FcRn Affinity-Pharmacokinetic Relationship Of Five Human IgG4 Antibodies Engineered For Improved In Vitro FcRn Binding Properties In Cynomolgus Monkeys

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Running Title: Pharmacokinetics of Antibodies With Different Fc Mutations

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Number of text pages: 33
Number of tables: 3
Number of figures: 6
Number of references: 28
Number of words in the Abstract: 250
Number of words in the Introduction: 1,253
Number of words in the Discussion: 2193

List of abbreviations: IgGs, immunoglobulins; FcRn, neonatal Fc receptor; mAbs, monoclonal antibodies; β2m, β2-microglobulin; PK, pharmacokinetic; PD, pharmacodynamic; C-FcRn, cynomolgus monkey FcRn; SPR, surface plasmon resonance; PBS, phosphate-buffered saline; RU, resonance units; Kd, equilibrium dissociation constant; ELISA, enzyme linked immunosorbent assay; pH50, pH at which 50% of the FcRn-antibody complexes dissociate; HRP, horseradish peroxidase; IV, intravenous; LLOQ, lower limit of quantitation; pI, isoelectric point; cIEF, capillary isoelectric focusing; Tm, thermal stability; DSC, differential scanning calorimetry; AUC0-∞, area under the plasma concentration curve from zero to infinity; CL, clearance; Cmax, maximal observed plasma concentration; t1/2, elimination half-life; koff, dissociation rate
ABSTRACT

The pH-dependent binding of immunoglobulins (IgGs) to the neonatal Fc receptor (FcRn) plays a critical role in regulating IgG homeostasis in vivo. Enhancing interactions between Fc and FcRn via protein engineering has been successfully used as an approach for improving the pharmacokinetics of monoclonal antibodies (mAbs). While the quantitative translatability of the in vitro FcRn affinity enhancement to an in vivo pharmacokinetic benefit has been supported by several studies, there are also published reports indicating a disconnect in this relation. The body of literature suggests there are likely additional biochemical and biophysical properties of the mAbs along with their FcRn affinity that influences the in vivo pharmacokinetics. Herein, we more broadly evaluate the in vitro Fc:FcRn interactions and biochemical properties of five humanized IgG4 antibodies each with two Fc variant sequences (T250Q/M428L and V308P) and their corresponding pharmacokinetics in cynomolgus monkeys. Our findings indicate that the FcRn affinity-pharmacokinetic relationship does not show a direct correlation either across different IgGs or between the two variant sequences within a platform. Other parameters that have been suggested to contribute to mAb pharmacokinetic properties, such as the pH-dependent dissociation of the FcRn:IgG complexes, mAb biophysical properties and non-specific/charge binding characteristics of the mAbs also did not independently explain the differing pharmacokinetic behaviors. Our results suggest that there is likely not a single in vitro parameter that readily predicts in vivo pharmacokinetics, but that the relative contribution and interplay of several factors along with the FcRn binding affinity are important determinants of mAb pharmacokinetic properties.
INTRODUCTION

It is widely accepted that the interaction of immunoglobulins (IgGs) with the neonatal Fc receptor (FcRn) plays a critical role in regulating IgG homeostasis in vivo (Ghetie et al., 1996; Ghetie and Ward, 1997; Ward et al., 2003). FcRn interacts with the C_{12}-C_{13} portion of the Fc domain of IgGs in a tightly regulated pH-dependent manner with high affinity binding occurring at an acidic pH (pH ~6) and weak to no binding interactions as the pH is raised to neutral (pH 7.4) (Goebel et al., 2008; Ober et al., 2004a; Ober et al., 2004b; Prabhat et al., 2004). The pH-sensitive nature of the interaction facilitates the FcRn-mediated protection of IgGs pinocytosed into cells from intracellular degradation by binding to the receptor within the acidic microenvironment of endosomes (Ward et al., 2003; Ward et al., 2005). Following salvage from degradation, FcRn facilitates the recycling of IgG to the cell surface and subsequent release into the blood upon exposure of the receptor:IgG complex to the neutral pH environment outside the cell (Ward et al., 2003; Ward et al., 2005).

Enhancing the pH-dependent binding interactions between Fc and FcRn via protein engineering has been used successfully as an approach for improving the pharmacokinetics of monoclonal antibodies (mAbs). A handful of specific Fc variants (T250Q/M428L, M428L, M252Y/S254T/T256E, M428L/N434S, N434A, N434H) have provided evidence that improving the IgG affinity for FcRn at pH 6 with little to no influence on the pH 7.4 interactions molecule can result in ~2- to ~4-fold longer in vivo elimination phase half-life in either cynomolgus or rhesus monkeys (Dall'acqua et al., 2002; Dall'acqua et al., 2006; Datta-Mannan et al., 2007a; Datta-Mannan et al., 2007b; Deng et al., 2010; Hinton et al., 2004; Hinton et al., 2006; Yeung et al., 2009; Yeung et al., 2010). While the translatability of the in vitro FcRn affinity enhancement to an in vivo pharmacokinetic benefit has been supported by the aforementioned studies, these studies have generally focused on modulation of the FcRn interaction within the context of a single antibody framework making the predictive translation to other antibody backbones somewhat tenuous. Along these lines, retrospective analyses of several humanized mAbs having similar human FcRn binding properties demonstrated that they had ~1.5- to ~2-fold differences in their elimination half-lives in humans suggesting a lack of a direct quantitative correlation with FcRn
binding (Suzuki et al., 2010). Similarly, Gurbaxani et al. were unable to directly correlate FcRn binding affinity to the pharmacokinetics of a number of IgGs in mice (Gurbaxani et al., 2006). The quantitative correlation of FcRn affinity improvement with in vivo pharmacokinetic parameters has also been ambiguous in primate studies of engineered mAbs (Datta-Mannan et al., 2012; Deng et al., 2010). Deng and coworkers, showed an anti-tumor necrosis factor-α mAb Fc variant (N434H) with ~3-fold higher FcRn affinity at pH 6 had similar pharmacokinetics in cynomolgus monkeys when compared to a lower receptor affinity variant constructed on the same mAb backbone (N434A) (Deng et al., 2010); although these findings may have been compromised by anti-drug antibodies (ADA). Likewise, Fc mutations (M252Y/S254T/T256E) that have increased receptor binding at pH 6 by ~10-fold have shown ~3- to 4-fold improvements in mAb half-life in monkeys (Dall'acqua et al., 2006), whereas, Fc mutations (T250Q/M428L) which display ~30 fold-increases in FcRn binding have shown only ~2.5-fold improvements in half-life (Hinton et al., 2004; Hinton et al., 2006). One of the challenges in establishing a systematic understanding of the FcRn affinity-pharmacokinetic relationship are the different methodologies/technologies that have been used to measure IgG:FcRn interactions which have included cell based approaches, isothermal calorimetry, immunoassays and surface plasmon resonance (SPR or BIAcore assay). The use of different techniques for measuring affinity adds to the ambiguity and makes a direct comparison of binding properties between studies difficult and likely influences the relation to the pharmacokinetic behavior.

There are several generally accepted key aspects of the human and non-human FcRn:IgG in vitro FcRn affinity-pharmacokinetic relationship that are critical for the successful improvement of IgG clearance in non-human primates (rhesus or cynomolgus monkeys): 1) higher in vitro IgG:FcRn affinity at pH 6 may translate to pharmacokinetic benefit in non-human primates if the increased affinity is predominantly attributable to the rate of dissociation of the complex (Datta-Mannan et al., 2007a; Datta-Mannan et al., 2007b); 2) higher in vitro IgG:FcRn affinity at pH 6 may not correlate with antibody pharmacokinetic benefits in non-human primates if increased affinity is driven predominantly by improvements in the rate of association of the complex (Datta-Mannan et al., 2007a; Datta-Mannan et al.,
2007b) and 3) significant enhancement of the direct pH 7.4 FcRn binding or slowing of the rate of the
dissociation of the IgG:FcRn complex in vitro translates a more rapid IgG clearance in vivo (Yeung et al.,
2009). However, in cases where these attributes are well understood there is still a quantitative and
qualitative discrepancy in the in vitro–in vivo relation indicating that there are likely additional factors or
properties of the mAbs along with their FcRn affinity that influences the in vivo pharmacokinetics. While
the interplay of target-mediated mAb clearance mechanisms is also acknowledged to contribute to some
of these discrepancies (Chaparro-Riggers et al., 2012; Yeung et al., 2010), a number of studies eliminated
this as a factor by using in vivo systems with low/no endogenous antigen or mAb doses that saturated
target. Recent reports have pointed to other clearance pathways and biochemical characteristics,
including the charge, stability, post-translational modifications, aggregation potential and the Fab region
of the molecule itself as having significant impact on antibody disposition (Igawa et al., 2010; Khawli et
al., 2010; Wang et al., 2011; Yeung et al., 2010). There remains, however, a paucity of data on the
characterization of multiple biochemical and biophysical factors in combination with FcRn affinity on the
in vivo disposition of mAbs.

In an effort to address these issues in the current work, we more broadly evaluated the FcRn affinity
and several biochemical/biophysical properties for five humanized IgGs each constructed with two Fc
variant sequences (T250Q/M428L and V308P)1 along with the pharmacokinetics of these molecules in
cynomolgu monkeys. The Fc variants were built on IgG4 antibody backbones with 100% identical C\textsubscript{H}1,
C\textsubscript{H}2, C\textsubscript{H}3 and hinge regions to allow for a more systematic head-to-head comparison of the in vitro
factors. Each of the antibody backbones also contains a specific mutation (serine to proline) in the hinge
region to inhibit heavy chain exchange (Stubenrauch et al., 2010). The five IgGs had sequence
differences in the heavy and light chain variable regions since each targeted a specific soluble antigen. In
each case, there were insignificant peripheral levels of target antigen in normal cynomolgu monkeys
with the intent of eliminating/limiting the influence of antigen binding on the kinetics or distribution of
the antibodies. We found that an a priori prediction of the quantitative translation of pharmacokinetic
properties based simply on the FcRn in vitro affinity-in vivo pharmacokinetic relationship is difficult both
within a series of Fc variants for a single mAb or for variants across mAbs. Importantly, our observations also suggest that the predictive value of a single in vitro parameter, including biochemical factors and its translation across published reports is unclear. Our data indicate that engineering strategies to improve the in vivo kinetic performance of an IgG need to consider the FcRn interaction with an integrated/multifactoral approach (biophysical properties, antigen load, glycosylation, proteolytic stability, non-specific binding, charge, chemical modification) to more completely predict the pharmacokinetic effect of an Fc variant when placed on a particular monoclonal antibody. Understanding these factors and the interplay of FcRn in the metabolism and survival of these constructs in vivo are critical to facilitate the rational optimization of the pharmacokinetic/pharmacodynamic (PK/PD) properties of mAbs.
MATERIALS AND METHODS

Cell culture

293EBNA cells were maintained at 37 °C under 5-8% CO₂ conditions in Dulbecco's modified Eagle's medium/F-12 (Gibco) supplemented with 20 mM HEPES (Gibco), 5 μg/mL nucellin (Eli Lilly and Company), 0.4 μg/mL tropolone (Sigma Aldrich), 0.075% (w/v) F68 (Gibco) and 50 μg/mL Geneticin (Sigma Aldrich).

Construction, expression and purification of recombinant proteins

The antibody variant was derived from a humanized IgG₄ Fc variant library created using a Kunkel-based strategy (Kunkel et al., 1987) described earlier (Datta-Mannan et al., 2007a; Datta-Mannan et al., 2007b). The T250Q/M28L and V308P variants were obtained by site-directed mutagenesis of the wild-type humanized IgG₄ clone using the QuikChange method (Stratagene) and confirmed by DNA sequencing. The wild-type and variants IgGs were expressed in 293EBNA cells and purified from culture supernatants using Protein-A Sepharose (GE Healthcare) affinity chromatography followed by size exclusion chromatography methods described previously (Datta-Mannan et al., 2007a; Datta-Mannan et al., 2007b).

Recombinant, soluble cynomolgus monkey FcRn was expressed in 293EBNA cells transfected with the plasmids encoding for the soluble portion αFcRn and β₂m and the protein was purified as previously described (Datta-Mannan et al., 2007a; Datta-Mannan et al., 2007b).

IgG:FcRn binding affinity measurements with surface plasmon resonance (BIAcore)

The interaction of the WT and variant IgGs with recombinant cynomolgus monkey FcRn was monitored by surface plasmon resonance (SPR) detection using a BIAcore T-100 instrument (Biacore Inc.). Briefly, a mouse anti human kappa antibody (BD Bioscience), with no binding to cynomolgus monkey and human FcRn, was immobilized to a CM5 chip at ~5000 Ru using the standard amine-coupling kit to use as a capture methodology. Antibodies samples were prepared at 5 ug/mL and were
injected for 20 seconds. Typically, 200 to 800 resonance units (RUs) of antibodies were captured on the chip. The running buffer for the binding experiments was phosphate buffered saline (PBS), 0.05% Tween 20 (w/v) pH 6. Soluble FcRn dilutions from 2.0 uM to 2.7 nM were prepared in the binding buffer and injected at flow rate of 100uL/min for 0.5 min over the antibody-captured sensor chip at 25°C. The dissociation phase was monitored for 15 minutes. The chip was regenerated with 10mM glycine hydrochloride, pH 1.5. The binding data were obtained by double reference subtraction. Kinetic binding constants were determined through global fits of the average of two data sets collected on separate days using Biacore T100 Evaluation, version 1.0. The kinetics (association and dissociation rates) were simultaneously fit to a heterogeneous ligand binding model to determine the high affinity and low affinity equilibrium dissociation constant (K_d) value for each FcRn:IgG interaction. The fitted curves to the sensorgrams had low residuals and \( \chi^2 \) values.

**pH-dependent dissociation enzyme-linked immunosorbent assay (ELISA) for the WT T250Q/M428L Fc variant antibodies**

Biotinylated cynomolgus monkey FcRn for ELISA assays was produced by reacting each purified soluble protein with EZ-Link® Sulfo-NHS-Biotin (Pierce Chemical Co.) using the conditions supplied by the vendor. The FcRn:biotin ratio was measured as 1:1, using the EZ™ Biotin Quantitation Kit (Pierce Chemical Co.). The pH-dependent ELISA for the interaction of the FcRn with the five wild-type IgGs, five T250Q/M428L and five V308P Fc variant antibodies was performed as described in earlier studies with other IgGs (Datta-Mannan et al., 2007a; Datta-Mannan et al., 2007b). Briefly, after washing and blocking neutravidin-coated plates, 0.5 μg of biotinylated FcRn was added to each well and allowed to bind the neutravidin for 1 h at 25°C. Wells were washed after FcRn binding and 1 μg/well of each antibody, dissolved in 100 mM NaPO₄, pH 6.0, 0.05% Tween 20 (v/v), 0.1% ovalbumin (m/v), was added to each well and incubated for 1 h at 25°C. Following antibody binding, wells were washed three times with 1 to 3 minute incubations at 25°C between each wash using 100 mM NaPO₄, 0.05% Tween 20 (v/v) buffer having pH values from 6.0 to 8.0 in 0.2 pH increments. The remaining bound antibodies were
detected with a horseradish peroxidase (HRP)-conjugated goat (Fab’)2 anti-human-Fab (Jackson Labs). Optical density data were analyzed by the same four-parameter nonlinear regression fit as described previously (Datta-Mannan et al., 2007a; Datta-Mannan et al., 2007b) to determine the midpoint (pH50) of the titration curve (the pH at which 50% of the FcRn:antibody complexes dissociates). At each pH, data are expressed as the percentage of the total antibody bound at pH 6.

**Evaluation of mAb Isoelectric Points (pIs)**

The pIs of the mAbs were determined by capillary isoelectric focusing (cIEF), PA 800 plus Pharmaceutical Analysis System (Beckman Coulter). Samples were prepared by mixing 5-10 μg sample with 200 μL of 3 M urea-cIEF gel, 12.0 μL of Pharmalyte 3-10 (GE Healthcare), 20.0 μL of cathodic stabilizer (500 mM arginine), 2.0 μL of anodic stabilizer (200 mM iminodiacetic acid), 2.0 μL of each of 5 pI markers (pI 10.0, 9.5, 7.0, 5.5, and 4.1), and vortexing for 15 seconds. The electrophoresis and data collection were performed using a focusing step voltage 25 kilovolts for 15 minutes, a chemical mobilization step voltage 30 kilovolts for 30 minutes, a UV detection at a wavelength 280 nm and a data collection rate of 2 hertz. The cartridge temperature was 20 °C and the sample storage temperature was 10 °C. The data were analyzed by using 32 Karat program (Beckman Coulter).

**Evaluation of thermal stability (Tm)**

Thermal stability of samples was measured using a TA Instruments NanoDSC equipped with an autosampler. Samples diluted to 0.5 mg/mL in PBS were heated from 20 to 110°C at a rate of 1°C/min under 45 psi of pressure. Sample scans were buffer blank subtracted, converted to molar heat capacity, and fit to a two-state scaled model with three transitions representing the Cα2, Cα3 and Fab domain unfolding to obtain Tm.

**Evaluation of interactions with heparin**

Heparin binding ELISA plates (BD Biosciences) were coated with 0.5 μg/well of heparin (Sigma Chemical Co.) diluted in PBS, pH 7.4, at room temperature overnight. After washing and blocking, 0.1 μg of each IgG diluted in PBS casein (Thermo Scientific) was added to each well and allowed to bind the
heaprin-coated wells for 1 h at 25°C. After washing, the bound antibodies were detected with a HRP-conjugated mouse anti-human IgG (Southern Biotechnology Associates). The amount of antibody that remained bound to heparin was expressed as a percentage of the background signal of wells containing no antibody.

**Cynomolgus monkey pharmacokinetic studies**

Two independent cynomolgus monkey pharmacokinetic studies were performed. In both studies, five male cynomolgus monkeys (2.8-3.8 kg) were assigned to one of two study groups. Doses were administered as a single intravenous (IV) injection of a solution formulation containing all five wild-type molecules or five IgG4 variant molecules for a total of a 5 mg/kg dose (1 mg/kg of each mAb) in each study. In the first study, each animal received a single IV dose of the five wild-type or five T250Q/M428L variant IgG dissolved in PBS (pH 7.4) at 1.0 mg/kg. In the second study, each animal received a single IV dose of the five wild-type or five V308P variant IgG dissolved in PBS (pH 7.4) at 1.0 mg/kg. Blood samples were collected from femoral vein prior to dosing and at 0.083, 0.25, 0.5, 1, 3, 6, 12, 24, 48, 72, 96, 120, 168, 240, 312, 384, 456, 528, 600, 672, 840, 1008, 1176, 1344, 1512, 1680, 1848, and 2016 hours after administration of the dose in both studies. The blood samples were allowed to clot at ambient temperature prior to centrifugation to obtain serum.

**Bioanalytical assays and pharmacokinetic data analysis**

Concentrations of the wild-type IgGs and T250Q/M428L and V308P variant IgGs in cynomolgus monkey serum were determined using one of five validated antigen capture ELISAs for each of the five mAb backbones. Briefly, for the antigen capture ELISAs, each well of a Immulon 4 microtiter plate (Thermo Electron Corporation) was coated with the antigen of interest at 4°C overnight. After washing and blocking, standards and samples were added to the wells in a volume of 0.1 mL and incubated for 1 h at room temperature. The five mAb backbones (as either wild-type IgG or T250Q/M428L or V308P mutants) were selective for a single antigen and showed no cross reactivity/nonspecific binding to non-cognate antigens in the ELISAs when examined individually or as a mixture of the five wild-type or five T250Q/M428L or V308P variant IgGs. After washing, the bound antibodies were detected with a HRP-
conjugated mouse anti-human light chain antibody (Southern Biotechnology Associates, Birmingham, AL). The wild-type, T250Q/M428L and V308P IgG standards were prepared in cynomolgus monkey serum using a standard curve range of 0.78 to 50 ng/mL for mAbs A1 and B1 and 1.56 to 100 ng/mL for mAbs C1, D1 and E1. The lower limit of quantitation (LLOQ) was defined as 2 ng/mL for mAbs A1 and B and 4 ng/mL for mAbs C1, D1 and E1.

Pharmacokinetic parameters were calculated using the WinNonlin Professional (Version 3.2) software package (Pharsight Corporation, Mountain View, CA). Serum concentration-time data were calculated using a model-independent approach based on the statistical moment theory. The parameters calculated included the maximum serum concentration (C\text{max}), area under the curve (AUC\text{0-∞}), clearance (CL), and elimination half-life (t\text{1/2}). For the wild-type IgG groups, the pharmacokinetic parameters are presented as a mean (N=10 animals) across the two cynomolgus monkey studies.
RESULTS

Characterization of the binding interactions and kinetics of the wild-type and T250Q/M428L and V308P Fc variant antibodies with cynomolgus monkey FcRn by surface plasmon resonance (SPR) assays.

A representative SPR sensorgram of the interaction of wild-type and Fc variant constructs of antibody A1 with cynomolgus monkey FcRn at pH 6 are shown in Figure 1. These data show the T250Q/M428L and V308P Fc variants improve the affinity of the mAb to FcRn predominantly via slowing the rate of dissociation (k_{off}) (Figure 1). Sensorgrams for the other mAbs displayed a similar pattern (data not shown). The data were fit to a heterogeneous ligand binding model, thus both low and high binding affinity FcRn:IgG interactions were observed (Supplementary Table 1). Since the high affinity K_{D} accounted for a greater fraction of the binding (Supplementary Table 1), it was used for all the subsequent analyses. The high affinity rate constants (k_{off}) and binding affinities (K_{D}) values for the interaction of all the IgGs with cynomolgus FcRn at pH 6.0 are reported in Table 1. Our finding show the five wild-type IgGs bind to cynomolgus monkey FcRn with considerable differences even though they have identical CH1, CH2 and CH3 regions. The high affinity K_{D} of the five wild-type antibodies to cynomolgus monkey FcRn showed up to ~3-fold differences and ranged from ~90 to ~200 nM (Table 1). Our pH 6 binding results (Table 1) also showed that both variant sequences enhanced the FcRn K_{D} for each of the five antibody platforms differentially (Table 1). The pH 6 FcRn binding affinity improvements for the T250Q/M428L and V308P variants were ~11- to ~115-fold and ~43- to ~390-fold, respectively. We observed the binding affinity enhancements for our five wild-type IgGs were more marked for the interaction of the V308P Fc variants with FcRn than the T250Q/M428L mutants. Although, the magnitude of the K_{d} improvement of the each of the Fc variant antibodies spanned a broad range, within either the five T250Q/M428L or five V308P mutants, the antibodies have comparable affinities for cynomolgus monkey FcRn (T240Q/M428L K_{d} values of ~3 to 13 nM and V308P K_{d} values of ~0.5 to 1.5 nM), indicating these mutations within each IgG backbone make similar free energy contributions to FcRn binding. Furthermore, our findings showed the differences in the affinity of mutant antibodies for cynomolgus monkey FcRn at pH 6 are predominately due to slowed rates of dissociation (k_{off} values)
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(Table 1); an important characteristic of enhanced FcRn binding implicated in improving IgG pharmacokinetics (Datta-Mannan et al., 2007a; Datta-Mannan et al., 2007b). We found our increases in FcRn binding affinity for the T250Q/M428L and V308P variants were within a comparable range to those observed in earlier studies which reported FcRn binding affinity enhancements with other variant sequences (range from ~2- to 400-fold) (Datta-Mannan et al., 2007a; Datta-Mannan et al., 2007b; Suzuki et al., 2010; Yeung et al., 2009; Yeung et al., 2010). The binding data of the Fc variant mAbs with human FcRn showed a similar pattern improved affinity receptor driven predominately by a slowed rate of dissociation relative to each IgGs wild-type antibody counterpart (data not shown).

**Determination of the pH-dependent dissociation of cynomolgus monkey FcRn and wild-type, T250Q/M428L and V308P Fc variant antibody complexes**

The pH-dependency of the FcRn:mAb interaction was measured *in vitro* by forming IgG:receptor complexes at pH 6 and monitoring the degree (pH50 or the pH at which half of the IgG:FcRn complexes dissociate) and extent (amount of mAb that remains bound to FcRn at pH 8.0) of dissociation of the preformed complexes exposed to increasing pH using an ELISA method described previously (Datta-Mannan et al., 2007a; Datta-Mannan et al., 2007b). The 15 preformed wild-type: and variant:cynomolgus monkey-FcRn complexes dissociated with pH50 values of ~6.2 to 7.1 (Table 1). The amount of mAb that remained bound to cynomolgus FcRn across the 15 IgGs at pH 8.0 ranged from ~5% to ~61%. The amount of each mAb that remained bound to FcRn at pH 7.2 to 7.4 was similar to pH 8.0 (data not shown). When compared to their respective wild-type mAb counterparts, the Fc variant constructs generally displayed higher pH50 values and a greater extent of dissociation from FcRn at pH 8.0. The WT and variant IgG pH-dependent interactions with human FcRn were similar to the cynomolgus monkey FcRn (data not shown).

**Characterization of the biochemical and biophysical properties of the wild-type and T250Q/M428L and V308P Fc variant antibodies.**
The majority of the pIs of the wild-type humanized IgG molecules ranged from 7.41 to 7.75 (Table 3) with the exception of antibody E1 which displayed a lower pI value of 6.64 (Table 3). The Fc variants had no significant influence on the pI relative to each antibody wild-type IgG counterpart (Table 3). The change in the Tm values of the antibodies spanned a range of ~-3°C to -8°C, ~-2°C to 3.9°C and 0°C to -0.8°C for the C1H2, C1H3 and Fab regions, respectively (Table 3). The V308P and T250Q/M428L mutations had the largest influence on the C1H2 region and generally lowered the Tm by ~7.5°C and ~4.5°C, respectively (Table 3). Across the fifteen mAbs, we observed varied responses to heparin binding ranging from no binding to 63% increase in heparin interactions relative to background (Table 3). Within an antibody platform, the V308P and T250Q/M428L Fc variants displayed reasonably similar heparin binding interactions relative to their wild-type antibody counterparts, with the exceptions of A1 T250Q/M428L and D1 T250Q/M428L which showed 29% and 28% higher heparin binding than their wild-type IgG, respectively (Table 3).

Pharmacokinetics of the wild-type and T250Q/M428L and V308P Fc variant antibodies in cynomolgus monkeys after a single IV administration.

In order to understand how the in vitro FcRn binding properties observed for the IgG:cynomolgus monkey FcRn interactions and biophysical characteristics of antibodies correlate to antibody kinetics in vivo, we investigated the pharmacokinetics of the fifteen IgGs (5 each of wild-type antibodies and T250Q/M428L and V308P Fc variant mAbs) in cynomolgus monkeys after IV administration. As an approach to minimize the number of animals used for our pharmacokinetic studies, we choose to conduct our in vivo evaluations of these antibodies by leveraging dose formulation solutions comprised of mixtures of the five wild-type, five T250Q/M428L or five V308P IgG4s. At the concentrations in the mixed solutions (1 mg/mL/antibody), assessments of our formulations showed no soluble or insoluble antibody aggregates (data not shown), indicating the heterogeneity of the solutions did not negatively impact the solubility of the individual antibody components. Thus, each cynomolgus monkey was
administered a formulation containing all five wild-type molecules, five T250Q/M428L IgG4s or five V308P variants via an intravenous injection for a total of a 5 mg/kg dose (1 mg/kg of each mAb).

After a single IV administration of the mixed wild-type mAb or a Fc variant IgG formulation, the five wild-type and five of each of the T250Q/M428L and V308P variant mAbs were cleared from the circulation, showing a rapid distribution phase followed by a prolonged elimination phase characteristic of antibodies with insignificant peripheral levels of target antigen. The average serum concentration-time profiles for each wild-type antibody and the two Fc variants are shown in Figures 2 and 3. When compared within a humanized IgG platform, the serum profiles of the wild-type and variant antibodies displayed significant differences (Figures 2 and 3). Both the T250Q/M428L and V308P mutations improve the pharmacokinetics (slow CL and/or increase T1/2) when placed on each wild-type IgG platform (Figures 2 and 3 and Table 2). The degree of pharmacokinetic improvement (fold enhancement of a pharmacokinetic parameter) varies across the five IgGs (Figures 2 and 3 and Table 2). The T250Q/M428L mutations showed mean clearance and elimination half-live improvements of 1.9-fold (range of 1.2- to 2.3-fold) and 1.3-fold (range of 0.9- to 1.5-fold), respectively, whereas the V308P showed mean clearance and elimination half-live improvements of 1.5-fold (range of 0.8- to 2.2-fold) and 2.4-fold (range of 1.8- to 3.0-fold), respectively, relative to the wild-type IgGs. Analysis of the relationship of the \textit{in vitro} IgG:FcRn interactions with \textit{in vivo} clearance/half-life for the wild-type and each variant across the entire population of fifteen IgGs and for each individual IgG platform show there is not a direct \textit{in vitro} FcRn binding interaction/pharmacokinetic correlation for the IgGs (Figures 4-6).
DISCUSSION

A subject of considerable interest for therapeutic antibody engineering is optimizing the in vivo serum kinetics of mAbs. It is highly desirable from a therapeutic drug development perspective to identify in vitro assay(s) or molecule characteristics that can be used to reasonably predict the in vivo pharmacokinetics and guide protein optimization/engineering strategies. There are several factors that have been clearly shown to influence the pharmacokinetics of a mAb in vivo. The class of therapeutic target, including whether membrane bound or soluble, and the density or turnover of the target antigen both play a major role in governing disposition (Chaparro-Riggers et al., 2012; Yeung et al., 2010). In addition, the biochemical properties of the antibody (i.e. biophysical properties, antigen affinity, glycosylation, charge proteolytic stability) (Igawa et al., 2010; Khawli et al., 2010; Wang et al., 2011; Yeung et al., 2010) in combination with the target characteristics previously described, influence the kinetics of a particular mAb. The interaction of a mAb with FcRn and the effectiveness of the FcRn-mediated antibody recycling mechanism for salvaging an IgG from intracellular degradation has also been clearly shown to modulate mAb disposition (Dall'acqua et al., 2002; Dall’acqua et al., 2006; Datta-Mannan et al., 2007a; Datta-Mannan et al., 2007b; Deng et al., 2010; Hinton et al., 2004; Hinton et al., 2006; Yeung et al., 2009; Yeung et al., 2010). The relative contribution of each of these factors underlies the basic in vivo characteristics of an antibody and heavily influence strategies aimed at optimizing pharmacokinetic/pharmacodynamic properties of the molecule.

Studies in FcRn knockout mice have clearly demonstrated the physiological relevance of the FcRn receptor system in regulating the persistence of endogenous and exogenously administered IgG (Christianson et al., 1997; Ghetie et al., 1996; West, Jr. and Bjorkman, 2000). Since Fc engineering to modify FcRn interactions can be applied to a wide variety of mAbs with different biochemical and biophysical properties, this strategy is a very attractive approach by which to engineer therapeutic antibodies for improved pharmacokinetic properties. In this regard, Fc engineering to improve FcRn binding properties of IgGs has been demonstrated to extend the half-life of mAbs in non-human primates (Dall’acqua et al., 2002; Dall’acqua et al., 2006; Datta-Mannan et al., 2007a; Datta-Mannan et al., 2007b; Deng et al., 2010; Hinton et al., 2004; Hinton et al., 2006; Yeung et al., 2009; Yeung et al., 2010).
2007b; Deng et al., 2010; Hinton et al., 2004; Hinton et al., 2006; Yeung et al., 2009; Yeung et al., 2010). However, several inconsistencies reported in the literature, make it less apparent that *in vitro* binding assays aimed at characterizing features of the FcRn:IgG interaction can be used to predict a reasonably acceptable pharmacokinetic behavior *in vivo* and remains a relatively uncharacterized frontier (Gurbaxani et al., 2006; Suzuki et al., 2010). Moreover, the majority of published reports have predominately focused on the *in vitro-* *in vivo* relationship in a single mAb with one and/or multiple independent Fc mutations which ignores or minimizes the intermolecule complexities described above (Igawa et al., 2010; Khawli et al., 2010; Wang et al., 2011; Yeung et al., 2010). Thus, in an effort to implement or validate a strategy that can be more widely applied, it may be important to develop a more thorough understanding of the FcRn binding interactions in the context of the biochemical/biophysical attributes of various antibodies. Towards this end, we developed a series of five humanized IgGs each constructed with the two Fc variant sequences (T250Q/M428L and V308P) and measured their interactions with FcRn *in vitro*, characterized the influence of the mutations on the biophysical properties of each IgG and determined pharmacokinetics of each mAb in cynomolgus monkeys. The Fc variants were built on IgG4 antibody backbones with 100% identical CH1, CH2, CH3 and hinge regions to allow for a more systematic head-to-head comparison. The five IgGs had sequence differences in the heavy and light chain variable regions since each targeted a specific soluble antigen. The target antigens were at insignificant levels in cynomolgus monkeys with the intent of circumventing/limiting antigen binding influencing the peripheral clearance of the antibodies for each of the tested mAbs.

In the present study, we examined the relationship of the *in vitro* IgG:FcRn interaction with *in vivo* clearance/half-life for the wild-type and each variant across the entire population of fifteen IgGs and for each individual IgG platform (i.e. wild-type, T250Q/M428L and V308P for mAbs A1, B1, C1, D1 and E1) (Figure 4). The fifteen IgGs (five each of wild-type and T250Q/M428L and V308P Fc variants) displayed a broad range of FcRn binding affinities at pH 6 from 0.5 to 195 nM (Table 1) allowing us to examine the pharmacokinetic effects over an ~400-fold $K_d$ span. Across the fifteen IgGs, we observed the general trends of improved serum half-life and slower clearance with antibodies that displayed
enhanced FcRn binding at pH 6. The T250Q/M428L and V308P mutations provided similar improvements in the overall mean clearance across the five antibodies with approximately 1.9-fold (range 1.2- to 2.3-fold) and 1.5-fold (range 0.8- to 2.2-fold) increases relative to the wild-type IgGs, respectively. The mean half-life improvement was greater for the V308P variants (~2.4-fold relative to the wild-type antibodies with a range of 1.8- to 3.0-fold) compared to the T250Q/M428L variants (~1.3-fold relative to the wild-type antibodies with a range of 0.9- to 1.5-fold). Despite the general trend of improved pharmacokinetics of the Fc variants with enhanced receptor affinity, there are clear outliers that make a direct correlation of the FcRn binding affinity at pH 6 or rate of dissociation ($k_{off}$) with half-life or clearance unclear (Figure 4). For instance, the A1 V308P and B1 V308P antibodies both show larger than anticipated improvements in half-life based simply on their in vitro FcRn binding interactions than the rest of the IgGs examined. Similarly, the wild-type B1 and wild-type E1, have similar FcRn binding affinities (195 nM and 160 nM, respectively) yet display ~4.2-fold differences in clearance (Tables 1 and 2). Furthermore, across the fifteen IgGs there was a relatively modest effect on the range of values observed for both half-life and clearance (~6- and ~12-fold, respectively) even though our IgG:FcRn binding affinity range at pH 6 spanned ~400-fold. When the outliers are accounted for, the majority of the antibodies are within even narrower observed half-life and clearance ranges of ~3- and ~7-fold, respectively. Our evaluation of the FcRn affinity-pharmacokinetic relationship across five antibody platforms with two Fc variant sequences suggests that there may be an upper limit or diminishing relative improvements observed in an antibody’s pharmacokinetics which disconnects from the much larger improvements that can be achieved in the in vitro FcRn binding affinity.

It has been previously shown that mAbs/Fcs that directly bind to FcRn at neutral pH or incompletely dissociate from the receptor at neutral pH have increased clearance and shorter half-lives in vivo (Datta-Mannan et al., 2007a; Yeung et al., 2009). In this regard, none of the wild-type or variant antibodies tested in this study bound directly to cynomolgus monkey FcRn at pH 7.4 (data not shown) which likely does not account for the disconnect between receptor affinity and pharmacokinetics for our IgGs. We did observe differences in the pH-dependent dissociation of the mAb:FcRn complexes. Notably, the wild-
type antibodies dissociated more readily from FcRn at lower pH values and maintained a higher degree of association at pH 8 than the Fc variant antibodies (Table 1). From a physiological perspective, these data suggest the wild-type antibodies may not stay tightly associated with FcRn within the endosomal compartment as the pH increases during recycling or dissociate well from the receptor when exposed to the extracellular environment. This is in contrast to the Fc variant antibodies that stayed associated with FcRn at higher range of acidic pHs and more completely dissociated at neutral pH. These factors could contribute to enhanced intracellular degradation of the wild-type mAbs resulting in faster clearance and shorter half-life. Although these trends around the pH-dependency of the FcRn:mAb interactions make reasonable sense, there are inconsistencies that make the quantitative correlation of these parameters with half-life or clearance quite tenuous (Figure 5). Coupled with our FcRn binding affinity data, these observations suggest factors other than FcRn may affect antibody kinetics and are not readily predictable from simply characterizing IgG:FcRn binding interactions.

We also examined the FcRn affinity/pH-dependent interactions and pharmacokinetic relationship within an IgG platform to minimize the influence of factors (i.e., biochemical/biophysical properties) discussed above other than FcRn interactions on kinetics. The underlying assumption was that the contribution of additional/unknown clearance mechanisms would be normalized between the Fc variants and their wild-type counterpart. As a result, the primary difference influencing mAb kinetics within a platform would be driven by FcRn-mediated interactions. The results indicate that both the T250Q/M428L and V308P Fc variants improve one or both of these kinetic parameters for each IgG relative to the each antibody’s wild-type counterpart (Figure 6). We observed a reasonably translatable relationship in the rank order of the steepness of the affinity-clearance and affinity-half-life slope parameters across the two Fc variants (Figure 6); however, the magnitude of the effects of the enhanced FcRn affinity on clearance and half-life are different across the five IgG platforms (Figure 6). For instance, antibodies D1 and E1 grafted with either the T250Q/M428L or V308P Fc mutations show greater changes in clearance and half-life (improvements in both) relative to the other three antibodies. These data suggest that FcRn mediated clearance mechanisms play a less important role in the
pharmacokinetics of the wild-type D1 and E1 antibodies relative to the other three. The larger relative improvements observed for the variant D1 and E1 antibodies appeared to be a consequence of the relatively poor kinetics (rapid clearance) of the wild-type counterparts, as the absolute kinetic values were not necessarily improved compared to other three Fc antibody platforms (Figure 6 and Table 2). Consistent with this interpretation is the observation that mAbs D1 and E1 showed the greatest pharmacokinetic improvements with both Fc mutations although they did not show the greatest *in vitro* enhancement in FcRn binding. In the cases of the other three antibodies (A1, B1 and C1), the beneficial effects of the T250Q/M428L or V308P mutations are somewhat mixed, with either marginal or no improvements in the observed half-life and/or clearance relative to their wild-type antibody counterpart. When placed on antibody C1, the T20Q/M428L or V308P mutations improved half-life and clearance by less than two-fold. Antibodies A1 and B1 showed improvements in both of the kinetic parameters with only one of the Fc variants (Table 3 and Figure 6). Taken together, these data suggest that antibodies A1, B1 and C1 were being protected by FcRn nearly optimally. Thus, further increasing the FcRn binding affinity of the A1, B1 and C1 antibodies did not result in as great of an improvement in pharmacokinetics of the mutant versions of these antibodies relative to their wild-type counterparts. The observations imply that additional factors, influence the kinetics of mAbs A1, B1 and C1 to a greater extent than FcRn. The data indicate that the disconnect between the FcRn affinity-pharmacokinetic relationship across IgG platforms is likely governed by a multitude of physiologic mechanisms and biochemical properties of the mAbs.

Several reports have suggested the biochemical characteristics of a mAb itself can have significant impact on its clearance and disposition (Igawa et al., 2010; Khawli et al., 2010; Wang et al., 2011; Yeung et al., 2010). To this point, we also examined several biophysical properties of our molecules including the pI values, Tm of each molecule and their heparin binding interactions as a surrogate measure to reflect potential charge based interactions with cell surfaces which may result in the clearance of the mAbs from circulation (Table 3). Our results do not indicate that a single one of these biophysical parameters readily predicts *in vivo* pharmacokinetics either within or across mAb platforms. While our data suggest
comparable biophysical properties between the mAbs tested (pI, T_m) and no clear evidence of a pattern of non-specific binding to cells in vitro (Table 3), we cannot fully preclude the possibility that unknown differences in the in vivo chemical stabilities or non-target related binding properties of the mAbs dissociate the relationship.

In summary, our results conflict with the simple perspective that greater increases in the affinity of an IgG for FcRn at pH 6 continue to translate to larger improvements in in vivo properties, even while maintaining or the improving pH-dependency of the FcRn interaction. Our data are consistent with previous suggestions that there are diminishing kinetic improvements in the context of improved FcRn binding properties. The current findings indicate that it is difficult to define a universal set of in vitro parameters which can be used to quantitatively predictive of in vivo benefits. Our finding coupled with literature suggest both the characteristics of the antibody (i.e., biophysical properties, antigen affinity, glycosylation, proteolytic stability) and its therapeutic target (membrane bound or soluble antigen, antigen load) can influence disposition and elimination. It is logical to suggest that many factors, in addition to FcRn-mediated antibody recycling, will need to be considered when designing such engineering strategies. It is possible that a set of parameters, when tailored to a particular mAb (or mAbs with similar properties), can be used to guide the optimization of the pharmacokinetic properties of that therapeutic monoclonal but, these factors may not be broadly transferrable to other antibodies.
ACKNOWLEDGMENTS

The authors thank Selina Estwick for measuring the concentrations of the mAbs, Robin Brown for determining the pH-dependency of the mAb:FcRn interactions, Peter McDonnell and Stacy Torgerson for measuring the heparin binding interactions, Yu Tian for determining the pI values of the antibodies, Jeffrey Boyles for measuring the Tm values and Ryan Hansen for insightful discussions.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Datta-Mannan, Chow, Dickinson, Driver, Lu, Witcher and Wroblewski

Conducted experiments: Datta-Mannan, Driver, and Chow

Contributed new reagents or analytic tools: Driver, Chow, Lu and Witcher

Performed data analysis: Datta-Mannan and Chow

Wrote or contributed to the writing of the manuscript: Datta-Mannan and Wroblewski
REFERENCES


FOOTNOTES

1Residues are numbered according to the Eu numbering system. T250Q/M428L indicates mutation of both Thr$^{250}$ to Glu$^{250}$ and Met$^{428}$ to Leu$^{428}$. V308P indicates mutation of Val$^{308}$ to Pro$^{308}$.
FIGURE LEGENDS

Figure 1. BIAcore sensorgrams of the interaction of (A) wild-type mAb A1 and the (B) T250Q/M428L and (C) V308P A1 variants with cynomolgus monkey FcRn at pH 6. Sensorgrams display the response values for duplicates measurements at the cynomolgus monkey FcRn concentrations of 0.0027 μM to 2.0 μM. Data collected are shown in red while the heterogeneous ligand model fits are shown in black.

Figure 2. The T250Q/M428L Fc mutations improve the pharmacokinetics for five mAbs. (A-E) Pharmacokinetic profiles of the five wild-type IgG4s (○) and T250Q/M428L (■) mAbs after intravenous administration to male cynomolgus monkeys. A1, B1, C1, D1 and E1 denote the five mAbs tested. Doses were administered as a single intravenous injection of a solution formulation containing all five wild-type molecules or five T250Q/M428L IgG4 variants for a total of a 5 mg/kg dose (1 mg/kg of each mAb). Serum concentrations were determined using a validated antigen capture ELISA for each mAb. The five mAb backbones (as either wild-type IgG or T250Q/M428L variant mAbs) were selective for a single antigen and showed no cross reactivity/nonspecific binding to non-cognate antigens in the ELISAs when examined individually or as a mixture of the five wild-type or variant IgGs. Data are the mean ± SD of three animals/timepoint.

Figure 3. The V308P Fc mutations improve the pharmacokinetics for five mAbs. (A-E) Pharmacokinetic profiles of the five wild-type IgG4s (○) and V308P (▲) variant mAbs after intravenous to male cynomolgus monkeys. A1, B1, C1, D1 and E1 denote the five mAbs tested. Doses were administered as a single intravenous injection of a solution formulation containing all five wild-type molecules or five V308P IgG4 variants for a total of a 5 mg/kg dose (1 mg/kg of each mAb). Serum concentrations were determined using a validated antigen capture ELISA for each mAb. The five mAb backbones (as either wild-type IgG or V308P variant mAbs) were selective for a single antigen and showed no cross reactivity/nonspecific binding to non-cognate antigens in the ELISAs when examined individually or as a mixture of the five wild-type or variant IgGs. Data are the mean ± SD of three animals/timepoint.
reactivity/nonspecific binding to non-cognate antigens in the ELISAs when examined individually or as a
mixture of the five wild-type or variant IgGs. Data are the mean ± SD of three animals/timepoint.

**Figure 4.** Correlation of the cynomolgus monkey pharmacokinetics with the cynomolgus monkey FcRn
binding affinity parameters at pH 6 across the 15 mAbs (WT, wild-type; V variant, V308P and T variant,
T250Q/M428L). Correlation of the half-life with (A) $K_d$ and (B) $k_{off}$. Correlation of the clearance with
(C) $K_d$ and (D) $k_{off}$. The figure legend in panel A applies to panels B, C and D.

**Figure 5.** Correlation of the cynomolgus monkey pharmacokinetics with the cynomolgus monkey FcRn
pH-dependent binding interactions across the 15 mAbs (WT, wild-type; V variant, V308P and T variant,
T250Q/M428L). Correlation of the half-life with the (A) percent mAb that remains bound to FcRn at pH
8 and (B) $pH_{50}$ (the pH at which 50% of the FcRn:antibody complexes dissociates). Correlation of the
clearance with the (C) percent mAb that remains bound to FcRn at pH 8 and (D) $pH_{50}$. The figure legend
in panel A applies to panels B, C and D.

**Figure 6.** Comparison of the cynomolgus monkey pharmacokinetics with the cynomolgus monkey FcRn
binding affinity parameters at pH 6 for each of the five IgGs. Comparison of the *in vivo* clearance versus
the *in vitro* FcRn $K_d$ of the five wild-type mAbs with their respective (A) T250Q/M428L and (B) V308P
Fc variant counterparts. Comparison of the *in vivo* half-life versus the *in vitro* FcRn $K_d$ of the five wild-
type mAbs with their respective (C) T250Q/M428L and (D) V308P Fc variant counterparts.
TABLE 1

*In vitro* interaction of the humanized mAbs with FcRn.

<table>
<thead>
<tr>
<th>mAb</th>
<th>(K_d^a) (nM)</th>
<th>(k_{off}^b) (\times 10^{-4}) (1/s)</th>
<th>(pH_{50}^c)</th>
<th>% Bound at pH 8 (d^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1 Wild-type</td>
<td>65</td>
<td>39.5</td>
<td>6.39</td>
<td>29</td>
</tr>
<tr>
<td>A1 V308P</td>
<td>1.5</td>
<td>2.0</td>
<td>6.87</td>
<td>25</td>
</tr>
<tr>
<td>A1 T250Q/M428L</td>
<td>2.7</td>
<td>2.7</td>
<td>6.64</td>
<td>17</td>
</tr>
<tr>
<td>B1 Wild-type</td>
<td>195</td>
<td>141</td>
<td>6.25</td>
<td>46</td>
</tr>
<tr>
<td>B1 V308P</td>
<td>0.5</td>
<td>1.0</td>
<td>6.77</td>
<td>19</td>
</tr>
<tr>
<td>B1 T250Q/M428L</td>
<td>1.7</td>
<td>1.9</td>
<td>6.77</td>
<td>22</td>
</tr>
<tr>
<td>C1 Wild-type</td>
<td>66</td>
<td>52.5</td>
<td>6.22</td>
<td>41</td>
</tr>
<tr>
<td>C1 V308P</td>
<td>0.6</td>
<td>1.0</td>
<td>6.52</td>
<td>12</td>
</tr>
<tr>
<td>C1 T250Q/M428L</td>
<td>4.7</td>
<td>4.3</td>
<td>6.41</td>
<td>8</td>
</tr>
<tr>
<td>D1 Wild-type</td>
<td>91</td>
<td>5.5</td>
<td>6.36</td>
<td>45</td>
</tr>
<tr>
<td>D1 V308P</td>
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<td>1.0</td>
<td>7.10</td>
<td>28</td>
</tr>
<tr>
<td>D1 T250Q/M428L</td>
<td>8.5</td>
<td>8.9</td>
<td>6.73</td>
<td>18</td>
</tr>
<tr>
<td>E1 Wild-type</td>
<td>160</td>
<td>176</td>
<td>6.39</td>
<td>66</td>
</tr>
<tr>
<td>E1 V308P</td>
<td>1.4</td>
<td>3.0</td>
<td>6.71</td>
<td>20</td>
</tr>
<tr>
<td>E1 T250Q/M428L</td>
<td>13.4</td>
<td>1.5</td>
<td>6.67</td>
<td>25</td>
</tr>
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</table>

\(^a\)\(K_d\) \((k_{off}/k_{on} \times 10^9)\), data determined from surface plasmon resonance kinetic analyses at pH 6.0. No direct binding of FcRn to the IgGs was observed at pH 7.4 in surface plasmon resonance experiments at FcRn concentrations as high as 5 \(\mu\)M.

\(^b\)\(k_{off}\), rate of dissociation of the FcRn:antibody complexes determined from surface plasmon resonance kinetic analyses at pH 6.0.

\(^c\)\(pH_{50}\), pH at which 50% of preformed FcRn:antibody complexes dissociate.

\(^d\)Percentage of the total antibody in preformed complexes that remained FcRn bound at pH 8 as determined by ELISA.
TABLE 2
Pharmacokinetic parameters for five humanized wild-type mAbs and the T250Q/M428L and the V308P Fc variants in cynomolgus monkeys after intravenous administration

<table>
<thead>
<tr>
<th>mAb</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (μg/mL)</th>
<th>AUC&lt;sub&gt;0-∞&lt;/sub&gt; (μg·hr/mL)</th>
<th>CL (mL/h/kg)</th>
<th>t&lt;sub&gt;1/2β&lt;/sub&gt; (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1 Wild-type</td>
<td>27 ± 2</td>
<td>4264 ± 1242</td>
<td>0.25 ± 0.06</td>
<td>328 ± 162</td>
</tr>
<tr>
<td>A1 V308P</td>
<td>38 ± 8</td>
<td>6972 ± 1311</td>
<td>0.15 ± 0.04</td>
<td>637 ± 127</td>
</tr>
<tr>
<td>A1 T250Q/M428L</td>
<td>22 ± 5</td>
<td>5069 ± 1063</td>
<td>0.20 ± 0.05</td>
<td>285 ± 59</td>
</tr>
<tr>
<td>B1 Wild-type</td>
<td>41 ± 5</td>
<td>5705 ± 2622</td>
<td>0.21 ± 0.11</td>
<td>268 ± 50</td>
</tr>
<tr>
<td>B1 V308P</td>
<td>34 ± 10</td>
<td>7651 ± 1505</td>
<td>0.14 ± 0.03</td>
<td>526 ± 154</td>
</tr>
<tr>
<td>B1 T250Q/M428L</td>
<td>30 ± 4</td>
<td>7850 ± 1080</td>
<td>0.13 ± 0.02</td>
<td>336 ± 146</td>
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<tr>
<td>C1 Wild-type</td>
<td>25 ± 5</td>
<td>2843 ± 192</td>
<td>0.35 ± 0.02</td>
<td>126 ± 17</td>
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<tr>
<td>C1 V308P</td>
<td>20 ± 2</td>
<td>3503 ± 320</td>
<td>0.26 ± 0.05</td>
<td>289 ± 88</td>
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<tr>
<td>C1 T250Q/M428L</td>
<td>17 ± 2</td>
<td>3324 ± 507</td>
<td>0.31 ± 0.04</td>
<td>263 ± 59</td>
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<tr>
<td>D1 Wild-type</td>
<td>28 ± 5</td>
<td>1905 ± 453</td>
<td>0.55 ± 0.14</td>
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<tr>
<td>D1 V308P</td>
<td>19 ± 3</td>
<td>2588 ± 341</td>
<td>0.40 ± 0.05</td>
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<tr>
<td>D1 T250Q/M428L</td>
<td>24 ± 0.6</td>
<td>2910 ± 587</td>
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<tr>
<td>E1 Wild-type</td>
<td>18 ± 4</td>
<td>1347 ± 640</td>
<td>0.88 ± 0.44</td>
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<tr>
<td>E1 V308P</td>
<td>26 ± 5</td>
<td>5426 ± 697</td>
<td>0.19 ± 0.02</td>
<td>310 ± 49</td>
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<tr>
<td>E1 T250Q/M428L</td>
<td>23 ± 3</td>
<td>3863 ± 849</td>
<td>0.27 ± 0.06</td>
<td>246 ± 115</td>
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</table>

<sup>a</sup>Serum concentrations determined using validated antigen capture ELISAs.

<sup>b</sup>Data are the mean ± SD of the pharmacokinetic parameters determined from five monkeys per group for the V308P and T250Q/M428L Fc variants and ten monkeys (across two studies with N=5 monkeys/study) for the wild-type IgGs.

<sup>c</sup>Determined from non-compartmental pharmacokinetic analyses.

C<sub>max</sub>, maximal observed serum concentration; AUC<sub>0-∞</sub>, area under the serum concentration curve from zero to infinity; concentration; CL, clearance; t<sub>1/2β</sub>, elimination half-life.
TABLE 3

Biochemical/Biophysical Characterization of the humanized mAbs $^{a,b,c}$.

<table>
<thead>
<tr>
<th>mAb</th>
<th>$T_m$1</th>
<th>$T_m$2</th>
<th>$T_m$ Fab</th>
<th>pI$^b$</th>
<th>Heparin Binding$^c$</th>
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<tr>
<td>A1 Wild-type</td>
<td>67.7</td>
<td>71.4</td>
<td>75.1</td>
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<td>71.2</td>
<td>81.2</td>
<td>7.64</td>
<td>5</td>
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<td>C1 Wild-type</td>
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<td>C1 V308P</td>
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</tr>
<tr>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>D1 T250Q/M428L</td>
<td>61.7</td>
<td>64.5</td>
<td>68.0</td>
<td>7.57</td>
<td>63</td>
</tr>
<tr>
<td>E1 Wild-type</td>
<td>69.7</td>
<td>74.0</td>
<td>78.3</td>
<td>6.64</td>
<td>0</td>
</tr>
<tr>
<td>E1 V308P</td>
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<td>73.0</td>
<td>78.1</td>
<td>6.56</td>
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<td>73.7</td>
<td>78.0</td>
<td>6.64</td>
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</table>

$^a$ $T_m$, $T_m$1, $T_m$2 and $T_m$ Fab show the $T_m$ values for the C H2, C H3 and Fab regions of each mAb, respectively

$^b$pI, Isoelectric point

$^c$Heparin binding, data show percent of IgG that remains bound to heparin relative to background well containing no antibody.

NA, data not available
Figure 2

Graphs showing the immunoreactivity (ug/mL) over time (hr) for different variants after administration.
Figure 3
Figure 4
Figure 5
Figure 6
FcRn Affinity-Pharmacokinetic Relationship Of Five Human IgG4 Antibodies Engineered For Improved In Vitro FcRn Binding Properties In Cynomolgus Monkeys

Amita Datta-Mannan, Chi-Kin Chow, Craig Dickinson, David Driver, Jirong Lu, Derrick R. Witcher and Victor J. Wroblewski

Drug Metabolism and Disposition
## SUPPLEMENTARY TABLE 1

Binding interactions of the humanized mAbs with cynomolgus monkey FcRn.

<table>
<thead>
<tr>
<th>mAb</th>
<th>$K_d^1$ (nM)</th>
<th>$F_{binding}^1$</th>
<th>$K_d^2$ (nM)</th>
<th>$F_{binding}^2$</th>
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<tr>
<td>A1 Wild-type</td>
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<td>78</td>
<td>866</td>
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<tr>
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<td>75</td>
<td>817</td>
<td>25</td>
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<tr>
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<td>77</td>
<td>1040</td>
<td>23</td>
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<tr>
<td>B1 T250Q/M428L</td>
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<td>66</td>
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<tr>
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<td>13.4</td>
<td>66</td>
<td>547</td>
<td>34</td>
</tr>
</tbody>
</table>

$^aK_d$ ($k_{off}/k_{on} \cdot 1 \times 10^9$), high binding affinity data. Determined from surface plasmon resonance kinetic analyses at pH 6.0.

$^bF_{binding}$, fraction of the binding sites corresponding to $K_d$.

$^cK_d$ ($k_{off}/k_{on} \cdot 1 \times 10^9$), low binding affinity data. Determined from surface plasmon resonance kinetic analyses at pH 6.0.

$^dF_{binding}$, fraction of the binding sites corresponding to $K_d$. 
No direct binding of FcRn to the IgGs was observed at pH 7.4 in surface plasmon resonance experiments at FcRn concentrations as high as 5 µM.