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Human IgG lacking effector functions demonstrate lower FcRn-binding and reduced transplacental transport

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ABSTRACT

We have previously generated human IgG1 antibodies that were engineered for reduced binding to the classical Fcγ receptors (FcγRI–III) and C1q, thereby eliminating their destructive effector functions (constant region G1Δnab). In their potential use as blocking agents, favorable binding to the neonatal Fc receptor (FcRn) is important to preserve the long half-life typical of IgG. An ability to cross the placenta, which is also mediated, at least in part, by FcRn is desirable in some indications, such as feto-maternal alloimmune disorders. Here, we show that G1Δnab mutants retain pH-dependent binding to human FcRn but that the amino acid alterations reduce the affinity of the IgG1:FcRn interaction by 2.0-fold and 1.6-fold for the two antibodies investigated. The transport of the modified G1Δnab mutants across monolayers of human cell lines expressing FcRn was approximately 75% of the wild-type, except that no difference was observed with human umbilical vein endothelial cells. G1Δnab mutation also reduced transport in an ex *vivo* placenta model. In conclusion, we demonstrate that, although the G1Δnab mutations are away from the FcRn-binding site, they have long-distance effects, modulating FcRn binding and transcellular transport. Our findings have implications for the design of therapeutic human IgG with tailored effector functions.

1. Introduction

In order to provide an inert IgG Fc region for use in blocking antibodies or fusion proteins where no killing of the target cells should occur, we previously engineered a human IgG1 constant region to reduce its interactions with effector molecules. This was achieved by substituting key motifs of IgG1with residues that are in equivalent positions in the highly homologous but less active constant regions of IgG2 and IgG4. This approach, which substitutes IgG1 residues with equivalents from IgG2 (E233P, L234V, L235A and G236 deleted) (Δb) and from IgG4 (A327G, A330S, P331S) (Δa), minimizes the potential to create new immunogenic epitopes. The locations of these residue changes are illustrated in Fig. 1. The constant region, G1Δab, has been combined with anti-RhD (Fog-1) variable regions to give an antibody that shows minimal binding to FcγRI and III and reduced FcγRII binding (Armour et al., 1999; Armour et al., 2003; Armour et al., 2000). RBC sensitised with the Fog-1 G1Δab do not trigger either ADCC or monocyte activation and the antibody can inhibit activation of these

Abbreviations: FcγR, Fc-gamma receptor; FcRn, neonatal Fc-receptor; FMAIT, fetomaternal alloimmune thrombocytopenia; HPA, human platelet antigens; IVIG, intravenous immunoglobulin; HuVEC, human umbilical vein endothelial cells; JAR, human choriocarcinoma cells

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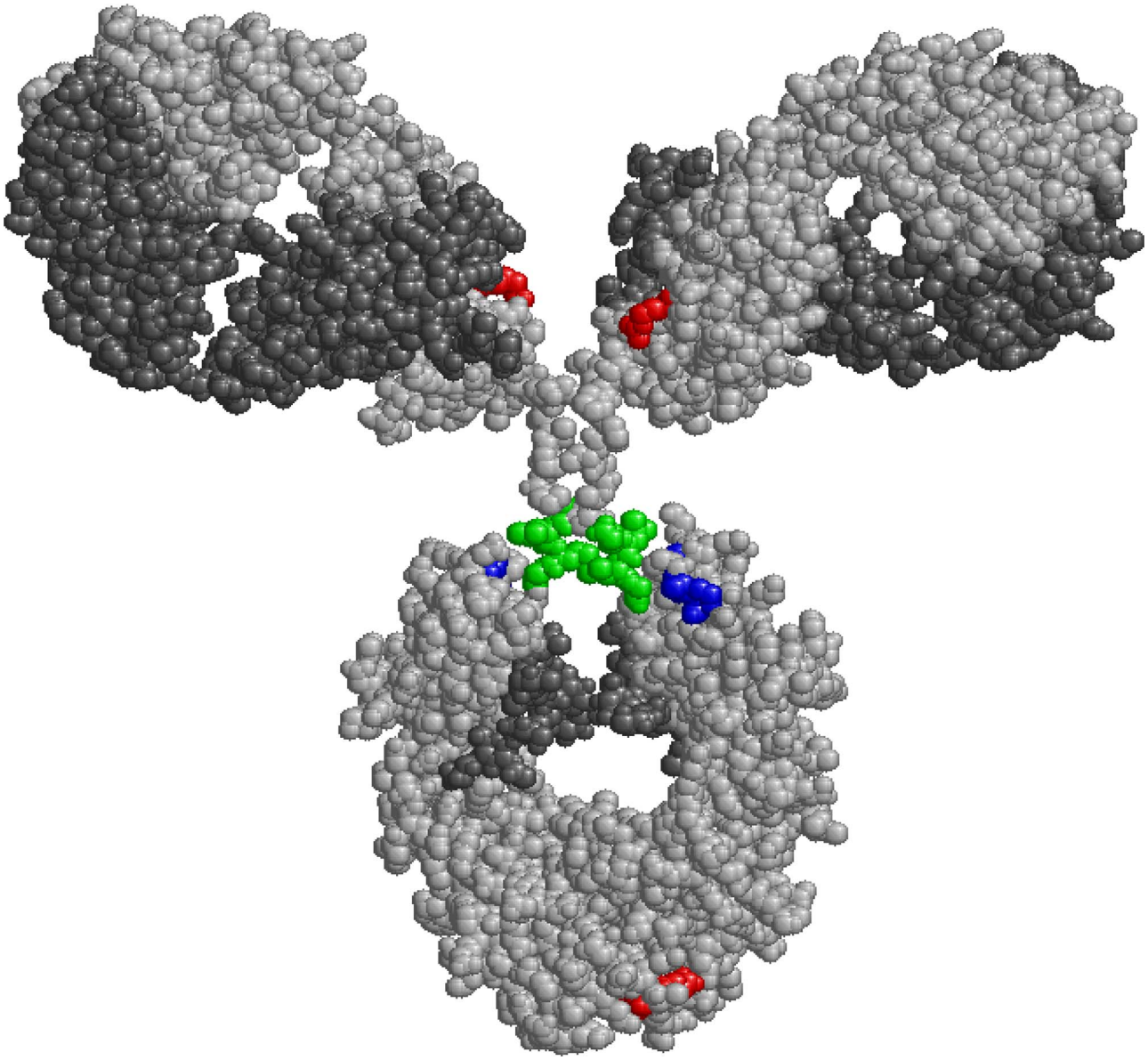

Fig. 1. A structural illustration of human IgG1showing the locations of the residues that were mutated to produce the G1ˆnab constant region. IgG1 heavy chains are shown in light grey with the light chains and Fc-associated carbohydrate in dark grey. The red residues are those altered by the Δn mutation to substitute the IgG1 G1m(1,17) allotypic residues with the corresponding IgG2 residues (K214T, D356E and L358M in the CH1 and CH3). The blue amino acids are changed to IgG4 residues by the Δa mutation (A327G, A330S, P331S in the CH2) and the green residues were substituted with the corresponding amino acids of IgG2 by the Δb mutation (E233P, L234V, L235A, G236 deleted in the lower hinge region of the CH2). The image was generated from the PDB file of an IgG1 model (Clark, 1997) using RasMol V2.7.3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mechanisms by Fog-1 IgG1 or clinical anti-RhD sera (Armour et al., 1999; Armour et al., 2000). In contrast, IgG4 activates monocytes and both IgG2 and IgG4 can mediate ADCC with effector cells from some donors. Thus, the G1Δab constant region is less immunologically activating than native IgG2 or IgG4. Further modification (Δn) has removed one allotypic residue from the CH1 region (K214T) and two allotypic residues from the CH3 region (D356E, L358M) without changing the properties of the constant region (Armour et al., 2006). RBC sensitised with the antibody, Fog-1 G1Δnab, have longer survival *in vivo* in humans than Fog-1 G1-sensitised cells (Armour et al., 2006).

One of our goals, in designing an inert constant region, is to produce a therapeutic antibody for the treatment of fetomaternal alloimmune thrombocytopenia (FMAIT). This condition is caused by the alloimmunisation of pregnant woman against human platelet antigens (HPA) and occurs in approximately 1 in 1500 pregnancies with 85% of these being due to IgG against the HPA-1a antigen on β3 chain of the platelet integrin αIIbβ3 (GPIIbIIIa) (Davoren et al., 2002; van den Akker and Oepkes, 2008). In the most severe cases, intracranial haemorrage causes death or disability. Treatment strategies are currently not ideal. They include increased maternal care, IVIG (with or without steroids) and, less commonly, intrauterine platelet transfusions (van den Akker and Oepkes, 2008). We have a high affinity, highly specific human IgG for HPA-1a (B2) (Griffin and Ouwehand, 1995; Garner et al., 2000) to use as the basis of a therapeutic IgG. Such an antibody could be administered maternally, cross the placenta and bind HPA-1a on fetal platelets, where it could block binding of the maternal cytotoxic anti-HPA-1a IgG1. If the antibody had an inert constant region, the blocking would achieve a reduction in fetal platelet destruction. Indeed, a modified version of the anti-HPA-1a antibody, B2 G1Δnab, reduced monocyte activation in response to platelets sensitised with 18 maternal samples of anti-HPA-1a sera (Ghevaert et al., 2008) by at least 75% and, importantly, did not affect the function of HPA-1a-expressing platelets

(Joutsi-Korhonen et al., 2004). In a study in humans, B2 G1Δnab-sensitised, autologous platelets showed the same intravascular survival as non-sensitised platelets, while the survival of platelets coated with a mixture of B2 IgG1 and B2 G1Δnab antibodies was prolonged relative to that of platelets coated with B2 IgG1 alone (Ghevaert et al., 2013). Similar results have been obtained from mouse studies using a deglycosylated IgG1 anti-HPA-1a that does not bind FcγRs except for weak binding to FcγRIa (Bakchoul et al., 2013).

It is not yet known how the *in vivo* half-life of G1Δnab antibodies compares to the 21-day half-life of wildtype IgG1 or how efficiently they are transported across the placenta, which is particularly relevant to use in FMAIT. Both attributes depend at least in part on the kinetics of binding to the neonatal Fc receptor, FcRn (Roopenian and Akilesh, 2007). FcRn is an MHC class I-like heterodimer, consisting of a 38 kDa α-chain and non-covalently associated 12–14 kDa β2-microglobulin (Simister and Mostov, 1989; Claypool et al., 2002), which binds to the CH2-CH3 interface of IgG molecules (Martin et al., 2001), a region distinct from that mutated in our G1Δnab antibodies (Shields et al., 2001). Since a high affinity interaction between FcRn and IgG depends on Fc histidine residues (particularly H310 and H435) (Kim et al., 1999; Kim et al., 1994) being protonated, binding occurs at pH < 6.5 but not at physiological pH (Martin et al., 2001; Burmeister et al., 1994; Raghavan et al., 1995; Vaughn and Bjorkman, 1998). Transport across the human placenta requires IgG to negotiate the syncytiotrophoblast cell layer, which covers the chorionic villi, and the endothelium of the fetal capillaries (Simister et al., 1996). The syncytiotrophoblasts internalise maternal serum into endosomes that acidify, thus allowing binding of the maternal IgG to FcRn (Claypool et al., 2004). The IgG is transcytosed across the cell (Dickinson et al., 1999) until the vesicles fuse with the basal membrane whereupon the IgG is released due to the physiological pH of the villus interstitium (Lencer and Blumberg, 2005). The method by which the IgG crosses the fetal endothelium into the fetal blood is less clear but is thought to involve either passive or receptor mediated transcytosing vesicles or compartments (Leach et al., 1996; Story et al., 1994; He et al., 2008; Ober et al., 2004). This routing is similar to that described for protection of IgG from catabolism, which prolongs its half-life. This is thought to occur mainly in the vascular endothelium and myeloid cells (Akilesh et al., 2007; Vidarsson et al., 2006) and FcRn functions similarly in transport and catabolic rescue (Roopenian and Akilesh, 2007; Kim et al., 2009). IgG is internalized along with other serum proteins but is routed away from the lysosomal pathway by binding to FcRn upon acidification. The IgG may be recirculated to the cell surface and released or transcytosed into the tissue (Lencer and Blumberg, 2005; Antohe et al., 2001).

Here, we have compared the binding of wild-type and mutant IgG1 to human FcRn using ELISA and surface plasmon resonance. In addition, we compared their ability to be transported across monolayers of human polarized cells. Finally, we used an *ex vivo* placental perfusion transfer model to investigate the transfer of mutant antibodies from the maternal circulation right through to the fetal circulation.

2. Results

2.1. The G1Δnab mutants bind human FcRn in a pH dependent manner

To investigate if the wild-type and engineered G1Δnab variants of both the B2 and Fog-1 antibodies retained the ability to bind human FcRn in a pH dependent manner, binding was measured by ELISA at both pH 6.0 and 7.4, as to mimic the pH inside an acidified endosome (Fig. 2A) and the extracellular milieu (Fig. 2B). The results showed that the modified antibodies bind the receptor in a strictly pH dependent manner with strong binding at acidic pH and only negligible binding at neutral pH. No major differences between the variants were detected. This shows that the pH dependency of the wild-type IgG-FcRn interaction is retained in the G1Δnab mutants with both specificities.

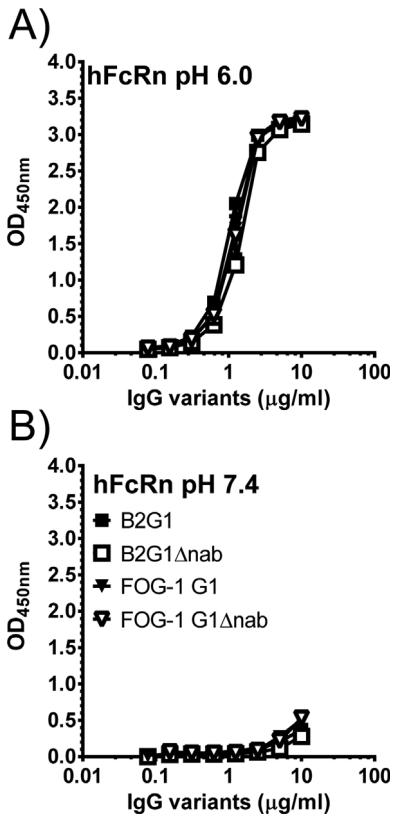

Fig. 2. IgG1 and G1Δnab variants bind to FcRn at pH 6.0 and not at pH7.4. ELISA results showing the binding of soluble FcRn-GST fusion protein to wild-type B2 G1, B2 G1Δnab, wt Fog-1 G1 or Fog-1 G1Δnab at pH 6.0 (A) and pH 7.4 (B). Bound receptor was detected using an anti-GST antibody. No differences were observed between the antibodies. Data are mean ± SEM of one representative experiment out of three.

2.2. The G1Δnab mutants show altered human FcRn binding kinetics

To address if the engineered variants affect the binding kinetics in more detail, we immobilized human FcRn on biosensor chips followed by injection of serial dilutions of human IgG1 and G1Δnab variants at pH 6.0. When the obtained sensorgrams were fitted to a bivalent binding model, we found that engineering of the antibodies had slightly altered the binding kinetics toward human FcRn (Fig. 3, Table 1). Both G1Δnab mutants had slower on-rates than their wild-type counterparts and Fog-1 G1Δnab also had a faster off-rate. This resulted in a decrease in avidity of binding for the G1Δnab variants compared to the equivalent IgG1, shown as a 1.6- to 2-fold increase in K_D values.

2.3. Δnab mutations affect apical to basolateral transport of IgG1 in human cells

To address whether altered binding kinetics of the G1Δnab affected transcellular transport, FcRn-(α-chain)-transfected melanoma cells (A375-FcRn) were seeded in a transwell system as previously described (Stapleton et al., 2011). Less B2 G1Δnab antibody than wild-type antibody was transported from the apical to the basolateral side (Fig. 4A, $p = 0.026$). As expected, non-transfected A375 cells did not significantly transport either antibody, confirming the requirement for FcRn (Fig. 4B). When using choriocarcinoma cells (JAR), which endogenously express FcRn, the trend for higher transport of the B2 G1 wild-type antibody compared to the G1Δnab antibody continued. However, the difference in transport between the two antibodies did not reach significance in this case (Fig. 2C, $p = 0.189$). Similar results were obtained when we measured the transport of Fog-1 G1 and Fog-1 G1Δnab (anti-Rh D) antibodies across A375-FcRn cells where 28.6%

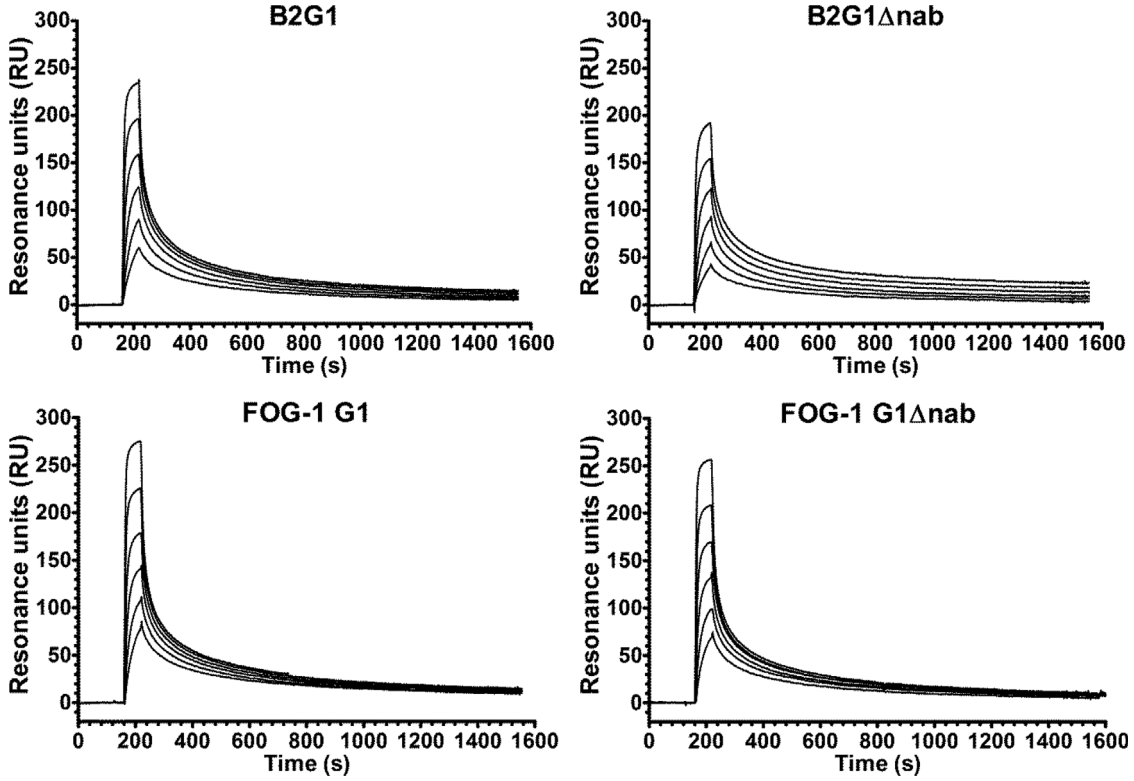

Fig. 3. Reversible FcRn binding of IgG1and G1Δnab variants at pH 6.0. Representative SPR sensorgrams showing injection of titrated amounts of (A) wild-type B2 G1, (B) B2 G1Δnab, (C) wt Fog-1 G1 and (D) Fog-1 G1Δnab over immobilized FcRn at pH 6.0. Injections were performed at 25 °C and the flow rate was 50 μl/min.

Table 1
Kinetics of the IgG interactions with human FcRn.[a]

Analyte	ka1 (10^5/Ms)[b]	kd1(10^{-3}/s)[b]	KD (nM)
B2 G1	5.0 ± 0.1	14.5 ± 0.4	29
B2 G1Δnab	2.5 ± 0.1	14.2 ± 1.4	57
Fog-1 G1	7.7 ± 0.1	14.4 ± 0.1	19
Fog-1 G1Δnab	5.5 ± 0.2	17.2 ± 0.2	31

[a] Dilutions of IgG variants were injected over immobilized human FcRn.
[b] The kinetic rate constants were obtained using the bivalent ligand model supplied by the BIAevaluation 4.1 software. The kinetic values represent the average of triplicates.

(± 0.9%) of WT Fog-1 G1 and 23.0% (± 0.8%) of Fog-1 G1Δnab were transported in 2 h experiments.

2.4. Δnab mutations do not affect transport of IgG1 in HuVEC

Next, we tested transport of B2 G1 and B2 G1Δnab antibodies across human umbilical vein endothelial cells (HuVEC), representing the placental endothelium. In contrast to what was seen with A375-FcRn and JAR cells, the Δnab mutations did not significantly affect transport in HuVEC, either in the apical to basolateral (Fig. 5A) or the reverse direction (Fig. 5B). HuVEC cells may be expected to preferentially transport IgG in the basolateral to apical direction as would occur in the placental endothelium. The percent IgG transported from the apical to basolateral side seem higher (Fig. 5A and B) but when the data are expressed as absolute amounts of IgG variants transported, correcting for the compartment volumes, no preferential direction of transport was found (Fig. 5C).

2.5. Δnab mutations affect transport of human IgG1 across placental tissues in an ex vivo perfusion model

We then studied the effect of the Δnab mutations in a placental perfusion model, which offers a safe alternative for studying various parameters of human pregnancy (Porter et al., 2016). For these

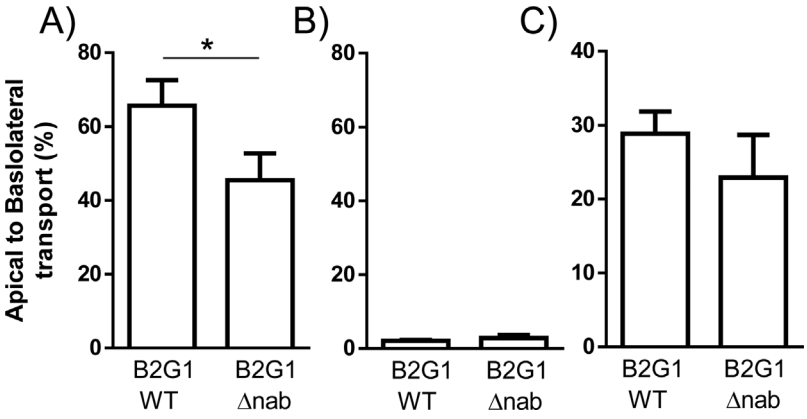

Fig. 4. G1Δnab variants show lowered transcytosis. Apical to basolateral transport of B2 G1 and B2 G1Δnab over a monolayer of A) FcRn-(α-chain)-transfected A375 (melanoma) B) wild-type A375, and C) choriocarcinoma (JAR) cells, naturally expressing FcRn. Samples were taken at 2 h. Each bar represents the mean and SD of 3 experiments and was analysed using a two-way student *T*-test.

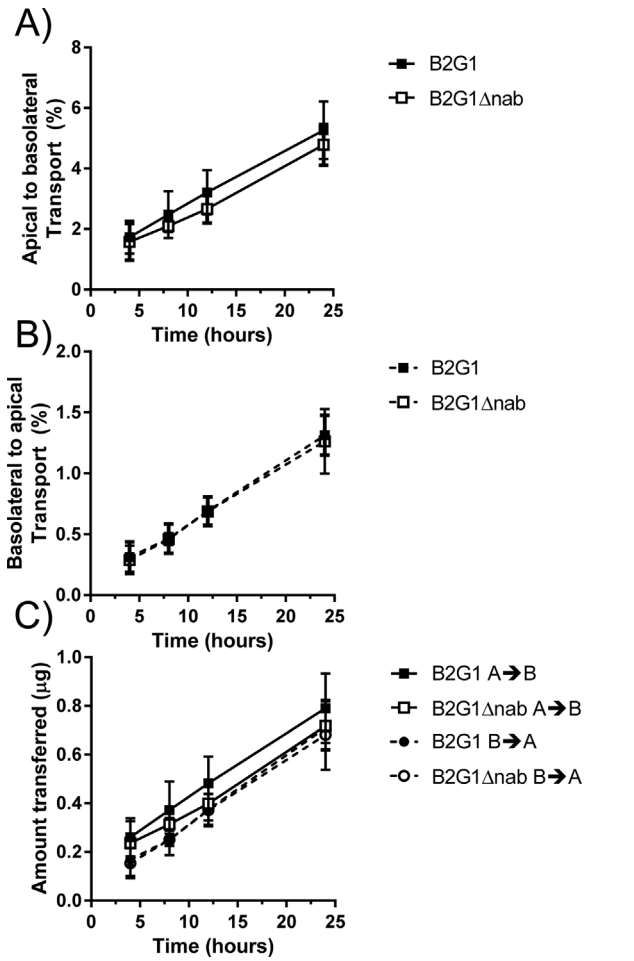

Fig. 5. No transport defect of G1Δnab antibodies is observed across monolayers of HuVEC cells. A) Apical to basolateral transport and B) basolateral to apical transport during 24 h transwell experiments. The amount of antibody that has been transferred is expressed as a percentage of the input amount of antibody. C) Absolute amounts of B2 G1 and B2 G1Δnab antibodies transported in the apical to basolateral (A → B) and basolateral to apical (B → A) directions after correcting for compartment sizes. Each datapoint represents average of three independent experiments and each experiment consisted of triplicate, independent wells and each measurement was analysed in duplicate in the aforementioned ELISA. Data was analysed by a Non-parametric Kruskal-Wallis test and a Bonferonni-post correction.

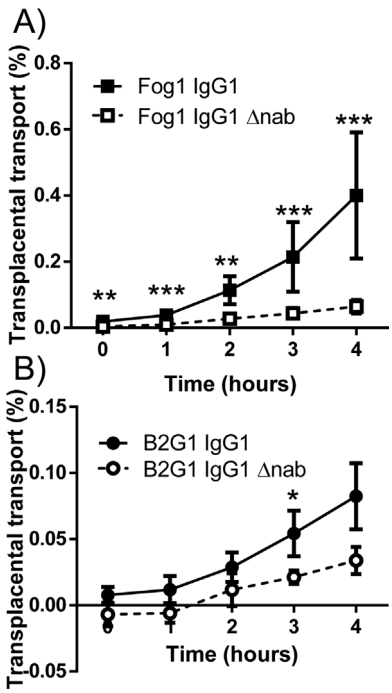

Fig. 6. Δnab-variants show a defect in transplacental transport. A) *Ex vivo* placental perfusion of Fog-1 G1 and Fog-1 G1Δnab antibodies in 8 independent experiments for each antibody. Experiments were run for up to 5 h and samples were taken at hourly intervals. Data were analysed by unpaired *t*-tests at each time point. B) *Ex vivo* combination placental perfusion cross-over experiments with either native Fog-1 G1/mutant B2 G1Δnab or native B2 G1/and mutant Fog-1 G1Δnab antibodies (n = 6 of each combination). Experiments were run for up to 5 h and samples were taken at hourly intervals. Data were analysed by unpaired *t*-tests at each time point.

experiments, we used both Fog-1 and B2 antibodies, although there are reports that anti-HPA-1a antibodies also recognizes the vitronectin receptor on placental cells due to it having an integrin β3 subunit (Kumpel et al., 2008). Wild-type or Δnab IgG1 antibodies were added to the maternal side and samples were collected from the fetal side over time to quantify antibody transport across the tissues. The Fog-1G1Δnab variant was transported significantly less than its wildtype counterpart (Fig. 6a). The B2 antibodies took longer to transport across the placenta than the Fog-1 antibodies, suggesting binding to HPA-1a on placental tissue (9/11 placentas were genotyped as HPA1a/1a and 2 were HPA-1a/1b; data not shown). However, a similar trend was apparent with reduced transport of the B2 G1Δnab variant compared to its wild type, although this was not statistically significant (Fig. 6B).

We then studied the effect of the Δnab mutations in a placental perfusion model, which offers a safe alternative for studying various parameters of human pregnancy (Porter et al., 2016). Wild-type or Δnab IgG1 antibodies were added to the maternal side and samples were collected from the fetal side over time to quantify antibody transport across the tissues. The Fog-1G1Δnab variant was transported significantly less than its wildtype counterpart (Fig. 6a). In a second series of experiments, we used both Fog-1 and B2 antibodies, although

there are reports that anti-HPA-1a antibodies also recognize the vitronectin receptor on placental cells due to it having an integrin β3 subunit (Kumpel et al., 2008). Here, each perfusion experiment measured the transport of Fog-1 G1 and B2 G1Δnab or B2 G1 and Fog-1 G1Δnab. The B2 antibodies took longer to transport across the placenta than the Fog-1 antibodies, suggesting binding to HPA-1a on placental tissue (9/11 placentas were genotyped as HPA1a/1a and 2 were HPA-1a/1b; data not shown). However, a similar trend was apparent with reduced transport of the B2 G1 Δnab variant compared to its wild type, although this was not statistically significant (Fig. 6B).

3. Discussion

Here we show that human IgG1, which has been engineered by engrafting amino acids derived from IgG2 and IgG4 in order to lower its effector functions, still has the capacity to bind and be transported by FcRn. However, the transport was unexpectedly shown to be reduced compared to wild-type IgG1.

The mutations incorporated into the G1Δnab constant region are located in the lower hinge region at the N-terminal of the CH2 domain (residues 233–236) and in a loop that lies close to the lower hinge region in the three-dimensional structure (residues 327–331). Further, amino acid residues (214 in the CH1 domain and 356/358 in the CH3 domain) were altered to remove allotypic epitopes, which are potential immunogenic residues (Armour et al., 2006; Vidarsson et al., 2014). Shields et al. found by ELISA that an IgG1 molecule with the Δb changes in its lower hinge had lower binding to human FcRn, whereas mutation at 327, 330, 331 (all Δa), 356 or 358 (allotypic variants) had no effect on FcRn binding (Shields et al., 2001). It therefore appears most likely that the lower hinge (Δb) mutations are causing the slight decrease in affinity for FcRn that we observed.

Since the crystal structures of FcRn/Fc complexes show the FcRn

interaction site to involve residues of the CH2-CH3 interface (Martin et al., 2001; Burmeister et al., 1994), it is unlikely that the mutations of G1Δnab have altered the direct FcRn-contact surface. As we found that the on-rate of binding to human FcRn is negatively affected by the mutations, it is possible that the mutations reduce the ability of the IgG to adopt an optimal conformation for FcRn binding (Moal and Bates, 2012). Similar long-distance effects have been described in the YTE/MST mutant where changes were designed to alter FcRn binding but also seem to affect FcγR binding (Grevys et al., 2015).

The ability of an IgG molecule to be transported by FcRn may be dependent not only on its affinity for FcRn at pH 6.0 but also on its binding kinetics throughout the pH gradient of the endosomal pathway. Various groups have found that the *in vivo* half-lives of IgG molecules did not correlate with their affinity for FcRn at acidic pH but instead correlated with their efficiency of dissociation from FcRn at neutral pH (Dall' Acqua et al., 2002; Schoch et al., 2015; Wang et al., 2011). Part of this pH-dependent sensitivity for FcRn-binding is mediated by the variable region charge state, which differs from antibody to antibody, and affects FcRn binding and transport (Schoch et al., 2015). This affect may also be responsible for the slightly altered transport level we see in A375-FcRn cells using antibodies with different specificities (Stapleton et al., 2011). We described a change in pH-dependency for human IgG3 for which the affinity at neutral pH is slightly higher than IgG1 but the affinity at binding pH (pH 6.5) is lower, resulting in reduced competitiveness in the presence of IgG1 (Stapleton et al., 2011). Although we found no difference in binding at neutral pH between the variants, suggesting altered pH-sensitivity of the mutant is not an issue, this aspect can only be resolved with more sensitive techniques, which are unavailable to us at this moment.

The amount of G1Δnab antibodies transported across JAR cells, which naturally express FcRn, or A375-FcRn cells, which overexpress the receptor, was 70–80% that of wild-type IgG1. The overexpression might be responsible for the increased transport levels seen with the A375-FcRn cells. In addition, we show that reduced transport for the engineered variants is also the case in an *ex vivo* placental perfusion system. Interestingly, however, no significant difference in transport was measured in HuVECs in either the apical to basolateral or basolateral to apical direction. Although a trend was observed in the apical to basolateral direction, which matched that seen in all the other systems with G1Δnab transport being slightly reduced, this may not be relevant as the placental endothelial cell layer is expected to transport IgG in the basolateral to apical direction. It is unclear why HuVECs didn't show any clear preference for direction of transport, although this may indicate that the rate of transplacental transport is not primarily dependent on transendothelial transport.

When we compared transport of the antibodies in *ex-vivo* placentas, a reproducible slower transport capacity was detected for the Fog-1 Δnab mutant compared to the wild-type, even though the transport rates between the different placentas varied greatly. Both anti-HPA-1a antibodies, B2 G1 and B2 G1Δnab, behaved very differently to the anti-RhD antibodies in this model: very low transport was observed after a long lag period, presumably due to platelet antigen expression in the placental tissue itself (Kumpel et al., 2008). Placental antigen expression may impact any potential FMAIT treatment but the effect might be mitigated by the presence of maternal anti-HPA-1a antibody, as well as the therapeutic antibody, to saturate the antigen sites on the placenta and transport would be occurring over a long time period.

Transfer of IgG across the syncytiotrophoblasts is believed to involve FcRn since it has been detected in syncytiotrophoblasts by *in situ* hybridization and immunohistochemistry (Simister, 2003). In support of FcRn being critical for transport across this cellular layer, introducing a H435A mutation in CH2-CH3 interface of the Fc decreased binding to FcRn at pH below 6.5 and also impaired transfer in choriocarcinoma JAR cells (Stapleton et al., 2011) and in perfused placentas (Firan et al., 2001). In contrast, there is a lack of clarity about which receptor is responsible for transporting IgG across the endothelial cell layer into the fetal circulation. Part of this uncertainty may stem from dissimilar patterns of receptor expression being detected at different points along the villus vascular tree. In humans, FcγRIIIa and FcγRIIIb are unlikely candidates as these receptors discriminate between fucosylated and non-fucoyslated antibodies, but no difference in these glycoforms are seen in placental transport (Einarsdottir et al., 2013). However, FcγRIIb2 has been detected in the villus endothelium of the full-term placenta at the mRNA level and by immunohistology (Mishima et al., 2007; Lyden et al., 2001). The intensity of staining decreased with progression from the terminal villus capillaries through the intermediate villus vessels and the stem villus vessels to become nil in the cord, whereas the converse is true for FcRn (Lyden et al., 2001). Studies with an endothelial cell line (Antohe et al., 2001; Gafencu et al., 2003), which was thought to have been isolated from the small vessels of a term placenta, detected FcRn mRNA but no mRNA encoding classical FcγR (Antohe et al., 2001). However, one of these studies also claimed to have found an unidentified 55 kDa receptor on the cell-surface which binds IgG independently of pH. Using various immunofluorescence and electron microscopy techniques on ≤100 nm-thick term placenta sections, Takizawa et al. (2005) found FcγRIIb2 to be expressed on an unidentifiable organelle of endothelial cells. 90% of these organelles, which are as abundant as caveolae, were intracellular with 5% near each surface. Half of these structures also contained IgG, which accounted for 80% of the IgG in the cells. At least some of the remaining IgG looked destined for degradation, whilst there was none in the caveolae. Immunostaining also detected a high concentration of IgG in the villus interstitium, which suggests that FcRn-mediated transport of IgG across the syncytiotrophoblast layer could create a concentration gradient for IgG across the endothelial cell layer. A concentration gradient could mean that a passive mechanism of non-specific transcytosis would achieve near-unidirectional IgG transport through the endothelial cells. Equally, a high IgG concentration could overcome the problem of the low affinity of FcγRIIb (K_a of 2×10^4–2×10^5 depending on IgG subclass; (Bruhns et al., 2009)). In another system, the transfer of IgG across the mouse yolk sac is independent of FcγRIIb2, whereas FcRn is necessary for transfer of IgG across the endoderm, just as it is across the syncitiotrophoblast, the equivalent layer in the human placenta (Mohanty et al., 2010). Finally, antibodies lacking FcγR binding are transported across perfused placentas as normal, while antibodies or fragments thereof without efficient FcRn binding show lowered placental transport in both the perfusion model (Porter et al., 2016) and in humans (Einarsdottir et al., 2014a; Mathiesen et al., 2013). Together, the current data suggest FcRn to be involved in transporting IgG from mother to fetus but participation of alternative receptors cannot be fully excluded, although FcγR are unlikely candidates.

In summary, these data suggest that residues originating from the lower hinge of IgG2 negatively affect binding to FcRn and transplacental transport. This is in agreement with the fact that IgG2 is generally considered to be transported to a lower degree than IgG1 (Einarsdottir et al., 2014b; Malek et al., 1996). As these residues are not part of the core FcRn binding site (Burmeister et al., 1994; West and Bjorkman, 2000), it is likely that the residues affect the tertiary structure of IgG2 and thereby accessibility to FcRn. It is important that this information is used in the design of potential therapeutic antibodies if they are not to show compromised transplacental transport or, more generally, reduced half-life. To retain FcRn functionality whilst silencing the effector functions of IgG, the effects of individual IgG2 residue mutations could be examined or the use of alternative mutations explored.

4. Materials and methods

4.1. Production of antibodies

We have previously described the generation of transfected rat

myeloma cell lines that secrete Fog-1 G1, Fog-1 G1Δnab (Armour et al., 2006), B2 G1 and B2 G1Δnab (Joutsi-Korhonen et al., 2004) antibodies. Antibodies were prepared from culture supernatants as described (Joutsi-Korhonen et al., 2004).

4.2. Construction, production and purification of recombinant humanFcRn

A vector containing a truncated version of human FcRn HC cDNA encoding the three ectodomains (α1–α3) genetically fused to a cDNA encoding the Schistosoma japonicum glutathione S-transferase (GST) has been described (Berntzen et al., 2005). The vector, denoted pcDNA3-hFcRn-GST-hβ2m-oriP, also contains cDNAs encoding human β2-microglobulin and the Epstein-Barr virus origin of replication (oriP). Secreted recombinant soluble FcRn was produced by transient transfection of HEK 293E cells and purified using a GSTrap column as described (Berntzen et al., 2005).

4.3. Surface plasmon resonance assays

All SPR experiments were performed as previously described (Stapleton et al., 2011). Briefly, a Biacore 3000 instrument, CM5 biosensor chips and amine coupling were used as described by the manufacturer (GE Healthcare). Injections were done using phosphate buffer at pH 6.0. Kinetic evaluations were performed using immobilized soluble human FcRn–GST (~1000 RU) and IgG variants (2–500 nM) injected.

4.4. pH dependent ELISA

96-well plates (Nunc) were coated with serial dilutions of the antibodies (10.0–0.07 μg/ml) and incubated overnight at 4 °C followed by washing three times with PBS/Tween pH 7.4. The wells were blocked with 4% skimmed milk (Neogen Europe Ltd.) for 1 h at room temperature (RT) and then washed in PBS/Tween pH 6.0. Purified human FcRn-GST (1 μg/ml) was diluted in 4% skimmed milk, PBS/Tween pH 6.0 and pre-incubated with an HRP-conjugated anti-GST antibody (GE Healthcare) diluted 1:5000 and added to the wells. The plates were incubated for 1 h at RT and washed with PBS/Tween pH 6.0. Bound receptor was detected by adding 100 μl of the substrate 3,3′,5,5′-tetramethylbenzidine substrate (Calbiochem) followed by addition of 50 μl of 1 M HCl. The absorbance was measured at 450 nm using a Sunrise TECAN spectrophotometer. The assay was also performed using PBS/Tween pH 7.4 in all steps.

4.5. Cell culture

Human choriocarcinoma cells (JAR; ATCC, VA) were grown in IMDM medium (Cambrex) and melanoma cells (A375; FcRn− β2m+, ATCC, VA) in RPMI 1640 medium (Invitrogen/Gibco), both supplemented with L-glutamine (300 μg/ml, Invitrogen), penicillin (100 U/ml, PAA Laboratories), streptomycin (100 μg/ml, PAA) and 10% foetal calf serum (FCS). A375-FcRn cells were generated by transfecting A375 with the human FcRn α-chain vector as previously described (Stapleton et al., 2011). HuVEC-C (ATCC, VA) were grown in RPMI 1640 medium (Invitrogen/Gibco), supplemented with L-glutamine (300 μg/ml, Invitrogen), penicillin (100 U/ml, PAA Laboratories), streptomycin (100 μg/ml, PAA) and 10% HI FCS.

4.6. IgG transcytosis

Transcytosis experiments with A375 (wild-type and human FcRn) and JAR cells were performed as previously described (Stapleton et al., 2011). Briefly, 12 mm polycarbonate Transwell filters (0.4 μm pore size, Costar/Corning) were inoculated with 5×10^5 cells, grown overnight to confluence, washed with PBS and fresh medium added (IMDM at pH 7.4 with supplements as stated above). Mixtures of IgG also contained streptavidin-HRP (Sanquin) to assess background transport. Apical to basolateral transport was calculated according to ([IgG]basolateral × 1.5 ml)/([IgG]input × 0.5 ml) × 100%. All experiments were performed in triplicate and samples were taken at 2 h.

For HuVEC transcytosis experiments, 12 mm polyester Transwell filters (0.4 μm pore size, Fisher Scientific, UK) were inoculated with 1×10^5 cells which were grown for 3 days to achieve an optically confluent monolayer. The monolayers were subsequently washed with PBS and medium was replaced: 1.8 ml medium basolaterally and 0.5 ml apically. Mixtures of IgG (30 μg/ml of monoclonal anti-RhD) added to the medium contained 200 μg/ml FITC-BSA to assess background transport. Samples were taken at time-points up to 24 h and the concentrations of B2 IgG in the receiving compartments were quantified by sandwich ELISA, using goat anti-human IgG (Fc-specific) antibodies (Sigma, Poole, Dorset, UK) to capture the B2 IgG. 1–20 μl test samples (made-up to 20 μl with HBSS + 10% FBS) were added to the wells with 80 μl PBS/0.05% Tween 20. Samples from the input compartments at 0 h (30 μg/ml) were similarly applied in dilution series to provide standards. Detection reagents were HRPO-conjugated goat anti-human λ chain antibodies (Rockland Immunochemicals) or, for greater sensitivity, biotinylated goat anti-human λ chain antibodies (AbD Serotec) followed by avidin-HRPO (Sigma). The concentration of IgG in each test sample was determined from ELISA readings for at least two different dilutions of the sample by comparison to curves fitted to the signals for the titrated standards.

4.7. Placental perfusion model

Freshly delivered human placentae, free from any complications, were collected as approved (North of Scotland Research (NORES) Ethical Committee (Ethics Reference 09/S0801/006) and National Health Service (NHS) Grampian Research and Development Office approval). The obtained placentas were mounted in the perfusion apparatus at 37 °C as previously described (Urbaniak et al., 1997). Viability of the placenta was validated during perfusions by measuring fetal venous return, maintenance of glucose consumption, and progesterone production in the perfused lobule (Duncan et al., 1995). The fetal and maternal circulations of a single lobule of placenta were perfused within 30 min of delivery with perfusate consisting of RPMI medium minus phenol red and L-glutamine (Sigma), containing 3% dextran (83,000 M, wild-type; Sigma), gentamicin (12 mg/l; Roussel, Dublin 2, Rep. of Ireland), co-trimoxazole (80 mg/l; David Bull Laboratories Pty. Ltd., Mulgrave, Victoria, 3170, Australia) and 10 IU/ml heparin. Perfusate was supplied at 6 ml/min to the fetal circulation, and at 20 ml/min to the maternal circulation. The maternal perfusate was gassed with 95% O_2/5% CO_2 and the fetal perfusate with 95% N_2/5% CO_2 *via* gas oxygenators. Protein (endogenous IgG) and erythrocyte washout proceeded for one hour after which both circuits were closed to recirculate.

Closed circuit perfusion was performed using 200 ml sampling reservoirs containing 12.9 g/l BSA (Sigma) in the closed fetal circuit and 30ug/ml of each test antibody (equivalent to 15,000 IU of anti-RhD or 6 mg of IgG/200 ml) in the maternal circuit. Antibodies tested were Fog-1 G1 or Fog-1 G1Δnab alone, Fog-1 G1 and B2 G1Δnab in combination or B2 G1 and Fog-1 G1Δnab in combination. Perfusion experiments were conducted for up to 5 h, with hourly samples (1.5 ml) taken from each circuit. Erythrocytes were removed by centrifugation and samples were stored at −20 °C.

For the Fog-1 experiments, the concentration of anti-RhD IgG in each perfusate sample was determined by flow cytometric quantification using RhD positive red cells and by comparison to curves fitted to the signals from their respective titrated standards, fetal test samples were analysed undiluted and maternal samples pre-diluted to avoid agglutination. Concentrations of anti-RhD/IgG in both circuits were corrected for the release of endogenous placental IgG, determined from control perfusions, and for variations in reservoir volumes at the time of

sampling. For the B2 perfusion experiments, the concentration of platelet-specific IgG in each test sample was determined from flow cytometric quantification using HPA-1a positive platelets and by comparison to curves fitted to the signals from their respective titrated standards (500 ng/ml–0 ng/ml).

The transfer of IgG from maternal to fetal perfusate was calculated as the transfer fraction (Tf%) using the equation:-

$$Tf\% = [IgG]f \times 100/[IgG]m$$

where [IgG]f and [IgG]m are the concentrations of specific IgG in the fetal and maternal perfusates, respectively

4.8. Statistical analysis and data sets

All data represent the mean and standard deviation of at least three independent experiments. All transcytosis assays consisted of three replicates. GraphPad Prism for Windows (GraphPad Software) was used for all statistical analysis. Significance was set at $P < 0.05$, and is indicated on all figures as $*P < 0.05$.

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