

FcRn Affinity-Pharmacokinetic Relationship of Five Human IgG4 Antibodies Engineered for Improved In Vitro FcRn Binding Properties in Cynomolgus Monkeys[§]

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ABSTRACT:

The pH-dependent binding of 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). Although 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 influence 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 nonspecific/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 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_H2-C_H3 portion of the Fc domain of IgGs in a tightly regulated pH-dependent manner with high affinity binding occurring at an acidic pH (~6) and weak to no binding interactions as the pH is raised to neutral (7.4) (Ober et al., 2004a,b; Prabhat et al., 2004; Goebel et al., 2008). 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, 2005). After 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, 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, and 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, 2006; Hinton et al., 2004, 2006; Datta-Mannan et al., 2007a,b; Yeung et al., 2009, 2010; Deng et al., 2010). Although 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-

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ABBREVIATIONS: FcRn, neonatal Fc receptor; mAbs, monoclonal antibodies; SPR, surface plasmon resonance; WT, wild type; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; K_d , equilibrium dissociation constant; pH₅₀, pH at which 50% of the FcRn-antibody complexes dissociate; pI, isoelectric point; T_m , thermal stability; k_{off} , dissociation rate.

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). Likewise, Gurbaxani et al. (2006) were unable to directly correlate FcRn binding affinity to the pharmacokinetics of a number of IgGs in mice. The quantitative correlation of FcRn affinity improvement with in vivo pharmacokinetic parameters has also been ambiguous in primate studies of engineered mAbs (Deng et al., 2010; Datta-Mannan et al., 2012). Deng et al. (2010) showed an anti-tumor necrosis factor- α mAb Fc variant (N434H) with \sim 3-fold higher FcRn affinity at pH 6 had similar pharmacokinetics in cynomolgus monkeys compared with a lower receptor affinity variant constructed on the same mAb backbone (N434A), although these findings may have been compromised by anti-drug antibodies. Likewise, Fc mutations (M252Y/S254T/T256E) that have increased receptor binding at pH 6 by \sim 10-fold have shown \sim 3- to 4-fold improvements in mAb half-life in monkeys (Dall'Acqua et al., 2006), whereas Fc mutations (T250Q/M428L) that display \sim 30-fold increases in FcRn binding have shown only \sim 2.5-fold improvements in half-life (Hinton et al., 2004, 2006). One of the challenges in establishing a systematic understanding of the FcRn affinity-pharmacokinetic relationship is 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, 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 nonhuman FcRn-IgG in vitro FcRn affinity-pharmacokinetic relationship that are critical for the successful improvement of IgG clearance in nonhuman primates (rhesus or cynomolgus monkeys): 1) higher in vitro IgG-FcRn affinity at pH 6 may translate to pharmacokinetic benefit in nonhuman primates if the increased affinity is predominantly attributable to the rate of dissociation of the complex (Datta-Mannan et al., 2007a,b); 2) higher in vitro IgG-FcRn affinity at pH 6 may not correlate with antibody pharmacokinetic benefits in nonhuman primates if increased affinity is driven predominantly by improvements in the rate of association of the complex (Datta-Mannan et al., 2007a,b); 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 to 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 influence the in vivo pharmacokinetics. Although the interplay of target-mediated mAb clearance mechanisms is also acknowledged to contribute to some of these discrepancies (Yeung et al., 2010; Chaparro-Riggers et al., 2012), 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. Published 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 a significant impact on antibody disposition (Igawa et al., 2010; Khawli et al., 2010; Yeung et al., 2010; Wang et al., 2011). However, there remains 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), along with the pharmacokinetics of these molecules in cynomolgus monkeys. (Residues are numbered according to the European Union numbering system. T250Q/M428L indicates mutation of both Thr250 to Glu250 and Met428 to Leu428. V308P indicates mutation of Val308 to Pro308.) The Fc variants were built on IgG4 antibody backbones with 100% identical C_H1, C_H2, C_H3, 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 because each targeted a specific soluble antigen. In each case, there were insignificant peripheral levels of target antigen in normal cynomolgus 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 and for variants across mAbs. 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/multifactorial approach (biophysical properties, antigen load, glycosylation, proteolytic stability, nonspecific binding, charge, chemical modification) to more completely predict the pharmacokinetic effect of an Fc variant when placed on a particular mAb. Understanding these factors and the interplay of FcRn in the metabolism and survival of these constructs in vivo is critical to facilitate the rational optimization of the pharmacokinetic/pharmacodynamic properties of mAbs.

Materials and Methods

Cell Culture. 293EBNA cells were maintained at 37°C under 5 to 8% CO₂ conditions in Dulbecco's modified Eagle's medium/F12 (Invitrogen, Carlsbad, CA) supplemented with 20 mM HEPES (Invitrogen), 5 μ g/ml nuclellin (Eli Lilly & Co., Indianapolis, IN), 0.4 μ g/ml tropolone (Sigma-Aldrich, St. Louis, MO), 0.075% (w/v) F68 (Invitrogen), and 50 μ g/ml Geneticin (G418) (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 previously (Datta-Mannan et al., 2007a,b). The T250Q/M28L and V308P variants were obtained by site-directed mutagenesis of the wild-type (WT) humanized IgG4 clone using the QuikChange method (Agilent Technologies, Santa Clara, CA) and were confirmed by DNA sequencing. The WT and variant IgGs were expressed in 293EBNA cells and purified from culture supernatants using Protein-A Sepharose (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) affinity chromatography followed by size exclusion chromatography methods described previously (Datta-Mannan et al., 2007a,b). Recombinant, soluble cynomolgus monkey FcRn was expressed in 293EBNA cells transfected with the plasmids encoding for the soluble portion α FcRn and β ₂-microglobulin, and the protein was purified as described previously (Datta-Mannan et al., 2007a,b).

IgG-FcRn Binding Affinity Measurements with SPR (BIAcore AB). The interaction of the WT and variant IgGs with recombinant cynomolgus monkey FcRn was monitored by SPR detection using a BIAcore T-100 instrument (BIAcore AB, Uppsala, Sweden). In brief, a mouse anti-human kappa antibody (BD Biosciences, Santa Clara, CA), with no binding to cynomolgus monkey and human FcRn, was immobilized to a CM5 chip at \sim 5000 resonance units using the standard amine-coupling kit as a capture methodology. Antibody samples were prepared at 5 μ g/ml and were injected for 20 s. In general, 200 to 800 resonance units 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 μM to 2.7 nM were prepared in the binding buffer and injected at a flow rate of 100 $\mu\text{l}/\text{min}$ for 0.5 min over the antibody-captured sensor chip at 25°C. The dissociation phase was monitored for 15 min. The chip was regenerated with 10 mM 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 (BIAcore AB). 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 χ^2 values.

pH-Dependent Dissociation Enzyme-Linked Immunosorbent Assay for the WT T250Q/M428L Fc Variant Antibodies. Biotinylated cynomolgus monkey FcRn for enzyme-linked immunosorbent assays (ELISAs) was produced by reacting each purified soluble protein with EZ-Link Sulfo-NHS-Biotin (Thermo Fisher Scientific, Waltham, MA) using the conditions supplied by the vendor. The FcRn/biotin ratio was measured as 1:1, using the EZ Biotin Quantitation Kit (Thermo Fisher Scientific). The pH-dependent ELISA for the interaction of the FcRn with the five WT IgGs, five T250Q/M428L, and five V308P Fc variant antibodies was performed as described in previous studies with other IgGs (Datta-Mannan et al., 2007a,b). In brief, after washing and blocking NeutrAvidin-coated plates (Thermo Scientific, Rockford, IL), 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 $\mu\text{g}/\text{well}$ of each antibody, dissolved in 100 mM NaPO_4 , pH 6.0, 0.05% Tween 20 (v/v), and 0.1% ovalbumin (m/v), was added to each well and was incubated for 1 h at 25°C. After antibody binding, wells were washed three times with 1- to 3-min incubations at 25°C between each wash using 100 mM NaPO_4 , 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 ImmunoResearch Laboratories, Inc., West Grove, PA). Optical density data were analyzed by the same four-parameter nonlinear regression fit as described previously (Datta-Mannan et al., 2007a,b) to determine the midpoint (pH₅₀) of the titration curve (the pH at which 50% of the FcRn-antibody complexes dissociate). At each pH, data are expressed as the percentage of the total antibody bound at pH 6.

Evaluation of mAb Isoelectric Points. The isoelectric points (pIs) of the mAbs were determined by capillary isoelectric focusing, PA 800 plus Pharmaceutical Analysis System (Beckman Coulter, Fullerton, CA). Samples were prepared by mixing a 5- to 10- μg sample with 200 μl of 3 M urea-capillary isoelectric focusing 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 five pI markers (10.0, 9.5, 7.0, 5.5, and 4.1), and vortexing for 15 s. The electrophoresis and data collection were performed using a focusing step voltage 25 kV for 15 min, a chemical mobilization step voltage 30 kV for 30 min, a UV detection at a wavelength of 280 nm, and a data-collection rate of 2 Hz. 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. Thermal stability (T_m) of samples was measured using a TA Instruments NanoDSC (TA Instruments, New Castle, DE) equipped with an autosampler. Samples diluted to 0.5 mg/ml in PBS were heated from 20–110°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 C_{H1}2, C_{H1}3, and Fab domain unfolding to obtain T_m .

Evaluation of Interactions with Heparin. Heparin binding ELISA plates (BD Biosciences) were coated with 0.5 $\mu\text{g}/\text{well}$ of heparin (Sigma-Aldrich) 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 Fisher Scientific) was added to each well and allowed to bind to the heparin-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, Birmingham, AL). The amount of antibody that re-

mained 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 injection of a solution formulation containing all five WT molecules or five IgG4 variant molecules for a total of a 5 mg/kg dose (1 mg/kg each mAb) in each study. In the first study, each animal received a single intravenous dose of the five WT or five T250Q/M428L variant IgGs dissolved in PBS (pH 7.4) at 1 mg/kg. In the second study, each animal received a single intravenous dose of the five WT or five V308P variant IgGs dissolved in PBS (pH 7.4) at 1 mg/kg. Blood samples were collected from the femoral vein before 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 h after administration of the dose in both studies. The blood samples were allowed to clot at ambient temperature before centrifugation to obtain serum.

Bioanalytical Assays and Pharmacokinetic Data Analysis. Concentrations of the WT 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. For the antigen-capture ELISAs, each well of an Immulon 4 microtiter plate (Thermo Fisher Scientific) 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 were incubated for 1 h at room temperature. The five mAb backbones (as either WT IgG or T250Q/M428L or V308P mutants) were selective for a single antigen and showed no cross-reactivity/nonspecific binding to noncognate antigens in the ELISAs when examined individually or as a mixture of the five WT 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). The WT, 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 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 software package (version 3.2; Pharsight, 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 maximal observed serum concentration (C_{max}), area under the plasma concentration curve from zero to infinity, clearance, and elimination half-life ($t_{1/2}$). For the WT 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 WT and T250Q/M428L and V308P Fc Variant Antibodies with Cynomolgus Monkey FcRn by SPR Assays. A representative SPR sensorgram of the interaction of WT and Fc variant constructs of antibody A1 with cynomolgus monkey FcRn at pH 6 are shown in Fig. 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}) (Fig. 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 (Supplemental Table 1). Because the high-affinity K_d accounted for a greater fraction of the binding (Supplemental Table 1), it was used for all the subsequent analyses. The high-affinity rate constant (k_{off}) and binding affinity (K_d) values for the interaction of all the IgGs with cynomolgus FcRn at pH 6.0 are reported in Table 1. Our findings show the five WT IgGs bind to cynomolgus monkey FcRn with considerable differences even though they have identical C_{H1}1, C_{H1}2, and C_{H1}3 regions. The high-affinity K_d of the five WT antibodies to cynomolgus monkey FcRn showed up to ~3-fold differences and ranged from ~90 to ~200 nM

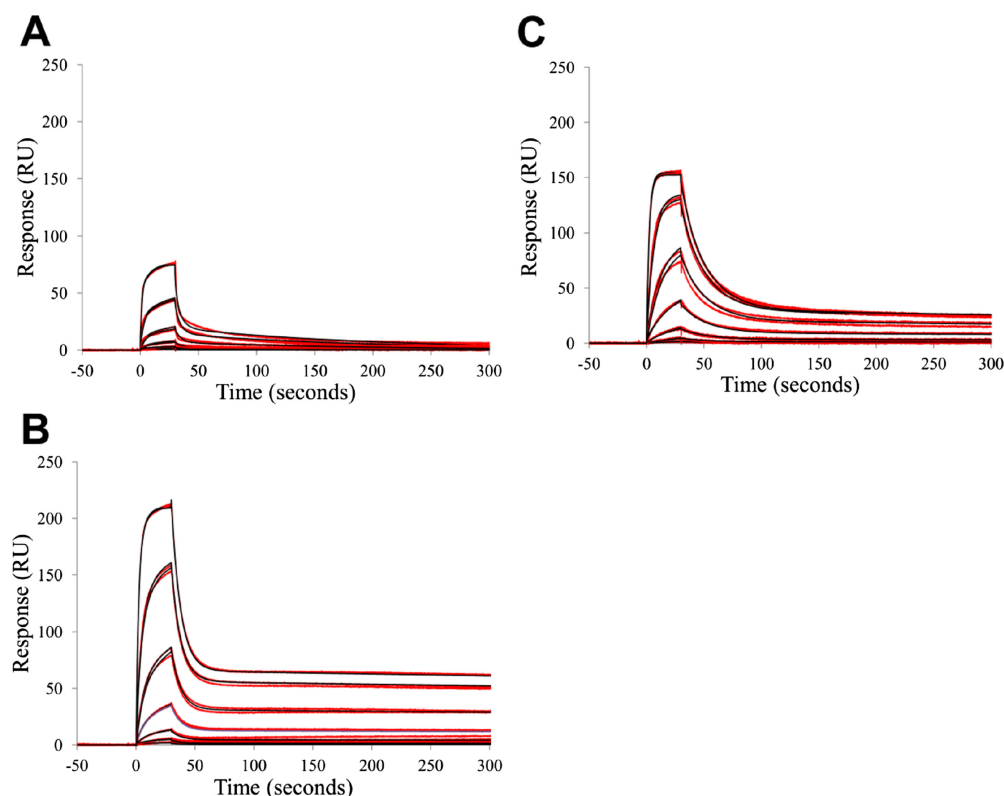


FIG. 1. BIAcore sensorgrams of the interaction of WT mAb A1 (A) and the T250Q/M428L (B) and V308P A1 (C) variants with cynomolgus monkey FcRn at pH 6. Sensorgrams display the response values for duplicate measurements at the cynomolgus monkey FcRn concentrations of 0.0027 to 2.0 μM . Data collected are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

(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 that the binding affinity enhancements for our five WT 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 the five V308P mutants, the antibodies have comparable affinities for cynomolgus mon-

key FcRn (T240Q/M428L K_d values of ~ 3 –13 nM and V308P K_d values of ~ 0.5 –1.5 nM), indicating these mutations within each IgG backbone make similar free energy contributions to FcRn binding. In addition, our findings showed that the differences in the affinity of mutant antibodies for cynomolgus monkey FcRn at pH 6 are predominantly due to slowed rates of dissociation (k_{off} values) (Table 1), an important characteristic of enhanced FcRn binding implicated in improving IgG pharmacokinetics (Datta-Mannan et al., 2007a,b). 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,b; Yeung et al., 2009, 2010; Suzuki et al., 2010). The binding data of the Fc variant mAbs with human FcRn showed a similar pattern improved affinity receptor driven predominantly by a slowed rate of dissociation relative to each IgG WT antibody counterpart (data not shown).

Determination of the pH-Dependent Dissociation of Cynomolgus Monkey FcRn and WT, T250Q/M428L, and V308P Fc Variant Antibody Complexes. The pH dependence of the FcRn-mAb interaction was measured in vitro by forming IgG-receptor complexes at pH 6 and monitoring the degree (pH_{50} 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,b). The 15 preformed wild-type: and variant:cynomolgus monkey-FcRn complexes dissociated with pH_{50} 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 that at pH 8.0 (data not shown). Compared with their respective WT mAb counterparts, the Fc variant constructs generally displayed higher pH_{50} values and a

TABLE 1

In vitro interaction of the humanized mAbs with FcRn

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

^a K_d ($k_{\text{off}}/k_{\text{on}} \cdot 1 \times 10^9$); data determined from SPR kinetic analyses at pH 6.0. No direct binding of FcRn to the IgGs was observed at pH 7.4 in SPR experiments at FcRn concentrations as high as 5 μM .

^b Determined from SPR kinetic analyses at pH 6.0.

^c Percentage of the total antibody in preformed complexes that remained FcRn-bound at pH 8 as determined by ELISA.

TABLE 2

Biochemical/biophysical characterization of the humanized mAbs

T_m , T_{m1} , T_{m2} , and T_m Fab show the T_m values for the C_{H2} , C_{H3} , and Fab regions of each mAb, respectively.

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

mAb	T_m			pI	Heparin Binding
	T_{m1}	T_{m2}	T_m Fab		
	°C				
A1 WT	67.7	71.4	75.1	7.75	20
A1 V308P	60.4	70.6	74.8	7.69	22
A1 T250Q/M428L	63.2	70.8	75.0	7.70	49
B1 WT	69.8	70.0	81.3	7.58	3
B1 V308P	61.5	73.9	81.3	7.61	0
B1 T250Q/M428L	65.4	71.2	81.2	7.64	5
C1 WT	66.8	69.3	71.8	7.41	0
C1 V308P	60.3	68.6	71.4	7.48	13
C1 T250Q/M428L	62.4	67.7	71.3	7.54	4
D1 WT	64.7	66.8	68.8	7.57	35
D1 V308P	N.A.	N.A.	N.A.	N.A.	0
D1 T250Q/M428L	61.7	64.5	68.0	7.57	63
E1 WT	69.7	74.0	78.3	6.64	0
E1 V308P	61.8	73.0	78.1	6.56	4
E1 T250Q/M428L	64.9	73.7	78.0	6.64	0

N.A., data not available.

greater extent of dissociation from FcRn at pH 8.0. The WT and variant IgG pH-dependent interactions with human FcRn were similar to those of the cynomolgus monkey FcRn (data not shown).

Characterization of the Biochemical and Biophysical Properties of the WT and T250Q/M428L and V308P Fc Variant Antibodies.

The majority of the pIs of the WT humanized IgG4 molecules ranged from 7.41 to 7.75 (Table 2) with the exception of antibody E1, which displayed a lower pI value of 6.64 (Table 2). The Fc variants had no significant influence on the pI relative to each antibody WT IgG counterpart (Table 2). The change in the T_m values of the antibodies ranged from ~ -3 to -8°C , ~ -2 to 3.9°C , and 0 to -0.8°C for the C_{H2} , C_{H3} , and Fab regions, respectively (Table 2). The V308P and T250Q/M428L mutations had the largest influence on the C_{H2} region and generally lowered the T_m by ~ 7.5 – 4.5°C , respectively (Table 2). Across the 15 mAbs, we observed varied responses to heparin binding ranging, from no binding to 63% increase in heparin interactions relative to background (Table 2). Within an antibody platform, the V308P and T250Q/M428L Fc variants displayed reasonably similar heparin binding interactions relative to their WT antibody counterparts, with the exceptions of A1 T250Q/M428L and D1 T250Q/M428L, which showed 29 and 28% higher heparin binding than their WT IgG, respectively (Table 2).

Pharmacokinetics of the WT and T250Q/M428L and V308P Fc Variant Antibodies in Cynomolgus Monkeys after a Single Intravenous Administration. 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 15 IgGs (five each of WT antibodies and T250Q/M428L and V308P Fc variant mAbs) in cynomolgus monkeys after intravenous administration. To minimize the number of animals used for our pharmacokinetic studies, we chose to conduct our in vivo evaluations of these antibodies by using dose formulation solutions composed of mixtures of the five WT, five T250Q/M428L, or five V308P IgG4s. At the concentrations in the mixed solutions ($1 \text{ mg} \cdot \text{ml}^{-1} \cdot \text{antibody}^{-1}$), assessments of our formulations showed no soluble or insoluble antibody aggregates (data not shown), indicating the heterogeneity of the solutions did not negatively affect the solubility of the individual

antibody components. Thus, each cynomolgus monkey was administered a formulation containing all five WT 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 each mAb).

After a single intravenous administration of the mixed WT mAb or an Fc variant IgG formulation, the five WT 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 WT antibody and the two Fc variants are shown in Figs. 2 and 3. Compared within a humanized IgG platform, the serum profiles of the WT and variant antibodies displayed significant differences (Figs. 2 and 3). Both the T250Q/M428L and V308P mutations improved the pharmacokinetics (slowed clearance and/or increased $t_{1/2}$) when placed on each WT IgG platform (Figs. 2 and 3; Table 3). The degree of pharmacokinetic improvement (fold enhancement of a pharmacokinetic parameter) varied across the five IgGs (Figs. 2 and 3; Table 3). The T250Q/M428L mutations showed mean clearance and elimination half-life improvements of 1.9-fold (range, 1.2–2.3-fold) and 1.3-fold (range, 0.9–1.5-fold), respectively, whereas the V308P showed mean clearance and elimination half-life improvements of 1.5-fold (range, 0.8–2.2-fold) and 2.4-fold (range, 1.8–3.0-fold), respectively, relative to the WT IgGs. Analysis of the relationship of the in vitro IgG-FcRn interactions with in vivo clearance/half-life for the WT and each variant across the entire population of 15 IgGs and for each individual IgG platform showed that there is not a direct in vitro FcRn binding interaction/pharmacokinetic correlation for the IgGs (Figs. 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. Several factors 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 (Yeung et al., 2010; Chaparro-Riggers et al., 2012). In addition, the biochemical properties of the antibody (i.e., biophysical properties, antigen affinity, glycosylation, and charge proteolytic stability) (Igawa et al., 2010; Khawli et al., 2010; Yeung et al., 2010; Wang et al., 2011), in combination with the target characteristics described previously, 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 have also been clearly shown to modulate mAb disposition (Dall'Acqua et al., 2002, 2006; Hinton et al., 2004, 2006; Datta-Mannan et al., 2007a,b; Yeung et al., 2009, 2010; Deng et al., 2010). The relative contribution of each of these factors underlies the basic in vivo characteristics of an antibody and heavily influences 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 (Ghetie et al., 1996; Christianson et al., 1997; West and Bjorkman, 2000). Because 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

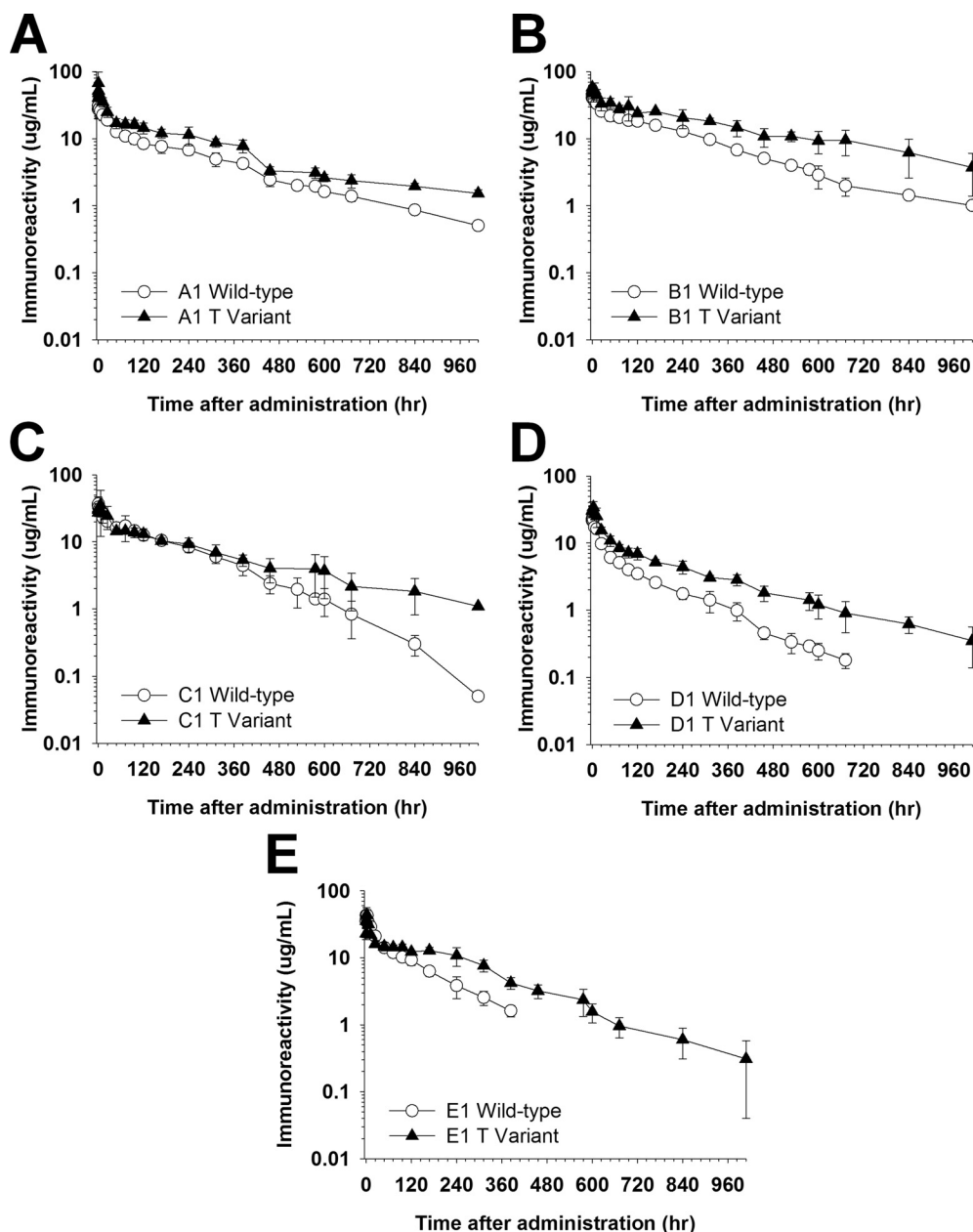


Fig. 2. The T250Q/M428L Fc mutations improve the pharmacokinetics for five mAbs. A–E, pharmacokinetic profiles of the five WT IgG4s (○) and T250Q/M428L (▲) mAbs after intravenous administration to male cynomolgus monkeys. A1, B1, C1, D1, and E1 denote the five mAbs tested. Doses were administered as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

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 nonhuman primates (Dall'Acqua et al., 2002, 2006; Hinton et al., 2004, 2006; Datta-Mannan et al., 2007a,b; Yeung et al., 2009, 2010; Deng 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 remain a relatively uncharacterized frontier (Gurbaxani et al., 2006; Suzuki et al., 2010). Moreover, the majority of published reports have predominantly 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; Yeung et al., 2010; Wang et al., 2011). 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 under-

standing of the FcRn binding interactions in the context of the biochemical/biophysical attributes of various antibodies. Toward 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 C_H1, C_H2, C_H3, 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 because 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 WT and

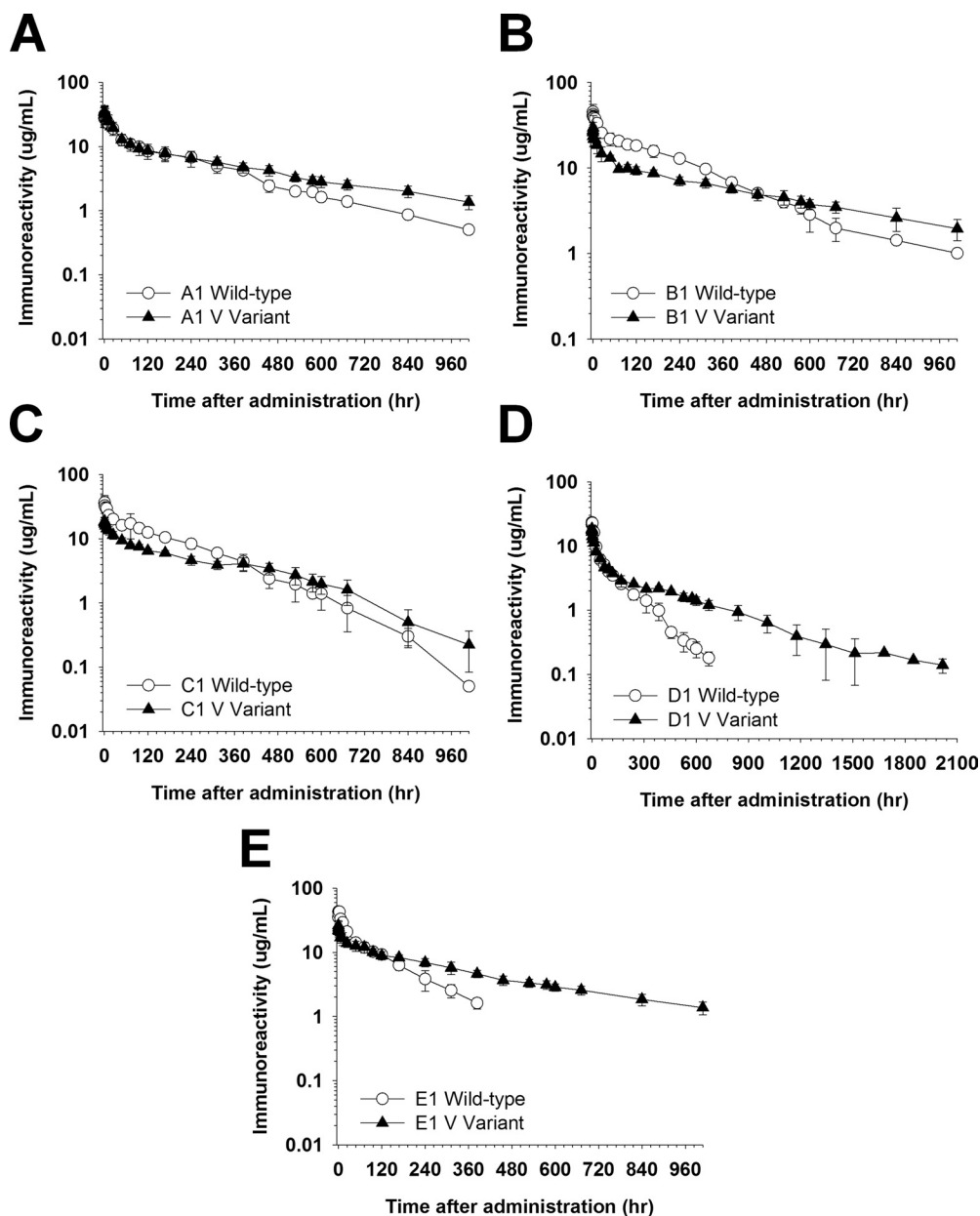


FIG. 3. The V308P Fc mutations improve the pharmacokinetics for five mAbs. A–E, pharmacokinetic profiles of the five WT IgG4s (○) and V308P (▲) variant mAbs after intravenous administration to male cynomolgus monkeys. A1, B1, C1, D1, and E1 denote the five mAbs tested. Doses were administered as a single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

each variant across the entire population of 15 IgGs and for each individual IgG platform (i.e., WT, T250Q/M428L, and V308P for mAbs A1, B1, C1, D1, and E1) (Fig. 4). The 15 IgGs (five each of WT 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 15 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, 1.2–2.3-fold) and 1.5-fold (range, 0.8–2.2-fold) increases relative to the WT IgGs, respectively. The mean half-life improvement was greater for the V308P variants (~2.4-fold relative to the WT antibodies with a range of 1.8–3.0-fold) compared with the T250Q/M428L variants (~1.3-fold relative to the WT antibodies with a range of 0.9–1.5-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 (Fig. 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. Likewise, the WT B1 and WT E1 have similar FcRn binding affinities (195 and 160 nM, respectively) yet display ~4.2-fold differences in clearance (Tables 1 and 2). Furthermore, across the 15 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

TABLE 3

Pharmacokinetic parameters for five humanized WT mAbs and the T250Q/M428L and the V308P Fc variants in cynomolgus monkeys after intravenous administration

Serum concentrations determined using validated antigen capture ELISAs. Data are the mean \pm S.D. of the pharmacokinetic parameters determined from five monkeys per group for the V308P and T250Q/M428L Fc variants and 10 monkeys (across two studies with $n = 5$ monkeys/study) for the WT IgGs. Determined from noncompartmental pharmacokinetic analyses.

mAb	C_{\max}	$AUC_{0-\infty}$	CL	$t_{1/2\beta}$
	$\mu\text{g/ml}$	$\mu\text{g} \cdot \text{h/ml}$	$\text{ml} \cdot \text{h}^{-1} \cdot \text{kg}^{-1}$	h
A1 WT	27 \pm 2	4264 \pm 1242	0.25 \pm 0.06	328 \pm 162
A1 V308P	38 \pm 8	6972 \pm 1311	0.15 \pm 0.04	637 \pm 127
A1 T250Q/M428L	22 \pm 5	5069 \pm 1063	0.20 \pm 0.05	285 \pm 59
B1 WT	41 \pm 5	5705 \pm 2622	0.21 \pm 0.11	268 \pm 50
B1 V308P	34 \pm 10	7651 \pm 1505	0.14 \pm 0.03	526 \pm 154
B1 T250Q/M428L	30 \pm 4	7850 \pm 1080	0.13 \pm 0.02	336 \pm 146
C1 WT	25 \pm 5	2843 \pm 192	0.35 \pm 0.02	126 \pm 17
C1 V308P	20 \pm 2	3503 \pm 320	0.26 \pm 0.05	289 \pm 88
C1 T250Q/M428L	17 \pm 2	3324 \pm 507	0.31 \pm 0.04	263 \pm 59
D1 WT	28 \pm 5	1905 \pm 453	0.55 \pm 0.14	126 \pm 46
D1 V308P	19 \pm 3	2588 \pm 341	0.40 \pm 0.05	351 \pm 52
D1 T250Q/M428L	24 \pm 0.6	2910 \pm 587	0.35 \pm 0.08	278 \pm 107
E1 WT	18 \pm 4	1347 \pm 640	0.88 \pm 0.44	94 \pm 51
E1 V308P	26 \pm 5	5426 \pm 697	0.19 \pm 0.02	310 \pm 49
E1 T250Q/M428L	23 \pm 3	3863 \pm 849	0.27 \pm 0.06	246 \pm 115

C_{\max} , maximal observed serum concentration; CL, clearance; $t_{1/2\beta}$, elimination half-life.

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 WT 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 WT 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 WT 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 a higher range of acidic pH values and more completely dissociated at neutral pH. These factors could contribute to enhanced intracellular degradation of the WT mAbs, resulting in faster clearance and shorter half-life. Although these trends around the pH dependence 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 (Fig. 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 under *Introduction*, 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 WT counterparts. 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 WT counterpart (Fig. 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 (Fig. 6); however, the magnitude of the effects of the enhanced FcRn affinity on clearance and half-life are different across the five IgG platforms (Fig. 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 WT 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 WT counterparts, because the absolute kinetic values were not necessarily improved compared with the other three Fc antibody platforms (Fig. 6; 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 WT antibody counterparts. When placed on antibody C1, the T20Q/M428L or V308P mutations improved half-life and clearance by less than 2-fold. Antibodies A1 and B1 showed improvements in both of the kinetic parameters with only one of the Fc variants (Fig. 6; Table 3). 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 WT 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 a significant impact on its clearance and disposition (Igawa et al., 2010; Khawli et al., 2010; Yeung et al., 2010; Wang et al., 2011). To this point, we also examined several biophysical properties of our molecules including the pI values, T_m of each molecule, and their heparin binding interactions as a surrogate measure to reflect potential charge-based interactions with cell surfaces that 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. Although our data suggest comparable biophysical properties between the mAbs tested (pI, T_m) and no clear evidence of a pattern of nonspecific binding to cells in vitro (Table 3), we cannot fully preclude the possibility that unknown differences in the in vivo chemical stabilities or nontarget-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 improving pH dependence 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

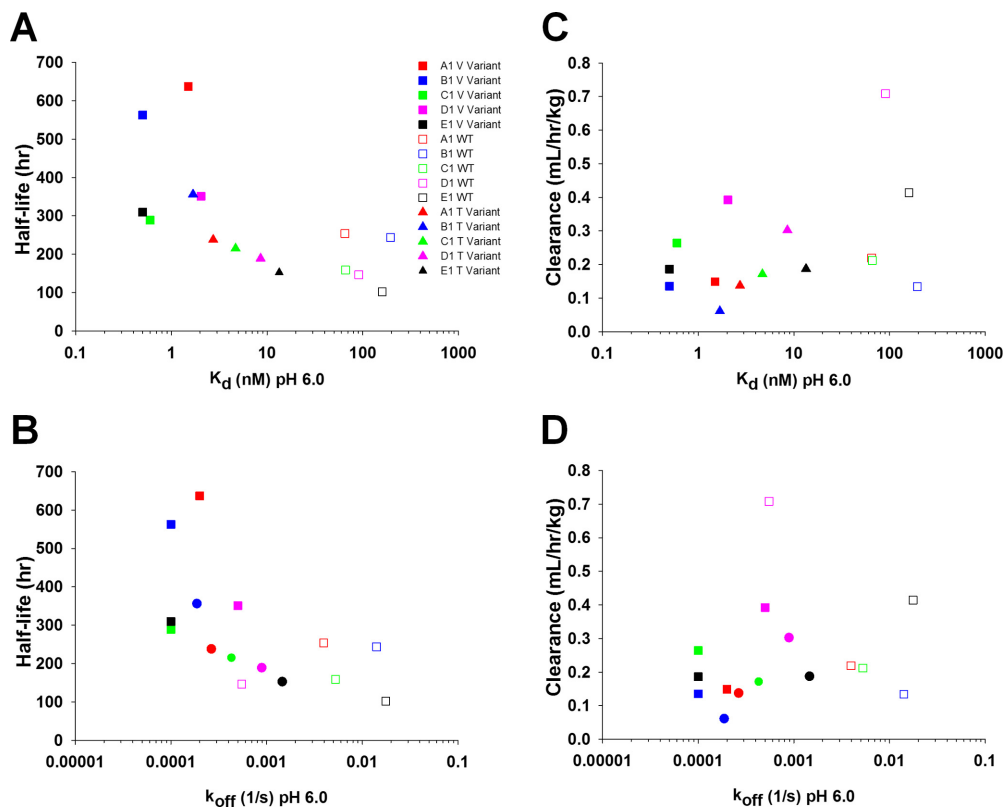


FIG. 4. Correlation of the cynomolgus monkey pharmacokinetics with the cynomolgus monkey FcRn binding affinity parameters at pH 6 across the 15 mAbs (V variant, V308P and T variant, T250Q/M428L). Correlation of the half-life with K_d (A) and k_{off} (B). Correlation of the clearance with K_d (C) and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

universal set of in vitro parameters that can be used to quantitatively predict in vivo benefits. Our finding, coupled with literature, suggests both the characteristics of the antibody (i.e., biophysical properties, antigen affinity, glycosylation, and proteolytic stability) and its ther-

apeutic 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

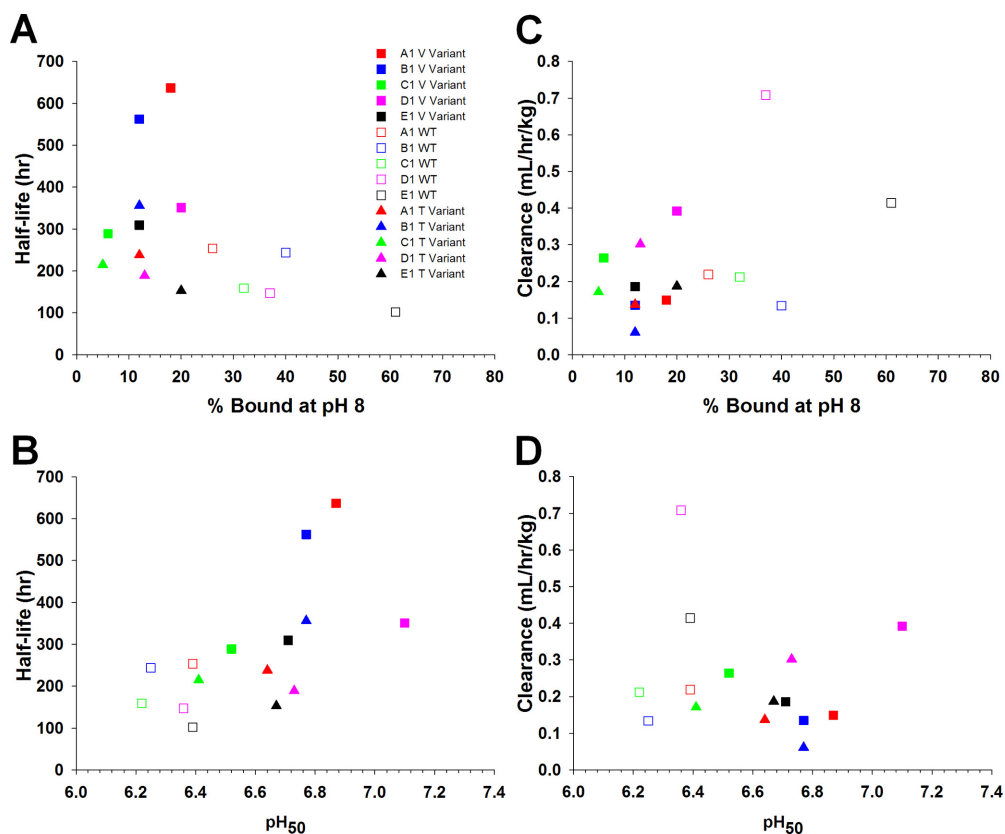


FIG. 5. Correlation of the cynomolgus monkey pharmacokinetics with the cynomolgus monkey FcRn pH-dependent binding interactions across the 15 mAbs (V variant, V308P and T variant, T250Q/M428L). Correlation of the half-life with the percentage of mAb that remains bound to FcRn at pH 8 (A) and pH_{50}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

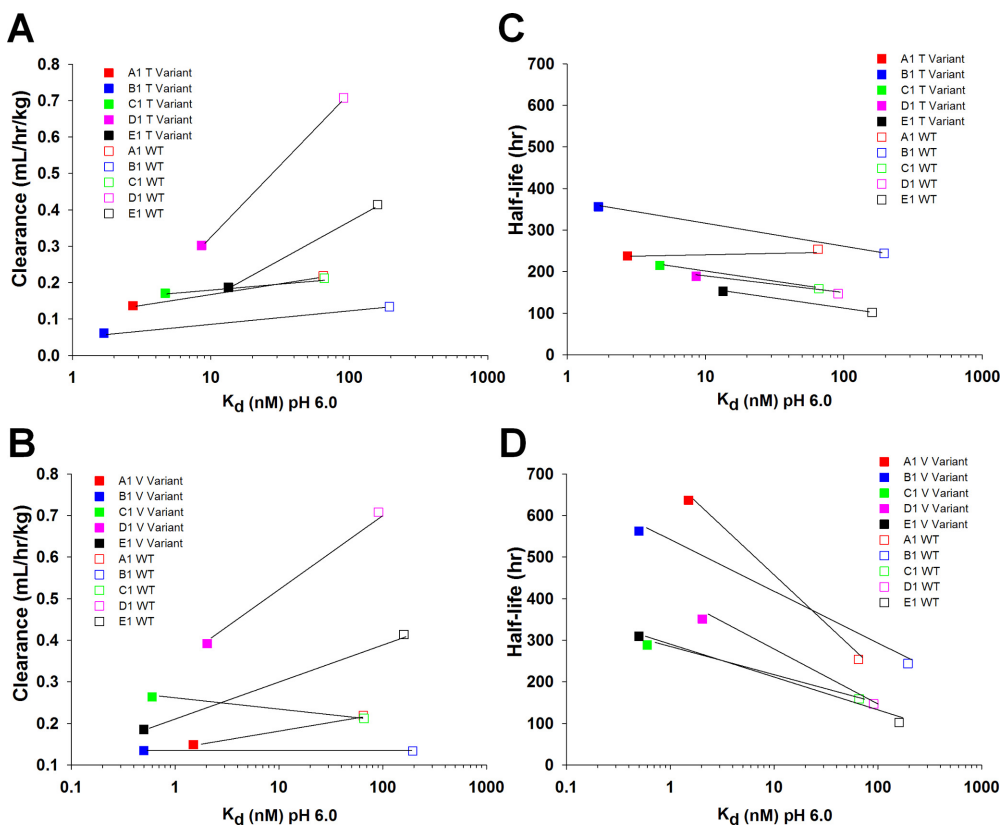


FIG. 6. Comparison of the cynomolgus monkey pharmacokinetics with the cynomolgus monkey FcRn binding affinity parameters at pH 6 for each of the five IgGs. Comparison of the in vivo clearance versus the in vitro FcRn K_d of the five WT mAbs with their respective T250Q/M428L (A) and V308P Fc variant counterparts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

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.

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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: Chow, Driver, Lu, and Witcher.

Performed data analysis: Datta-Mannan and Chow.

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

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