Monoclonal Antibody Clearance

IMPACT OF MODULATING THE INTERACTION OF IgG WITH THE NEONATAL Fc RECEPTOR*

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Monoclonal antibodies (mAbs)2 and Fc fusion proteins have become important therapeutic options in numerous disease indications, including cancer, inflammation, and autoimmune diseases (1). The proven efficacy of these molecular entities in combination with advances in protein engineering and directed evolution strategies has led to efforts to optimize the functional activity of these biologic agents. The goal of many of these approaches is to improve patient convenience and safety by reducing dose and/or dose frequency and potentially to improve efficacy (1). Many reports have suggested that improvement in the pharmacokinetic and pharmacodynamic properties of humanized monoclonal antibodies may be gained through modification of the interaction of the Fc region of IgGs with FcRn (2–6). It has been proposed that optimizing the properties of the receptor interaction may alter the intracellular trafficking of IgGs resulting in reduced clearance and increased half-life in vivo (7–9). Because these pharmacokinetic improvements can translate to enhanced or broadened therapeutic utility of a particular mAb, it has been of primary interest to understand aspects of the IgG/FcRn interaction that can serve as the basis of a rational and universal protein engineering strategy.

FcRn has been shown to be involved in the transcytosis of IgGs across epithelium allowing transfer of IgG from mother to offspring (10–13). Considerable evidence also exists to support the role of FcRn in regulating levels of circulating IgGs in rodents and higher species (2, 9, 14–17). The hallmark characteristic of the IgG/FcRn system is its strict pH binding dependence. Whereas IgG can bind to FcRn via the Fc region at pH 6.0, no direct binding occurs at neutral pH, and dissociation of the IgG-FcRn complex is also facilitated at neutral pH (13, 18–20). The pH dependence of this interaction has served as the basis for the currently proposed mechanism of intracellular IgG trafficking and recycling. In short, it is believed that IgG uptake into the cell occurs via fluid phase pinocytosis with IgG subsequently binding to FcRn in the acidic environment of the endosomal compartment (7–9). FcRn-bound antibody is thought to be protected from degradation by following a recycling pathway to the cell surface where the neutral pH facilitates dissociation and release of IgG into the circulation (7–9). Unbound IgG, in contrast, is believed to be directed down a degradative pathway resulting in proteolysis in lysosomes (7–9). The proportion of IgG processed through the recycling versus degradative pathways is believed to be important in determining the persistence of an IgG in the circulation (8).

The neonatal Fc receptor (FcRn) plays a critical role in regulating IgG homeostasis in vivo. There are mixed reports on whether modification of the interaction with FcRn can be used as an engineering strategy to improve the pharmacokinetic and pharmacodynamic properties of monoclonal antibodies. We tested whether the T250Q/M428L mutations, which improved the pharmacokinetics of humanized IgGs in the rhesus monkey, would translate to a pharmacokinetic benefit in both cynomolgus monkeys and mice when constructed on a different humanized IgG framework (anti-tumor necrosis factor-α (TNFα)). The T250Q/M428L anti-TNFα variant displayed an ~40-fold increase in binding affinity to cynomolgus monkey FcRn (C-FcRn) at pH 6.0, with maintenance of the pH binding dependence. We also constructed another anti-TNFα variant (P257I/Q311I) whose binding kinetics with the C-FcRn was similar to that of the T250Q/M428L variant. The binding affinity of the T250Q/M428L variant for murine FcRn was increased ~500-fold, with maintenance of pH dependence. In contrast to the interaction with C-FcRn, this interaction was driven mainly by a decrease in the rate of dissociation. Despite the improved in vitro binding properties of the anti-TNFα T250Q/M428L and P257I/Q311I variants to C-FcRn, the pharmacokinetic profiles of these molecules were not differentiated from the wild-type antibody in cynomolgus monkeys after intravenous administration. When administered intravenously to mice, the T250Q/M428L anti-TNFα variant displayed improved pharmacokinetics, characterized by an ~2-fold slower clearance than the wild-type antibody. The discrepancy between these data and previously reported benefits in rhesus monkeys and the inability of these mutations to translate to improved kinetics across species may be related to a number of factors. We propose extending consideration to differences in the absolute IgG-FcRn affinity, the kinetics of the IgG/FcRn interaction, and differences in the relative involvement of this pathway in the context of other factors influencing the disposition or elimination of monoclonal antibodies.

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Influence of FcRn Interaction on IgG Kinetics

Given the presumed mechanism of FcRn intracellular trafficking, the foundation of engineering strategies aimed at improving the pharmacokinetic characteristics of an antibody has been to increase the affinity of an IgG for FcRn at pH 6.0 while maintaining the pH dependence of the interaction (3, 4, 21, 22). Amino acid residues in the C_{14}2 and C_{14}3 domains of IgG Fc have been shown to be important to the pH dependence of the IgG/FcRn binding interaction (6, 12, 17, 22–25). Mutation of amino acid residues in this region has been the focus of a number of investigations attempting to characterize how modulating this interaction may influence antibody clearance mechanisms and the in vivo performance of therapeutic monoclonal antibodies (2–5, 21, 23–27). Several reports have suggested that Fc sequences or mutations in the Fc region associated with an increase in the affinity of the IgG/FcRn interaction at pH 6.0 directly relate to differences observed in the terminal phase half-life (t_{1/2}) of these antibodies in vivo (2–5, 23). However, results from a number of more recent investigations have provided data that appear to contradict this premise, putting into question whether a direct relationship exists between FcRn affinity and in vivo clearance (21, 22, 27). Given these discrepancies, the broader conclusions from these investigations remain equivocal. Adding to this ambiguity is the very limited data that have been published on the relevance of this receptor interaction to the in vivo kinetic properties of an antibody in primates. Recent reports have provided evidence that specific mutations (T250Q/M428L, M428L, and M525Y/S254T/T256E) in a humanized IgG1 and/or IgG2, which increased their affinity for FcRn at pH 6.0, resulted in a longer kinetic elimination phase half-life in either cynomolgus or rhesus monkeys (3, 4, 28). In a separate study, however, Fc mutations that imparted similar affinity improvements and in vitro binding properties to a humanized IgG1 did not translate to in vivo kinetic benefit in cynomolgus monkeys (21). Taken together, these data do not convincingly support the notion that a rational engineering strategy to positively influence the in vivo kinetics of a broad range of antibody therapeutics can be based on modulating characteristics of the IgG/FcRn interaction.

In this study, we tested whether the double Fc mutation (T250Q/M428L), which had been demonstrated previously to improve the pharmacokinetic properties of humanized IgGs in the rhesus monkey, would translate to a similar benefit when constructed on a different IgG framework. Fc variants were built on an anti-TNFα IgG1 antibody backbone with the intent of eliminating/limiting the influence of antigen binding on the properties to a humanized IgG1 did not translate to imparted similar affinity improvements and

**EXPERIMENTAL PROCEDURES**

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

Construction, Expression, and Purification of Recombinant Proteins—The P257I/Q311I antibody variant was derived from a humanized IgG1 Fc variant library created using a Kunkel-based strategy (29) described earlier for other anti-TNFα Fc variants (21). The T250Q/M428L variant was obtained by site-directed mutagenesis of the WT anti-TNFα clone using the QuikChange method (Stratagene) and confirmed by DNA sequencing. The WT, P257I/Q311I, and T250Q/M428L anti-TNFα 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 for other anti-TNFα antibodies (21).

Recombinant, soluble cynomolgus monkey FcRn (C-FcRn) and murine FcRn (M-FcRn) were expressed in 293EBNA cells transfected with the plasmids encoding for the soluble portion of each receptor species αFcRn and β₂-microglobulin, and the proteins were purified as described previously (21).

Isolation of Endogenous Cynomolgus Monkey and Murine IgG—Recombinant protein A-Sepharose (GE Healthcare) and protein G-Sepharose (GE Healthcare) were used to isolate IgG from preseed samples of cynomolgus monkey serum and CD-1 mouse plasma, respectively. Both protein A and G bind to each IgG subtype in the respective species. Briefly, columns were packed with either 2 ml of protein A- or protein G-Sepharose for isolation of primate or mouse IgG, respectively. Monkey serum or CD-1 mouse plasma (1 ml) was then allowed to flow over the column by gravity. The columns were washed with 1 mM potassium phosphate, 3 mM sodium phosphate, 0.150 M NaCl (pH 7.4) until the absorbance (A_{280}) returned to base line. Bound antibodies were eluted with 100 mM glycine (pH 3.2), and fractions were neutralized using 40 μl of 1 M Tris (pH 8.0) per ml of elution buffer. Fractions containing the antibodies were pooled and characterized by SDS-PAGE under reducing conditions. Coomassie Blue-stained gels indicated that the IgGs from both species were purified to greater than 95% homogeneity and displayed the characteristic heavy and light chain bands for each IgG. The purified IgGs were dialyzed into qualitatively and quantitatively similar to that of the anti-TNFα T250Q/M428L variant. However, the pharmacokinetic profiles of the anti-TNFα T250Q/M428L and P257I/Q311I were not differentiated from the wild-type (WT) anti-TNFα molecule after intravenous administration to cynomolgus monkeys. These observations suggest that engineering strategies to improve the in vivo kinetic performance of an IgG need to consider the FcRn interaction in appropriate context with other factors (biophysical properties, antigen load, glycosylation, and proteolytic stability), which may influence the disposition or elimination of a particular monoclonal antibody therapeutic.
PBS, and the samples were sterile-filtered (0.22 μm) and stored at 4 °C.

Quantification of the Endogenous (steady-state) IgG Concentrations in Cynomolgus Monkeys and CD-1 Mice—Total IgG levels in cynomolgus monkey serum and CD-1 mouse plasma were determined by ELISA using pre-dose samples from the animals used in the pharmacokinetic studies. Briefly, for determining the IgG levels in mouse or primate, each well of an Immulon 4 microtiter plate (Thermo Electron Corp.) was coated overnight at 4 °C with either 0.2 μg of rabbit anti-mouse IgG (heavy chain-specific; Zymed Laboratories Inc.) or donkey anti-human IgG (Fcy chain-specific; Jackson ImmunoResearch), respectively. 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. Dilutions (1:10,000–1:160,000) of cynomolgus monkey serum or mouse plasma samples were made in 1% (w/v) casein in PBS prior to addition to the appropriate wells. Bound mouse and monkey IgGs were detected using horseradish peroxidase-conjugated rabbit anti-mouse IgG (Zymed Laboratories Inc.) and goat anti-human IgG (Jackson ImmunoResearch), respectively. The goat anti-human IgG (Jackson ImmunoResearch) purified recombinant murine and human IgG1 were used to generate standard curves for IgG (heavychain-specific; Zymed Laboratories Inc.) or donkey anti-human IgG (Jackson ImmunoResearch), respectively. The goat anti-human IgG (Jackson ImmunoResearch) purified recombinant murine and human IgG1 were used to generate standard curves for determining the concentrations of endogenous IgG in CD-1 mice and cynomolgus monkeys, respectively. The dilutions for both the primate and murine samples displayed linearity indicating there was no assay interference with the reagents used for sample capture or detection. The concentration of isolated endogenous murine and cynomolgus monkey IgG was also determined by measuring the ultraviolet absorbance at 280 nm and using an extinction coefficient of 1.4 mg/ml for each IgG. The endogenous murine and cynomolgus IgG concentrations determined by ELISA and ultraviolet spectrophotometry were equivalent, indicating the ELISA reagents used to quantify the IgG concentrations reacted adequately with the murine and cynomolgus monkey IgG.

IgG/FcRn Binding Affinity Measurements with Surface Plasmon Resonance (BLAcore)—The interaction of WT anti-TNFα and the P257I/Q311I and T250Q/M428L variants with recombinant, immobilized human-FcRn, C-FcRn, and M-FcRn was monitored by surface plasmon resonance (SPR) detection using a BLAcore 2000 instrument (Biacore Inc.) as described previously (21). The interactions with isolated endogenous C-IgG and endogenous M-IgG were characterized in the same fashion. In short, C-FcRn, and M-FcRn were immobilized to flow cells 3 and 4, respectively, of a CM5 sensor chip using amine-coupling chemistry. The surface density of each receptor species was 275–350 response units. The first flow cell was used as a blank control surface lacking FcRn and was prepared similarly to the other flow cells (21). All binding experiments were performed with the WT and Fc variant IgGs dissolved in PBS (pH 6.0), 0.005% (v/v) Tween 20 over a concentration range of 0.0033 to 4 μM as described for previous studies (21). The binding data were obtained by subtracting the signal of flow cell 1 from the other two flow cells. Kinetic binding constants were determined through global fits of the average of two data sets collected on separate days using BIAevaluation, version 3.1. The kinetics (association and dissociation rates) were simultaneously fit to a heterogenous binding model to determine the equilibrium dissociation constant (Kd) value for each FcRn/IgG interaction (30). The data curves for each phase of the sensorgrams had low residuals and χ² values.

pH-dependent Dissociation ELISA for the WT, P257I/Q311I, and T250Q/M428L Anti-TNFα Antibodies—Biotinylated C-FcRn and M-FcRn for ELISAs were produced by reacting each purified soluble protein with EZ-Link™ Sulfo-NHS-Biotin (Pierce) using the conditions supplied by the vendor, and the FcRn:biotin ratio was measured as 1.0 and 1.0, respectively, using the EZ™ biotin quantitation kit (Pierce). The pH-dependent ELISA for the interaction of each receptor species with the WT, P257I/Q311I, and T250Q/M428L anti-TNFα antibodies was performed as described in earlier studies with other variant anti-TNFα IgGs (21). Optical density data were analyzed by the same four-parameter nonlinear regression fit as described previously (21) to determine the midpoint (pH 50, where 50% of the FcRn-antibody complexes dissociate) 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 6.0.

Cynomolgus Monkey Pharmacokinetic Studies—Two independent cynomolgus monkey pharmacokinetic studies were performed. In both studies, six male cynomolgus monkeys (2.8–3.8 kg) were assigned to one of two study groups. In the first study, each animal received a single intravenous dose of anti-TNFα WT or P257I/Q311I dissolved in PBS (pH 7.4) at 0.5 mg/kg. In the second study, each animal received a single intravenous dose of anti-TNFα WT or T250Q/M428L dissolved in PBS (pH 7.4) at 0.75 mg/kg. For both studies, blood samples were collected from the femoral vein prior to dosing and at 0.25, 0.5, 1, 3, 6, 12, 24, 48, 72, 96, 120, 168, 240, 312, 384, 456, and 528 h after administration of the dose. Blood samples were allowed to clot at ambient temperature prior to centrifugation to obtain serum.

Murine Pharmacokinetic Studies—Pharmacokinetic studies with anti-TNFα WT and T250Q/M428L were also conducted in male CD-1 mice (20–30 g) (Harlan, Indianapolis, IN). A single intravenous dose of the anti-TNFα WT or T250Q/M428L dissolved in PBS (pH 7.4) was administered via the tail vein at a dose level of 1 mg/kg. Blood samples were collected from three or four animals per treatment group per time point at 0.08, 0.25, 1, 6, 12, 24, 72, 96, 144, 168, 192, 216, 288, 360, and 432 h after administration. The samples were collected by saphenous vein or by tail clip into tubes containing potassium EDTA as anticoagulant and processed to plasma.

Bioanalytical Assays and Pharmacokinetic Data Analysis—Concentrations of the WT anti-TNFα and the P257I/Q311I and T250Q/M428L variants in cynomolgus monkey serum and mouse plasma were determined using validated anti-human IgG, and/or TNFα capture (R & D Systems) ELISAs described previously (21). The WT and T250Q/M428L standards were prepared in cynomolgus monkey serum or mouse plasma using a standard curve range of 0.78 to 50 ng/ml. The lower limit of quantitation (LLOQ) was defined as 2 ng/ml. The P257I/Q311I variant standards were prepared similarly to the other IgGs, using a curve range from 1.56 to 100 ng/ml. The LLOQ was
defined as 4 ng/ml. The standard curve range and LLOQ for each antibody was the same for both assay formats.

Pharmacokinetic parameters were calculated using the WinNonlin Professional (version 3.2) software package (Pharsight Corp., 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 area under the plasma concentration curve from zero to infinity (AUC∞,0), clearance, volume of distribution (Vd), and elimination half-life (t1/2).

Statistical Analyses—Statistical differences in pharmacokinetic parameters for the wild-type and Fc variants in cynomolgus monkey were determined using standard analysis of variance methods. In vitro binding data (KD and pH50) were also analyzed using analysis of variance. For mouse plasma concentration data, a linear mixed effect model was used, which included time-by-group and quadratic terms for time-by-group as fixed effects.

RESULTS

Table 1 Interaction of the Humanized Anti-TNFα Antibodies with C-FcRn

| Parameter   | Wild type | P257I/Q311I | T250Q/M428L |
|-------------|-----------|-------------|-------------|
| Kd (nM)     | 209 ± 11  | 2.6 ± 0.5   | 5.2 ± 0.1   |
| kcat/[Km] (1/s) | 0.22      | 6.5         | 0.61        |
| koff/[Km] (1/s) | 4.6      | 0.25        | 0.68        |
| pH50        | 6.3 ± 0.1 | 6.5 ± 0.2   | 6.3 ± 0.1   |
| % Bound pH 7.4 | 0.5 ± 3   | 7 ± 2       | 0.7 ± 0.1   |

* Data were reported previously (21).
* Values are p < 0.01 compared with wild type.
* Half-life of the IgG-FcRn complexes is at pH 6.0 (ln2/kd)/60.
* pH at which 50% of the IgG-FcRn complex is dissociated as determined from a four-parameter nonlinear regression fit of the ELISA data.
* Percentage of the total antibody in preformed complexes that remained FcRn-bound at pH 7.4 as determined by ELISA.

Table 2 Interaction of the Humanized Anti-TNFα Antibodies with M-FcRn

| Parameter   | Wild type | P257I/Q311I | T250Q/M428L |
|-------------|-----------|-------------|-------------|
| Kd (nM)     | 118 ± 7   | 4.7 ± 0.9   | 0.23 ± 0.05 |
| kcat/[Km] (1/s) | 0.39      | 1.3         | 0.28        |
| koff/[Km] (1/s) | 4.6      | 0.61        | 0.0065      |
| pH50        | 6.3 ± 0.1 | 7.3 ± 0.2   | 7.0 ± 0.1   |
| % Bound pH 7.4 | 6.3 ± 3   | 14 ± 2      | 14 ± 2      |

* Data were reported previously (21).
* Values are p < 0.01 compared with wild type.
* pH at which 50% of the IgG-M-FcRn complex is dissociated as determined from a four-parameter nonlinear regression fit of the ELISA data.
* Percentage of the total antibody in preformed complexes that remained FcRn-bound at pH 7.4 as determined by ELISA.

We previously reported the binding affinity (KD values) of the WT and P257I/Q311I antibodies for C-FcRn and M-FcRn (21). The KD values of the WT interaction with C-FcRn and M-FcRn was 209 ± 11 and 118 ± 7 nM, respectively (Tables 1 and 2). The T250Q/M428L and P257I/Q311I variants showed an increase in affinity to C-FcRn at pH 6.0 of ~40- and ~80-fold relative to the WT, respectively. The T250Q/M428L and P257I/Q311I variant also displayed ~500- and ~25-fold increases in binding affinity to M-FcRn relative to the WT antibody, respectively (Tables 1 and 2). Previously published data from our laboratory has indicated that IgGs in complexes that do not retain pH-dependent dissociation from FcRn are rapidly cleared from the circulation. We studied this parameter in vitro by forming IgG-receptor complexes at pH 6.0 and monitoring the degree of dissociation of the preformed complexes exposed to increasing pH values using an ELISA method described previously (21).
The preformed WT- and variant-C-FcRn complexes dissociated similarly and maintained pH-dependent dissociation characteristic of the Fc/FcRn interaction as indicated by the pH$_{50}$ values and the percent of antibody that remained bound to C-FcRn at pH 7.4 (Fig. 2 and Table 1). The pH$_{50}$ values of these interactions were $\sim$6.3–6.5, and the percentages of antibody that remained bound to C-FcRn was $\sim$0.5–7%. The complexes of WT and the T250Q/M428L variant with M-FcRn dissociate with pH$_{50}$ values of 6.6 and 7.0, respectively. Additionally, the >85% of the WT complexes dissociate from the M-FcRn at pH 7.4. However, the preformed M-FcRn-anti-TNFα antibody complexes have different pH$_{50}$ values. The pH$_{50}$ at which the complex of P257I/Q311I with M-FcRn dissociates with a pH$_{50}$ value near neutral pH (pH 7.3), with approximately $\sim$50% remaining associated at pH 7.4 (Fig. 2 and Table 2). As expected, the P257I/Q311I variant did clear very rapidly in vivo (not shown), and the murine in vivo results are not discussed further in this report.

The enhanced affinity of the variants for C-FcRn relative to the WT were predominantly driven by increased association kinetics ($k_{on}$ values) with modest influences on rates of dissociation ($k_{off}$ values) (Table 1). In contrast, the increased affinity of the T250Q/M428L variant for M-FcRn at pH 6 was attributed exclusively to a slower rate of dissociation (Table 2). The increased affinity of P257I/Q311I variant was attributable to effects on both the rate of association and dissociation (Table 2). The improved affinity of these variant IgG complexes is consistent with previous reports indicating the involvement of the C$_4$2 and C$_4$3 Fc regions in the interaction of IgG with FcRn (6, 12, 17, 18, 21, 22, 24, 31–36). At the highest IgG concentration tested for pH 6.0 binding ($\sim$15 nM), neither the WT nor variant anti-TNFα mAbs bound at measurable levels to any species of FcRn at pH 7.4 (data not shown), indicating that these mutations did not influence the characteristic pH binding dependence of the FcRn/IgG interaction (22).

**Endogenous IgG Levels and Binding Affinities for FcRn**—The influence of the concentration of endogenous IgG and their binding affinities for FcRn has generally not been considered in relation to engineering strategies aimed at modulating the pharmacokinetic properties of therapeutic mAbs through the FcRn interaction. Characterizing these factors may provide insight to help explain species differences in in vivo properties of a mAb or binding characteristics necessary to achieve benefit.

The concentrations of endogenous IgG in the circulation of cynomolgus monkeys and CD-1 mice were determined to be $\sim$11 and 0.2 mg/ml, respectively (Table 3). After purification by affinity chromatography from serum or plasma, the binding affinity and interaction kinetics of the C-IgG and M-IgG were measured using SPR with C-FcRn and M-FcRn, respectively (Fig. 3). The C-IgG bound C-FcRn with an affinity of 132 $\pm$ 6 nM (Table 3), which is $\sim$2-fold better than that of the WT anti-TNFα molecule interaction with C-FcRn ($K_a$, WT-C-FcRn $\sim$200 nM) (Tables 1 and 3). The C-IgG was also kinetically different, displaying rates of association and dissociation $\sim$9- and $\sim$15-fold slower than the WT anti-TNFα antibody (Tables 1 and 3). In addition, whereas the variant IgGs bound C-FcRn with $\sim$25–50-fold greater affinity than C-IgG, the dissociation kinetics of the variant-C-FcRn complexes were $\sim$5–10 times faster than for the C-IgG/C-FcRn interaction (Tables 1 and 3).

M-IgG bound to M-FcRn with an affinity of 243 $\pm$ 15 nM (Table 3), which is $\sim$2-fold weaker than the affinity of WT anti-TNFα for M-FcRn (Table 3). Similar to the C-IgG/C-FcRn interaction, the M-IgG/M-FcRn complexes also display slowed association ($\sim$50-fold) and dissociation ($\sim$24-fold) kinetics relative to the WT anti-TNFα/M-FcRn interaction. The anti-TNFα variants bound M-FcRn with $\sim$50–1000-fold higher affinity than the M-IgG; the P257I/Q311I variant had an $\sim$50-fold increase in affinity for M-FcRn relative to M-IgG, driven predominantly by an increased rate of association ($\sim$167-fold increase).

### TABLE 3

**Interaction of endogenous cynomolgus monkey IgG (C-IgG) and murine IgG (M-IgG) with homologous species FcRn**

|                | C-IgG/C-FcRn | M-IgG/M-FcRn |
|----------------|--------------|--------------|
| [IgG]$_a$ (mg/ml) | 11.3         | 0.2          |
| $K_a$ (nM)      | 132 $\pm$ 6  | 243 $\pm$ 15 |
| $k_{on}$ ($10^4$/s) | 25           | 7.8          |
| $k_{off}$ ($10^{-3}$/s) | 3.3          | 1.9          |
| $t_{1/2}$ (min) | 3.5          | 6.1          |

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*a Endogenous IgG concentration in serum or plasma was determined by ELISA.

*b $K_a$ ($k_{on}$/($k_{on} + k_{off}$)) indicates data determined from kinetic analysis of three independent binding experiments (mean $\pm$ S.D.).

Half-life of the IgG-FcRn complexes is at pH 6.0 ($\ln(2)/k_{off}/60$).
greater). The T250Q/M428L variant bound to M-FcRn with an ~1000-fold higher affinity than M-IgG contributed to both an increased association rate (~36-fold) and slowed dissociation rate (~30-fold).

Pharmacokinetics of Anti-TNFα WT and Fc Variant Antibodies in Cynomolgus Monkeys—The relative improvement in FcRn binding affinity imparted to the anti-TNFα mAb by the T250Q/M428L mutations was similar to that reported previously with a different monoclonal antibody (3, 4). We also observed a similar affinity enhancement and effects on binding kinetics with a second set of mutations (P257I/Q311I). Although we showed translation of the effect of the T250Q/M428L mutations on in vitro affinity, we wanted to test the hypothesis proposed in earlier work (3, 4) that these specific mutations could generically result in pharmacokinetic benefit when transferred to a variety of antibody backbones. Along the same lines, we also wanted to understand the in vivo influence of the P257I/Q311I mutations, which resulted in similar in vitro FcRn binding characteristics as T250Q/M428L mutations. To evaluate these questions, we studied the pharmacokinetics of the two variants and the WT anti-TNFα IgG1 in cynomolgus monkeys.

After intravenous administration of 0.5 or 0.75 mg/kg, the WT and two variant mAbs were cleared from the circulation in a biphasic manner. The serum profiles of the WT and variant mAbs were very similar to each other (Fig. 4). No apparent

![FIGURE 3. BIAcore sensorgrams of the interaction of endogenous cynomolgus monkey (A) and murine (B) IgG with C-FcRn and M-FcRn, respectively. Sensorgrams display the response values for two measurements at the antibody concentrations of 0.0033 to 4.0 μM.](image)

![FIGURE 4. Pharmacokinetic profiles of the WT anti-TNFα (B) and the P257I/Q311I (C) and T250Q/M428L (D) variants in cynomolgus monkeys. Antibodies were administered as a single intravenous (IV) injection of 0.5 (A) and 0.75 mg/kg (B), respectively. Serum concentrations were determined using a validated TNFα-capture ELISA. Data are the mean ± S.D. of three animals/group.](image)

**TABLE 4**

| Dose (mg/kg) | Wild type | P257I/Q311I | Wild type | T250Q/M428L |
|--------------|-----------|-------------|-----------|-------------|
| C<sub>max</sub> (μg/ml) | 20 ± 14 | 27 ± 11 | 32 ± 10 | 34 ± 21 |
| AUC<sub>0-∞</sub> (μg/ml) | 484 ± 118 | 553 ± 61 | 943 ± 274 | 977 ± 579 |
| CL (ml/h/kg) | 1.1 ± 0.3 | 0.9 ± 0.1 | 0.8 ± 0.2 | 0.9 ± 0.4 |
| V<sub>s</sub> (ml/kg) | 115 ± 21 | 115 ± 19 | 138 ± 27 | 147 ± 71 |
| t<sub>1/2</sub> (h) | 144 ± 18 | 114 ± 9 | 121 ± 16 | 112 ± 11 |

* Data were reported previously (21).
influence of differential proteolysis or by complexation with the target antigen. 

**Pharmacokinetics of Anti-TNFα WT and T250Q/M428L Variant in Normal Mice—**The pharmacokinetic behavior of the anti-TNFα WT and T250Q/M428L variant was examined in mice to investigate whether the differences observed in the in vitro binding with M-FcRn correlated with improved kinetics (Fig. 5).

The WT and T250Q/M428L anti-TNFα antibodies were administered as a single intravenous dose of 1 mg/kg. The antibodies were cleared from the circulation in a biphasic manner (Fig. 5). Differences were noted in the clearance and volume of distribution of the two antibodies (Table 5). The clearance of the T250Q/M428L variant was −2.3-fold slower and its distribution volume −2-fold smaller than the WT. The terminal phase $t_{1/2}$ (dissociation half-life) of the WT and variant antibody was comparable, 15.3 versus 16.7 days (Table 5).

**DISCUSSION**

Studies in FcRn knock-out mice have clearly demonstrated the role of the FcRn receptor system in regulating the persistence of endogenous and exogenously administered IgG (14, 37, 38). In addition, mutations in the Fc region that eliminate binding of IgG to FcRn dramatically increase the clearance of an IgG in vivo (17, 39). From a pragmatic perspective, these observations make this receptor interaction an obvious target by which to engineer therapeutic antibodies for improved pharmacokinetic and pharmacodynamic properties. Current data suggest that eliminating or substantially decreasing the influence of this receptor pathway leads to a marked negative impact on the clearance and $t_{1/2}$ of antibodies (14, 37, 38, 40, 41). However, inconsistencies reported in the literature make it less apparent that modulating this receptor system can be used conclusively as an approach to improve the in vivo pharmacokinetic properties of an IgG (21, 22, 27). In this context, to implement or validate such a strategy, it is important to translate observations made in rodent systems to that of a higher species such as primate. Until recently, there had been no published information describing the role of FcRn in regulating IgG levels or the influence of Fc engineering on IgG pharmacokinetics in primates. In recent studies, Hinton et al. (3, 4) engineered a single and double mutation (M428L and T250Q/M428L) into the Fc region of a humanized IgG1 and IgG2 (OST-577). These mutations resulted in improved pharmacokinetic properties, characterized by a slower clearance (2–2.5-fold) and increased $t_{1/2}$ (2–2.5-fold), when administered to rhesus monkeys by intravenous injection. The molecules with the T250Q/M428L mutation demonstrated binding increases of 28–29-fold relative to the WT OST-577 molecules, using a competitive binding format with human FcRn at pH 6.0 (3, 4). The single mutant, M428L, demonstrated binding increases of 8–11-fold in vitro and a similar pharmacokinetic profile as the T250Q/M428L variant in the rhesus monkey (only tested on an IgG2 backbone; the IgG1 version of M428L was not tested in animals) (3). In addition, both the M428L and T250Q/M428L variants maintained the characteristic pH dependence of binding and pH-related complex dissociation (3). These observations led to the broader speculation that a range of antibodies, subtypes, and Fc fusion proteins could be engineered to have longer serum half-lives by transferal of this particular mutation to these molecules (3). Interestingly, although transferal of the T250Q/M428L mutations to our anti-TNFα also imparted a 40-fold increase in FcRn binding at pH 6.0 with maintenance of the characteristic pH dependence of binding and pH-related complex dissociation (Table 1), we were not able to demonstrate pharmacokinetic benefit in cynomolgus monkeys (Fig. 4 and Table 4). Similarly, a second anti-TNFα variant (P257I/Q311I) that demonstrated the same magnitude of increase in in vitro affinity and binding characteristics as the anti-TNFα T250Q/M428L variant did not show pharmacokinetic benefit in cynomolgus monkeys (Fig. 4 and Table 4). These results conflict with the concept that increasing the affinity of an IgG for FcRn at pH 6.0 necessarily translates to improved in vivo properties. The data strongly suggest that the in vivo benefit resulting from the T250Q/M428L sequence changes is not universally transferable to other IgGs. In addition, these observations bring into question the concept that a common set of mutations can be used to impart pharmacokinetic benefit to a broad range of therapeutic antibodies.

It is not entirely clear as to why Fc mutations that impart similar in vitro FcRn binding characteristics to an IgG do not result in similar changes to in vivo pharmacokinetic properties.
In our study, the in vivo clearance and elimination $t_{1/2}$ of the WT sequence anti-TNFα in cynomolgus monkeys were 8-fold faster and 2.5-fold shorter, respectively, than that determined for the WT OST-577 in rhesus monkeys (3) (Fig. 4 and Table 4). This observation could be related to differences in mechanisms involved in the clearance and elimination of these two antibodies. A plausible explanation for the discrepancy in the effect of the Fc changes is that recycling via FcRn is only one of several processes, or is not the predominant mechanism, involved in regulating the clearance of the anti-TNFα mAbs in primates. In the situation where other prevalent mechanisms influence the clearance of a mAb, it is possible that improvements in FcRn binding affinity may not always result in beneficial effects on in vivo properties. On the other hand, if the more favorable baseline in vivo properties of WT OST-577 or another mAb were due to a primary role of FcRn recycling, its in vivo kinetic behavior may be more sensitive to sequence alterations that affect receptor affinity. Because the WT anti-TNFα cleared more slowly and had a longer $t_{1/2}$ in the mouse than in primate (Tables 4 and 5), this mechanism would also be consistent with the observed beneficial effect of the T250Q/M428L anti-TNFα mutation on in vivo clearance in mice (Fig. 5 and Table 5). Given that there is no evidence to indicate differences in the proteolytic stability, blood cell binding, or physical characteristics (i.e., aggregation) of the WT or variant anti-TNFα molecules that could potentially modulate clearance (data not shown), it is not clear what alternative mechanisms could be involved in clearance in the primate. Because TNFα exists as a very small pool in normal animals (26, 42, 43), it is unlikely that clearance is driven by binding to antigen or clearance of antigen-antibody complex. However, the possibility that a nonspecific binding mechanism contributes disproportionately to the clearance of the anti-TNFα mAb in primates and masks the potential benefit of improved FcRn binding cannot be excluded.

The current set of data together with other recently published observations (21, 22, 27) indicate that, at least from an antibody engineering perspective, a generalized relationship between FcRn binding affinity and improved in vivo properties is difficult to establish. In this regard, further investigation of other sequence changes, such as the M252Y/S254T/T256E mutations (28), that have been demonstrated to provide pharmacokinetic improvement to specific IgG platforms is warranted. In relation to the M252Y/S254T/T256E mutations, a humanized IgG, Fc variant, MEDI-524-M252Y/S254T/T256E, demonstrated an ~10-fold enhanced FcRn binding affinity relative to the WT molecule at pH 6.0 (28). Although the observed fold increase in binding affinity of MEDI-524-M252Y/S254T/T256E for the primate receptor was less than that reported previously for other IgG variants (21, 22), the serum half-life increase of ~4-fold for this variant IgG constitutes the greatest in vivo kinetic benefit presently described in the literature (28). Together, the observations in these reports (3, 4, 21, 22, 28) suggest that some fundamental characteristic of antibody clearance may be influencing the in vivo outcome of engineering strategies aimed at modulating the IgG/FcRn interaction.

Although the lack of pharmacokinetic correlation is very striking in the case of our mAbs, the M428L and T250Q/M428L variants of OST-577 were also indistinguishable from each other pharmacokinetically despite a 3–4-fold difference in their affinities for rhesus FcRn (3). However, the ability of Fc mutations that enhance affinity of FcRn binding to translate to improved in vivo properties may also be related to the base-line affinity of an Fc sequence for FcRn. In this study, the WT anti-TNFα mAb was determined to have a $K_D$ of ~100–200 nM, similar to the range that has been reported for the binding of other IgGs to FcRn (21, 22, 34, 41). It is conceivable that relative to the base-line affinity the binding improvements (~40-fold) we imparted are not large enough to influence the in vivo behavior of this mAb in primates. The WT OST-577 molecule had better in vivo kinetics in the rhesus monkey, which could be consistent with it having a much higher affinity for FcRn. Unfortunately, the $K_D$ values of WT or variants of OST-577 for rhesus monkey FcRn were not reported because of the assay format employed (3, 4). Additionally, although the FcRn binding affinity was reported for MEDI-524-M252Y/S254T/T256E, the FcRn binding kinetics were not (28). Also, it is difficult to compare the binding affinity determined for MEDI-524 (reported in the micromolar range) in the context of our observations, because of the methodological differences in the assay formats (28). In relation to increased affinity, however, changes that produce a similar fold binding increase could translate to improved pharmacokinetic properties, particularly if FcRn recycling was the predominant modulator of clearance. This interpretation would not be inconsistent with the much greater binding affinity enhancement (~500-fold) necessary to translate to the in vivo benefit observed with the T250Q/M428L anti-TNFα variant in mice (Table 2).

The influence of the absolute affinity of an IgG for FcRn is also important to consider in the context of the concentration of endogenous IgGs and their relative FcRn binding affinities. In cynomolgus monkey, we determined that the endogenous IgGs had an overall affinity for FcRn that was ~2-fold higher than that of the WT anti-TNFα mAb, and the circulating concentrations were determined to be in the range of 11 mg/ml (Table 3). In the circumstance where endogenous IgG concentrations are in at least 1000-fold excess, it may be difficult to observe the potential in vivo effects with the extent of affinity improvements imparted against C-FcRn. In contrast, the endogenous murine IgG had an overall decreased affinity (~2-fold) for M-FcRn relative to that of the WT anti-TNFα mAb, and its concentrations were determined to be the range of 0.2 mg/ml (Table 3). It seems logical that the lower endogenous IgG concentrations coupled with the lower FcRn affinity in mice would favor demonstration of in vivo benefit particularly in combination with the more marked affinity improvement of the T250Q/M428L anti-TNFα variant seen against M-FcRn. As discussed previously, although the P257I/Q311I displayed increased affinity for M-FcRn, it was rapidly cleared from the circulation in mice because of its lack of dissociation at neutral pH once it binds FcRn (21).

Regarding the impact of FcRn recycling on the in vivo performance of an IgG, it may also be more important to consider the interaction of IgG and FcRn in terms of dissociation rates (21). In combination with affinity, the rate of dissociation of the FcRn-IgG complexes after formation at endosomal pH may be a factor determining the proportion of mAb processed through
the recycling or degradative pathways within the cell, and thus related to in vivo properties. In the case of the variant anti-TNFα mAbs, the improved $K_D$ value for C-FcRn was predominantly a function of increases in association rate (~30-fold) with only modest decreases observed in rates of dissociation (~1.4–3-fold) (Table 1). The differences we observed in the dissociation rate of the variant and WT anti-TNFα mAbs may not be large enough to result in distinguishable alterations in pharmacokinetic characteristics. Because binding kinetics were not determined, it is impossible to establish whether the association or dissociation rate was more significantly affected by the T250Q/M428L mutations. It is also plausible that affinity or kinetic differences could be driven through conformational influences of different Fab regions.

In summary, the observations in this report suggest that it may be difficult to define a common set of parameters that can be used to guide the optimization of the pharmacokinetic properties of a broad range of therapeutics monoclonal antibodies. Because 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 likely that many factors in addition to FcRn-mediated antibody recycling will need to be considered when designing such engineering strategies.

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REFERENCES

1. Kim, S. J., Park, Y., and Hong, H. J. (2005) Mol. Cells 20, 17–29
2. Ghetie, V., Popov, S., Borvak, J., Rudu, C., Matesoi, D., Medesan, C., Ober, R. J., and Ward, E. S. (1997) Nat. Biotechnol. 15, 637–640
3. Hinton, P. R., Johifs, M. G., Xiong, J. M., Hanestad, K., Ong, K. C., Bullock, C., Keller, S., Tang, M. T., Tso, J. Y., Vasquez, M., and Tsurushita, N. (2004) J. Biol. Chem. 279, 6213–6216
4. Hinton, P. R., Xiong, J. M., Johifs, M. J., Tang, M. T., Keller, S., and Tsurushita, N. (2006) J. Immunol. 176, 346–356
5. Medesan, C., Cianga, P., Mummert, M., Stanescu, D., Ghetie, V., and Ward, E. S. (1998) Eur. J. Immunol. 28, 2092–2100
6. Vaughn, D. E., Milburn, C. M., Penny, D. M., Martin, W. L., Johnson, J. L., and Bjorkman, P. J. (1997) J. Mol. Biol. 274, 597–607
7. Ober, R. J., Martinez, C., Lai, X., Zhou, J., and Ward, E. S. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 11076–11081
8. Ober, R. J., Martinez, C., Vaccaro, C., Zhou, J., and Ward, E. S. (2004) J. Immunol. 172, 2021–2029
9. Ward, E. S., Zhou, J., Ghetie, V., and Ober, R. J. (2003) Int. Immunol. 15, 187–195
10. Cianga, P., Medesan, C., Richardson, J. A., Ghetie, V., and Ward, E. S. (1999) Eur. J. Immunol. 29, 2515–2523
11. Cianga, P., Cianga, C., Cosma, L., Ward, E. S., and Carasevici, E. (2003) Hum. Immunol. 64, 1152–1159
12. Medesan, C., Radu, C., Kim, J. K., Ghetie, V., and Ward, E. S. (1996) Eur. J. Immunol. 26, 2533–2536
13. Wallace, K. H., and Rees, A. R. (1980) Biochem. J. 188, 9–16
14. Ghetie, V., Hubbard, J. G., Kim, J. K., Tsien, M. F., Lee, Y., and Ward, E. S. (1996) Eur. J. Immunol. 26, 690–696
15. Ghetie, V., and Ward, E. S. (1997) Immunol. Today 18, 592–598
16. Junghans, R. P., and Anderson, C. L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5512–5516
17. Kim, J. K., Firan, M., Radu, C. G., Kim, C. H., Ghetie, V., and Ward, E. S. (1999) Eur. J. Immunol. 29, 2819–2825
18. Raghavan, M., Bonagura, V. R., Morrison, S. L., and Bjorkman, P. J. (1995) Biochemistry 34, 14649–14657
19. Raghavan, M., and Bjorkman, P. J. (1996) Annu. Rev. Cell Dev. Biol. 12, 181–220
20. Rodewald, R. (1976) J. Cell Biol. 71, 666–669
21. Datta-Mannan, A., Witcher, D. R., Tang, Y., Watkins, J., and Wroblewski, V. J. (2007) Drug Metab. Dispos. 35, 1–9
22. Dall’Acqua, W. F., Woods, R. M., Ward, E. S., Palaszynski, S. R., Patel, N. K., Brewah, Y. A., Wu, H., Kiener, P. A., and Langermann, S. (2002) J. Immunol. 169, 5171–5180
23. Kim, J. K., Tsien, M. F., Ghetie, V., and Ward, E. S. (1994) Eur. J. Immunol. 24, 542–548
24. Raghavan, M., Chen, M. Y., Gastinel, L. N., and Bjorkman, P. J. (1994) Immunity 1, 303–315
25. Sinistre, N. E., and Mostow, K. E. (1989) Nature 337, 184–187
26. Verderi, F., Aujoulat, M., Condeyaux, F., and Descotes, J. (1995) Toxicology 105, 81–90
27. Gurbaxani, B., Dela Cruz, L. L., Chintalacharuvu, K., and Morrison, S. L. (2006) Mol. Immunol. 43, 1462–1473
28. Dall’Acqua, W. F., Kiener, P. A., and Wu, H. (2006) J. Biol. Chem. 281, 23514–23524
29. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 154, 367–382
30. Martin, W. L., and Bjorkman, P. J. (1999) Biochemistry 38, 12639–12647
31. Burmeister, W. P., Gastinel, L. N., Simister, N. E., Blum, M. L., and Bjorkman, P. J. (1997) Nature 382, 336–343
32. Burmeister, W. P., Huber, A. H., and Bjorkman, P. J. (1994) Nature 372, 379–383
33. Martin, W. L., West, A. P., Jr., Gan, L., and Bjorkman, P. J. (2001) Mol. Cell 7, 867–877
34. Shields, R. L., Namenuk, A. K., Hong, K., Meng, Y. G., Rae, J., Briggs, J. X., D., Lai, J., Stallan, A., Li, B., Fox, J. A., and Presta, L. G. (2001) J. Biol. Chem. 276, 6591–6604
35. Vaughn, D. E., and Bjorkman, P. J. (1998) Structure (Lond.) 6, 63–73
36. West, A. P., Jr., and Bjorkman, P. J. (2000) Biochemistry 39, 9698–9708
37. Christianson, G. J., Brooks, W., Vekasi, S., Manolfi, E. A., Niles, J., Roope-nian, S. L., Roths, J. B., Rothlein, R., and Roopenian, D. C. (1997) J. Immunol. 159, 4781–4792
38. Israel, E. J., Patel, V. K., Taylor, S. F., Marshak-Rothstein, A., and Simister, N. E. (1995) J. Immunol. 154, 6246–6251
39. Firan, M., Bawdon, R., Rudu, C., Ober, R. J., Eaken, D., Antohe, F., Ghetie, V., and Ward, E. S. (2001) Int. Immunol. 13, 993–1002
40. Vaccaro, C., Zhou, J., Ober, R. J., and Ward, E. S. (2005) Nat. Biotechnol. 23, 283–1288
41. Wani, M. A., Haynes, L. D., Kim, J., Bronson, C. L., Chaudhury, C., Mo-hanty, S., Waldmann, T. A., Robinson, J. M., and Anderson, C. L. (2006) Nat. Protoc. 1, 1058–1061
42. Raponi, G., Keller, N., Rozenberg-Arksa, M., Lun, M. T., Mancini, C., and Verhoef, J. (1993) Infect. Immun. 61, 3976–3980
43. Reuben, S., Sumi, M. G., Mathai, A., Nair, M. D., and Radhakrishnan, V. V. (2003) Neurol. India 51, 487–489