Cross-species analysis of Fc engineered anti-Lewis-Y human IgG1 variants in human neonatal receptor transgenic mice reveal importance of S254 and Y436 in binding human neonatal Fc receptor

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ABSTRACT

IgG has a long half-life through engagement of its Fc region with the neonatal Fc receptor (FcRn). The FcRn binding site on IgG1 has been shown to contain I253 and H310 in the CH2 domain and H435 in the CH3 domain. Altering the half-life of IgG has been pursued with the aim to prolong or reduce the half-life of therapeutic IgGs. More recent studies have shown that IgGs bind differently to mouse and human FcRn. In this study we characterize a set of hu3S193 IgG1 variants with mutations in the FcRn binding site. A double mutation in the binding site is necessary to abrogate binding to murine FcRn, whereas a single mutation in the FcRn binding site is sufficient to no longer detect binding to human FcRn and create hu3S193 IgG1 variants with a half-life similar to previously studied hu3S193 F(ab)2, (t1/2b, S254A, 119.9 h; Y436A, 162.1 h; wild-type, 163.1 h). These variants had minimal effect on half-life in BALB/c nu/nu mice (t1/2b, S254A, 119.9 h; Y436A, 162.1 h; wild-type, 163.1 h). These results provide insight into the interaction of human Fc by human FcRn, and are important for antibody-based therapeutics with optimal pharmacokinetics for payload strategies used in the clinic.

Abbreviations A fast, amplitude of the fast clearance component; A slow, amplitude of the slow clearance component; AUC, area under the curve; CHX-A, DTPA, C-functionalized trans-cyclohexyldiethylenetriaminepentaacetic acid; FcRn, neonatal Fc receptor; huFcRn, human neonatal Fc receptor; 125I, iodine-125; 111In, indium-111; %ID, percentage injected dose; LeY, Lewis y antigen; muFcRn, murine neonatal Fc receptor; PBS-T, PBS containing 0.05% v/v Tween20; t1/2b, half-life, elimination half-life; Tg, transgenic

Introduction

The Fc portion of IgG interacts with the neonatal Fc receptor (FcRn, Brambell receptor), which prevents it from being degraded in the lysosomes and gives antibodies long serum persistence. Other biological roles of FcRn include perinatal transfer of IgG, antibody-mediated presentation of antigen and IgG transfer across epithelial and endothelial barriers. Altering the IgG:FcRn interaction has increasingly been explored to improve the therapeutic or diagnostic applications of antibodies. Reducing the half-life of intact IgG or Fc-containing antibody fragments can improve payload delivery properties of antibodies. Alternatively, human IgG1 with increased affinity for huFcRn at pH 6.0, but not at pH 7.4, shows increased serum half-life, which can reduce dosing amount or frequency. In contrast, human IgG1 with increased affinity at pH 7.4 inhibits FcRn function and can be used to reduce the biologic effects of endogenous pathology-causing IgGs.

Crystal structures of human FcRn, rat FcRn and Fc:FcRn complexes have been used to identify the residues on the human IgG1-Fc:FcRn interface involved in the Fc:FcRn binding. Site-directed mutagenesis has also been used to identify the amino acids at the CH2-CH3 domain interface of mouse IgG1 and human IgG1, which are involved in murine FcRn binding. These studies identified I253 and H310 in the CH2 domain, and H435 in the CH3 domain as the key residues involved in murine and human IgG1-Fc binding to murine FcRn. Changing these residues into alanine resulted in abrogation of binding to FcRn in vitro and reduced half-lives of these IgG variants in mice.

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While mice are often used in the preclinical evaluation of biodistribution and pharmacokinetic properties of therapeutic IgG, cross-species differences of FcRn are known to cause remarkable differences between human IgG1 binding to mouse and human FcRn. In general, wild-type human IgG1 binds murine FcRn (mufcRn) more strongly than human FcRn (huFcRn).26,27 ScFv-Fc IgG1 wild-type and variants (e.g., I253A, H310A or H310Q) showed approximately 5-fold faster blood clearance in human FcRn transgenic mice compared to BALB/c mice.28 In addition to residues I253, H310 and H435 critical to human IgG1:huFcRn binding, alanine variants at positions S254 and Y436 have been shown to strongly reduce the binding of human IgG1 to huFcRn in vitro,26 but the importance of residues S254 and Y436 have not been examined in a huFcRn transgenic mouse model.27,28

Hu3S193 is a humanized IgG1 anti-Lewis Y (Le') monoclonal antibody.29 The Le' antigen is a type 2 blood group-related difucosylated oligosaccharide with the chemical structure Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-R, and has been shown to be expressed by 60–90% of human carcinomas of epithelial cell origin. The high frequency of Le' expressing tumors, its high density and altered expression on the surface of tumor cells, and relatively homogenous expression in primary and metastatic lesions have led to its selection as an antigenic target for solid tumor immunotherapy.30 The structural basis for hu3S193 targeting of Le' antigen on tumor cells has been defined by crystallography of Fab complexes with the minimal Le'-tetrasaccharide epitope.11,12 Hu3S193 has potent immune effector function, including complement-dependent cytotoxicity (CDC) (IC50 0.5 μg/ml) and antibody-dependent cell-mediated cytotoxicity (ADCC) (IC50 0.5 μg/ml) against Le'-expressing tumor cells.29 Hu3S193 has also been shown to have significant anti-tumor effect in animal models, particularly for delivery of payloads, including131I, 90Y and 177Lu.33–36 Several clinical Phase 1 studies have shown the therapeutic potential of hu3S193 in different solid tumors (e.g., breast, colorectal, ovarian, non-small-cell lung cancer) with uptake of111In-hu3S193 in biodistribution studies demonstrated in cutaneous lesions, lymph node lesions and hepatic metastases.37–40 Hu3S193 has also been explored in a Phase 1 trial as an antibody-drug conjugate.39

In this study, we demonstrate for the first time the importance of residues S254 and Y436 in the human IgG1 FchuFcRn interaction by evaluating the blood clearance of these mutants in huFcRn transgenic mice. This study builds on previous work examining cross-species binding differences of mouse and human FcRn to IgG1 variants.27,28 A series of IgG1 residues previously shown to be involved in binding to huFcRn in vitro25 (I253, S254, H310, N434, H435 and Y436) were changed in the Fc part of hu3S193 by site-directed mutagenesis and pharmacokinetics were studied in BALB/c nu/nu mice and human FcRn transgenic mice. Our data demonstrate that alanine replacement at S254 and Y436 can increase blood clearance rates compared to wild-type IgG1 in human FcRn transgenic mice, but not in BALB/c nu/nu mice. In huFcRn transgenic mice the S254A and Y436A variants clear slower than I253A, H310A or H435A variants. In addition, in huFcRn transgenic mice double mutations S254A/Y436A could further reduce binding to huFcRn and enhance the blood clearance, but not to the extent of I253A, H310A or H435A. This study shows that alterations in residues S254 and Y436 can offer an intermediate range of elimination half-lives compared to wild-type IgG1 or mutants with undetectable huFcRn binding. These results are important for the design of antibody-based therapeutics with optimal pharmacokinetics for payload strategies used in the clinic.

Results

Antibody production and purification

Based on the in vitro study by Shields et al.,25 hu3S193 S254A, H435A and Y436A were generated to determine if binding to both human and mouse FcRn are abrogated in vitro and in vivo. A recent study reported markedly reduced in vitro and in vivo binding of I253A and H310A anti-carcinoembryonic antigen (CEA) scFv-Fc antibody fragments to FcRn, and therefore hu3S193 I253A and H310A were generated as positive controls.28 The hu3S193 N434A variant was generated as a control because it was previously reported that this mutation can

![Figure 1. Computational analysis of the FcRn interaction with hu3S193 Fc. (A) Ribbons-style representation of the model of the huFcRn complex with hu3S193 Fc. FcRn is composed of a MHC-like binding chain (red) and β-2-microglobulin (white). A single heavy chain of hu3S193 Fc (blue) with the residues involved in the interface with FcRn displayed as CPK spheres. (B) Free energy contribution plot for the hu3S193 Fc interacting with huFcRn and mufcRn, respectively. Data were fitted to a linear relationship with an R^2 value of 0.82.](image-url)
increase the binding to huFcRn in vitro, thus extending the half-life in vivo. The following double mutants were generated: I253A/H310A, I253A/H435A, H310A/H435A, S254A/Y436A.

To assess structural consequences of mutations of hu3S193 Fc, we modeled the complexes with murine and human FcRn (Fig. 1). Predicted contributions to binding energy by Fc residues were correlated \( R^2 = 0.82 \) between FcRn species, but there were also some notable differences, such as S254 and Y436 that were only predicted to contribute to binding huFcRn \( (\Delta G_{\text{calc}} < -1.0 \text{ kcal/mol}) \). Computational mutagenesis was also performed (data not shown) to assess all interface residues as alanine and selected residues as non-alanine mutations (I253 and H310). Non-alanine variants were selected (I253D, I253P, H310D, H310E and H310Q) because they were predicted to affect muFcRn and huFcRn binding the most (i.e., greater increase in calculated Gibbs free energy values compared with the equivalent alanine substitution).

All antibodies were purified to greater than 98% purity prior to in vitro and in vivo characterization as determined by SDS-PAGE and size-exclusion chromatography. Fluorescence-activated cell sorting (FACS) analysis using A431 cells demonstrated that all variants retained binding to the Le^a antigen.

**Figure 2.** ELISA analysis at pH 6.0 of binding of hu3S193 wild-type and variants to FcRn. Each panel compares binding of FcRn to wild-type hu3S193 with a series of different hu3S193 variants at different concentrations. (A-D) Binding obtained from hu3S193 wild-type and variants to muFcRn. (E-H) Binding obtained from hu3S193 wild-type and variants to huFcRn. \( n = 2 \); bars, SD.
**Binding analyses of hu3S193 IgG1 variants to soluble FcRn via ELISA**

To compare the binding properties of the hu3S193 variants to soluble FcRn, biotinylated human and murine FcRn were immobilized onto streptavidin-coated plates and binding of antibodies was measured at pH 6.0. Wild-type hu3S193 showed stronger binding to muFcRn (EC50, 0.018 ± 0.002 μg/ml) compared to huFcRn (EC50, 0.40 ± 0.02 μg/ml). The following single mutants of hu3S193 did not show binding to human and murine FcRn in the ELISA: I253A, I253D, I253P, H310A, H310D, H310E, H310Q, and H435A (Fig. 2).

Table 1 summarizes the differences in binding of the hu3S193 variants to muFcRn and huFcRn. S254A largely lost binding to muFcRn, but retained over half-maximal binding to huFcRn. H433A showed a modest reduction in binding to muFcRn, but binding to huFcRn was similar to wild-type IgG1. N434A displayed improved binding to huFcRn, but not to muFcRn. Y436A mutant showed a reduction in binding to huFcRn, but not to muFcRn. The double mutant S254A/Y436A displayed almost no binding to muFcRn, but retained some binding to huFcRn.

| Location on Fc | Variants       | mufcRn* mean (SD) | huFcRn* mean (SD) |
|---------------|----------------|-------------------|------------------|
| CH2           | I253A          | <0.05             | <0.05            |
| CH2           | I253D          | <0.05             | <0.05            |
| CH2           | I253P          | <0.05             | <0.05            |
| CH2           | S254A          | 0.14 (0.01)       | 0.54 (0.07)      |
| CH2           | H310A          | <0.05             | <0.05            |
| CH2           | H310D          | <0.05             | <0.05            |
| CH2           | H310E          | <0.05             | <0.05            |
| CH2           | H310Q          | <0.05             | <0.05            |
| CH3           | H433A          | 0.76 (0.02)       | 1.15 (0.12)      |
| CH3           | N434A          | 0.71 (0.10)       | 1.75 (0.03)      |
| CH3           | N435A          | <0.05             | <0.05            |
| CH2/CH3       | Y436A          | 0.95 (0.10)       | 0.57 (0.03)      |
| CH2/CH3       | I253A/H310A    | <0.05             | <0.05            |
| CH2/CH3       | I253A/H310Q    | <0.05             | <0.05            |
| CH2/CH3       | S254A/Y436A    | 0.08 (0.04)       | 0.42 (0.03)      |
| CH2/CH3       | H310A/H435A    | <0.05             | <0.05            |

*Values are the ratio of binding of the hu3S193 variants to that of wild-type at 0.04 μg/ml.

**Interactions of mutant and wild-type hu3S193 antibodies with muFcRn and huFcRn analyzed using surface plasmon resonance**

Biotinylated forms of soluble muFcRn and huFcRn were immobilized at high densities (6.0 ng/mm²) on different channels of a neutravidin-coated chip. Antibodies were run at 667 nM concentration at acidic conditions and binding was monitored in response units (RU) (Fig. 3). In general, as observed in the ELISA study, hu3S193 wild-type and mutants bound muFcRn stronger than huFcRn (RU hu3S193 wild-type: muFcRn, 2545 ± 34; huFcRn, 1066 ± 47). Mutants binding to muFcRn could be categorized in 3 groups: 1) high binders (1500 < RU < 2500; wild-type, S254A, H433A, N434A, Y436A and S254A/Y436A); 2) low binders (RU < 500; I253A, H310A, H310Q and H435A); and 3) non-binders (e.g., I253A/H310A). Of the high binders, significantly reduced binding to wild-type hu3S193 was observed for S254A (RU, 2047 ± 37; p < 0.05), H433A (RU, 2009 ± 75) and S254A/Y436A (RU, 1630 ± 35; p < 0.01).

Hu3S193 mutants binding to huFcRn could be categorized in 2 groups: binders (500 < RU < 1500; wild-type, S254A, H433A, N434A, Y436A and S254A/Y436A) and non-binders (e.g., I253A). No differences were observed between single and double non-binding hu3S193 mutants in the human system with all non-binders showing complete abrogation of binding to huFcRn. Significantly reduced IgG1 binding of huFcRn compared to wild-type (RU wild-type, 1066 ± 47; p < 0.01) was observed for S254A (RU, 819 ± 19; p < 0.05) and S254A/Y436A (RU, 532 ± 8; p < 0.01). N434A showed significantly higher binding to huFcRn (RU N434A, 1568 ± 16; p < 0.01) compared to wild-type, which was less pronounced with respect to muFcRn binding to human IgG1 (RU N434A, 2792 ± 59; RU wild-type, 2545 ± 34; p < 0.05).

All non-alanine mutants in key residues I253 and H310 were also analyzed for binding to muFcRn and huFcRn on Biacore (Fig. 3). Compared to alanine mutants, substitutions of I253 and H310 for D, E, P or Q completely abrogated binding for mutants I253D, I253P, H310D, H310E to muFcRn (Figs. 3A, 3B), with the exception of H310Q (RU, 246 ± 30), which showed a slight increase in binding compared to H310A (RU, 135). In the human system, no binding for any of these mutants was observed to huFcRn (Figs. 3C, 3D). However, this is not surprising since the alanine mutants of I253 and H310 in IgG1 also failed to interact with huFcRn.

To obtain more quantitative binding data, kinetic evaluations were explored by fitting the data to the bivalent ligand binding model (Table S1). All antibodies bound muFcRn stronger than huFcRn. Wild-type and Y436A bound muFcRn 50 times more strongly than huFcRn. H433A bound muFcRn 40 times more strongly. S254A and S254A/Y436A bound muFcRn 20 times more strongly than huFcRn and N434A bound muFcRn 2.6 times more strongly. Interestingly, the binding of N434A to huFcRn is almost as strong as the binding of wild-type hu3S193 to muFcRn. The reduced affinity of the antibodies to huFcRn is mainly due to faster dissociation rates of the antibodies observed with huFcRn.

**Pharmacokinetics of hu3S193 IgG1 variants in BALB/c nu/nu mice and huFcRn transgenic mice**

Blood clearance studies using 111In-CHX-A" DTPA-labeled hu3S193 mutants and wild-type were performed in BALB/c nu/nu mice and in a huFcRn transgenic mouse model. Fig. 4 shows the blood clearance curves observed from both mouse models. Pharmacokinetic parameters of all antibodies are shown in Table 2. The blood clearance of 125I-hu3S193 (2.5–5 μg) wild-type antibody was used as an internal control to exclude inter-variability between mice due to reasons such as antibody-drug-responses and age differences of bred huFcRn transgenic mice. Mice with 125I-hu3S193 (%ID/mL) values within the 95% CI of mean as shown in Table S2 were accepted for evaluation of half-life and AUC of co-injected 111In-CHX-
"DTPA mutants. Statistical analysis of the pharmacokinetic parameters, serum elimination half-life ($t_{1/2b}$) and area under the curve (AUC) are shown in Fig. 5. Serum distribution half-life ($t_{1/2a}$) and amplitude of the fast ($A_a$) and slow ($A_b$) clearance component are shown in Fig. S1.

Consistent with a strongly reduced binding to muFcRn in vitro, the following mutants were found to have a significantly shorter $t_{1/2b}$ and AUC in BALB/c nu/nu mice compared to the wild-type hu3S193: I253A, I253D, I253P, H310A, H310D, H310E, H310Q, I253A/H310A, ($p < 0.0001$). Changing
Table 2. Blood clearance parameters of hu3S193 variants and wild-type in BALB/c nu/nu mice and huFcRn transgenic mice.

| Mouse strain | Variants | $A_a$ | $A_b$ | $t_{1/2a}$ | $t_{1/2b}$ | AUC* |
|--------------|----------|-------|-------|------------|------------|------|
| BALB/c nu/nu | Wild-type | 37.36 | 36.31 | 2.44       | 163.1      | 7029 |
|              | I253A    | 33.94 | 44.13 | 2.88       | 61.2       | 4792 |
|              | I253D    | 25.19 | 39.99 | 1.10       | 8.9        | 663  |
|              | I253P    | 18.40 | 44.94 | 0.91       | 9.0        | 731  |
|              | S254A    | 39.67 | 32.80 | 2.58       | 119.9      | 6145 |
|              | H310A    | 38.2  | 46.95 | 1.42       | 22.7       | 2069 |
|              | H310D    | 25.11 | 36.04 | 1.49       | 17.9       | 1221 |
|              | H310E    | 25.78 | 46.58 | 1.01       | 15.0       | 1326 |
|              | H310Q    | 35.79 | 42.39 | 0.96       | 37.1       | 3980 |
|              | N434A    | 38.45 | 35.24 | 2.28       | 169.0      | 7193 |
|              | H435A    | 38.83 | 38.31 | 1.82       | 56.3       | 4175 |
|              | Y436A    | 38.66 | 34.90 | 2.48       | 162.1      | 7110 |
|              | I253A/H310A | 21.42 | 59.26 | 1.07       | 9.6       | 903  |
|              | S254A/Y436A | 41.79 | 31.56 | 2.38       | 122.8     | 6074 |
| huFcRn Tg    | Wild-type | 24.08 | 17.61 | 3.68       | 86.73      | 2540 |
|              | I253A    | 17.98 | 31.65 | 0.93       | 12.23      | 680  |
|              | I253D    | 14.88 | 37.92 | 0.40       | 10.71      | 675  |
|              | I253P    | 23.93 | 28.58 | 1.12       | 11.41      | 541  |
|              | S254A    | 21.77 | 22.33 | 3.26       | 42.06      | 1466 |
|              | H310A    | 30.16 | 25.06 | 1.30       | 12.94      | 678  |
|              | H310D    | 18.03 | 29.70 | 1.06       | 11.19      | 562  |
|              | H310E    | 22.75 | 34.94 | 0.61       | 12.08      | 673  |
|              | H310Q    | 19.45 | 33.07 | 1.88       | 13.06      | 714  |
|              | N434A    | 18.54 | 27.52 | 1.61       | 143.4      | 5264 |
|              | H435A    | 11.82 | 27.49 | 1.41       | 12.91      | 652  |
|              | Y436A    | 14.86 | 25.70 | 4.21       | 39.34      | 1547 |
|              | I253A/H310A | 20.87 | 28.40 | 1.24       | 11.18      | 643  |
|              | S254A/Y436A | 13.93 | 23.88 | 3.35       | 34.67      | 1378 |

*Amplitude of the fast clearance component;  
Amplitude of the slow clearance component, and the sum of $A_a$ and $A_b$ equals the total injected dose (%ID/mL);  
Serum distribution half-life (h);  
Serum elimination half-life (h);  
Area under the curve is a time integral of the blood clearance curve (%ID/mL × h).

Figure 5. Key pharmacokinetic parameters calculated from single non-linear regressions (2-phase decay) from individual blood clearance curves obtained from BALB/c nu/nu mice and huFcRn transgenic mice. (A) BALB/c nu/nu mice: $t_{1/2a}$; (B) huFcRn Tg mice: $t_{1/2b}$; (C) BALB/c nu/nu mice: AUC; (D) huFcRn Tg mice: AUC. n = 3–6; bars, SEM.
alanine at position 253 into P or D further shortened the half-life of these mutants (t_{1/2, b} of L253A, 61.23 ± 19.37 h; L253D, 8.85 ± 1.83 h; L253P, 8.97 ± 2.10 h; p < 0.0001), confirming the reduced in vitro binding of L253D and L253P compared to L253A as seen with BIACore. Consistent with minimally reduced in vitro binding seen with both ELISA and BIACore, S254A (t_{1/2, b}, 119.9 ± 22.58 h, p < 0.01; AUC, 6145 ± 805 %ID/g × h; p = 0.3679) and S254A/Y436A (t_{1/2, b}, 122.8 ± 23.42 h, p < 0.05; AUC, 6074 ± 796 %ID/g × h; p = 0.2372), but not Y436A (t_{1/2, b}, 162.1 ± 37.83 h; AUC, 7110 ± 957 %ID/g × h; p = 0.2372) were found to have a significantly lower t_{1/2, b} compared to wild-type (t_{1/2, b} of I253A, 163.1 ± 34.88 h; AUC, 7029 ± 954 %ID/g × h). However, no significant differences in AUC were seen with these mutants compared to wild-type, indicating that the observed changes in half-life were minimal. Finally, changing alanine at position 310 into P or D further shortened the half-life of H310E compared to H310A (t_{1/2, b} of H310A, 22.7 ± 2.50 h; H310E, 14.96 ± 1.68 h; p < 0.05). The H310Q mutant demonstrated a longer half-life compared to H310A (t_{1/2, b} of H310A, 22.7 ± 2.50 h; H310Q, 37.1 ± 6.74 h; p < 0.0001), which confirms the higher in vitro binding of H310Q compared to H310A seen with BIACore. Based on the AUC values, the pharmacokinetic rankings of hu3S193 wild-type and mutants in BALB/c nu/nu mice were: wild-type = N434A = S254A = S254A/Y436A > H435A > I253A > H310Q > H310A > H310E = H310D = I253D = I253P = I253A/H310A.

In the huFcRn transgenic mouse model, all variants showed a half-life that was significantly different from wild-type (Table 2 and Fig. 5) confirming the differences seen in vitro with BIACore and ELISA. As reported previously by Petkova et al.,41 the N434A variant showed a significantly longer half-life compared to wild-type (t_{1/2, b} of wild-type, 83.15 ± 20.11 h; N434A, 143.4 ± 41.14 h; p < 0.0001) and higher AUC values (AUC; wild-type, 2585 ± 491 %ID/g × h; N434A, 5264 ± 206 %ID/g × h, p < 0.0001). Y436A did not show a significantly reduced half-life compared to wild-type in the BALB/c nu/nu mice, but did in the huFcRn transgenic mice (t_{1/2, b} of wild-type, 83.15 ± 20.11 h; Y436A, 143.4 ± 41.14 h; p < 0.0001).

In addition, the AUC values of S254A (AUC; 1556 ± 341 %ID/g × h; p < 0.0001), Y436A (AUC; 1665 ± 299 %ID/g × h; p < 0.0001) and S254A/Y436A (AUC; 1387 ± 118 %ID/g × h; p < 0.0001) were significantly lower than wild-type (AUC; 2585 ± 491 %ID/g × h) in huFcRn transgenic mice. All other mutants with changes to residues I253, H310 or H435 showed t_{1/2, b} values that were comparable to the fastest clearance rates observed in BALB/c nu/nu mice with no differences observed between mutants. Based on the AUC values, the ranking of hu3S193 wild-type and mutants in huFcRn transgenic mice was: N434A > wild-type > S254A = Y436A = S254A/Y436A > H435A = I253A = H310Q = H310A = H310E = H310D = I253D = I253P = I253A/H310A.

In both mouse models, good correlation was found between in vitro binding (RUmax values obtained via BIACore) and in vivo parameters (t_{1/2, b} and AUC values) (Fig. 6).

**Discussion**

Previous reports have shown that I253, H310 and H435 are key residues involved in the human IgG1-FcRn interaction, and the importance of these sites have been confirmed both with in vitro and in vivo studies using human FcRn transgenic mice.25,27,28 In contrast, the role of S254 and Y436 has been unclear. Original rat co-crystal structure analysis predicted S254 as an interface residue, but not Y436.16 In vitro binding studies suggested that alanine variants at positions S254 and Y436 abrogate the binding of human IgG1 to human FcRn similarly to I253A or H435A.25

The Y436A variant in murine IgG1 has been studied in vivo in BALB/c mice, and did not affect half-life of murine IgG1.21 No in vivo studies of S254A as a single mutation have been published. One triple mutant including mutation S254T (YTE) showed improvement in human FcRn binding and longer serum half-lives in primates, suggesting that changes in the S254 residue can alter human FcRn binding.42 In this study, we used huFcRn transgenic mice to analyze the pharmacokinetics of a

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**Figure 6.** Correlation between RUmax values obtained by BIACore in vitro, and in vivo parameters (elimination half-lives (t_{1/2, b}) and AUC) obtained by blood clearance studies of hu3S193 IgG1 wild-type and variants in BALB/c nu/nu and huFcRn transgenic mice. (A) BALB/c nu/nu mice: RUmax and t_{1/2, b}; (B) huFcRn Tg mice: RUmax and t_{1/2, b}; (C) BALB/c nu/nu mice: RUmax and AUC; (D) huFcRn Tg mice: RUmax and AUC.
series of hu3S193 variants, and demonstrated that residues S254 and Y436 contribute in the human IgG1-FcRn interaction as predicted by molecular modeling, and are potentially important sites to examine during the development of therapeutic antibodies for payload delivery in humans.

Similar to Andersen et al., good correlation was found between binding parameters obtained from the in vitro FcRn binding studies and in vivo blood clearance using huFcRn transgenic mice and BALB/c nu/nu mice. Low binding corresponded to fast clearance. However, BIAcore measurements for FcRn binding could more accurately define pharmacokinetic differences between constructs in in vivo studies. As an example, mutants with complete loss of binding to muFcRn in ELISA (I253A, H310A and H435A) showed some binding to muFcRn with BIAcore analysis, correlating with the enhanced blood clearance observed in BALB/c nu/nu mice, but slower than mutants that showed no binding at all (e.g., I253A/H310A). Our data suggests that measuring differences in RU binding levels by BIAcore predicts in vivo half-life of antibodies better than ELISA. While binding to muFcRn shows identical $K_D$ values for Y436A and wild-type, the S254A and Y436A mutants show a 2-fold reduced affinity and 2.5-fold faster dissociation rate (Table S1). BIAcore allows measurement of direct interaction between analyte and ligand, whereas ELISA involves multiple wash steps and the need for a secondary antibody for detection. A faster off rate for S254A and S254A/Y436A compared to wild-type and Y436A might therefore reduce the binding of S254A and S254A/Y436A compared to wild-type and Y436A in BALB/c nu/nu mice. These data suggests that binding affinity and binding kinetics can influence the sensitivity of detection measured by BIAcore versus ELISA.43

Kinetic analysis of binding at 667 nM concentration of antibodies showed that wild-type hu3S193 bound muFcRn ($K_D$, 106 nM) more strongly than huFcRn ($K_D$, 5595 nM), and this correlated with a longer half-life of hu3S193 wild-type in BALB/c nu/nu mice compared to huFcRn transgenic mice, confirming previous studies. Alanine changes S254A, Y436A and S254A/Y436A detected in ELISA, whereas minimal reduction by BIAcore is observed, which corresponds better with the half-life measured in BALB/c nu/nu mice. Similarly, Lofgren et al. show that binding affinity and binding kinetics can influence the sensitivity of detection measured by BIAcore versus ELISA. Previous reports show that different amino acid changes in position H435 can affect the half-life differently in huFcRn transgenic mice; e.g., the elimination half-life of T84.66 scFv-Fc H435R ($t_{1/2}$ (h), 42.02) was significantly longer than T84.66 scFv-Fc H435Q ($t_{1/2}$ (h), 10.25). Here, we investigated the amino acid changes in position I253 and H310 that would have the biggest effect on elimination half-life. Although significantly reduced half-lives were observed for I253D, I253P, H310D and H310E in BALB/c nu/nu mice, no further reduction in half-lives were observed in huFcRn transgenic mice. This supports the finding that in the huFcRn transgenic model, a single change made in I253, H310 and H435 results in the fastest elimination half-life observed with the huFcRn transgenic model, similar to the fast half-lives observed with double mutant I253A/H310A in the BALB/c nu/nu mouse model.

The huFcRn transgenic model has reduced endogenous levels of IgG. Reduced endogenous blood levels of murine IgG in the huFcRn transgenic mice, might affect the blood clearance results obtained with the huFcRn transgenic model. Therefore, more subtle differences between the fast clearing mutants might be observed in the presence of higher endogenous IgG levels.

The data presented in this paper shows for the first time in a huFcRn transgenic mouse model that mutations in the human IgG1-Fc in position S254 and Y436 reduce IgG1 half-lives compared to wild-type IgG1. These findings emphasize the importance of testing the therapeutic utility of antibodies with altered pharmacokinetics in appropriate preclinical models when predicting pharmacokinetics in humans. Immunodeficient mice transgenic for huFcRn have been described and could be useful to study tumor uptake with these reagents.

Materials and methods

Structural models of hu3S193 Fc with human and murine FcRn

Comparative template-based models of hu3S193 Fc in complex with huFcRn and muFcRn were generated based on the crystal
structure of a rat IgG-Fc:FcRn complex\textsuperscript{45} using the Modeler algorithm\textsuperscript{46} as implemented in Discovery Studio, Version 1.6 (Accelrys, USA). The contribution of residues of human IgG1 in binding human and mouse FcRn were assessed using the Fast Contact scoring function.\textsuperscript{47}

**Generation, expression and purification of hu3S193 IgG1 variants**

The construction and production of hu3S193 has been described before.\textsuperscript{29} The hu3S193 kappa light chain was ligated into the pEE14.4 mammalian expression vector (GS Gene expression system, Lonza Biologics) and the hu3S193 IgG1 heavy chain was ligated into the pEE6.4 mammalian expression vector via a HindIII/EcoRI double digest. The expression vector for the hu3S193 heavy chain was used as a template for site-directed mutagenesis [GeneTailor\textsuperscript{TM} Site-Directed Mutagenesis kit (Agilent Technologies, 200521)] to introduce the following alanine substitutions in the CH2 and CH3 domains: I253A, S254A, H310A, H433A, N434A, H435A, Y436A, I253A/H310A, I253A/H435A, S254A/Y436A and H310A/H435A. Primers were designed according to the manufacturer’s instructions (Table S3). For the generation of the double mutants, the expression vector for the hu3S193 heavy chain with one mutation was used as a template to introduce the second mutation. Light and mutated heavy chain genes were cloned into a double-gene vector using a NotI/ PvuI double digest to allow equal expression of hu3S193 IgG1 light chain and heavy chain. Freestyle-293F cells were cultured and transient transfections with double-gene vectors were done according to the manufacturer’s instructions (Invitrogen, K9000-01). Supernatants were harvested at 96 hours post transfection. Harvested supernatants were centrifuged to remove cell debris and sterile-filtered (0.22 \(\mu\)m bottle top filter, Nalgene) before purification. Hu3S193 antibodies were purified using HiTrap Kappa-Select columns (GE Healthcare Life Sciences, 17-5458-11). Eluted fractions were neutralized using 0.1 M Tris-HCl buffer (pH 8.0), dialyzed against phosphate-buffered saline (PBS) and concentrated using Amicon Ultra-15 concentrators (Merck Millipore, UFC905024).

**Generation, expression and purification of murine and human soluble FcRn**

Human FcRn cDNA (SC117595) and \(\beta_2\)m cDNA (SC117632) clones were obtained from OriGene Technologies. Murine FcRn cDNA and \(\beta_2\)m cDNA clones were obtained from GeneArt Gene Synthesis (Life Technologies). Extracellular domains of FcRn heavy chain and full length \(\beta_2\)m were cloned into the pEE14.4 and pEE6.4 vectors, respectively (GS Gene expression system, Lonza Biologics), similar to the method of Gastinel et al.\textsuperscript{48} Truncated huFcRn heavy chain was created by inserting a stop codon after amino acid 267. Truncated murine FcRn heavy chain was created by inserting a stop codon after amino acid 269. Two tandemly located affinity tags, c-myc epitope (EQKLISEEDL) and His\(_6\) (HHHHHHH), were added at the protein C-terminus of human and murine FcRn. This was achieved by inserting truncated FcRn without a stop codon in a pEE14.4/FLAG His\(_6\) vector. The FLAG and His\(_6\) containing cDNA was formed by annealing a c-myc-His\(_6\) forward oligonucleotide, 5\' - AATTCCGACGAAAGCTCATCGCGAG-GAGGACCTGCACCACCCATCATCATTGATGATAAT-3\' and a c-myc-His\(_6\) reverse oligonucleotide, 5\' - GATCATTATCAT-CAATGGTGATGTGGTGGTGGTCCTCTGCCGTAT-GAGCTTCTGTCCG -3\’ which was introduced into pEE6.4 as an EcoRI/BclI cDNA fragment. Two tandemly located affinity tags, FLAG epitope (DYKDDDK) and His\(_6\) (HHHHHHH), were added at the protein C-terminus of human and murine FcRn. This was achieved by inserting truncated FcRn without a stop codon in a pEE14.4/FLAG His\(_6\) vector. The FLAG and His\(_6\) containing cDNA was formed by a FLAG oligo forward, 5\' - AATTCCGACGAAAGCGAGGG-ACGACGAGGACCCACCCATCATCATTGATGATAAT-3\' and a FLAG oligo reverse, 5\' - GATCATTATCATAATGGTGATGTGGTGGTGGTCCTCTGCCGTAT-GAGCTTCTGTCCG -3\’ which was introduced into the pEE14.4 vector as an EcoRI/BclI restriction fragment. Four double gene vectors were generated to produce huFcRnHu\(_6\) (huFcRn), muFcRnMu\(_6\) (muFcRn), huFcRnFLAGHis\(_6\)Hu\(_6\)-mc-mycHis\(_6\) (huFcRn-tagged) and muFcRnFLAGHis\(_6\)Mu\(_6\)-mc-mycHis\(_6\) (muFcRn-tagged).

**Quality control of hu3S193 IgG1 variants, hu3S193 IgG1-Fc and soluble FcRn receptors**

Purified proteins were analyzed by size-exclusion chromatography on a Superdex 200 HR 10/30 column (GE Healthcare Life Sciences, 17-1088-01) using 0.01 M sodium phosphate and 0.15 M sodium chloride (pH 7.2) as elution buffer. A 0.05 mL column was prepared and eluted at 0.4 mL/min flow rate. Protein was detected by absorbance at 214 nm and 280 nm. A molecular weight calibration curve was generated using standard markers containing blue dextran, ferritin, aldolase, ovalbumin and chymotrypsinogen (GE Healthcare life sciences, 28-4038-42). In addition, SDS-PAGE was used to evaluate the size and integrity of expressed IgG1 mutants and soluble FcRn.

**FACS analysis**

Binding of hu3S193 variants to Le\(^+\) antigen was done on Le\(^+\) positive A431 epidermoid carcinoma cells (ATCC, CRL-1555). Aliquots of 2 \times 10\(^5\) cells were incubated with hu3S193 antibodies (200 nM) in DMEM/F12 medium (Invitrogen) containing 10% fetal bovine serum (FBS; Sigma) on ice for 1 hour. After washing the cells with PBS, cells were incubated with a goat phycoerythrin (PE)-conjugated anti-human IgG (Sigma-Aldrich, P9170) or a goat fluorescein isothiocyanate (FITC)-conjugated anti-human IgG (Sigma-Aldrich, F6380) and incubated on ice for 30 min. Cells were washed with PBS and resuspended in a final volume of 200 \(\mu\)L. In control samples, primary antibody was omitted. Flow cytometric analysis was done using a Guava EasyCyte Plus flow cytometer (Guava Technologies). Cancer cell populations were gated based on forward and side scatter variables. Data analysis was done using WinMDI (Joseph Trotter).

**Biotinylation of soluble FcRn**

For biotinylation, biotinamidopropionate N-hydroxysuccinimide ester (Sigma-Aldrich, B2643) was used. Soluble FcRn (human
or murine) was buffer exchanged into 0.1 M sodium bicarbonate before biotin was added in a 2 mol biotin/mole protein ratio. Biotinylated FcRn was purified through a P-6DG desalting column (Bio-Rad, 150-0738) equilibrated with 0.1 M sodium bicarbonate. Biotinylated FcRn was eluted with sodium chloride injection BP 0.9% w/v (Pfizer).

**ELISA to assess mouse and human FcRn binding to hu3S193 antibodies**

MaxiSorp 96-well plates (Thermo Fisher Scientific, NUN430341) were coated with 50 μL of soluble streptavidin (Sigma-Aldrich, S0677) at a concentration of 6 μg/mL in PBS (pH 7.4, Invitrogen, 14190144) overnight at 37°C. Plates were washed with PBS containing 0.05% v/v Tween20 (PBS-T, pH 7.4), and then blocked with 220 μL of 3% v/v IgG-depleted PBS in PBS-T. For the remaining steps, all buffers were changed to pH 6.0 to allow for the acidic binding requirements of FcRn to IgG-Fc. Following the 1 h incubation of the blocking buffer at room temperature, the plates were washed and 50 μL of biotinylated FcRn (human or murine) was added at 3 μg/mL in PBS-T. After 1 h incubation at room temperature, plates were washed and 3-fold serial dilutions of hu3S193 mutants (0.0137–30 μg/mL) were added at 50 μL in PBS-T. After 1 h incubation, plates were washed and bound IgG was detected by adding 100 μL of peroxidase-conjugated goat F(ab)² anti-human IgG LC and HC (Abcam, ab98525) in 1% v/v IgG-depleted FCS in PBS-T. After a final 1 h incubation, plates were washed and the substrate 3,3',5,5'-tetramethyl benzidine (Sigma-Aldrich, T0440) was added for 10 min. The reaction was stopped by adding 2 M sulfuric acid and absorbance was read at 450nm.

**Surface plasmon resonance**

Interaction studies between hu3S193 antibodies and FcRn receptors were conducted using a BIAcore 2000 biosensor. Biotinylated FcRn (huFcRn-Bt) were immobilized onto a carboxymethyldextran (huFcRn-Bt) were immobilized onto a carboxymethyldextran hydrogel sensor chip (Xantec Bioanalytics, CMD500L) derivatized with NHS/EDC chemistry. An immobilization level of 6.0 ng/mm² was obtained for both receptors. Binding analyses were performed by injecting 30 μL of hu3S193 antibodies (100 μg/mL) at 10 μL/min in 10 mM HEPES containing 3.4 mM EDTA, 0.15 mM sodium chloride and 0.005% Tween 20 (HBS) at pH 6.0. Binding levels measured as response units (RU) were obtained at the end of the association phase, using a NeutrAvidin derivatized blank channel as control. Surface regeneration was performed following binding and dissociation using HBS buffer at pH 8.0. The bivalent binding model (BIA-evaluation 4.1.1) was used to fit the binding curves run at 667 nM. Refractive index was set at 0. K_D values were calculated from the kinetic parameters k_on and k_off (K_D = k_off/k_on).

**Chelation and radiolabeling of hu3S193 IgG1 variants**

Hu3S193 antibodies were labeled separately with 2 isotopes, ¹²³I or ¹¹¹In. Iodine-125 was obtained from Perkin Elmer (NEZ033H002MC). ¹¹¹In was obtained from MDS Nordion (Canada).

Radioiodination was performed using pH neutralised isotopes, catalyzed by Pierce iodination beads (Pierce, 28665). After a brief 10 min incubation period, the reaction was purified through a Sephadex G50 desalting column (Sigma-Aldrich, G5080) equilibrated with PBS.

Radiolabeling of the hu3S193 antibody constructs with Indium-111 was performed by using the bifunctional metal ion chelate C-functionalized trans-cyclohexyldienetriamine-pentaacetic acid (CHX-A” DTPA) (Macrocyclics, B-355). Che- lation of proteins with CHX-A”, DTPA and subsequent radiolabeling with ¹¹¹In were performed as described before.⁴⁰-⁵² Radiolabeling was performed on the day of injection into mice. Prior to injection, the percentage of unbound radionuclide content was determined by instant thin layer chromatography (ITLC) as previously described.⁴⁹ Determination of the immunoreactivity of radiolabeled hu3S193 antibody constructs was performed by a single-point binding assay, where 1 × 10⁷ Leu² positive A431 cells were incubated with 20 ng of ¹¹¹In-CHX-A”. DTPA-hu3S193 antibody constructs for 45 min at room temperature with continuous mixing throughout to keep the cells in suspension. Cells were washed 3 times, and pellets were measured in a gamma counter (Cobra II, Model 5002, Packard Instruments, Canberra, Australia). Three samples of radiolabeled antibody at the same concentration as that initially added to the cells were measured at the same time of the cell pellets and immunoreactivity was calculated: (cpm cell pellet/mean cpm radioactive antibody standards) × 100. Serum stability was analyzed by ITLC on the day of injection, at 48 h and after 7 d. Radiolabeled antibody (20 ng) was incubated in human serum at 37°C.

**Blood clearance studies in BALB/c and huFcRn transgenic mice**

Female athymic mice (BALB/c nu/nu; 6 weeks) were obtained from the Animal Resources Center (WA, Australia). FcRn-deficient B6-FcgrTm1Dcr mice (muFcRn⁵⁻) and mice from a huFcRn transgenic line 276, isogenic on a B6 background (huFcRn (276) Tg), were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice deficient in muFcRn and carrying huFcRn transgene (Tg) (muFcRn⁵⁻/¹ huFcRn Tg) were generated by crossing muFcRn-deficient mice (FcRn⁵⁻) with mice expressing huFcRn (huFcRn (276) Tg). All animal studies were approved by the Austin Hospital Animal Ethics Committee and were conducted in compliance with NHMRC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

BALB/c nu/nu mice were injected with 5 μCi ¹¹¹In-CHX-A” DTPA mutant or ¹¹¹In-CHX-A” DTPA wild-type (2.5–5 μg). HuFcRn transgenic mice were co-injected in the tail vein with 5 μCi ¹²³I-hu3S193 (2.5–5 μg) wild-type antibody and either 5 μCi ¹¹¹In-CHX-A” DTPA mutant or ¹¹¹In-CHX-A” DTPA wild-type (2.5–5 μg). Blood samples (10–20 μL) were collected from groups of 4 or 5 mice at 0.5, 1, 2, 4, 8, 24, 48, 72, 120, 168, 240 and 336 h after injection of radioactive antibodies. Samples were counted in a gamma counter (Cobra II). Standards prepared from injected material were counted each time with blood samples enabling calculations to be corrected for physical decay of the isotope.
The results of the serum were expressed as % injected dose per milliliter (%ID/mL). A two-phase exponential decay curve was fitted to serum data for each animal using Graphpad Prism 6.03 (Graphpad Software Inc., La Jolla, CA). Estimates were determined for the pharmacokinetic parameters: $\alpha$ half-life ($t_{1/2a}$), $\beta$ half-life ($t_{1/2b}$), and AUC.

**Statistical analysis**

Differences in calculated parameters and measurements between mutants and wild-type were analyzed using a one-way ANOVA. Differences of pharmacokinetic half-lives of each antibody in BALB/c nu/nu mice vs. transgenic mice were compared using an unpaired t-test. Differences were considered significant at $p < 0.05$. All analyses were performed using Graphpad Prism version 6.03.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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