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Short communication

Generation of two high affinity anti-mouse FcRn antibodies: Inhibition of IgG recycling in wild type mice and effect in a mouse model of immune thrombocytopenia

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ABSTRACT

Primary immune thrombocytopenia (ITP) is an autoimmune disease characterized by pathogenic immunoglobulin G (IgG) autoantibodies that bind to platelets, causing their phagocytic removal and leading to reductions in platelet number. The neonatal Fc receptor (FcRn) selectively salvages and recycles IgG, including pathogenic IgG, thereby extending the half-life of IgG in plasma. Two anti-mouse FcRn monoclonal antibodies (mAb) (4470 and 4464) were generated to evaluate the effect of inhibiting IgG recycling. Statistically significant reductions in plasma IgG concentration were observed upon administration of 4470 (10, 30 and 100 mg/kg) in wild-type mice. In a passive mouse model of ITP, 4464 alleviated the reduction in platelet number and/or preserved newly produced platelets when dosed prophylactically as well as in a therapeutic dosing regimen once platelet numbers had already been reduced. These results support the investigation of anti-FcRn therapy as a potential treatment for ITP.

1. Introduction

Immune thrombocytopenia (ITP) is a disease defined by low platelet number that, in turn, leads to increased risk of bleeding incidents [1]. In healthy adult human blood, platelet counts range from 150×10^9 to 450×10^9/L. Patients with ITP have platelet counts below 100×10^9/L [1]; counts of $< 30 \times 10^9$/L can be associated with hemorrhagic strokes and potentially fatal bleeding events [2].

ITP is an autoimmune disease that, like many other autoimmune diseases, is characterized by the presence of pathogenic autoantibodies (mostly of the immunoglobulin [Ig] G subclass). In ITP these pathogenic antibodies are commonly against platelet proteins e.g. glycoprotein IIb-IIIa [3] (also known as CD41/CD61, or integrin $\alpha_{IIb}\beta_3$). The pathogenic antibodies can act to reduce production of platelets by megakaryocytes. However, it is more common for the autoantibodies to opsonize the platelets, which are then phagocytosed, notably by reticuloendothelial cells in the spleen and liver. These activities result in low platelet numbers.

Reducing the concentration of pathogenic antibodies is understood to have a positive therapeutic effect in autoimmune disease. Indeed, plasmapheresis (the physical removal of antibodies from the blood) and intravenous immunoglobulin (IVIg; pooled IgG from thousands of human donors) are already used as first-line therapies in a number of autoimmune diseases [4]. IVIg has been shown to be effective in treating ITP; although its mechanism of action is not clear, and multiple pathways have been proposed [5].

One leading hypothesis is that IVIg causes a decrease in the concentration of endogenous IgG, by disrupting the function of the neonatal Fc receptor (FcRn). In normal physiology, IgG (and albumin) is salvaged and recycled by FcRn, such that IgG has a long plasma half-life of about 3 weeks in humans [6,7]. Large doses of IVIg are given to patients undergoing therapy for ITP, which increases total plasma IgG concentration above the normal level. It is believed that IVIg competes with endogenous IgG (including pathogenic IgG) for FcRn, resulting in an overall increase in the rate of IgG catabolism. Consequently, the concentration of endogenous IgG, including that of endogenous pathogenic IgG, is reduced [8]. However, evidence also exists to suggest that in ITP, IVIg may function in an FcRn-independent manner, since

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Crow [9] reported that in a murine model of ITP, IVIg can be therapeutic in the absence of FcRn.

Another potentially more specific approach to reducing endogenous IgG concentration is through direct blockade of FcRn (either via a monoclonal antibody (mAb) or antibody engineering to increase the affinity of IgG Fc to FcRn) and inhibition of the IgG-FcRn interaction, such that IgG is not salvaged and recycled. This therapeutic approach has been shown to be effective in several animal models of autoimmune diseases [10–13].

We have developed rozanolixizumab (UCB7665), an anti-human FcRn IgG4P mAb designed for the treatment of autoimmune diseases [14]. Rozanolixizumab does not bind mouse FcRn, hence, efficacy testing was conducted to support its clinical use, using pharmacologically equivalent mouse anti-FcRn IgG1 antibodies in mouse disease models.

2. Materials and methods

To generate 4470, the heavy and light chain variable (V) regions from the parent rabbit antibody were cloned into mammalian expression vectors containing a mouse gamma-1 constant (C) region or a mouse kappa C-region, respectively. 4464 was generated by murinization; complementarity determining regions (CDRs) from the rabbit V-regions were grafted onto mouse germline antibody V-region acceptor frameworks (IMGT, http://www.imgt.org/). Mouse V-region IGKV15-103 plus JK2 J-region was selected as the acceptor framework for the light chain CDRs; the heavy chain CDRs were grafted onto mouse V-region IGHV2S4 plus JH3 J-region acceptor framework. The CDRs were indicated according to the Kabat definition of sequence hypervariability [17], apart from CDR-H1 where a combination of the sequence hypervariability and structural loop definitions was employed, encompassing residues H26–H35. Genes encoding the murinized heavy and light chain V-region antibody sequence were synthesized by DNA2.0 (Newark, USA). The heavy and light chain genes were cloned into mammalian expression vectors containing a mouse gamma-1C-region or a mouse kappa C-region, respectively. To express the recombinant, mouse IgG1 antibodies, the heavy and light chain vectors for each mAb (4470 and 4464) were co-transfected into Chinese hamster ovary-SXE cells [18]; these were assessed for their affinity to mouse FcRn.

To assess antibody cross-blocking, Amine Reactive Second-Generation (AR2G) biosensors (Pall ForteBio) were coated with goat anti-mouse Fcγ-specific $F(ab')_2$ antibody (115-006-071; Jackson ImmunoResearch Lab Inc., UK) following the manufacturer's protocol. An antibody cross-blocking experiment was done on the ForteBio Octet Red384 device to demonstrate that the binding sites of antibodies 4464 and 4470 on murine FcRn were similar. After immobilization of the anti-mouse IgG capture agent, two separate experiments were undertaken that involved a baseline step (60 s) in kinetics buffer (ForteBio), loading of the first antibody (either 4464 [experiment 1] or 4470 [experiment 2]) at 15 μg/mL (600 s), baseline step (60 s), loading of murine FcRn at 4.6 μg/mL (600 s), baseline step and finally a second test antibody at 15 μg/mL (600 s). All protein sample dilutions were made in kinetics buffer and a shake speed of 1000 rpm was used throughout. In both experiments, the second test antibody was either 4464, 4470 or 4473 (a positive non-competitive anti-murine FcRn control antibody). Binding response (nm) was plotted against time to produce a sensogram. Individual test antibodies were tested on separate biosensors. The presence of a signal with the second test antibody suggested a non-competitive binding site whereas lack of signal suggested binding to a similar or overlapping epitope on murine FcRn.

To assess the effect of single doses of 4470, female wild-type C57BL/6 mice (> 8 weeks of age at study start; n = 8 per group) received 150 μL doses of 4470 at 10, 30, or 100 mg/kg IV, or PBS (vehicle) at time (T) = 0. Blood was taken from a peripheral vein on Day −1 and further serial bleeds were taken 8, 24, 48, 72, 144, and 528 h post-dosing. Serum was separated and analyzed for IgG levels by ELISA using mouse IgG ELISA kits (Abcam, UK; ab151276) as per the manufacturer's instructions. Data are presented as a percentage of the pre-dose plasma IgG concentration. To assess the effect of multiple 4470 doses, the 30 mg/kg and vehicle (PBS) groups were dosed again, as above, at monthly intervals for a further 4 doses. Blood was taken after each dose and plasma IgG levels were analyzed as previously described.

The presence or absence of ADA was investigated using a non-quantitative meso scale discovery (MSD)-based assay [19]. Briefly, sulfo-TAG conjugated anti-murine FcRn mAb and biotin-labelled anti-murine FcRn mAb were incubated with serum samples in a 96-well plate overnight at room temperature. Samples were transferred to a streptavidin-coated MSD plate and incubated for 1 h at room temperature. MSD plates were washed, 150 μL/well Read Buffer was added and the plate was read immediately with MSD Sector Imager 600. Serum from mice immunized with 4470 in the presence of adjuvant (shown to contain high titers of ADA) was included as a non-quantitative positive control.

For the mouse ITP model [15], Alzet osmotic minipumps, with a flow rate of 0.5 μL/h, were implanted subcutaneously on Day 0 into male BALB/c mice (> 8 weeks of age at the start of studies; n = 5–12 per group). Approximately 1 μg of anti-CD41 antibody (eBioscience, UK) was delivered into the circulation per day throughout the experiment. Mice were dosed with 4464 IV, isotype-matched control mouse IgG1 or vehicle (PBS) either prophylactically from Day 0 or therapeutically from Day 3. Human IVIg (wild-type polyclonal IgG, 1000 mg/kg) (Global Rx) was administered on Day 3. A pre-bleed was taken from a peripheral vein prior to insertion of the osmotic minipumps. Further serial bleeds from the peripheral vein were taken and a final blood sample was taken by cardiac puncture, under terminal anesthesia. Quantification of platelets was carried out using flow cytometry. Briefly, heparinized whole blood was stained with anti-CD45 PerCPCy5.5 (Ebioscience, UK) and anti-CD42d PE (Ebioscience, UK). Samples were then acquired using a BD FACS Canto II (BD Biosciences, UK) and analysis was carried out using FlowJo software (FlowJo LLC, USA). Data are presented as percentage of initial platelet number. Differences between groups were analyzed by one-way ANOVA and Sidak's post-hoc test.

Female wild-type C57BL/6 mice for the 4470 experiments, and male BALB/c mice for the 4464 experiments in a mouse ITP model, were both provided by Charles River, UK. Animals were housed and studies carried out in accordance with the requirements of the Animals (Scientific Procedures) Act 1986.

3. Results

To assess whether FcRn blockade with rozanolixizumab could be an effective treatment for ITP, two high affinity anti-mouse FcRn IgG1 mAbs (4470 and 4464) were isolated from two immunized rabbits. 4470 was engineered to be a chimeric rabbit-mouse IgG1, and 4464 a fully murinized IgG1 antibody. Surface plasmon resonance (SPR) has shown these mAbs (4470 and 4464) bind to murine FcRn (K_D ~ 600 pM and ~700 pM at pH 7.4, and ~200 pM and ~100 pM at pH 6.0, respectively) and block binding of IgG but not albumin. Octet studies demonstrated that 4470 and 4464 cross-block each other, inferring that they bind a similar epitope on murine FcRn (Supplementary Fig. 1). Given the closely matched affinity, identical inhibition profile and likely similar epitope specificity, these two anti-murine FcRn antibodies can be considered to be comparable reagents for studying mouse FcRn biology. This study aimed to evaluate the effect of single and multiple doses of 4470 on plasma IgG in wild-type mice; the effect of 4464 on platelet levels was also explored in a mouse model of ITP.

Female wild-type mice (C57BL/6) received a single dose of 4470 intravenously (IV) and by 48 h after administration plasma IgG concentrations were significantly decreased, 47.1%, 76.7% and 69.1% following 10 mg/kg, 30 mg/kg or 100 mg/kg doses respectively, in the presence of continued IgG synthesis (Fig. 1A). These decreases in

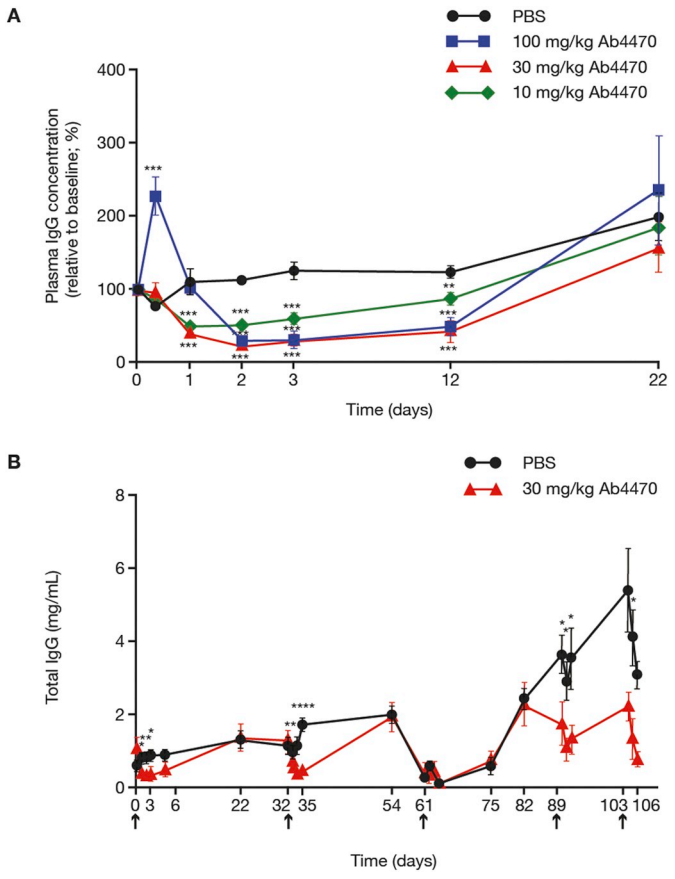

Fig. 1. (A) The effect of anti-murine FcRn antibody 4470 (single doses of 10, 30, and 100 mg/kg IV) on plasma IgG concentrations (given as percentage of concentration at time zero for each animal) in wild-type mice. n = 8 in each group; data are mean ± SEM. Statistical analysis (one-way ANOVA and Dunnett's post-test): **P < 0.01, ***P < 0.001 significance of difference from vehicle (PBS)-treated animals. (B) The effect of 4470 on plasma IgG concentration in wild-type C57BL/6 mice, dosed at approximately monthly intervals (indicated by arrows). Dose was 30 mg/kg IV. n = 8 in each group; data are mean ± SEM. Statistical analysis (one way ANOVA and Dunnett's post hoc test): *P < 0.05, **P < 0.01, ****P < 0.0001. IgG concentration was assayed by ELISA.
FcRn, neonatal Fc receptor; IgG, immunoglobulin G; IV, intravenous; PBS, phosphate buffered saline.

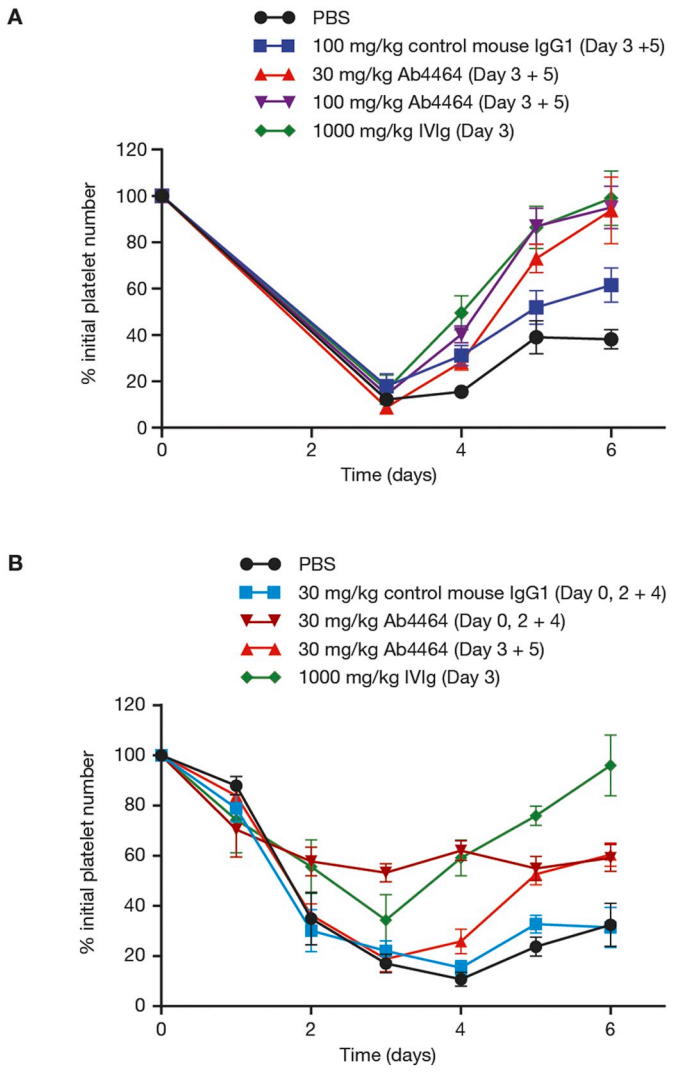

Fig. 2. (A) The effect of anti-murine FcRn Ab4464 on plasma platelet number in a mouse model of ITP. 4464, isotype-matched control mouse IgG1, or vehicle (PBS) were administered IV, therapeutically at Days 3 and 5; positive control human IVIg was administered IV at Day 3. n = 11–12 in each group, combined data from two identical experiments; data are mean ± SEM. Statistical analysis (one way ANOVA and Sidak's multiple comparisons): P < 0.05 versus IVIg and PBS controls. (B) The effect of 4464, control mouse IgG1 or human IVIg on plasma platelet number by prophylactic or therapeutic regimens (starting dosing at Day 0 [the day that induction of ITP commenced] or Day 3). n = 5–6 in each group; data are mean ± SEM. Statistical analysis (one way ANOVA and Sidaks multiple comparisons): P < 0.05 versus IVIg and no treatment controls. FcRn, neonatal Fc receptor; IgG, immunoglobulin G; IVIg, intravenous immunoglobulin; ITP, immune thrombocytopenia; PBS, phosphate buffered saline; SEM, standard error of the mean.

plasma IgG concentration were sustained at levels significantly below baseline for 144 h post-dosing. A small, non-significant and brief reduction in plasma IgG concentration was observed after injection of the phosphate buffered saline (PBS) vehicle in control animals, probably due to the dilution effect of the PBS volume (150 µL, or ~15% of total blood volume); plasma IgG returned to baseline within 24 h. A temporary increase in total mouse IgG 8 h after dosing was noted for the 100 mg/kg doses. This is thought to be due to detection of 4470 by the mouse IgG assay, which was not specific for endogenous IgG and detects all IgG in the plasma including 4470. IgG concentration returned to control levels around Day 10 (240 h). By Day 22 (528 h) IgG concentration was above baseline for all animals (Fig. 1A). Due to limitations in sample volume, plasma albumin concentrations were not measured in these experiments.

To investigate the effect of multiple dosing, including the potential for generation of anti-drug antibodies (ADA), the mice initially receiving a single dose of 30 mg/kg were subsequently dosed, at approximately monthly intervals, with additional doses of 4470 (30 mg/kg IV) (Fig. 1B). Monthly 30 mg/kg IV doses provided repeated reductions in plasma IgG concentrations even against an increase of plasma IgG concentration and variability observed over time as the mice aged, as seen in vehicle-treated animals (Fig. 1B). ADAs were not detected in any mouse treated with up to five doses of 4470 (30 mg/kg IV), suggesting that 4470 binding to FcRn did not in itself promote the generation of ADAs.

To investigate the potential therapeutic effects of FcRn blockade in autoimmune disease, the anti-murine FcRn Ab 4464 was evaluated in a mouse model of ITP. Having demonstrated an IgG-reducing effect with the chimeric anti-FcRn antibody (4470), the fully murinized anti-FcRn (4464) was utilized in the ITP disease model. Induction of transient ITP, by administration of anti-CD41, caused a marked decrease in platelet concentration by Day 3 as expected [15], after which platelet numbers gradually recovered (Fig. 2). 4464 administered therapeutically on Days 3 and 5 at 30 mg/kg or 100 mg/kg IV, or an IVIg positive control

on Day 3 at 1000 mg/kg, increased the rate of platelet recovery compared with an isotype-matched control mouse IgG1, administered on Day 3 and 5 at 100 mg/kg (Fig. 2A). The magnitude of effects achieved with 4464 100 mg/kg and IVIg were similar (Fig. 2A). Significant differences were observed for the 4464 100 mg/kg and IVIg groups versus both the isotype-matched control mouse IgG1 and vehicle- (PBS) treated group on Day 5. For the 4464 30 mg/kg dose, a significant difference was observed on Day 5 compared with the vehicle- (PBS) treated group (all $P < 0.05$). On Day 6, significant differences were observed for IVIg and both doses of 4464 (30 and 100 mg/kg) versus the vehicle- (PBS) treated group.

Prophylactic treatment with 4464 30 mg/kg from Day 0 (the day that induction of ITP commenced) reduced the rate of platelet loss (Fig. 2B; significant differences were observed versus both the isotype-matched control mouse IgG and vehicle- (PBS) treated group on Days 4 and 5). Therapeutic treatment on Days 3 and 5 again resulted in an improved platelet recovery rate (Fig. 2B; significant differences were observed versus isotype-matched control mouse IgG1 and vehicle- (PBS) treated groups on Day 5). There were no significant differences between isotype-matched control mouse IgG1 and vehicle- (PBS) treated groups. The concentration of circulating CD41 was not measured because it was below the level of detectability in all samples.

4. Discussion

In summary, our data show that inhibition of FcRn by a mAb reduces plasma IgG in wild-type mice, as well as preventing platelet loss and improving platelet recovery rates in the ITP model. Due to the complex nature of the IgG recycling process, blockade of FcRn (by an anti-FcRn mAb) also promotes the clearance of the circulating anti-FcRn mAb; however, the reductions of plasma IgG observed in wild-type mice following administration of 4470 validate the antibody dosing and exposure level, resulting in a strong PD effect. ADAs were not observed in normal mice dosed repeatedly with 4470, indicating that blockade of FcRn per se did not cause or potentiate generation of ADA.

In the mouse model of autoantibody-driven ITP, at least some of the SC-infused anti-CD41 autoantibody would be expected to rapidly bind to platelets without being recycled by FcRn [9]. Despite this, 4464 was effective at increasing the rate of platelet recovery (compared with vehicle and control mouse IgG1 antibody at the same dose). This was the case whether the anti-FcRn was injected before or after the anti-CD41, the latter more closely representing the situation in ongoing disease in humans. A previous study of IVIg effects on antibody clearance in mice showed that IVIg increased the clearance of an anti-platelet antibody in wild-type mice, but not in mice lacking expression of FcRn, indicating that IVIg increases antibody elimination via saturation of FcRn [16].

The studies described here were carried out to support the development of rozanolixizumab (UCB7665), an anti-human FcRn mAb designed for the treatment of autoimmune diseases including ITP. Rozanolixizumab does not bind mouse FcRn so could not be used in the current studies. In a healthy volunteer study (n = 48), dose-dependent reductions in serum IgG were observed, and an acceptable safety profile was demonstrated, with single doses of rozanolixizumab subcutaneous and IV [14]. These data provided the first insight into the potential therapeutic effect of rozanolixizumab, which is currently in phase II trials for patients with primary ITP (NCT02718716).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2018.11.040.

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Disclosure of conflicts of interest

BS, LC, AC, AE, KG, DL, AS, KT and FRB were all employees of UCB Pharma when the work was completed and may hold stock and/or stock options.

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