Effects of altered FcγR binding on antibody pharmacokinetics in cynomolgus monkeys

Maya K Leabman*, Y Gloria Meng†, Robert F Kelley*, Laura E DeForge*, Kyra J Cowan‡, and Suhasini Iyer*

†Department of Pharmacokinetics and Pharmacodynamics; Genentech, Inc; San Francisco, CA USA; ‡Department of Biochemical and Cellular Pharmacology; Genentech, Inc; San Francisco, CA USA; *Department of Antibody Engineering; Genentech, Inc; San Francisco, CA USA; †Department of BioAnalytical Sciences; Genentech, Inc; San Francisco, CA USA

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Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; AF, afucosylated; anti-LTα, anti-lymphotoxin alpha; ATA, anti-therapeutic antibodies; AUC, exposure; CHO, Chinese hamster ovary; CL, clearance; DIG, digoxigenin; ELISA, enzyme linked immunosorbent assay; Fab, fragment antigen binding; Fc, fragment crystallizable; FcRn, neonatal Fc receptor; FcγRs, Fcγ receptors; GST, glutathione S-transferase; HRP, horseradish peroxidase; IgG1, immunoglobulin G1; LALA substitution, L234A/L235A substitution; mAb, monoclonal antibody; MSD, Meso Scale Discovery; PK, pharmacokinetics; T1/2, terminal half-life; TMB, 3,3',5,5' tetramethyl benzidine; WT, wild type

Antibody interactions with Fcγ receptors (FcγRs), like FcγRⅢA, play a critical role in mediating antibody effector functions and thereby contribute significantly to the biologic and therapeutic activity of antibodies. Over the past decade, considerable work has been directed towards production of antibodies with altered binding affinity to FcγRs and evaluation of how the alterations modulate their therapeutic activity. This has been achieved by altering glycosylation status at N297 or by engineering modifications in the crystallizable fragment (Fc) region. While the effects of these modifications on biologic activity and efficacy have been examined, few studies have been conducted to understand their effect on antibody pharmacokinetics (PK). We present here a retrospective analysis in which we characterize the PK of three antibody variants with decreased FcγR binding affinity caused by amino acid substitutions in the Fc region (N297A, N297G, and L234A/L235A) and three antibody variants with increased FcγRⅢA binding affinity caused by afucosylation at N297, and compare their PK to corresponding wild type antibody PK in cynomolgus monkeys. For all antibodies, PK was examined at a dose that was known to be in the linear range. Since production of the N297A and N297G variants in Chinese hamster ovary cells results in aglycosylated antibodies that do not bind to FcγRs, we also examined the effect of expression of an aglycosylated antibody, without sequence change(s), in E. coli. All the variants demonstrated similar PK compared with that of the wild type antibodies, suggesting that, for the six antibodies presented here, altered FcγR binding affinity does not affect PK.

Introduction

Monoclonal antibodies (mAbs) are a rapidly growing class of therapeutics. Approximately 30 antibodies have been marketed in the European Union and United States for use in a variety of indications, and it is estimated that hundreds more are in clinical development.1 The success of antibodies as a class of therapeutics is due in large part to their ability to bind with high affinity and specificity to their target antigen, thereby eliciting targeted and specific responses.

Therapeutic antibodies are most commonly based on the immunoglobulin G1 (IgG1) isotype that is composed of two identical heavy chains and two identical light chains.2 The heavy and light chains assemble via covalent and non-covalent interactions to form a structure consisting of three functional domains: two antigen-binding domains (referred to as FabS) and one constant crystallizable fragment (Fc) domain. While the Fab domains confer antigen specificity, the Fc region mediates intrinsic biologic properties via interaction with Fc receptors, e.g., antibody transport, clearance (CL), effector functions. The neonatal Fc receptor (FcRn) is the primary Fc receptor involved in antibody transport and homeostasis. Fc gamma receptors (FcγRs) play a critical role in eliciting antibody effector functions like antibody-dependent cell-mediated cytotoxicity (ADCC), and thus ultimately contribute significantly to the biologic and therapeutic activity of many antibodies. FcγRs also play a role in the elimination of immune complexes, via binding and internalization by phagocytic cells.3

Considerable work has been done to alter antibody interactions with FcγRs in order to modulate therapeutic activity. Antibodies are complex glycoproteins that contain a ubiquitous N-linked glycan at position N297 of the Fc domain (Eu numbering).
Studies have demonstrated that altering glycosylation at N297 can modify interactions with FcγRs and thereby affect antibody effector functions.5,6 While the absence of the glycan at N297 abolishes binding to FcγRs and antibody effector functions, specific glycosylation patterns at N297, notably absence of the core fucose (afucosylation), can increase binding affinity to FcγRIIIA and, thereby, increase effector functions like ADCC activity.7,8

In cases where antibody effector functions may not be necessary (i.e., neutralizing, agonistic or antagonistic mAbs) or may be undesirable (e.g., to prevent unwanted killing of non-target cells),9 antibodies with altered FcγR binding can be developed by engineering the Fc region or via production in specific hosts or cell lines. For example, in addition to the well-documented amino acid substitutions at position N297 that remove glycosylation at this position and abolish FcγR binding, studies have demonstrated that the double substitution, L234A/L235A (LALA), greatly reduces binding to FcγRs.10,11 Aglycosylated antibodies produced in E. coli also have minimal binding to FcγRs and can provide a simplified and more economical antibody production platform.12,13 In cases where enhanced effector function is a desired property of the therapeutic antibody, afucosylated (AF) or reduced fucose-containing antibodies that increase affinity between the Fc region and FcγRIIIA have been produced in engineered cell lines or by using defucosylating enzymes.2

With the increased development of antibodies with altered glycosylation status and FcγR binding, it is important to better understand the effect of these modifications on pharmacokinetics (PK). Several studies have been conducted to improve understanding of the relationship between glycosylation at N297 and PK.14-19 Much of the PK data gathered to date, however, was obtained from rodents and may not be translatable to humans because of the differences in human IgG binding to rodent vs. human FcγRs.12 Lastly, the effect of other modifications that alter FcγR binding affinity, such as aglycosylation, afucosylation or Fc substitutions, has not been well-characterized.

Here, we present a comprehensive analysis of the effect of altered FcγR binding affinity on PK. For all antibodies, PK was assessed at doses in the linear range, where target-independent mechanisms (i.e., FcRn) dominate clearance. Our report includes PK data in cynomolgus monkeys for a number of different antibody variants with altered binding affinity to FcγRs. This includes data on multiple aglycosylated antibodies developed by engineering at the N297 position, aglycosylated antibodies produced in E. coli, an antibody with a LALA substitution, and AF antibodies. Together, our data suggests that changes in FcγR binding affinity cause minimal effects in antibody PK in cynomolgus monkeys.

### Results
We examined the PK of six antibodies with altered FcγR binding affinity following single or multiple intravenous (IV) doses in cynomolgus monkeys. The antibodies targeted different antigen types, including highly expressed multi-transmembrane receptors (e.g., anti-CD20 2H7.v16),10 soluble cytokines (e.g., anti-lymphotoxin α (anti-LTα)),21 and cell surface proteins and ligands (e.g., anti-X, anti-Y and anti-OX40L).22 One of the antibodies studied, anti-gD, targets a viral envelope protein and has no cross-reactive target in cynomolgus monkeys.

**FcγR binding affinity of variants.** Using antibodies that are either the same or representative of the antibodies used in the PK studies, we assessed the binding affinity of each of our variants to human FcγRs (FcγRI, FcγRIIA, FcγRIIB, and FcγRIIIA) using an in vitro enzyme linked immunosorbent assay (ELISA). For FcγRIIIA, binding affinity to both the F158 and V158 polymorphic forms was assessed. The relative binding affinities of each variant to human FcγRs compared with that of a wild type (WT) or IgG1 isotype control antibody are shown in Table 1.

Consistent with previous reports,5,10,11 the N297A and LALA variants exhibited minimal or no measurable binding to human FcγRs (Table 1). Because this is the first report of an N297G variant, we assessed binding of the N297G variant to all cynomolgus monkey FcγRs and no measurable binding to human FcγRs (data not shown) and human FcγRs (Fig. 1). These data demonstrate that antibodies binding affinity to the N297G variant to the human or cynomolgus FcγRs and no measurable ADCC or complement-dependent cytotoxicity activity (data not shown). Neither the N297A nor N297G substitutions significantly affected FcRn binding (Table S1).

Since it is well-documented that aglycosylated antibodies produced in E. coli do not bind to FcγRs, FcγR binding affinity of the WT anti-Y expressed in E. coli was not included here.12,13 As shown in Table 1, the AF variants (2H7.v16 AF and anti-LTα AF) had increased binding affinity to both the F158 and V158 forms of FcγRIIIA. The relative binding affinity of the AF variants to FcγRIIIA-F158 was ~15–46 times greater than that of control, and the relative binding affinity to FcγRIIIA-V158 was ~6–12 times greater than that of control. Anti-X, another AF

### Table 1. Relative binding affinity of antibody variants to human FcγRs

| Antibody     | Antibody Type | hFcγRI | hFcγRIIA (H131) | hFcγRIIA (R131) | hFcγRIIB | hFcγRIIIA (F158) | hFcγRIIIA (V158) |
|--------------|---------------|--------|----------------|----------------|-----------|----------------|----------------|
| Anti-OX40L   | N.S.          | N.S.   | 0.035          | 0.19           | 0.074     | 0.014          |
| Anti-Y      | N297A         | N.S.   | N.S.          | N.S.           | N.S.      | N.S.           |
| Anti-gD      | N297G         | N.S.   | N.S.          | N.S.           | N.S.      | N.S.           |
| 2H7.v16     | AF            | 1.1    | 0.84          | N.S.           | 1.8       | 46             | 12             |
| Anti-LTα     | AF            | 1.0    | 1.0           | N.S.           | 1.1       | 15             | 5.9             |

AF, afucosylated; N.S., no measurable binding. *Binding affinity expressed relative to wild type (WT) control (i.e., WT anti-OX40L, WT 2H7.v16 or WT anti-LTα). Binding affinity expressed relative to wild type (WT) isotype control (Trastuzumab). Relative binding affinity of N297G variant was assessed on 2H7.v16 backbone (i.e., 2H7.v16 N297G binding affinity / 2H7.v16 WT binding affinity).

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antibody, also had greatly increased binding affinity to both the F158 and V158 forms of FcγRIIA (data not shown). In contrast, the AF variants had similar binding affinity to FcγRI, FcγRIIA (H131 and R131 allotypes) and FcγRIIB (relative binding affinities ranged from ~1- to 2-fold; data not shown). Consistent with previous reports, afucosylation did not significantly affect binding to FcRn (Table S1). 5,23

Effect of decreased FcγR binding affinity on PK. The PK of four antibody variants with reduced or abolished FcγR binding affinity (e.g., anti-gD N297G, anti-Y N297A, anti-Y produced from E. coli, and anti-OX40L LALA) were examined in cynomolgus monkeys following single or multiple IV doses. For each variant, PK data was compared with that of the WT version. Serum concentration–time curves are shown in Figure 2. PK parameters are shown in Table 2.

For the anti-OX40L and anti-gD antibodies, both the WT antibodies and N297G/LALA variants exhibited biphasic disposition with a rapid distribution phase followed by a slower elimination phase. PK parameters for the WT and variant antibodies are shown in Table 2. These data show that the anti-OX40L LALA variant and the anti-gD N297G variant had PK similar to their WT counterparts. Specifically, CL and terminal half-life (t1/2) of the WT and LALA versions of anti-OX40L were 3.64 and 3.42 mL/day/kg and 16.0 and 19.7 d, respectively, following five weekly IV doses of 7.5 mg/kg. CL and t1/2 of WT and N297G anti-gD were also similar to each other following a single IV dose of 10 mg/kg. Anti-therapeutic antibodies (ATAs) were detected in 1 of 5 animals administered N297G anti-gD. Mean CL in the four animals without detectable ATAs was similar to that of the WT anti-gD group (3.57 mL/day/kg) (Table 2A).

For the anti-Y antibodies, following a single IV dose of 20 mg/kg, ATAs were observed in most animals, preventing a robust analysis of CL and t1/2. Therefore, partial exposures (AUC) through day 7 and 14 were calculated to compare PK of the WT and variant antibodies. As shown in Table 2B, AUC0–7 and AUC0–14 were similar for all three antibodies, indicating no major differences in PK either between WT and N297A anti-Y or between the anti-Y produced from Chinese hamster ovary (CHO) and E. coli.
Here, we present a retrospective analysis of the effect of altered interactions with FcγRs on antibody PK. We characterized the PK of six variant antibodies with altered FcγR binding affinity in cynomolgus monkeys and compared it to that of the WT counterparts. For all antibodies, PK was assessed at doses in the linear range, where target-independent mechanisms (i.e., FcRn) dominate clearance. Our panel of antibodies encompasses a wide range of targets with varying levels of expression, including soluble targets (LTα) and cell surface targets (CD20), thus allowing for a broad understanding of the potential effect of altered FcγR binding affinity on PK. Furthermore, in contrast to previous studies, we conducted our PK comparisons in cynomolgus monkeys, the preferred species for preclinical PK studies because of their high homology of the target antigen sequence with humans and the similar binding affinity of human IgG for human and cynomolgus monkey Fc receptors.25,26

We compared the PK of three antibodies that were engineered for reduced FcγR binding compared with their WT counterparts in cynomolgus monkeys following single or multiple IV doses. One of the antibodies, anti-OX40L, contained L234A and L235A substitutions (i.e., LALA variant) that are known to greatly reduce FcγR binding.11 The other two antibodies, anti-Y and anti-gD, contained amino acid substitutions at N297. Anti-Y contained the well-studied N297A substitution that is known to eliminate Fc glycosylation and thereby abolish FcγR binding and ADCC activity. The anti-gD variant contained a N297G amino acid substitution that also prevents Fc glycosylation and abolishes ADCC activity. For anti-Y, we also compared the PK of a WT version produced in CHO to the aglycosylated WT version produced in E. coli. This panel of antibodies allowed us to examine the effects of aglycosylation, impaired FcγR binding and production host on PK. For all studies, doses were chosen within the linear PK range in which the target antigen is saturated and non-specific CL mechanisms dominate PK.

The results demonstrate that in all cases, the PK of the aglycosylated/reduced FcγR binding variants was similar to their WT counterparts. This is the first report on the PK of an N297G variant and our data suggests that this amino acid substitution has acceptable PK properties.

While some published reports describe the PK of variants with reduced FcγR binding affinity through aglycosylation or engineered amino acid substitutions, the data available to date is fairly limited. Similar to our findings with the anti-OX40L LALA variant, Hessell et al.10 reported similar t1/2 (7.8 ± 1.7 d and 6.7 ± 3.2 d, respectively) in macaques for a WT and LALA variant of b12, a human mAb that targets a conserved epitope overlapping the CD4-binding site of gp120. A complete PK data and analysis, however, was not included. The PK of TRX1, an anti-CD4 antibody with the N297A amino acid substitution, was examined in a Phase 1 study in healthy volunteers at doses ranging from 1–10 mg/kg. Because the PK of TRX1 was highly nonlinear over this dose range, modeling was employed to estimate Vc (volume of distribution to the central compartment) and t1/2 in the absence of receptor-mediated CL. This modeling suggested that the non-specific CL of TRX1 was within the range of a typical human IgG1.27 More recently, the PK of an anti-IL6 antibody (ALD518) with the N297A substitution was studied in healthy male subjects and patients with advanced cancer.28 The t1/2 of ALD518 at all

### Table 2. Pharmacokinetic parameters of WT and FcγR binding variants

| Antibody Type | Clearance (mL/day/kg) | t1/2 (day) |
|---------------|----------------------|------------|
| WT            | 3.64 ± 1.22          | 16.0 ± 8.94|
| LALA          | 3.43 ± 0.57          | 19.7 ± 5.6 |
| WT            | 3.68 ± 0.566         | 16.1 ± 3.14|
| N297G         | 3.93 ± 1.16          | 14.1 ± 7.27|
| WT            | 3.61 ± 0.950         | 14.8 ± 4.19|
| AF            | 4.19 ± 0.817         | 9.88 ± 2.61|
| WT            | 11.0 ± 2.01          | 3.63 ± 0.39 |
| AF            | 10.2 ± 2.44          | 3.18 ± 0.86 |
| AF 1 mg/kg    | 3.61 ± 0.78          | 12.9 ± 2.63 |
| AF 10 mg/kg   | 3.27 ± 0.35          | 14.0 ± 1.77 |

AUC, exposure; AF, afucosylated; CHO, Chinese hamster ovary; WT, wild type; t1/2, terminal half-life.

**Effect of afucosylation on PK.** We also examined the PK of three AF antibodies in cynomolgus monkeys (Fig. 3). For two of these antibodies (2H7:v16 and anti-LTα), PK of both the WT and AF version were obtained; for the other antibody (anti-X), PK of the AF version was compared with that of a typical human IgG1 in cynomolgus monkeys. Serum concentration–time curves for the WT and AF versions are shown in Figure 3. For WT and AF 2H7:v16, CL and t1/2 following four weekly IV doses of 50 mg/kg were similar and were 3.61 and 4.19 mL/day/kg and 14.8 and 9.88 d, respectively. ATAs were detected in 2 of 4 animals administered WT 2H7:v16 and 1 of 16 animals administered AF 2H7:v16; however, in both cases, ATAs were not detected until several months after dosing (>64 d) and did not appear to affect CL. For anti-LTα, CL of the WT and AF variant was somewhat faster than that expected for a human IgG1 in cynomolgus monkeys. ATAs were observed in all animals by day 43, which may have contributed to the slightly faster than expected CL; however, the PK behavior of the WT and AF anti-LTα were similar following a single dose of 1.5 mg/kg and did not appear to be dramatically affected by the ATAs.

Lastly, the PK of AF anti-X was studied in cynomolgus monkeys following single doses of 1 and 10 mg/kg. PK for AF anti-X was linear over this dose range and in line with that expected for IgG1 in cynomolgus monkeys, with a CL of 3.27 mL/day/kg and a t1/2 of 14.0 d following a single dose of 10 mg/kg.24

**Discussion**

Here, we present a retrospective analysis of the effect of altered interactions with FcγRs on antibody PK. We characterized the PK of six variant antibodies with altered FcγR binding affinity in cynomolgus monkeys and compared it to that of the WT counterparts. For all antibodies, PK was assessed at doses in the linear range, where target-independent mechanisms (i.e., FcRn) dominate clearance. Our panel of antibodies encompasses a wide range of targets with varying levels of expression, including soluble targets (LTα) and cell surface targets (CD20), thus allowing for a broad understanding of the potential effect of altered FcγR binding affinity on PK. Furthermore, in contrast to previous studies, we conducted our PK comparisons in cynomolgus monkeys, the preferred species for preclinical PK studies because of their high homology of the target antigen sequence with humans and the similar binding affinity of human IgG for human and cynomolgus monkey Fc receptors.25,26

We compared the PK of three antibodies that were engineered for reduced FcγR binding compared with their WT counterparts in cynomolgus monkeys following single or multiple IV doses. One of the antibodies, anti-OX40L, contained L234A and L235A substitutions (i.e., LALA variant) that are known to greatly reduce FcγR binding.11 The other two antibodies, anti-Y and anti-gD, contained amino acid substitutions at N297. Anti-Y contained the well-studied N297A substitution that is known to eliminate Fc glycosylation and thereby abolish FcγR binding and ADCC activity. The anti-gD variant contained a N297G amino acid substitution that also prevents Fc glycosylation and abolishes ADCC activity. For anti-Y, we also compared the PK of a WT version produced in CHO to the aglycosylated WT version produced in E. coli. This panel of antibodies allowed us to examine the effects of aglycosylation, impaired FcγR binding and production host on PK. For all studies, doses were chosen within the linear PK range in which the target antigen is saturated and non-specific CL mechanisms dominate PK.

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While some published reports describe the PK of variants with reduced FcγR binding affinity through aglycosylation or engineered amino acid substitutions, the data available to date is fairly limited. Similar to our findings with the anti-OX40L LALA variant, Hessell et al.10 reported similar t1/2 (7.8 ± 1.7 d and 6.7 ± 3.2 d, respectively) in macaques for a WT and LALA variant of b12, a human mAb that targets a conserved epitope overlapping the CD4-binding site of gp120. A complete PK data and analysis, however, was not included. The PK of TRX1, an anti-CD4 antibody with the N297A amino acid substitution, was examined in a Phase 1 study in healthy volunteers at doses ranging from 1–10 mg/kg. Because the PK of TRX1 was highly nonlinear over this dose range, modeling was employed to estimate Vc (volume of distribution to the central compartment) and t1/2 in the absence of receptor-mediated CL. This modeling suggested that the non-specific CL of TRX1 was within the range of a typical IgG1.27 More recently, the PK of an anti-IL6 antibody (ALD518) with the N297A substitution was studied in healthy male subjects and patients with advanced cancer.28 The t1/2 of ALD518 at all
reduced fucose-containing antibody that has entered the clinic is GA201/RG7160, a humanized, glyco-engineered IgG1 anti-EGFR mAb. In a Phase 1 study in patients with advanced solid tumors, GA201 exhibited linear PK and CL ranging from 3.8–5.8 mL/hr at doses greater than 700 mg. These data suggest that, at high doses where target antigen is saturated, the PK of GA201 is similar to that of other IgG1 mAbs and is not impacted by the Fc-modifications which reduce fucose levels. However once again, direct comparison of PK to the WT version has not been reported.

AF antibodies are increasingly being used in the clinic where enhanced ADCC may be desirable, e.g., in cancer indications. One such antibody is GA101, a type II anti-CD20 antibody with reduced fucose in the Fc region, which enhances ADCC activity and potentially enhances efficacy in vivo. The PK of GA101 was recently reported in a small Phase 1 study in Japanese patients. While the authors report linear PK over the dose range examined (200–1200 mg) with CL ranging from 220–347 mL/day, they also note a high degree of inter-patient variability in PK that they ascribe to the small number of patients per group (n = 3 per group) and inter-patient heterogeneity in tumor load. These factors, together with the lack of a direct comparison to the WT version, make it difficult to form a robust assessment of the effect of reduced fucose in GA101 on its PK. Another example of a reduced fucose-containing antibody that has entered the clinic is GA201/RG7160, a humanized, glyco-engineered IgG1 anti-EGFR mAb. In a Phase 1 study in patients with advanced solid tumors, GA201 exhibited linear PK and CL ranging from 3.8–5.8 mL/hr at doses greater than 700 mg. These data suggest that, at high doses where target antigen is saturated, the PK of GA201 is similar to that of other IgG1 mAbs and is not impacted by the Fc-modifications which reduce fucose levels. However once again, direct comparison of PK to the WT version of GA201 has not been reported.
We report here the PK of three AF antibodies, anti-LTα, 2H7.v16 and anti-X, in cynomolgus monkeys. For anti-LTα and 2H7.v16, the PK of the AF antibody was similar to the WT version of each antibody, as evidenced by similar serum concentration–time curves, as well as similar CL and t½ values. For anti-X, PK following single doses of 1 and 10 mg/kg was similar to that of other IgG1s in humans. Together with the existing clinical data, our data further supports the concept that increasing FcγRIIIA binding affinity via afucosylation does not affect PK.

Given the increasing development of antibodies with altered FcγRIIIA binding affinities, it is important to understand the effect of these changes on antibody PK. We report a comprehensive study of the effects of FcγR binding affinity on PK in non-human primates. Our data suggest that, for the six antibodies studied here, altered FcγRIIIA binding affinity does not affect PK. These data are also consistent with data from early site-directed mutagenesis studies\(^5\) demonstrating that FcγR binding sites are distinct from the FeRn binding site; substitutions or alterations at these sites are therefore unlikely to affect PK. We included a broad range of antibody targets within our panel (i.e., soluble targets, membrane targets, and cell surface receptors) in an effort to increase the likelihood that these findings translate to other antibodies. To fully understand the effect of these alterations, however, we need to continue to evaluate the PK of these antibodies in humans and robustly assess the PK relative to unmodified antibodies.

### Materials and Methods

**Protein production.** Antibodies were produced in CHO cell lines at Genentech, Inc using standard techniques\(^32\) and were provided in clear liquid form for in vivo PK studies in cynomolgus monkeys. AF antibodies were produced in the fucosyltransferase 8 knock out CHO cell line.\(^33\) The anti-Y antibody was produced in *E. coli* using standard techniques as described in Simmons et al.\(^13\) All antibodies were IgG1 of the non-α allotype as described in Carter et al.\(^34\)

**In vitro FcγR binding assays.** Binding of WT and AF anti-OX40L, 2H7.v16 or anti-LTα IgG to the extracellular domains of human FcγRs was measured by ELISA as described previously.\(^35\) Briefly, anti-glutathione-S-transferase (anti-GST, Genentech) coated on the ELISA plates was used to capture GST-tagged FcγR extracellular domains (Genentech). Serial dilutions of IgG (for FcγRI binding) or IgG in complex with anti-kappa light chain antibody (MP Biomedicals, catalog number 08630682), for FcγRII and FcγRIIIA binding, were added to the plates. Bound IgG was detected using horseradish peroxidase (HRP) conjugated anti-human F(ab’), (Jackson ImmunoResearch, catalog number 109-036-097) using 3,3′,5,5′-tetramethyl benzidine (TMB) (Kirkegaard and Perry Laboratories, catalog numbers 50-65-02 and 50-76-02) as the substrate. The absorbance was read and the concentrations corresponding to the mid-point absorbance of the WT IgG titration curve were calculated. Relative affinities were calculated by dividing the WT concentration by the variant concentration.

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**Figure 3.** Serum concentration–time curves for variants with increased FcγRIIIA binding. Mean (± SD) serum concentration vs. time profiles in cynomolgus monkeys following administration of (A) four weekly IV doses of 50 mg/kg of WT or AF 2H7.v16; n = 8–12 animals/group, (B) a single IV dose of 1.5 mg/kg of WT or AF anti-LTα; n = 4 animals per group, and (C) single IV doses of 1 and 10 mg/kg of AF anti-X; n = 4 animals per group. For graphical presentation and PK analysis, serum concentrations that were LTR were excluded. For time points with ≤2 animals per group, only mean values are depicted in the graph. LTR, less than reportable; SD, standard deviation; WT, wild type.
Studies in cynomolgus monkeys. The PK studies in cynomolgus monkeys were approved by the Institutional Animal Care and Use Committee and were performed at the following sites: SNBL USA, Ltd. Everett, WA 98203; Charles River Laboratories, Preclinical Services Nevada, Reno, NV 89431; Roche Palo Alto, Palo Alto, CA. Studies included 4–12 cynomolgus monkeys per group. Doses administered ranged from 1–75 mg/kg and were chosen to be within the linear PK range. Blood samples for PK analysis were collected at multiple time points during the dosing phase and were collected for at least 4 weeks following the last dose. Non-compartmental analysis of serum concentration-time data from each animal was conducted using an IV bolus input model (Model 201, WinNonlin Pro, version 4.0.1, Pharsight Corporation). Nominal sample collection times were used in the analysis. CL and $t_{1/2}$ were calculated for each animal. Data are reported as group mean ± standard deviations.

**PK and ATA assays.** Concentrations of 2H7.v16 and anti-gD were determined using a generic ELISA that specifically detects human IgG in cynomolgus serum.36 Circulating anti-Y, anti-LTα, anti-OX40L, and anti-X were quantitated by antigen-binding assays using standard immunoassay techniques. For the anti-Y and anti-LTα assays, plates were coated with recombinant protein antigen produced at Genentech. Recombinant OX40L (produced by Roche) was biotinylated and captured onto streptavidin-coated plates (Thermo Scientific, catalog number 15500 or equivalent). For anti-X, plates were coated with a 15-amino acid peptide corresponding to the sequence of the antibody binding epitope. Cynomolgus serum samples were diluted to a minimum of 1:10 (2H7.v16 and anti-OX40L) or 1:100 (all other assays) and added to plates along with standard curves and assay controls. Bound antibody was detected using a monkey adsorbed HRP-labeled antibodies. Complexes were then detected by an electrochemiluminescent readout through either BioVeris (2H7.v16)37,38 or MSD (anti-LTα)39 instruments, or by an ELISA format (anti-Y).38,39 Samples with signals above the pre-determined assay cutpoints were considered positive for ATA.

**Disclosure of Potential Conflicts of Interest.**

All authors are current or former employees of Genentech, a member of the Roche group, and hold a financial interest in Roche.

**Supplemental Materials.** Supplemental materials may be found here: www.landesbioscience.com/journals/mabs/article/26436

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**References.**

1. Reichert JM. Masked therapeutic antibodies: combinations. MAbs 2012; 4:413-5; PMID:22531442; http://dx.doi.org/10.4161/mafs.19931

2. Jefferis R. Glycosylation as a strategy to improve antibody-based therapeutics. Nat Rev Drug Discov 2009; 8:226-34; PMID:19247305; http://dx.doi.org/10.1038/nrd2804

3. Deng R, Jin F, Prabhu S, Iyer S. Monoclonal antibody-based therapeutics. Nat Rev Drug Discov 2012; 11:635; PMID:22899547; http://dx.doi.org/10.1038/nrd3495

4. Edelman GM, Cunningham BA, Gall WE, Gotlib RR, Rutishauser U, Waxdal MJ. The covalent structure to the Fab region of recombinant human IgG (produced at Genentech) followed by avidin-HRP (Vector Laboratories, catalog number A-2004) for anti-Y. For anti-OX40L, digoxigenin (DIG)-labeled anti-human IgG was followed by addition of HRP-conjugated anti-DIG Fab (Roche Applied Science, catalog number 11633716001). Color was developed using HRP substrates (2, 3′-azino-diethylbenzthiazoline-sulfonic acid (KPL, catalog number 50-66-06 or equivalent) for anti-OX40L and TMB (KPL 50-76-00 or equivalent) for all others).

5. ATAs against 2H7.v16, anti-Y, and anti-LTα were detected using bridging-type assays as described earlier.37-39 An aliquot of each antibody was labeled with biotin (Pierce, catalog number 21338) and another aliquot labeled with either ruthenium (2H7.v16 and anti-LTα; BioVeris, catalog number 110015, and Meso Scale Discovery [MSD], catalog number R91AN-2, respectively) or with DIG (anti-Y; Invitrogen, catalog number A2952). Serum samples were incubated with a cocktail of the biotinylated and either the ruthenium- or DIG-labeled antibodies to allow any ATA present in the sample to bind and form a bridge with the labeled antibodies. Complexes were then detected by an electrochemiluminescent readout through either BioVeris (2H7.v16)37,38 or MSD (anti-LTα)39 instruments, or by an ELISA format (anti-Y)38,39 Samples with signals above the pre-determined assay cutpoints were considered positive for ATA.

6. Shinkawa T, Nakamura Y, Yamane N, Shoji-Hosaka E, Kanda Y, Sakurada M, Uchida K, Anazawa H, Satoh M, Tamasaki M, et al. The absence of fucose but not the presence of galactose or bisecting N-acetylgalactosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. J Biol Chem 2003; 278:3466-73; PMID:12427744; http://dx.doi.org/10.1074/jbc.M210665200

7. Hristodorov D, Fischer R, Linden L. With or without sugar? (A)glycosylation of therapeutic antibodies. Mol Biotechnol 2013; 54:1056-68; PMID:23097175; http://dx.doi.org/10.1007/s12033-012-9612-x

8. Hessell AJ, Hangartner L, Hunter M, Havernitz CE, Beurskens EJ, Bakker JM, Lanigan CM, Landucci G, Fornhal DN, Parren PW, et al. Fc receptor but not complement binding is important in antibody protection against HIV. Nature 2007; 449:101-4; PMID:17805298; http://dx.doi.org/10.1038/nature06106

9. Brinkmann V, Margulies A, Schreiber L, Collisson E, Chisholm B, trays L, Gray JD, Rampling R, Fazekas de St Groth B, et al. Interleukin-6-producing plasmacytoid dendritic cells induce regulatory T cells that inhibit autoimmunity. Nature 2005; 434:113-8; PMID:15927017; http://dx.doi.org/10.1038/nature03535

10. Hessell AJ, Hangartner L, Hunter M, Havernitz CE, Beurskens EJ, Bakker JM, Lanigan CM, Landucci G, Fornhal DN, Parren PW, et al. Fc receptor but not complement binding is important in antibody protection against HIV. Nature 2007; 449:101-4; PMID:17805298; http://dx.doi.org/10.1038/nature06106
11. Hazheet M, Hessell AJ, Jensen RC, van de Winkel JG, Parren PW. Effector function activities of a panel of mutants of a broadly neutralizing antibody against human immunodeficiency virus type 1. J Virol 2001; 75:12161-8; PMID:11711607; http://dx.doi. org/10.1128/JVI.75.24.12161-12168.2001

12. Simmons LC, Reilly D, Klimowski L, Raju TS, Nimmerjahn F, Ravetch JV. Fcgamma receptors as regulators of immune responses. Nat Rev Immunol 2008; 8:34-47; PMID:18064051; http://dx.doi.org/10.1038/ nri2206

13. Jansson JL, Grogan JL, Gurney AL, McCutcheon M, Marian M, Harris R. The impact of Fc glycans on the properties of human IgG1s engineered for enhanced binding to the neonatal Fc receptor (FcRn). J Biol Chem 2006; 281:23154-24; PMID:16793771; http://dx.doi.org/10.1074/jbc.M604292200

14. Chen X, Liu YD, Flynn GC. The effect of Fc glycans on human IgG2 antibody clearance in humans. Glycobiology 2009; 19:240-9; PMID:18974198; http://dx.doi.org/10.1093/glycob/cwn120

15. Millward TA, Heintzmann M, Bill K, Lángel U, Schumacher P, Forster K. Effect of constant and variable domain glycosylation on pharmacokinetics of therapeutic antibodies in mice. Biologicals 2006; 34:87-90; PMID:17012310; http://dx.doi.org/10.1016/S0022-1759(05)00036-6

16. Wright A, Morrison SL. Effect of altered CH2-associated carbohydrate structure on the functional properties and in vivo fate of chimeric mouse-human immunoglobulin G1. J Exp Med 1994; 180:1087-96; PMID:8064227; http://dx.doi.org/10.1084/jem.180.3.1087

17. Wright A, Sato Y, Okada T, Chang K, Endo T, Morrison S. In vivo trafficking and catabolism of IgG1 antibodies with Fc associated carbohydrates of differing structure. Glycobiology 2000; 10:1347-55; PMID:11159927; http://dx.doi.org/10.1093/glycob/cwn120

18. Newkirk MM, Novick J, Stevenson MM, Fournier MJ, Zumwalt RE, Parren PW, Rebers F, Schumacher R, Forrer K. Effect of constant and variable domain glycosylation on pharmacokinetics of therapeutic antibodies in mice. Biologicals 2006; 34:87-90; PMID:17012310; http://dx.doi.org/10.1016/S0022-1759(05)00036-6

19. Sheehan F, Smith JT, Schuster M, Dragnev KH, Rigas JR. A humanized anti-IL-6 antibody (ALD518) in non-small cell lung cancer. Expert Opin Biol Ther 2011; 11:1663-8; PMID:21995322; http://dx.doi.org/10.1517/17471259.2011.627850

20. Bayliss TJ, Smith JT, Schuster M, Dragane KH, Rigas JR. A humanized anti-IL-6 antibody (ALD518) in non-small cell lung cancer. Expert Opin Biol Ther 2011; 11:1663-8; PMID:21995322; http://dx.doi.org/10.1517/17471259.2011.627850

21. Xiang H, Bender BC, Reyes AE, Merchant M, Jumbe NL, Romero M, Davancha T, Nijem I, Mai E, Young J, et al. Ovatumumab (MerMAb): Using nonclinical pharmacokinetic and concentration-effect data to support clinical development. [Epub ahead of print]. Clin Cancer Res 2013; 23:23894056; http://dx.doi.org/10.1158/1078-0432.CCR-13-0260

22. Ogura M, Tobinai K, Hatake K, Uchida T, Suzuki T, Kobayashi Y, Mori M, Terui Y, Yokoyama M, Hotta T. Phase I study of obinutuzumab (GA101) in Japanese patients with relapsed or refractory B-cell non-Hodgkin lymphoma. Cancer Sci 2013; 104:105-11; PMID:23046388; http://dx.doi.org/10.1111/cas.12040

23. Paz-Ares LG, Gomez-Roca C, Deland JP, Cervantes P, de la Iglesia P, Cebrian A, Coleman D, Good J, Lowe J, Rahman A, Yang J, et al. A novel homogeneous Biotin-digoxigenin based assay for the detection of human anti-therapeutic antibody rhuMAb2H7 in cynomolgus monkey serum using a Generic Immunoglobulin Pharmacokinetic (GRIIP) assay. J Immunol Methods 2008; 335:8-20; PMID:18402977; http://dx.doi.org/10.1016/j.jim.2008.01.016

24. Lanzendoerfer M, Parren PW, Rebers F, Schumacher R, Forrer K. Effect of constant and variable domain glycosylation on pharmacokinetics of therapeutic antibodies in mice. Biologicals 2006; 34:87-90; PMID:17012310; http://dx.doi.org/10.1016/S0022-1759(05)00036-6

25. Xiao Y, Veres JM, Chiang N, Ou Q, Ding J, Adams C, Hong K, Trioung BT, Ng D, Shen A, et al. Identification of IgG1 variants with increased affinity to FcγRIIIA and unaltered affinity to FcγRI and FcRn: comparison of soluble receptor-based and cell-based binding assays. J Immunol Methods 2011; 365:132-41; PMID:21185301; http://dx.doi.org/10.1016/j.jim.2010.12.014

26. Yang J, Ng C, Lowman H, Chestnut R, Schofield C, Sandland B, Ernst J, Bennett G, Quarmby V. Quantitative determination of humanized monoclonal antibody rhuMab2H7 in cynomolgus monkey serum using a Generic Immunoglobulin Pharmacokinetic (GRIIP) assay. J Immunol Methods 2008; 335:8-20; PMID:18402977; http://dx.doi.org/10.1016/j.jim.2008.01.016

27. Ng CM, Siefunken E, Anand BS, Faure S, Young J, et al. Onartuzumab (MetMAb): Using pharmacodynamic dose-escalation study of RG7160 in patients with advanced solid tumors. J Clin Oncol 2011; 29:3783-90; PMID:21900113; http://dx.doi.org/10.1200/JCO.2010.32.10.03

28. Omura M, Togai K, Hatake K, Uchida T, Suzuki T, Kobayashi Y, Mori M, Terui Y, Yokoyma M, Hotta T. Phase I study of obinutuzumab (GA101) in Japanese patients with relapsed or refractory B-cell non-Hodgkin lymphoma. Cancer Sci 2013; 104:105-11; PMID:23046388; http://dx.doi.org/10.1111/cas.12040

29. Rancatore P, et al. Expression of full-length immu- noglobulins in Escherichia coli: rapid and efficient DNA uptake in FUT8-deleted CHO cells for transient production of afucosylated antibodies. Biotechnol Bioeng 2010; 106:751-63; PMID:20564613; http://dx.doi.org/10.1002/bit.22749

30. Carter P, Prenta L, Gorman CM, Ridgway JB, Henner D, Wong WL, Rowland AM, Korts C, Carver ME, Shepard HM. Humanization of an anti-p185HER2 antibody for human cancer therapy. Proc Natl Acad Sci U S A 1992; 89:4285-9; PMID:1350088; http://dx.doi.org/10.1073/pnas.89.10.4285

31. Paz-Ares LG, Gomez-Roca C, Deland JP, Cervantes P, de la Iglesia P, Cebrian A, Coleman D, Good J, Lowe J, Rahman A, Yang J, et al. A novel homogeneous Biotin-digoxigenin based assay for the detection of human anti-therapeutic antibodies in autimmune murine serum. J Immunol Methods 2010; 362:101-11; PMID:20868690; http://dx.doi.org/10.1016/j.jim.2010.09.013

32. Peng K, Siradze K, Quarmby V, Fischer SK. Clinical immunogenicity specificity assessments: a platform evaluation. J Pharm Biomed Anal 2011; 54:629-35; PMID:21035975; http://dx.doi.org/10.1016/j. jpb.2010.09.035

33. Wong AW, Baginski TK, Reilly DE. Enhancement of DNA uptake in FUT8-deleted CHO cells for transient production of afucosylated antibodies. Biotechnol Bioeng 2010; 106:751-63; PMID:20564613; http://dx.doi.org/10.1002/bit.22749