Enhanced half-life of genetically engineered human IgG1 antibodies in a humanized FcRn mouse model: potential application in humorally mediated autoimmune disease

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Abstract

The MHC class I-like Fc receptor FcRn plays an essential role in extending the half-life ($t_{1/2}$) of IgG antibodies and IgG-Fc-based therapeutics in the circulation. The goal of this study was to analyze the effect of human IgG1 (hIgG1) antibodies with enhanced in vitro binding to FcRn on their in vivo $t_{1/2}$ in mice expressing human FcRn (hFcRn). Mutants of the humanized monoclonal Herceptin antibody (Hu4D5-IgG1), directed against human epidermal growth factor receptor 2 (p185 HER2), showed altered pH-dependent binding to hFcRn in vitro. Two engineered IgG1 mutants (N434A and T307A/E380A/N434A) showed a considerably extended $t_{1/2}$ in vivo compared with wild-type antibody in mice expressing an hFcRn transgene (Tg) but not in mice expressing the endogenous mouse FcRn. The efficiency of hFcRn-mediated protection was dependent on hFcRn Tg copy number. Moreover, when injected into FcRn-humanized mice at a concentration sufficient to partially saturate hFcRn, the engineered IgG1 mutants with an extended serum $t_{1/2}$ were most effective in reducing the $t_{1/2}$ of a tracer hIgG1 antibody. Finally, administration of mutant with high binding to hFcRn ameliorated arthritis induced by passive transfer with human pathogenic plasma. These results indicate that Fc regions modified for high binding affinity to hFcRn increases serum persistence of therapeutic antibodies, that the same approach can be exploited as an anti-autoimmune therapy to promote the clearance of endogenous pathogenic IgG and that FcRn-humanized mice are a promising surrogate for hIgG therapeutic development.

Introduction

IgG differs from other Ig classes in that it has the longest survival time in the circulation, with a half-life ($t_{1/2}$) ranging from 7 to 21 days in healthy humans (1–4). The Fc fragment has been implicated in the prolonged survival of IgG, due to the fact that Fc fragments have a $t_{1/2}$ similar to intact IgG, and much longer survival than Fab fragments (5, 6). This prolongation is explained by the existence of an Fc receptor, which rescues IgG from normal lysosomal degradation (7, 8). The class I MHC family molecule FcRn, comprised of the FcRn heavy chain and the β2-microglobulin light chain, is the Fc receptor responsible for IgG homeostasis (9–14). Functional studies with mutant murine IgG-Fc fragments demonstrated impaired binding to FcRn and abnormally short $t_{1/2}$ (15, 16). Furthermore, mouse IgG (mlgG) is cleared significantly faster in mice deficient in either the FcRn light or heavy chains (11, 12, 17, 18). The steady-state location of FcRn is endosomal, where it binds Fc with high affinity in an acidic environment (19, 20). Endocytic vesicles containing extracellular fluids fuse with the FcRn-containing vesicles, and in that acidic environment, easily protonated residues in the Fc CH2-CH3 domain interact with acidic FcRn residues (9, 21–24). IgG that binds FcRn is then recycled either apically or basolaterally to the...
plasma membrane, where upon exposure to a neutral pH it is released (20, 25–29). However, the rescue pathway can be overburdened by excess concentrations of IgG, thus explaining IgGs concentration–fractional catabolism relationship (7, 30).

FcRn’s maintenance of IgG homostasis has substantial implications. It is a primary reason why IgG is the most abundant serum Ig and the major serum Ig developed after immunization (11). Second, by enabling the maintenance of copious levels of pathogenic IgG, FcRn promotes the development of humoral autoimmunity (31, 32). Finally, FcRn-mediated homostasis is responsible for the extended serum $t_{1/2}$ of therapeutic IgG antibodies and IgG-Fc fusion proteins, which have emerged as a major treatment for a wide array of both neoplastic and autoimmune diseases (33).

Given the importance of therapeutic IgG and the fact that stabilization by FcRn is a key to their extended serum persistence, an attractive approach to improve therapeutic IgG’s $t_{1/2}$ would be to enhance the IgG–FcRn interaction. This improvement could conceivably reduce the dosage or frequency of therapeutic antibody administration without compromising their pharmacologic efficacy. Studies by the Ward laboratory were the first to show that specific mutations in the Fc portion of mIgG that binds mouse FcRn (mFcRn) can shorten or extend their serum $t_{1/2}$ (15, 28, 34, 35). More recent studies have evaluated human IgG (hIgG) antibodies with their Fc region engineered for enhanced acid-dependent binding to human FcRn (hFcRn) (36–39). However, it has been challenging to ascertain whether such antibodies show prolonged serum persistence in vivo. Recent studies with primates analyzing hlgG2 and hlgG1 mutants with increased affinity for hFcRn have shown promise, in that such mutants showed an increased serum $t_{1/2}$ (36, 40). However, rodent models have not proven as informative for evaluating the hlgG stabilization because of evolutionary differences in rodent IgG and FcRn and hlgG and hFcRn (35, 41). Mice lacking endogenous FcRn and transgenic for hFcRn are a potentially promising surrogate for evaluating the pharmacokinetics of hlgG therapeutics (11). Here we describe the use of a humanized FcRn mouse model to evaluate the pharmacokinetic efficacy of human Hu4D5-IgG1 mutant antibodies engineered to have enhanced binding to hFcRn. Such mutants show an extension of their serum $t_{1/2}$, increased ability to saturate the hFcRn protection pathway and increased ability to prevent autoimmune lesions caused by pathogenic hlgG.

**Methods**

**Antibodies**

The Hu4D5 wild-type (WT) IgG1 antibody was originally derived from the mouse mumAb4D5 antibody which was humanized using a gene conversion mutagenesis strategy (42). Hu4D5-IgG1 WT and the following FC mutants were used in the study: Hu4D5 Fc8 (I253A), Hu4D5 Fc42 (N434A) and Hu4D5 Fc270 (T307A/E380A/N434A) (numbering according to the EU index) (43). Hu4D5-IgG1 mutants were produced by site-directed mutagenesis, substituting solvent-exposed amino acids in the Fc CH2 and CH3 domains with Ala as previously described by Shields et al. (37, 42). Single amino acid-substituted mutants I253A, N434A and the combination mutant T307A/E380A/N434A were purified from large-scale transient CHO cell cultures using Protein A column (Prosep-A) (Millipore, Billerica, MA, USA) followed by an SP sepharose ion exchange column (Amersham Biosciences, Piscataway, NJ, USA) for use in in vitro and in vivo experiments. The humanized IgG1 antibody [hybridoma clone HuLy511, anti-hen egg-white lysozyme (HEL), a kind gift from Jeffery Foote, Fred Hutchinson Cancer Center, Seattle, WA, USA] (44) or mIgG1 anti-dinitrophenyl (DNP) hybridoma clone 1B7.11 [American Type Culture Collection (ATCC), Rockville, MD, USA] were used as tracer antibodies in vivo. Purified human Ig (Gamma Guard®, Baxter Healthcare Corp., Deerfield, IL, USA) was used for in vivo studies as well. The above-mentioned antibodies were injected intraperitoneally (i.p.) at the appropriate concentration after dilution with 1× PBS to a total final volume of 500 μl per mouse. The anti-hFcRn mAb (DVN24), which cross-reacts with mFcRn, was produced from a hybridoma generated in FcRn−/− mice immunized with hFcRn (D. C. Roopenian, in preparation).

**In vitro binding of Hu4D5 antibodies to FcRn**

Mouse fibroblast L cells (ATCC, Manassas, VA, USA) were stably transfected with either hFcRn plasmid or mFcRn plasmid (hFL and mFL) to direct FcRn expression on the plasma membrane rather than cytoplasmic endosomes. For mFL, a truncated mFcRn cDNA product was generated from C57BL/6J (B6) RNA isolated from neonatal proximal small intestine (collected on day 10) after amplification of cDNA product using the following oligonucleotide primers—forward: CCCCCCCCTTGAGGTCAAGACCCGCCGCCCGCCCA (CTCGAG Xhol site underlined) and reverse: CCCCCCGAATTCGTGCAGACCCGCCGCCCGCCCAACG (GAATTC EcoRI site underlined, premature stop codon in lowercase). CCCCCCGCCGCC nucleotides were added to the primers to improve restriction endonuclease cleavage of the PCR product. The 944-bp PCR product was digested with Xhol and EcoRI and was cloned into the corresponding sites of the pEGFP-C1 vector backbone (BD Biosciences, Franklin Lakes, NJ, USA) into which we had already inserted a 118-bp 5’ human signal FcRn sequence including those encoding the 23 amino acid hFcRn signal sequence 5’ of eGFP. This cloning step eliminated the eGFP coding sequence and positioned mFcRn directly downstream of the FcRn signal peptide. All PCR-amplified inserts were sequence verified bi-directionally across the cloning sites. Purified hFcRn and mFcRn plasmids were transfected into L cells using Lipofectin Plus (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol, and stable transfecteds were selected using 400 μg/ml G418 (Sigma-Aldrich, St Louis, MO, USA) in FBS-supplemented DMEM. The constructs lack the FcRn cytoplasmic endosomal targeting domain, and thus, transfected cells express high levels of hFcRn on their plasma membrane. Plasma membrane expression of mFcRn and hFcRn was verified using the anti-FcRn mAb (DVN24).

IgG binding to FcRn was detected by flow cytometry and all procedures were performed at 4°C. hFcRn-transfected L cells (hFL), mFcRn-transfected L cells (mFL) or untransfected L cells were washed with FACS buffer (pH 6, or pH 7.4 PBS containing 1% BSA, 0.05% NaN3). Cell aliquots (1 × 10^6) were then incubated in triplicate with 1 μg of Hu4D5-IgG1 antibodies, or mouse monoclonal IgG1 (1B7.11) in 50 μl FACS buffer at pH 6.0 or 7.4 in the round-bottomed wells of a 96-well plate.
After 60 min, the cells were washed with buffer (pH 6.0 or 7.4) and stained with either goat anti-human IgG conjugated to PE (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) or goat anti-mouse IgG conjugated to Alexa Fluor 488 (Invitrogen Corp., Carlsbad, CA, USA). After 30 min, the cells were washed with buffer (pH 6.0 or 7.4), acquired by gating on the top 10% brightest DNV24-AF647 positive events on a FACSCalibur and data were analyzed using Cell Quest software (BD Biosciences, San Jose, CA, USA). Propidium iodide (1 μg ml⁻¹) was used to exclude dead cells from the analysis. Results are presented as the mean ± SD of three replicates.

Mice

FcRn-deficient B6-Fcgrt<sup>−/+m1Dcr</sup> mice were generated as described (11, 45). Mice deficient in mFcRn and carrying hFcRn transgene (Tg) (FcRn<sup>−/+</sup> hFcRn Tg) were generated by crossing mFcRn-deficient mice (FcRn<sup>−/−</sup>) with mice expressing hFcRn (hFcRn Tg). The genomic hFcRn transgenic line 32 (hFcRn (32) Tg), isogenic on a B6 background, was produced using a 33-kb cosmid including the complete hFcRn gene and ~10-kb 5' and 3' flanking sequences, as described (11). The cdNA transgenic line 276 [hFcRn (276) Tg] expressing the hFcRn α-chain cloned into vector E carrying a CMV enhancer and a chicken β-actin promoter was produced as described (45). Expression of hFcRn in these FcRn<sup>−/+</sup> hFcRn Tg mice has been validated by reverse transcription (RT)–PCR, western blot and functional assays in vivo (45). Mice deficient in both murine receptors FcγRIIB and FcRn but expressing hFcRn Tg (FcγRIIB<sup>−/+</sup> FcRn<sup>−/+</sup> hFcRn (32) Tg mice) were produced by generating double-knockout FcRn<sup>−/+</sup> FcγRIIB<sup>−/+</sup> mice and crossing them with FcRn<sup>−/+</sup> hFcRn Tg mice. B6-FcγRIIB<sup>−/+</sup> mice were originally obtained from Taconic Farms (Germantown, NY, USA), FcγRIIB<sup>−/+</sup> and FcRn<sup>−/+</sup> mice were crossed to produce mice doubly homozygous for the mFcRn and FcγRIIB null alleles. B6-FcRn<sup>−/+</sup> FcγRIIB<sup>−/+</sup> mice were identified by RT-PCR for FcRn null allele as described (11) and by flow-cytometric analysis of peripheral leukocytes for FcγRIIB null allele as described (46). B6-FcγRIIB<sup>−/+</sup> FcγRIIB<sup>−/+</sup> hFcRn (32) Tg mice were then produced by crossing FcRn<sup>−/+</sup> FcγRIIB<sup>−/+</sup> with FcRn<sup>−/+</sup> hFcRn (32) Tg mice, and then selected among the progeny for mice heterozygous for the hFcRn Tg. The following PCR primer set was used for detection of genomic hFcRn forward: AGCCAAAGTCTCCGTGTC and reverse: TCAGAGATGCCAGTGTTCC. The amplified genomic product was 740 bp in size as analyzed on a 1.5% agarose gel. Sex matched (8- to 16-weeks old) mice were used in the study. All experiments were performed in pathogen-free Jackson Laboratory research mouse colony, with protocols approved by The Jackson Laboratory Animal Care and Use Committee. The FcRn<sup>−/+</sup> hFcRn transgenic mice described here are available on request.

In vivo elimination of Hu4D5 antibodies

The elimination of Hu4D5-IgG1 antibodies in vivo was tested using two different transgenic lines: FcRn<sup>−/+</sup> hFcRn (276) Tg (cDNA transgenic line 276) and FcRn<sup>−/+</sup> hFcRn (32) Tg (genomic transgenic line 32). Mice caring one (hFcRn Tg) or two copies (hFcRn Tg/Tg) of the hFcRn Tg were included in the study. Both transgenic and control mice (FcRn<sup>−/−</sup> and FcRn<sup>−/+</sup>) were injected on day 0 with a single i.p. injection of 100 μg of one of the following hlgG1 antibodies: WT, I253A, N434A or T307A/E380A/N434A diluted to final volume of 500 μl in sterile 1× PBS. The group of hFcRn (32) Tg mice was also pre-treated with 2 mg purified hIgG per animal on day −1 followed by tracer injection with Hu4D5-IgG1 antibodies on day 0. Each human test antibody was co-injected with control mouse anti-DNP IgG1 at the same concentration (100 μg per mouse). Blood samples were obtained from the retro-orbital plexus using non-heparinized capillary pipettes 5 min before injection and several time points after the tracer injection. A Her2 antigen-based ELISA was used to monitor the serum concentrations of the Hu4D5 antibodies. Briefly, 96-well plates were coated with 5 μg ml⁻¹ of Her2 (Genentech Inc., South San Francisco, CA, USA). The plates were blocked with 5% BSA in PBS, incubated with appropriately diluted serum samples (1:200), followed by mouse anti-human IgG-Fc-specific antibody (dilution 1:1000) conjugated to alkaline phosphatase (AP) (Southern Biotechnology Associates, Inc.). Activity was reported at 405 nm after development with colorimetric p-nitrophenyl phosphate (p-NPP) substrate at a concentration of 1 mg ml⁻¹ (Sigma–Aldrich). To determine the serum concentration of the 1B7.11 mlgG1 tracer bovine gammaglobulin-DNP (Calbiochem, La Jolla, CA, USA) was coated onto wells of 96-well plates at concentration of 5 μg ml⁻¹. Serum samples and varying concentrations of standard (mlgG1) were incubated on the plates and detected with goat anti-mouse IgG–AP antibody (Southern Biotechnology Associates, Inc.). The serum concentrations of hlgG1 antibodies or mlgG1 tracer antibodies were presented as percent remaining in the circulation at different time points after injection compared with day 1 values (100%), as described (11).

Competitive assay in vivo

To determine the ability of the Hu4D5 antibodies to promote the serum clearance of an hlgG1 or mlgG1 tracer, the serum clearance of hlgG1 or mlgG1 was analyzed in the presence of competitor Hu4D5 antibodies using FcRn<sup>−/+</sup> hFcRn (32) Tg mice. Animals (n = 3 per group) were infected i.p. with hlgG1 mAb (HULys11, anti-HEL) and control mlgG1 antibodies (1B7.11, anti-DNP) at concentrations of 100 μg per mouse diluted with 1× PBS to a 500-μl final volume. The same mice were injected 48 and 72 h later with competitor Hu4D5 antibody (WT, I253A, N434A or T307A/E380A/N434A) at one of the following concentrations: 1, 2 or 5 mg per 20 g body weight (2 × 1 mg, 2 × 2 mg and 2 × 5 mg). FcRn Tg mice were bled serially and after the injection of tracer and competitor antibody. Serum was collected and stored at −80°C until proceeding with the appropriate ELISA. To test the serum concentration of hlgG1 tracer (anti-HEL, HULys11 antibody), plates were coated overnight at 4°C with 5 μg ml⁻¹ of HEL (Sigma–Aldrich), washed four times with PBS, blocked with 5% BSA for 1 h at 37°C and incubated with serum samples at appropriate dilution. Mouse anti-human IgG (Fc specific) AP-conjugated antibody was used for detection at dilution 1:1000 in 1× PBS containing 1% BSA. After 1 h incubation at 37°C, plates were washed and incubated with the substrate p-NPP (Sigma–Aldrich). The serum concentrations of hlgG1 or mlgG1 tracer
antibodies were calculated based on serial dilutions of appropriate standards and are presented as percent remaining in the circulation at different time points.

**Testing the efficacy of Hu4D5-IgG1 antibodies in inducible model of arthritis**

In order to induce arthritis, FcγRIIB−/− FcRn−/− hFcRn (32) Tg mice were injected i.p. with plasma from a patient with active rheumatoid arthritis (RA). Clinical characteristics of the patient were included in Petkova et al. (47). A total of 2.5 ml of human plasma per mouse was administered as follows: 0.5 ml on day 0, 1 ml day 2 and 1 ml on day 7. Ankle diameter was inspected for inflammation and erythema by two observers. Joint swelling was scored by examination of both rear legs as follows: 0, unaffected; 1, questionable; 2, swelling of one rear leg and 3, severe swelling of both rear legs as previously described (31). To test the therapeutic efficacy of the Hu4D5 antibodies, the same mice were treated three (days 1, 3 and 8) or five times (days 1, 3, 8, 10 and 14) with either IgG1 WT or selected Fc mutant antibodies following the injections with human plasma (days 0, 2 and 7). The therapeutic antibodies were administered i.p. at a concentration 2.5 mg per 20 g body weight diluted with 1× PBS up to a final volume of 500 μL.

**Statistical analysis**

The \( t_{1/2} \) of the elimination \( \beta \)-phase for each test antibody was determined using a one-phase exponential decay model provided by JMP statistical software (SAS Institute, Boston, MA, USA), including data points between day 3 or 4 and day 12 or 16 post-injection. The model was fit for each individual mouse per experimental group. Once the \( t_{1/2} \) was calculated for each mouse, a \( t \)-test was used to test for differences in the average \( t_{1/2} \) for each group. Results are presented as mean \( t_{1/2} \) (days) \( \pm \) SD and a \( P \) value \( <0.05 \) was considered significant. Analysis of repeated measures was adopted for ankle measurements. Triplicate measurements for each rear ankle were averaged to produce an average ankle width for each mouse. Results were averaged for both legs of each animal and, subsequently, for each group of animals. Variation was always \( \leq 0.1 \) mm. Delta ankle thickness (millimeter) was calculated as mean difference between day 0 and each time point of the experiment and the \( P \) value was calculated using the Student’s \( t \)-test. A \( P \) value \( <0.05 \) was considered significant.

**Results**

**In vitro binding of Hu4D5-IgG1 antibodies to hFcRn and mFcRn**

A previous study used ELISA techniques to examine the acid-dependent binding of the humanized monoclonal Herceptin antibody, Hu4D5-IgG1 WT and mutant antibodies with amino acid substitutions in the CH2 and CH3 domains of the Fc region to hFcRn (37). The I253A mutant antibody exhibited substantially reduced binding. In contrast, the N434A mutant antibody showed 3.4-fold enhanced binding and the triply substituted mutant T307A/E380A/N434A exhibited 11.8-fold enhanced binding to hFcRn (37). To determine whether these affinity differences resulted in alterations in a more physiological setting, a cell-binding assay was developed. Murine fibroblast cells (L cells) expressing a truncated form of hFcRn (hFL cells) or mFcRn (mFL cells) were produced as described in Methods. The FcRn transfection constructs were engineered to lack the cytoplasmic endosomal targeting domain, causing the FcRn protein to localize to the plasma membrane rather than in the endosomes. As FcRn binds the Fc fragment of IgG at a slightly acidic but not at a neutral pH, flow cytometry-based binding assays were carried out at both pH 6.0 and 7.4 (Fig. 1). Significant binding of WT, N434A and T307A/E380A/N434A antibodies to hFL cells was observed at pH 6.0 with N434A and T307A/E380A/N434A mutants, respectively, exhibiting 1.6-fold (\( P = 0.007 \)) and 3.3-fold (\( P = 0.0001 \)) enhanced binding relative to the WT antibody (Fig. 1A). The Hu4D5 antibodies therefore bound to hFcRn expressed on the plasma membrane in a pattern consistent with that observed previously to soluble hFcRn (37). Binding of the WT and N434A antibodies was abolished at pH 7.4, with the exception that T307A/E380A/N434A demonstrated 2.4-fold increased binding to hFcRn as compared with WT antibody (\( P = 0.0002 \)). Moreover, mIgG1 failed to appreciably bind FcRn, a finding consistent with previous studies indicating that FcRn does not stabilize mIgG (35, 45).

Binding to mFcRn yielded a very different pattern for the functional Hu4D5 antibodies (WT, N434A and T307A/E380A/N434A) (Fig. 1B). These antibodies bound mFcRn at similarly high levels at pH 6.0 and there was no significant difference (\( P > 0.05 \)) in mean fluorescence intensity values between
the WT and high-affinity mutant antibodies (908 ± 28.4 for N434A, 1082 ± 136.5 for T307A/E380A/N434A and 989 ± 41.9 for WT antibody). In contrast, and as expected, substitution of the critical amino acid Ile at position 253 to Ala in the I253A mutant resulted in the abolishment of binding to mFcRn at both pH 6.0 and 7.4. Minimal binding of the WT and mutant Hu4DS antibodies to both hFcRn and mFcRn was observed at pH 7.4, thus confirming the acidic requirement for FcRn binding. However, as indicated in Fig. 3(B), T307A/E380A/N434A was exceptional in that it retained substantial binding to mFcRn at pH 7.4 (54-fold increase as compared with WT antibody; \( P < 0.0001 \)). This pattern was observed in three independent experiments (data not shown). There was minimal binding of human Hu4D5 antibodies to control L cells at both pHs (Fig. 1C).

**In vivo persistence of Hu4D5 antibodies**

We then examined whether the binding characteristics of the Hu4DS antibodies observed in vitro could result in differences in the serum persistence in vivo. The Hu4D5 antibodies were first analyzed in mice lacking mFcRn and expressing one copy of the cDNA hFcRn transgene [mFcRn/C0/C0/hFcRn (276) Tg]. The elimination curves of the three Hu4D5-IgG1 mutant antibodies were clearly distinct from that of the WT (Fig. 2A). As predicted by its failure to bind hFcRn in vitro, substitution of Ile at position 253 to Ala in the I253A mutant lead to enhanced clearance of this antibody compared with WT (\( t_{1/2} = 1.02 ± 0.13 \) days versus 1.72 ± 0.07 days, \( P \leq 0.01 \)) and was virtually undetectable on day 3 after post-injection. In contrast, mutants N434A and T307A/E380A/N434A demonstrated a substantial increase in serum persistence as compared with WT (Fig. 2A), with N434A and T307A/E380A/N434A showing a significant 2.2- to 2.5-fold increase of their \( t_{1/2} (t_{1/2} = 3.85 ± 0.55 \) days, \( P = 0.03 \) and 4.35 ± 0.53 days, \( P = 0.02 \), respectively, compared with the WT antibody (1.72 ± 0.07 days) (Table 1A).

The same trend was observed after injection of Hu4D5-IgG1 antibodies into cDNA Tg line 276 expressing two copies of the transgene [mFcRn−/− hFcRn (276) Tg/Tg] (Fig. 2B). In this case, the \( t_{1/2} \) of the WT, N434A and T307A/E380A/N434A antibodies was also extended (5.79 ± 0.34 days, 9.6 ± 0.5 days and 8.78 ± 1.55 days, respectively) compared with those tested in mFcRn−/− hFcRn (276) Tg mice (1.72 ± 0.07 days, 3.85 ± 0.55 days and 4.35 ± 0.53 days, respectively) (Table 1A). This extension caused by hFcRn Tg homozygosity indicates that the amount of hFcRn available for protection of administered antibodies contributes to the degree of protection from clearance in vivo.

**Fig. 2.** Elimination of Hu4D5-IgG1 antibodies in vivo. One hundred micrograms of each Hu4D5 antibody along with 100 μg of control mIgG1 antibody was injected i.p. on day 0 into mFcRn−/− hFcRn Tg, mFcRn+/+ and mFcRn−/− mice. The serum levels of the antibodies are presented as percent remaining in the circulation compared with day 1 (100%). Results were averaged for each test antibody and plotted as clearance curves ± SD. (A) mFcRn−/− hFcRn (276) Tg mice (\( n = 3 \) for each group). This experiment was performed three times with similar results. (B) mFcRn−/− hFcRn (276) Tg/Tg mice (\( n = 4 \)). (C) mFcRn−/− hFcRn (32) Tg mice (\( n = 3 \)); in this experiment, mice were pre-treated with 2 mg per 20 g body weight of hlG one day before the injection of Hu4D5-IgG1 antibodies to partially saturate hFcRn. This experiment was performed second time using 2.5 mg per 20 g of hlG pre-treatment and similar results were obtained (data not shown). (D) mFcRn−/− mice (\( n = 3 \)). Results obtained from in vivo elimination of control mIgG1 antibody were presented as \( t_{1/2} \) (see Table 1B).
We also evaluated the serum persistence of the Hu4D5 antibodies in mice by mixing one copy of the genomic hFcRn Tg [mFcRn⁻/⁻ hFcRn (276) Tg]. This mouse stock (line 32) was considerably more efficient in extending the serum t½ of hlgG compared with cDNA transgenic mice (line 276) (data not shown). Therefore, we failed to detect a significant difference in the normally long t½ of the Hu4D5 WT and high-affinity mutant antibodies in mFcRn⁻/⁻ hFcRn (32) Tg mice (data not shown). We attributed this failure to distinguish among the Hu4D5 antibodies to the overall high efficiency by which this genomic transgene protects hlgG from clearance, masking differences in FcRn-binding affinity during the period of measurement. To increase the sensitivity of detecting differences in serum t½, we argued that it might be possible to attenuate this high protection phenotype by partially saturating FcRn's protective function. mFcRn⁻/⁻ hFcRn (32) Tg animals were treated with hlgG1 (2 mg per 20 g body weight) one day before the infection with test Hu4D5-IgG1 antibodies (Fig. 2C). Mice were then bled and the clearance of the test Hu4D5-IgG1 antibodies was determined. The elimination curves of the WT and high-affinity mutants were clearly distinct from that of the I253A mutant. Moreover, WT hlgG1 antibody, single (N434A) and combination (T307A/E380A/N434A) mutants demonstrated an increase in serum persistence when tested in the mFcRn⁻/⁻ hFcRn (32) Tg (t½ = 6.48 ± 0.95 days, t½ = 10.66 ± 1.1 days and t½ = 9.7 ± 1.8 days) as compared with mFcRn⁻/⁻ hFcRn (276) Tg mice, more similar to that observed with the FcRn⁻/⁻ hFcRn (276) Tg/Tg mice (Table 1A). These overall results indicated that the high-affinity mutants showed a significant increase in the serum t½ compared with hlgG1 WT antibody, and that the level of FcRn expressed had a substantial effect in the overall t½ of these antibodies.

Since the Hu4D5 antibodies bind mFcRn (Fig. 1B) we analyzed their clearance in mFcRn⁻/⁻ mice (Table 1A). There was no statistical difference (P > 0.1) between the t½ of IgG1 WT, N434A and T307A/E380A/N434A antibodies (10.15 ± 0.49 days, 8.03 ± 1.88 days and 8.42 ± 1.8 days, respectively). Once again, I253A had the shortest t½ (1.56 ± 0.29 days). These results indicate that the high level of binding of the WT, N434A and T307A/E380A/N434A antibodies to FcRn in vitro results in the failure to realize differences in serum persistence in vivo. Finally, the persistence of the Hu4D5 antibodies was tested in mFcRn⁻/⁻ mice. As expected, the pharmacokinetic profiles of all antibodies in FcRn⁻/⁻ mice were similarly short, from 1.04 to 1.21 days (Fig. 2D and Table 1A). These results indicate that the extension in serum persistence of all the Hu4D5 antibodies is entirely dependent on FcRn.

Finally, we examined the tracer t½ of mlgG1 tracer in each mouse group analyzed above. In every case, the t½ of mlgG1 in mFcRn⁻/⁻ hFcRn Tg mice was greatly abbreviated compared with mice possessing a normal mFcRn allele, and as short as that found for mFcRn⁻/⁻ mice (Table 1B). These results confirm that FcRn does not extend the t½ of mlgG (35, 45).

Table 1. Half-lives of Hu4D5-IgG1 and mlgG1 antibodies in vivo

<table>
<thead>
<tr>
<th>Mice</th>
<th>WT</th>
<th>I253A</th>
<th>N434A</th>
<th>T307A/E380A/N434A</th>
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<tr>
<td>(A) Hu4D5-IgG1</td>
<td>mFcRn⁻/⁻ hFcRn (276) Tg</td>
<td>1.72 ± 0.075</td>
<td>1.02 ± 0.13 (0.003)</td>
<td>3.85 ± 0.55 (0.035)</td>
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<td>mFcRn⁻/⁻ hFcRn (276) Tg/Tg</td>
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<td>9.6 ± 0.5 (0.032)</td>
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<td></td>
<td>mFcRn⁻/⁻ hFcRn (32) Tg</td>
<td>6.48 ± 0.95</td>
<td>1.05 ± 0.5 (0.003)</td>
<td>10.6 ± 1.1 (0.009)</td>
</tr>
<tr>
<td></td>
<td>mFcRn⁻/⁻</td>
<td>10.1 ± 0.49</td>
<td>1.56 ± 0.29 (0.007)</td>
<td>8.03 ± 1.88 (0.26)</td>
</tr>
<tr>
<td>(B) mlgG1</td>
<td>mFcRn⁻/⁻ hFcRn (276) Tg</td>
<td>2.52 ± 0.68</td>
<td>2.05 ± 0.28</td>
<td>2.27 ± 0.32</td>
</tr>
<tr>
<td></td>
<td>mFcRn⁻/⁻ hFcRn (276) Tg/Tg</td>
<td>2.49 ± 0.28</td>
<td>1.95 ± 0.4</td>
<td>3.13 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>mFcRn⁻/⁻ hFcRn (32) Tg</td>
<td>2.31 ± 0.16</td>
<td>2.22 ± 0.45</td>
<td>2.36 ± 0.26</td>
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<tr>
<td></td>
<td>mFcRn⁻/⁻</td>
<td>9.49 ± 0.46</td>
<td>10.66 ± 0.57</td>
<td>10.04 ± 0.38</td>
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<tr>
<td></td>
<td>mFcRn⁻/⁻</td>
<td>1.7 ± 0.4</td>
<td>2.11 ± 0.2</td>
<td>1.8 ± 0.31</td>
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</tbody>
</table>
Antibody (hIgG1, anti-HEL HuLys11 antibody) was injected i.p. at a dose of 100 μg per mouse on day 0 (black arrow) followed by two injections with competitor Hu4D5 antibodies on days 2 and 3 (white arrows). Serum was collected and the amount of anti-HEL activity was determined. The following concentrations of competitor antibodies were used: (A) 2 × 1 mg per 20 g body weight, (B) 2 × 2 mg per 20 g body weight and (C) 2 × 5 mg per 20 g body weight. Five mice per group were used in this experiment.

Table 2. Half-lives of hlgG1 and mlgG1 tracers in presence of Hu4D5-IgG1 antibodies

<table>
<thead>
<tr>
<th>Tracer</th>
<th>Dose</th>
<th>Competitor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>(A) hlgG1</td>
<td>2 × 1 mg</td>
<td>5.25 ± 0.53</td>
</tr>
<tr>
<td></td>
<td>2 × 2 mg</td>
<td>5.24 ± 0.64</td>
</tr>
<tr>
<td></td>
<td>2 × 5 mg</td>
<td>2.94 ± 0.5</td>
</tr>
<tr>
<td>(B) mlgG1</td>
<td>2 × 1 mg</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>2 × 2 mg</td>
<td>1.84 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>2 × 5 mg</td>
<td>1.97 ± 0.44</td>
</tr>
</tbody>
</table>

β-phase *t*₁/₂ (days) of hlgG1 (A) and mlgG1 (B) tracer antibodies in the presence of different concentrations of Hu4D5 antibodies in mFcRn⁻/⁻ hFcRn (32) Tg mice (three to four mice per group). Group mean *t*₁/₂ (days) ± SD is shown for each tracer antibody (hlgG1 or mlgG1). Mice were co-injected with 100 μg of hlgG1 and mlgG1 tracers. *t*₁/₂ of tracer antibody in the presence of Hu4D5 variants (I253A, N434A and T307A/E380A/N434A) were compared with that of the same antibody in the presence of the WT antibody. *P*-values in parentheses. ND, not done. Control groups (data not shown) of mFcRn⁻/⁻ hFcRn (32) Tg mice were injected with hlgG1 or mlgG1 alone (without added competitor Hu4D5-IgG1 antibodies) yielded a *t*₁/₂ = 9.21 ± 1.31 days for hlgG1 and a *t*₁/₂ = 1.64 ± 0.29 days for mlgG1.

It is therefore possible that IgG antibodies with their Fc moiety engineered for high binding to hFcRn could have enhanced therapeutic potential by promoting even more efficient clearance of pathogenic IgG auto-antibodies. To test this possibility, we used a serum transfer model of experimental arthritis. Mice deficient in the inhibitory Fc receptor, FcγRIIB, fail to down-regulate pro-inflammatory Fc receptor signals and are thus hypersensitive to humoral stimuli (46). We have found that plasma or purified IgG from patients with active RA causes inflammatory joint lesions when transferred into FcγRIIB⁻/⁻ mice (47). We generated mice doubly deficient in mFcγRIIB and mFcRn that express the hFcRn transgene [mFcγRIIB⁻/⁻ hFcRn (32) Tg mice]. Several groups of mice were injected on days 0, 2 and 7 with human plasma collected from a patient with active RA in conjunction with Hu4D5-IgG1 antibodies (days 1, 3 and 8) (Fig. 4A and B) or days 1, 3, 8, 10 and 15 (Fig. 4C and D). The mice were then monitored for the ankle inflammation and swelling. Mice receiving plasma from the RA patient and treated with 3 × 2.5 mg of WT, N434A and T307A/E380A/N434A antibodies, all developed transient arthritis starting on day 7–8, and resolved the inflammation by day 20 (Fig. 4A). A minor reduction in delta ankle thickness

and 3.26 ± 0.19 days) compared with WT competitor antibody (*t*₁/₂ = 5.44 ± 0.64 days). However, on administration of 5 mg of Hu4D5-IgG1 antibodies (2 × 5 mg), discrimination of the WT, N434A and T307A/E380A/N434A antibodies was lost (*t*₁/₂ = 2.94 ± 0.5 days, 2.56 ± 0.71 days and 2.33 ± 0.5 days, respectively). Finally, as expected, because hFcRn does not efficiently engage mlgG (Fig. 1A) and (35, 45), the serum persistence of control tracer mlgG1 in the presence human Hu4D5-IgG1 antibodies did not appreciably deviate from its expected rapid elimination in mice not treated with Hu4D5-IgG1 antibodies (*t*₁/₂ = 1.64 ± 0.29 days) (Table 2B). These results indicate that mutant Hu4D5 antibodies that more efficiently bind hFcRn can be more effective than the WT in decreasing the hFcRn's IgG protection capability is partially compromised.

Amelioration of experimental arthritis by Hu4D5-IgG1 antibodies

Previous experiments have shown that high doses of IgG (20 mg per 20 g body weight) saturate the mFcRn protection pathway resulting in amelioration of the pathogenic activity of mlgG auto-antibodies (31).
between the groups of animals treated with WT antibody or the mutant N434A and T307A/E380A/N434A antibodies was observed, but only T307A/E380A/N434A mutant showed a significant reduction in ankle swelling on day 11 ($P = 0.05$) when compared with those treated with the WT antibody. Differences between the WT-treated mice compared with mutant antibodies-treated mice became more pronounced when analyzed using an overall arthritis score, which takes into consideration overall inflammation, in that there was a clear bifurcation on days 11 and 15 (Fig. 4B). We also tested the development of arthritis in a second group of mFcRn$^{-/-}$/mFcRn$^{-/-}$/hFcRn Tg mice, which were given two additional doses of Hu4DS-II antibodies (Fig. 4C and D). Mice received pathogenic RA plasma and were treated on days 1, 3, 8, 10 and 14 (open arrows) with the same concentration of WT, T307A/E380A/N434A or I253A mutant antibodies. Results represent mean delta ankle thickness and arthritis score ± SD.

**Fig. 4.** Amelioration of arthritis induced by transfer of human RA plasma in FcγRIIB$^{-/-}$/mFcRn$^{-/-}$/hFcRn (32) Tg mice after treatment with Hu4DS-IgG1 antibodies. (A and B) Mice were injected i.p. with human plasma from an RA patient as follows: 0.5 ml on day 0, 1 ml on day 2 and 1 ml on day 7 (black arrows) and treated on days 1, 3 and 8 (open arrows) with WT, N434A or T307A/E380A/N434A antibodies at a concentration 2.5 mg per mouse per injection. Results represent mean delta ankle thickness and arthritis score ± SD. (C and D) Groups of mice ($n = 3$) were injected with pathogenic serum (black arrows) and treated on days 1, 3, 8, 10 and 14 (open arrows) with the same concentration of WT, T307A/E380A/N434A or I253A mutant antibodies. Results represent mean delta ankle thickness and arthritis score ± SD.

**Discussion**

It is well established that FcRn is required in mammals for the greatly extended serum persistence of IgG antibodies. It does so by rescuing IgG from the normal catabolic fate to which other extracellular proteins are destined. This extension in half-life is a main reason why IgG-based therapeutics and other Fc-conjugated proteins are highly effective in the treatment of multiple diseases. Antibodies engineered to have a high affinity for FcRn may allow for a reduction in the dosage and/or frequency of their administration.

Mutagenesis studies have identified the critical role played by I253, S254, H435 and Y426 amino acids in mediating acid-dependent binding of hlgG1-Fc to hFcRn, based on the fact that replacement of these amino acids with Ala substantially reduced binding (38, 48). In contrast, substitutions at positions 380 and 434, located in the Fc portion of hlgG1 to alanine (E380A and N434A), resulted in improved binding to hFcRn, 2.2- and 3.5-fold, respectively, without affecting binding to FcγRs (37). Further improvement was documented when combination variants (K288A/N434A, E380A/N434A and T307A/E380A/N434A) showed a 2.9-, 8- and 11.8-fold increase, respectively, in binding activity to hFcRn at pH 6.0. We analyzed the effects of Hu4DS-IgG1 antibodies carrying the N434A and T307A/E380A/N434A substitutions on acid-dependent binding to hFcRn using a cell-based binding assay. A similar, but compressed, binding pattern of the N434A and T307A/E380A/N434A mutants was observed with L cells expressing hFcRn on their plasma membrane.
Remarkably, all discrimination among the Hu4D5 WT, and the N434A and T307A/E380A/N434A mutants was lost in binding to mFL. Previous studies document a high inherent affinity of mFcRn for many species forms of IgG, including humans (35). This high affinity may negate any potential binding advantages gained by these selective amino acid modifications. Indeed, we failed to find a clear discrimination in serum persistence among the Hu4D5 variants in mFcRn<sup>+/−</sup> mice. Moreover, the T307A/E380A/N434A mutant failed to demonstrate increased serum persistence as compared with WT IgG1 antibody when tested in BALB/c mice (49). These studies underscore the difficulties in extrapolating studies performed in conventional mouse models towards the development of improved hlgG-based therapeutics.

However, the question of greater interest was whether genetically engineering for high affinity interactions with hFcRn results in increased t<sub>1/2</sub> in vivo. Studies using primates showed that IgG1-Fc residues at positions 250 and 428 improved binding of IgG1, IgG2 and IgG4 to hFcRn at pH 6.0, when mutated to Q and L (T250Q and M428L). Most importantly, mutagenized hlG1 and hlG2 antibodies displayed an extension in their t<sub>1/2</sub> in primates (36, 40). Using mice deficient in mFcRn, but expressing hFcRn, we show that a ‘humanized’ mouse model can be used productively to discriminate the pharmacokinetics of hlG antibodies in vivo. Our data indicate a considerable extension in the serum t<sub>1/2</sub> for both the N434A and T307A/E380A/N434A mutants compared with the WT Hu4D5 antibody when tested in mFcRn<sup>+/−</sup> hFcRn (276) Tg mice. Similar results were obtained when antibodies were tested in a different Tg line [mFcRn<sup>+/−</sup> hFcRn (32) Tg], but only after pre-loading the mice with hlG to partially saturate hFcRn.

Despite the demonstrated differences in binding to FcRn in vitro, the serum t<sub>1/2</sub> of singly substituted N434A and the triply substituted T307A/E380A/N434A mutants were similar in vivo in mFcRn<sup>+/−</sup> hFcRn Tg mice. One possibility is that the triply substituted mutant was more immunogenic than the singly substituted mutant in that the former elicited a mouse anti-human response in vivo, thereby reducing its t<sub>1/2</sub> to that comparable with the singly substituted mutant. We view this possibility as unlikely because the acquisition of mouse anti-human antibodies would result in biphasic clearance kinetics, which were not observed for any of the Hu4D5 antibodies we tested. Moreover, ELISAs of sera from the T307A/E380A/N434A mutant antibody-injected mice failed to reveal detectable anti-human activity (data not shown). A second possibility is that maximal increase in hFcRn-mediated protection has been achieved by the single N434A substitution with no further extension by the increased affinity gained in the tripeptide. In this context, the triply substituted mutant retained some ability to bind hFcRn at a neutral pH (Fig. 1A). Moreover, the same antibody also demonstrated considerable binding to mFcRn at this neutral pH (Fig. 1B). Retention by hFcRn at a neutral pH may counteract any benefits of the increased avidity that this antibody compared with the singly substituted N434A mutant on the t<sub>1/2</sub> in the circulation. Such an argument would argue that there is an upper limit in the gains in serum persistence that can be realized by engineering therapeutic antibodies for high affinity binding to hFcRn.

Our studies also suggest a correlation between the levels of expression of hFcRn and the protection of hlgG. The efficiency of hlG1 protection was clearly impacted by doubling the transgenic dosage of hFcRn from one copy to two copies of the line 276 cDNA transgene, with a concomitant extension in the t<sub>1/2</sub> of the mutants, IgG1 N434A and IgG1 T307A/E380A/N434A over the IgG1 WT antibody. The highly efficient hFcRn genomic transgenic line 32 also resulted in a similar discrimination of the Hu4D5-IgG1 antibodies, but only after pre-loading the mice with hlG. These results underscore the quantitative importance of FcRn in relation to the maintenance of serum IgG, and raise the interesting possibility that genetic or environmentally induced variation resulting in differences in FcRn expression could profoundly influence the serum persistence of IgG.

Based on studies of mice deficient in either the FcRn heavy or light chains, we have previously argued that the blockade of FcRn could be advantageous for the treatment of autoimmune disease with a humoral component (31, 32, 50, 51). Moreover, many of the anti-inflammatory therapeutic benefits of high-dose IgG treatment can be attributed to the saturation of FcRn, thereby incapacitating its function and promoting the clearance of pathogenic IgG antibodies (31, 52, 53). However, the considerable expense, inconvenience and side-effects of high-dose IgG administration have restricted its use. IgG molecules or Fc fragments modified for high binding affinity for FcRn, thereby incapacitating FcRn’s normal protective function, could be a promising alternative to high-dose IgG therapy in the treatment of humoral autoimmune disease. Indeed, recent studies using hlG antibodies incorporating amino acid changes resulting in high FcRn binding efficiency have demonstrated enhanced elimination of hlG and the reduction of serum IgG in mice (39). However, as the studies were carried out in mice, a key remaining question is whether the enhanced elimination is also observed in the context of hFcRn and whether it leads to any therapeutic benefits. We found that Hu4D5 antibodies engineered for increased binding to FcRn more efficiently promote the in vivo elimination of hlG than the WT Hu4D5 antibody. However, this enhanced efficiency only gained statistical significance at a dosage range (2 × 2 mg), above which the discriminative effects were lost presumably because hFcRn was saturated. Nevertheless, the Hu4D5-IgG1 antibodies with the highest affinity binding was most effective compared in reducing inflammatory lesions caused by the administration of human RA patient plasma. As the Hu4D5 mutations were designed specifically to impact binding to hFcRn (37), this result is unlikely to be explained by engagement of pro-inflammatory FcγRs. This is a proof-of-principle that hFcRn blockade by engineered Fc-IgGs may be a valid approach in treating human autoimmune disease. However, further enhancements in FcRn binding and efficiency in vivo would be required to justify this therapeutic approach.

Given the profusion of IgG therapeutics, models that facilitate their pre-clinical pharmacokinetic evaluation are needed. It is becoming clear that standard rodent models are suboptimal for evaluating the pharmacokinetics of hlgG-based therapeutics because of inherent differences in the interaction sites and affinity of rodent FcRn and hFcRn for hlgG antibodies (35, 38, 41). Primates are an option, but are not suitable for routine use. The FcRn-humanized mouse model is a particularly attractive surrogate; however, the extent to which the pharmacokinetic patterns of the Hu4D5 antibodies patterns
observed in this model mirror their behavior in humans remains to be determined. Finally, FcRn is also considered to be responsible for perinatal transport of IgG across the placenta (54–56), and post-natal transepithelial IgG transport across the intestinal and the pulmonary epithelium (57, 58). Use of the FcRn-humanized model should indicate whether high affinity binding IgG antibodies are a more efficient means for transplacental or transepithelial delivery across these epithelial barriers.

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Abbreviations

- A
- AP
- ATCC
- B6
- DNP
- HEL
- hFcRn
- hFL
- hlgG
- i.p.
- mFcRn
- mFL
- mlgG
- p-NPP
- RA
- RT
- t1/2
- Tg
- WT

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