if transfused platelets were still detectable on day 3. For cohort 1B, in which study drug was administered 7 days before platelet transfusion, platelet analysis took place 15 minutes before platelet transfusion; 10, 20, 30, 40, and 50 minutes and 1, 2, 3, 4, and 24 hours after platelet transfusion; on day 3; and on day 7 if platelets were detected in a transfused individual on day 3.

2.6 | Flow cytometry

A validated flow cytometry–based method, developed by Vetlesen et al. [32] was used to determine the proportion of HLA-A2–positive platelets in the recipient at specified time points after administration of RLYB211 [33]. Optimization and validation of this method were performed by mixing small known numbers of HLA-A2–positive platelets with large known numbers of HLA-A2–negative platelets, and subsequently comparing the expected and observed proportions [33]. The lower limit of quantification was 0.015%, and linearity was 0.97. Approximately 1.5×10^6 platelets were resuspended in 150 μL of HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline and double stained with 6 μL of fluorescein isothiocyanate (FITC)–conjugated HLA-A2 antibodies (clone H0037; ONE Lambda Inc.) and 1.2 μL of PC5–conjugated CD41 antibodies (clone P2; Beckman Coulter). After 20 minutes of incubation in the dark at room temperature, 600 μL of fixation buffer (0.2% paraformaldehyde in phosphate-buffered saline) was added. One million events were collected at the lowest collection rate using a Canto II flow cytometer (*Becton Dickinson*). FACSDiva software (BD Bioscience) was used to determine the proportion of transfused platelets. Platelets were identified on scatter plots showing the forward scatter properties versus PC5 fluorescence, and the frequency of transfused platelets was assessed on scatter plots showing FITC fluorescence versus side scatter. Blood samples were analyzed consecutively. If transfused platelets could not be detected in 2 consecutive samples, samples from subsequent time points were not obtained or examined.

2.7 | Analysis of HPA-1a antibodies

To determine whether infusion of HPA-1a–positive platelets had induced HPA-1a antibody formation in the healthy participants, HPA-1a antibodies were quantified as described by Mörtberg et al. [34]. The World Health Organization International Standard anti–HPA-1a (100 IU; NIBSC code 03/152) was used as a calibrator [34]. The 5-parameter model was used for curve fitting and for calculating concentrations in study samples, using SoftMaxPro v. 7.1 software (Molecular Devices).

2.8 | Outcomes

The primary objective of the study was to establish whether RLYB211 could markedly accelerate the elimination of HPA-1ab platelets transfused into HPA-1a–negative healthy male participants. The primary endpoint was the half-life ($t_{1/2}$) of transfused HPA-1ab platelets in circulation after administration of RLYB211 or placebo. Proof of concept was prospectively defined as ≥90% reduction in $t_{1/2}$ of HPA-1a–positive platelets relative to placebo.

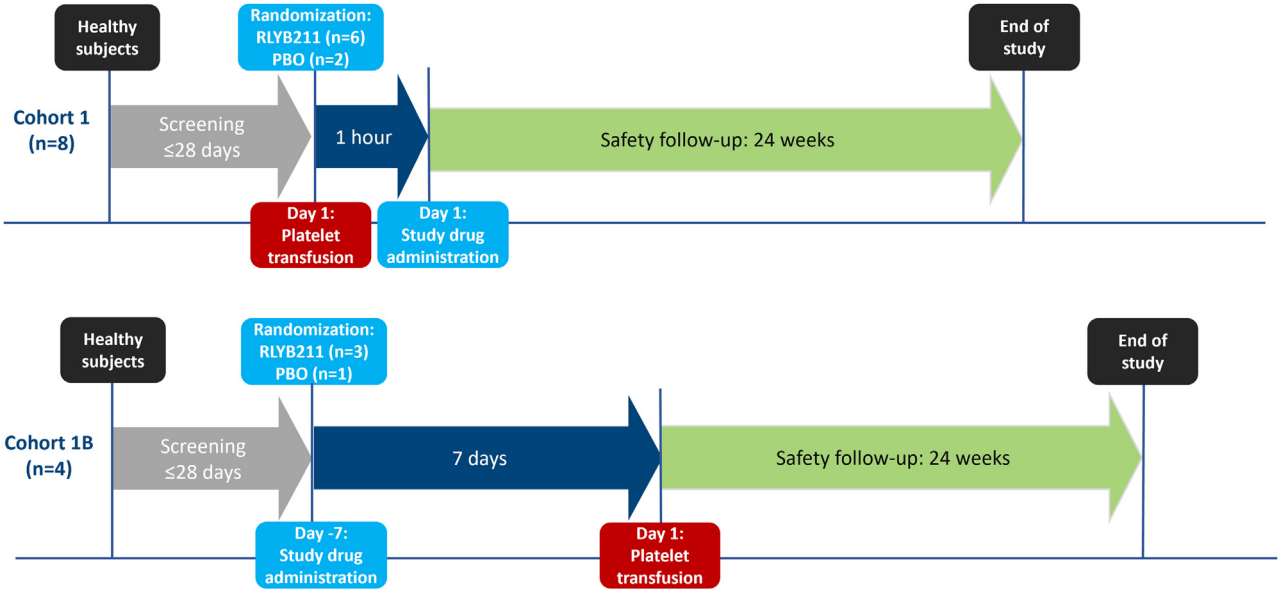

FIGURE 2 Study design. PBO, placebo.

Treatment–emergent adverse events (TEAEs) were defined as AEs that occurred from the time of study drug administration to 24 weeks of follow-up. TEAE incidence was presented by system organ class and preferred term, relationship to study drug, and severity. Safety data and an assessment for endogenous HPA-1a antibodies were reported throughout 24 weeks for cohort 1 and cohort 1B.

Other secondary endpoints assessed during the course of the study were vital signs (temperature, systolic and diastolic pressure, pulse, and respiratory rate), electrocardiogram (ECG), and clinical laboratory evaluations (hematology, serum chemistry, coagulation, and urinalysis). In addition, a 12-lead ECG was obtained to measure heart rate, PR, QRS, and QT intervals.

2.9 | Statistical methods

No formal sample sizing was conducted for this phase 1/2 study. Based on the prospectively defined proof-of-concept criteria (ie, ≥10× accelerated elimination of HPA-1a–positive platelets by RLYB211 compared with placebo), it was determined that 8 participants would be adequate for cohort 1 (6 RLYB211 and 2 placebo).

Based on the magnitude and consistency of effect size established for RLYB211 in cohort 1, it was then determined that 4 participants would be adequate to evaluate accelerated platelet elimination in cohort 1B (3 RLYB211 and 1 placebo).

Data were tabulated and presented graphically. Platelet kinetic analysis was performed on all participants for whom sufficient data were available to derive at least 1 of the platelet kinetic endpoints. The safety analysis set included all participants who received platelets and/or study drugs (RLYB211 or placebo) and was used for reporting safety, demographic characteristics, and exposure to treatment. Individual platelet proportions at each time point were normalized to the baseline assessment (100%), which was defined as the first flow cytometry data point after platelet transfusion, which was 1 hour for cohort 1 and 10 minutes for cohort 1B. Actual sampling time points relative to baseline were used for derivation of the noncompartmental analysis (NCA) and on the individual plots of platelet versus time. Flow cytometry–assessed platelet concentration values that were below the highest prebaseline value were excluded from the NCA. No formal analysis of "outliers" was performed. For the primary endpoint, the terminal elimination $t_{1/2}$ rate was calculated by NCA. The elimination phase was determined by visual inspection of the individual concentration curves. To estimate the slope (λ_z) of the log concentration-vs-time curve, at least 3 data points above the background FITC fluorescent signal before transfusion were used.

3 | RESULTS

Overall, 19 participants were screened, of whom 6 were ineligible for the study and 1 was eligible but not randomized. Therefore, from September 29, 2020, to October 12, 2021, 12 participants were allocated to cohort 1 or 1B, and overall, 9 were randomized to receive RLYB211 and 3 to receive a placebo (Figure 1). Table 1 shows baseline characteristics of the participants; all were White men, aged 23 to 65 years, with a BMI ranging from 21.4 to 32.3 kg/m^2.

All platelet units were harvested from donors of blood group O. The proportion of transfused HPA-1a–positive platelets in circulation after administration of RLYB211 and placebo is shown for cohorts 1 and 1B over 7 days in Figure 3A, and over 4 hours in Figure 3B. Representative flow cytometry plots for 1 participant receiving RLYB211 and 1 participant receiving placebo are shown in Figure 4. In both cohorts, administration of RLYB211 markedly accelerated the elimination of HPA-1a–positive platelets compared with placebo. For cohort 1, nearly all transfused platelets were eliminated after 2 hours,

TABLE 1 Baseline characteristics of the study population.

	Cohort 1 RLYB211 (*n* = 6)	Cohort 1B RLYB211 (*n* = 3)	Cohorts 1 and 1B placebo (*n* = 3)
Gender			
Male, *n*	6	3	3
Age in y, mean (SD)	46.8 (11.3)	46.7 (12.5)	39 (17.1)
Race, *n*			
White	6	3	3
Weight in kg, mean (SD)	93.6 (14.2)	91.4 (20.9)	92.7 (22.3)
BMI in kg/m^2, mean (SD)	28.6 (3.5)	28.0 (5.8)	27.3 (4.1)
ABO blood group, *n*			
A+	3	2	2
O+	3	1	1
Alcohol use, *n*			
Never	1	0	0
Current	5	3	3
Smoker, *n*			
Never	4	2	1
Former	2	1	2

BMI, body mass index; SD, standard deviation.

and the platelet elimination profile was consistent across all participants, with little interindividual variation (Figure 3 and Table 2).

The ability of RLYB211 to rapidly eliminate transfused platelets was also observed 7 days after a single dose administration. In cohort 1B, 2 out of 3 participants receiving RLYB211 achieved near-total elimination of platelets after 2 to 3 hours, and in the third participant, 36% of the transfused platelets could still be detected after 4 hours (Figure 3), resulting in a considerably longer platelet $t_{1/2}$. As no samples were scheduled between 4 and 24 hours after platelet transfusion, it was not possible to determine the exact platelet $t_{1/2}$ for this participant. A conservative estimate, assuming it took 24 hours to eliminate all transfused platelets, would result in a platelet $t_{1/2}$ of 2.6 hours, compared with 0.42 and 0.59 for the other 2 participants in cohort 1B. Despite the longer platelet $t_{1/2}$ in this 1 participant, all RLYB211-treated participants met the proof-of-concept criterion of elimination of HPA-1a–positive platelets by 10-fold or greater vs placebo, as defined by platelet $t_{1/2}$.

Vital signs, ECGs, and clinical laboratory evaluations were comparable between participants treated with RLYB211 and those given placebo (data not shown). TEAEs were infrequent (Table 3). Two TEAEs (headache and nausea) were deemed as possibly related to treatment, and both were in RLYB211-treated participants. In 1 participant, a TEAE of nausea occurred on the day of dosing and resolved the same day. In a second participant, a TEAE of headache occurred the day after dosing and resolved the same day. No intervention (concomitant medication or hospitalization) was required for these 2 AEs, for which severity was reported as mild. There was no discernible difference in the pattern of TEAEs between participants treated with RLYB211 and those treated with a placebo (Supplementary Table), and no serious TEAEs were reported. None of the participants had developed HPA-1a antibodies at 12 weeks (*n* = 12) or 24 weeks (*n* = 12).

4 | DISCUSSION

This study establishes proof of concept for the ability of 1000 IU of anti–HPA-1a to cause rapid and complete elimination of HPA-1ab platelets transfused into HPA-1a–negative individuals, based on a 10×10^9 platelet dose equivalent to the number of platelets in 30 mL of fetal blood, which simulates a large fetal-maternal bleed. This therapeutic effect persisted for at least 7 days after infusion of anti–HPA-1a. Rapid and complete platelet elimination was achieved in all participants after administration of RLYB211. These results are in line with those of Harrington et al. (1951), who demonstrated that transfusion of plasma from patients with immune thrombocytopenia caused severe thrombocytopenia in healthy individuals [35]. Experiments along the same line were later performed by Shulman et al. in 1965 [36]. The results from the current study are also consistent with previous proof-of-concept studies in murine models of FNAIT in which anti–HPA-1a, in addition to preventing alloimmunization [26,29], also caused rapid platelet elimination.

The results of the current study support the potential use of RLYB211 as a prophylaxis against HPA-1a immunization, where

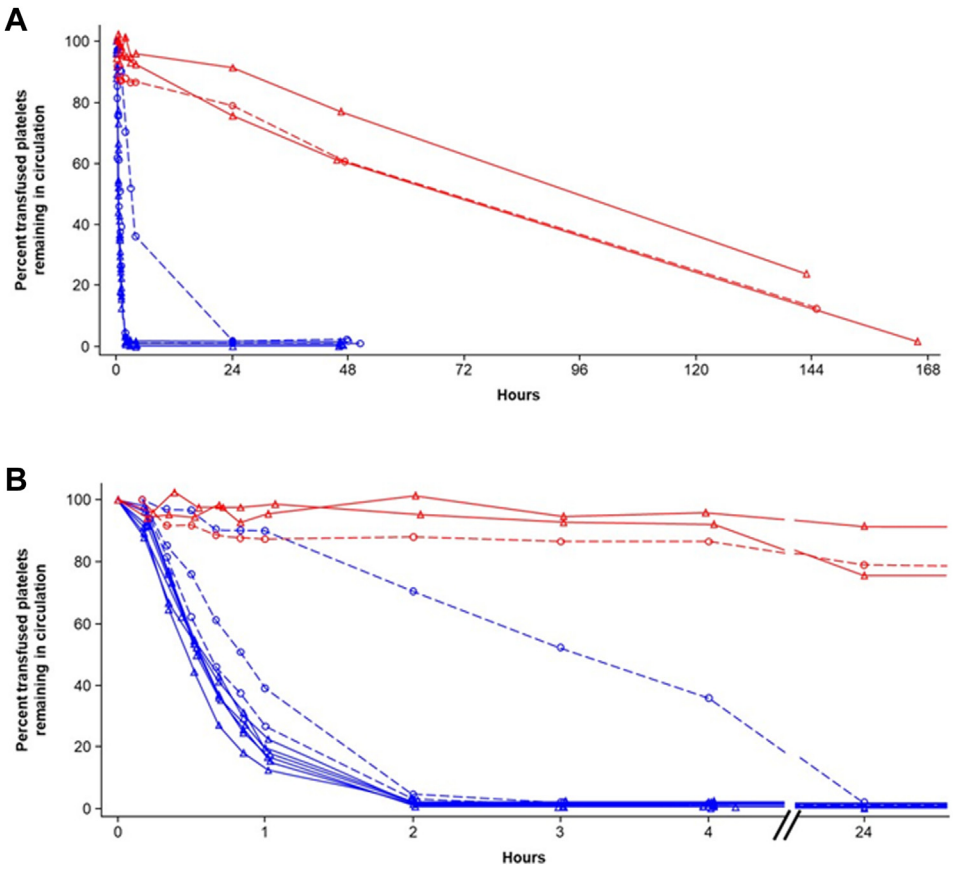

FIGURE 3 Proportion of transfused HPA-1a–positive platelets in circulation after administration of RLYB211 and placebo in cohorts 1 and 1B over 7 days (A) and 24 hours (B). ---Δ--- and ---○--- indicating the results of RLYB211 (blue). – –Δ– – and – –○– – indicating the results of placebo (red). Individual platelet proportions at each time point were normalized to the baseline assessment (100%), which was defined as the first flow cytometry data point after platelet transfusion—1 h for cohort 1 and 10 minutes for cohort 1B.

platelet elimination is being used as a surrogate marker for inhibition of HPA-1a alloimmunization. This assumption is based on the vast experience gained from the development of hyperimmune anti–D IgG drugs for the prevention of RhD immunization and HDFN. Numerous clinical studies have documented that transfused RhD-positive RBCs are rapidly eliminated after administration of RhD antibodies [37–41]. Moreover, the ability of hyperimmune anti–D products to suppress RhD immunization in RhD-negative pregnant women giving birth to an RhD-positive child, is well documented [42,43]. These trials have confirmed that elimination of RhD-positive RBCs is strongly associated with antibody-mediated immune suppression. Thus, today, all licensed polyclonal hyperimmune anti–D IgG preparations possess the ability to eliminate RhD-positive RBCs, and conversely, to our knowledge, there are no polyclonal, hyperimmune anti–D IgG preparations that can prevent RhD immunization without concurrent ability to eliminate RhD-positive RBCs from circulation.

RLYB211 was well tolerated, with only 2 TEAEs (headache and nausea) that were possibly related to treatment. Moreover, none of the participants became HPA-1a immunized as a result of participation in the study, probably because (1) the transfused dose of incompatible platelets was very small (only approximately 1/30 of a standard platelet unit); (2) HPA-1a is not particularly immunogenic, especially not in men in whom the immune balance between Th1 and Th2 is not skewed toward Th2, as it is in pregnant women [44]; and (3) all but 1 study participant were *HLA-DRB3*01:01* negative (data provided by the German Red Cross Blood Donor Service Baden-Württemberg–Hessen), greatly reducing the propensity to develop HPA-1a antibodies [45].

A more recently developed flow cytometry–based method to determine platelet survival was employed in our study as an alternative to the standard method, which involves tracking transfused platelets that have been labeled *ex vivo* with ^{51}Cr and/or ^{111}In [46,47]. These methods have several technical disadvantages, including labeling procedures that require substantial manipulation of the platelets and resultant uncertainty as to whether the labeled platelets are comparable with unmanipulated platelets [48,49]. Additionally, there are ethical, economic, and environmental concerns related to the use of this method [48,49]. To circumvent these inherent problems, we chose to use flow cytometry for assessing platelet survival. In a previous study [32], the median platelet $t_{1/2}$ of 5 platelet units (5–72 hours old) transfused to patients who underwent stem cell transplantation was 49 hours (range, 40–73 hours), which is in the same

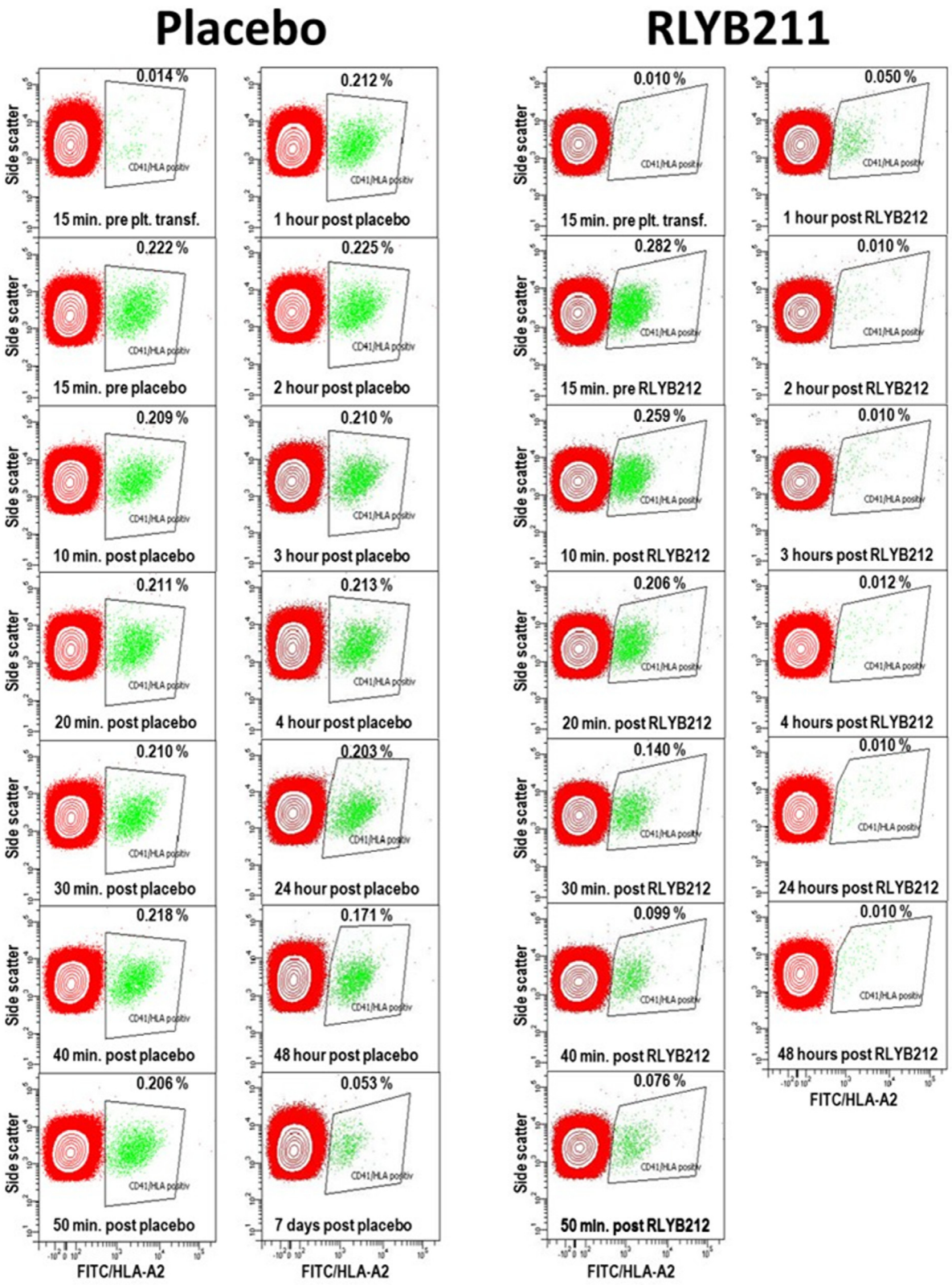

FIGURE 4 Representative flow cytometry plots for 1 participant given placebo and 1 RLYB211–treated participant.

range as the platelet $t_{1/2}$ in the 3 participants who received placebo in the current study. The low variability in the proportion of transfused HPA-1a–positive platelets in circulation within the first 4 hours after administration of platelets in the 3 participants who received placebo (Figure 3B) indicated that the method used in this study for determining platelet survival is robust.

TABLE 2 Summary of individual participant characteristics.

Participant[a]	Cohort	Treatment	Age (y)	BMI (kg/m^2)	Blood group of study participants	Platelet half-life (h)
1	1	Placebo	23	22.82	O	71.1
2	1	RLYB211	65	29.38	A	0.35
3	1	Placebo	57	28.43	A	60.0
4	1	RLYB211	53	29.31	A	0.32
5	1	RLYB211	36	31.70	O	0.28
6	1	RLYB211	38	23.19	O	0.37
7	1	RLYB211	50	26.02	O	0.30
8	1	RLYB211	39	32.25	A	0.34
9	1B	Placebo	37	30.79	A	49.3
10	1B	RLYB211	41	30.90	A	0.42
11	1B	RLYB211	38	31.75	A	0.59
12	1B	RLYB211	61	21.40	O	2.60

BMI, body mass index.

[a] None of the participants had human leukocyte antigen-specific antibodies.

In cohort 1B, 2 study participants had platelet $t_{1/2}$ of 0.42 and 0.59 hours, which is comparable to those found in cohort 1. One participant in cohort 1B had a considerably longer platelet $t_{1/2}$ (2.6 hours). However, compared with the half-lives observed in the participants treated with placebo (49.3, 60.0, and 71.1 hours), the platelet elimination in this participant still exceeded the success criterion for proof of concept, set to ≥90% reduction in $t_{1/2}$ compared with placebo. The reason for this discrepancy is unknown but it could not be attributed to baseline characteristics of the platelet donor, as the same donor was used for all 4 participants in cohort 1B. We could also not determine whether it was related to differences in anti–HPA-1a levels at the time of platelet transfusion, as these were not measured. Given that the interindividual variation in platelet elimination $t_{1/2}$ was very small for the other 7 individuals and the $t_{1/2}$ was much longer in this participant from cohort 1B, a possible explanation may be differences in the individual's immunological status, such as reduced number of macrophages in the participant's spleen that could have caused saturation of the phagocytic capacity, leading to longer survival of antibody-sensitized platelets; or reduced binding of the Fc part of IgG to the macrophages in the spleen, for instance due to a polymorphism in 1 of the Fcγ receptors.

These results establish proof of concept for prophylactic administration of RLYB211 to produce rapid elimination of HPA-1–mismatched platelets and support its potential use for the prevention of HPA-1a alloimmunization and occurrence of FNAIT. Because a substantial fraction of HPA-1a immunizations occur during an incompatible first pregnancy [16], a future prophylaxis against HPA-1a immunization would need to prevent the immunization cases occurring during pregnancy. Thus, it would be necessary to identify women at risk of becoming HPA-1a–immunized early in pregnancy. After an initial HPA-1a typing of the pregnant woman, there will be up to 3 downstream tests to inform whether prophylactic treatment with RLYB211 should be initiated: maternal *HLA-DRB3*01:01* status, fetal HPA-1a typing, and screening for maternal anti–HPA-1a [1]. These tests are available today and antenatal screening for the development of HPA-1a–related FNAIT could be implemented as part of the

TABLE 3 Summary of TEAEs—safety analysis set.

	Cohort 1 RLYB211 (*n* = 6) *n*/events	Cohort 1B RLYB211 (*n* = 3) *n*/events	Cohorts 1 and 1B placebo (*n* = 3) *n*/events
All TEAEs	5/15	3/10	3/11
Serious TEAEs	0	0	0
Severe TEAEs	2/2	0	0
TEAEs leading to withdrawal from study treatment	0	0	0
Related or possibly related TEAEs	1/1	1/1	0

n = number of participants with at least 1 event.

TEAE, treatment–emergent adverse event.

antenatal health care program, provided that screening and subsequent prophylaxis can be developed in a safe and cost-effective manner. Screening would have the potential to greatly reduce the incidence of FNAIT, analogously to the impact of antenatal screening and anti-RhD therapies on the incidence of HDFN [1].

Administration of RLYB211 periodically during pregnancy is broadly similar to antenatal administration of hyperimmune anti–D IgG for the prevention of RhD immunization late in pregnancy. The administered dose of RLYB211 would be well below an exposure level of 3 IU/mL, which is the level used in Norway for risk stratification of HPA-1a–immunized pregnant women [15,50]. Based on the assumption that the distribution volume of anti–HPA-1a is 4000 mL when i.v. RLYB211 is administered at a dose of 1000 IUs [51], the plasma concentration of anti–HPA-1a would not exceed 0.25 IU/mL, which is approximately 12 times below the 3 IU/mL safety threshold for harm to the fetus and neonate. It is worth noting that RhD prophylaxis is also given antenatally, with no documented adverse effects on the fetus, although it routinely causes a positive direct antiglobulin test [52].

A limitation of this study is the relatively small number of study participants, although the sample size was adequate to demonstrate the profound and durable effect of RLYB211 in eliminating mismatched transfused platelets. An additional limitation is that the study participants were healthy male volunteers. While appropriate for this proof-of-concept study, they are not representative of the ultimate target treatment population of high-risk pregnant women. Determining whether alloimmunization is prevented in the target population at the same dose of RLYB211 used in this study will require undertaking a future study in high-risk pregnant women, with prevention of alloimmunization as the primary endpoint.

The ability of a single-dose administration of RLYB211 to rapidly eliminate transfused platelets after 7 days in cohort 1B suggests that the treatment effect is durable and will last several weeks. This is based on the known typical $t_{1/2}$ for IgG, which ranges from 3 to 4 weeks. This finding is of particular importance where RLYB211 is used for antenatal prophylaxis, as it may be necessary to administer the drug periodically during the second and third trimesters to ensure a sufficient level of anti–HPA-1a to eliminate platelets entering the mother's circulation. Although the immunizing agent in cases of antenatal HPA-1a immunization is believed to be fetal platelets that enter the mother's circulation via a fetal-maternal bleeding, this source of immunization is unlikely to be involved in the rare cases where anti–HPA-1a appears during the first trimester; primarily because the fetal-maternal barrier in the first trimester comprises more layers than later in pregnancy [53]. The exact immunizing agent for these early cases of immunization is not known, but it has been speculated that HPA-1a–expressing microvesicles of trophoblastic origin could induce a maternal immune response against HPA-1a [54,55]. The elimination of HPA-1a–positive platelets, demonstrated in this study, suggests that administration of anti–HPA-1a during pregnancy would likely eliminate HPA-1a–positive microvesicles in a similar manner. These results establish clinical proof of concept for effecting rapid elimination of mismatched platelets and support the potential for HPA-1a antibodies as a possible prophylactic treatment for mothers at high risk of having an FNAIT-affected pregnancy.

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AUTHOR CONTRIBUTIONS

J.KK. conceptualized the study and wrote the first draft of the study protocol. The protocol was finalized by Rallybio after being reviewed by M.Kj., B.S., C.G., K.J.O., M.Ko., F.B., S.M.G.S., and C.W. prior to submission to the Goethe University ethics committee and the Paul Ehrlich Institut. J.KK., M.Kj., and B.S. were responsible for the plasma collection and for the production of the study drug. J.KK., C.G., E.F., and M.Kj. validated the method for determining transfused platelets. E.F. performed the flow cytometry analyses. All flow cytometry plots were approved by C.G., E.F., J.KK., and M.Kj. prior to entry into the study database. M.Ko. was the principal investigator of the study. Selection of platelet concentrates were performed by C.G. and E.F. Recruitment and screening of study participants was performed by C.G., E.F., S.B., M.Ko., S.M.G.S., C.W., and F.B. Anti–HPA-1a quantification was performed by A.M. and A.W. Administration of study drug, administration of platelets, and surveillance of study participants were performed by M.Ko., S.M.G.S., and F.B. Data were interpreted by J.KK., M.Kj., C.G., K.J.O., S.M.G.S., M.Ko., F.B., C.W., R.A., and Z.B. Statistics were performed by K.J.O. The manuscript was prepared by J.KK., C.G., K.J.O., R.A., and Z.B., with medical writing support provided by Chameleon Communications International and funding from Rallybio. F.B., E.F., S.B., M.Kj., M.Ko., A.M., E.S., S.M.G.S., C.W., and A.W. reviewed and revised the manuscript. All authors approved the final manuscript.

DECLARATION OF COMPETING INTERESTS

M.Kj., B.S., and J.KK. are stockholders of Prophylix AS, a Norwegian biotech company, which produced the study drug. M.Kj. and J.KK. are currently consultants for Rallybio, which is the sponsor of the study. R.A. and Z.B. are employees of Rallybio. J.KK. also provides consultancy services for ROS Therapeutics, Glycorex Transplantation AB, and Johnson & Johnson. German Red Cross Blood Donor Service Baden-Württemberg-Hessen gGmbH has a contract with Rallybio for the present study. The remaining authors declare no competing financial interests.

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SUPPLEMENTARY MATERIAL

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