Effector-attenuating Substitutions That Maintain Antibody Stability and Reduce Toxicity in Mice

Megan Lo, Hok Seon Kim, Raymond K. Tongo, Travis W. Bainbridge, Jean-Michel Vernes, Yin Zhang, Yuwen Linda Lin, Shan Chung, Mark S. Dennis, Y. Joy Yu Zuchero, Ryan J. Watts, Jessica A. Couch, Y. Gloria Meng, Javinder K. Atwal, Randall J. Brezski, Christoph Spiess, and James A. Ernst

Edited by Peter Cresswell

The antibody Fc region regulates antibody cytotoxic activities and serum half-life. In a therapeutic context, however, the cytotoxic effector function of an antibody is often not desirable and can create safety liabilities by activating native host immune defenses against cells expressing the receptor antigens. Several amino acid changes in the Fc region have been reported to silence or reduce the effector function of antibodies. These earlier studies focused primarily on the interaction of human antibodies with human Fc-γ receptors, and it remains largely unknown how such changes to Fc might translate to the context of a murine antibody. We demonstrate that the commonly used N297G (NG) and D265A, N297G (DANG) variants that are efficacious in attenuating effector function in primates allow a more accurate translation of results generated with an “effectorless” antibody between mice and primates. Further, we show that both human and murine antibodies containing the LALA-PG variant have increased beyond traditional monospecific antibodies. This is reflected in the rapid expansion of clinical trials with bispecific antibodies in recent years (3, 4). Additionally, many therapeutic approaches require modifications to the Fc region to silence antibody effector function or to extend antibody serum half-life. As these novel antibody formats and altered Fc properties are often combined, there is an increasing need to identify compatible amino acid changes in the antibody constant region. Furthermore, these changes in Fc need to translate between preclinical animal models and humans (5, 6).

The challenges in translating antibody properties from animals to humans have long been recognized and partially result from the protein sequence differences between human and murine antibodies (7). The most common human IgG isotype used in therapeutic development is IgG1 (1), which is highly abundant in human serum. IgG1 antibodies have a high level of native cytotoxic function and a long half-life and can be efficiently produced with recombinant technologies. In preclinical animal studies, particularly those conducted in mice with intact immune systems, species-specific antibodies are often used to avoid immunogenicity, and a murine isotype with Fc properties corresponding to the human antibody is selected. Murine IgG2a has high functional similarity to human IgG1 in terms of pharmacokinetics (PK) and Fc-mediated effector function and is therefore often chosen as a surrogate antibody for murine studies. In addition, the murine IgG2a isotype is convenient for PK assays, as this isotype is not expressed in the common laboratory mouse strain c57BL/6J in the absence of specific antigen stimulation (8). The cytotoxic potential of an antibody is primarily mediated through the Fc region of the antibody, either through the complement-dependent cytotoxicity (CDC) or antibody-dependent, cell-mediated cytotoxicity (ADCC) pathways.

The CDC response involves activation of a biochemical cascade through which protein components of serum directly attack a pathogen (9). The classical CDC pathway is initiated by binding of the C1q complex to the antibody-antigen complex,
which ultimately leads to C3 fixation and membrane attachment. Several components of the complement pathway can also directly recruit macrophages to opsonize cells in a process known as antibody-dependent cellular phagocytosis (ADCP) (10).

Activation and recruitment of lymphocytes during ADCC is mediated by binding of the antibody Fc region to Fc-γ receptors. The human Fc-γ receptor family consists of Fc-γ receptors I (CD64), IIa/b/c (CD32a/b/c), and IIIfa/b (CD16a/b). They are expressed on numerous cell types such as myeloid lineage cells, including macrophages. Fc-γ receptor I, IIa/c, and IIIfa are activating receptors, and Fc-γ receptor IIb is an inhibitory receptor. Fc-γ receptor IIb is unique among the human Fc-γ receptors because it does not have a transmembrane domain and is expressed on the surface of neutrophils by a glycosphatidylinositol anchor. The mouse Fc-γ receptor family consists of Fc-γ receptors I, IIb, III, and IV. Only Fc-γ receptor IIb is an inhibitory receptor. Fc-γRIV selectively binds the IgG2a/b antibodies (11–13). The most prevalent Fc-γ receptors on natural killer cells are Fc-γRIIla in humans, although a subset of human natural killer cells express Fc-γRIIc (14), or RIIB in mice (13, 15). With the exception of Fc-γRl, binding to all Fc-γ receptors is strongly enhanced by avidity effects following immune complex formation.

A number of strategies have been developed to reduce or eliminate antibody cytotoxicity (16). One effective strategy in human antibodies is the elimination of the N-linked glycosylation at residue Asn-297, achieved by substituting Asn-297 with an alanine, glycine, or aspartic acid (17, 18), or modification of the serine/threonine residue at position 299 (19). The elimination of glycosylation results in reduced antibody binding to C1q and Fc-γ receptors via allosteric changes in the antibody C1q domain. Although this strategy is effective at reducing both CDC and ADCC activity of human antibodies, the reduction in effector function is much less complete in mice. Aglycosylation and the associated allosteric changes also have the considerable disadvantage of reducing antibody stability. To further reduce the effector function of aglycosylated murine antibodies, changes that impair glycosylation at Asn-297 are often combined with the D265A mutation (20). The two most commonly used pairs of amino acid changes at these positions are designated D265A, N297A or D265A, N297G (DANG). Although the DANG substitutions are intended to eliminate effector functions of human and murine antibodies in mice, it has been shown recently that anti-transferrin receptor (TfR) bispecific antibodies containing DANG or NG substitutions can still lead to a potentially toxic reduction of TfR-expressing reticulocytes in mice via ADCP or CDC activity (21).

Alternative strategies have also been employed to mitigate antibody effector function, including substitutions of residues in the antibody lower hinge such as L234A and L235A (LALA) (22). These residues form part of the Fc-γ receptor binding site on the C1q2 domain (23), and the exchange of these residues between antibody isotypes with greater or lesser effector function identified their importance in ADCC. Although alanine substitutions at these sites are effective in reducing ADCC in both human and murine antibodies (24–26), these substitutions are less effective at reducing CDC activity. Another single variant, P329A, identified by a random mutagenesis approach to map the C1q binding site of the Fc, was shown to be highly effective at reducing CDC activity while retaining ADCC activity (27). The location of these amino acid changes on the Fc within 5 Å are colored gray in the surface rendering. The location of the Asp-265 and Asn-297 variants (DANG) that eliminate glycosylation and reduce effector functions are indicated in red. The locations of the Leu-234, Leu-235, and Pro-329 positions are highlighted in yellow. B, alignment of relevant regions of the hinge (gray fill) and C1q2 domain (cyan fill) of murine IgG2a and human IgG. The naturally effector-attenuated human IgG4 sequence is also included for reference. The positions of the LALA-PG and DANG variants are indicated by yellow and red, respectively. The numbering of the C1q2 residues for human IgG1 is according to Eu convention.
doses of antibody (3). Evaluations of effector-attenuation strategies in this study were based on this highly sensitive in vivo readout related to the fate of precursor red blood cells following anti-TfR antibody administration (21) within the context of the knobs-into-holes bispecific technology (30, 31). In addition, this work highlights a specific need for new methods that can significantly attenuate antibody effector function for murine bispecific antibodies without introducing undesirable production liabilities.

To identify effector silencing substitutions that could both improve the production of murine IgG2a-bispecific antibodies and effectively reduce effector function, we screened several antibody variants, including the recently identified combination of L234A, L235A, and P329A (LALA-PG) substitutions that have been shown to effectively silence the effector function of human IgG1 antibodies (32). In contrast to the allosteric effects of aglycosylation, the LALA-PG substitutions directly block the interaction of the Fc with the Fc-γ receptors and C1q. In addition to being more effective at reducing effector function than the allosteric mechanism of aglycosylation, they maintain antibody stability. In this work, we show that introduction of the LALA-PG substitutions into murine IgG2a antibodies vastly improves the expression and assembly of the knobs-into-holes half-antibodies, and the LALA-PG variant retains the thermal stability of the respective wild-type murine IgG2a and human IgG1 antibodies in contrast to the aglycosylated DANG and NG variants. In vitro assays demonstrate that the LALA-PG substitution in a murine IgG2a antibody highly attenuates Fc-γ receptor binding as well as complement fixation relative to both aglycosylated NG and DANG antibodies. Furthermore, our murine studies demonstrate that LALA-PG effector silencing translates to an in vivo setting and improves the safety profile of a TfR-targeting bispecific antibody. With the use of the murine IgG2a LALA-PG antibody variant, we have established a murine surrogate system to mimic the effector function of the LALA variants Retain a Similar Expression Level and Stability as Wild-type Antibodies—The production of knob-and-hole half-antibodies is the first step in the production of a bispecific antibody (21, 29, 33). After purification, the half-antibodies are assembled into the intact bispecific antibody. For an efficient production process, a high expression level of each half-antibody and efficient assembly of the two half-antibodies is necessary. Typical recovery of knob or hole murine IgG2a half-antibodies is 25–50 mg/liter in CHO. Unexpectedly, half-antibody expression is greatly attenuated in context with the DANG variant (Fig. 2A). We hypothesize that the impact on expression level could be a consequence of protein instability resulting from aglycosylation. To determine whether effectorless variants that retain the N-linked glycosylation at Asn-297 show improved expression over the DANG antibodies, we tested several previously identified variants that silence effector function. The expression of murine IgG2a half-antibody with LALA substitutions as well as in combination with either the D265A substitution (LALA-DA) or LALA-PG were tested. All variants express at levels at least comparable with the WT half-antibodies, with the exception of the LALA-DA, Hole half-antibody variant (Fig. 2A).

LALA-PG Variants Retain Assembly Properties of the Wild-type Half-antibodies—Murine IgG2a half-antibodies containing the effector function-reducing substitutions were evaluated for efficiency of assembly into intact bispecific antibody. Analogous to our observation with the expression of half-antibodies, the assembly into bispecific antibody from aglycosylated DANG and NG half-antibodies is dramatically impacted. The assembly efficiencies are reduced by 65–80% compared with the WT half-antibodies (Fig. 2B). In contrast, all of the glycosylated variants, LALA, LALA-DA and LALA-PG, retain an assembly efficiency that is similar to the wild-type antibody (Fig. 2B).

The LALA-PG Variant Retains a Thermal Stability Comparable with the Wild-type Antibody—We had speculated that the impact on expression and assembly could be related to reduced thermal stability of the aglycosylated antibodies. Differential scanning calorimetry (DSC) was performed with the murine WT and aglycosylated (NG) mlgG2a antibodies to confirm the...
impact of aglycosylation on stability (Fig. 3, A and B). The NG antibody shows a reduction in stability typically assigned to destabilization of the aglycosylated C_{H2} domain. To study whether murine and human LALA-PG antibodies retain the thermal stability of their respective wild-type antibodies, we repeated the DSC experiments with the annealed bispecific antibodies. Both the murine IgG2a-LALA-PG bispecific antibody (Fig. 3C) and the human IgG1-LALA-PG bispecific antibody (Fig. 3D) lack the characteristic reduction in stability associated with aglycosylated antibodies and demonstrate thermal stabilities consistent with previous reports of wild-type glycosylated human IgG1 and murine IgG2a antibodies (34–36). The calculated inflection points for the deconvoluted isotherms are shown in Fig. 3E.

**Figure 3. LALA-PG antibodies retain the stability of wild-type antibodies.** A–D, differential scanning calorimetry isotherms of wild-type murine IgG2a antibody (A; experimental data are shown in blue, and calculated isotherms are shown in orange), aglycosylated (NG) murine IgG2a (B; experimental data are shown in black, and calculated isotherms are shown in orange), murine IgG2a monospecific WT (blue) bispecific LALA-PG (red) antibodies (C), and human IgG1 and bispecific WT (blue) bispecific LALA-PG (red) antibodies (D). E, summary of the first and second thermal melt transition for murine (m) and human (h) Fc fragments and full-length antibodies.

The LALA-PG Variant Reduces the Effector Function of Murine IgG2a More Effectively Than LALA Alone—In murine serum, the LALA-PG variant displays reduced C1q binding (Fig. 4A) and C3 fixation (Fig. 4B) relative to both wild-type Fc and the DANG variants. The reduction in C1q binding with the LALA-PG antibody is even more pronounced with isolated murine C1q complex because of the absence of competitive binding by other proteins in serum. Experiments with human IgG1 did not show a reduction in serum C3 fixation (supplemental Fig. S1B) and very little difference between LALA-PG and NG variants in complement C1q binding in either the serum (supplemental Fig. S1A) or with isolated human C1q (supplemental Fig. S1C).

We further characterized the binding of effector-reduced antibodies to murine Fc-γ receptors by ELISA and observed residual binding of the murine IgG2a-NG antibodies to all Fc-γ receptors (Fig. 5). Addition of the D265A variant in conjunction with the NG variant further reduces binding to the Fc-γRI receptor and eliminates residual binding to all other Fc-γ receptors in the assay. In contrast, the LALA-PG variant shows residual Fc-γ binding only to the Fc-γRIII receptor. However, this binding is attenuated by more than 50-fold from the wild-type murine IgG2a antibody (Fig. 5). In human IgG1 antibodies, both the LALA-PG and the NG variants are equally effective at...
eliminating Fc-γ receptor binding (supplemental Fig. S2) and ADCC activity (supplemental Fig. S3). The LALA-PG and NG variants in human IgG1 were also assessed for macrophage-mediated ADCP activity in a highly avid assay format where aglycosylated antibodies have previously demonstrated activity comparable with IgG1 (37). Consistent with the previous report, the human IgG1 and its NG variant demonstrated comparable ADCP activity, whereas the LALA-PG variant had low to undetectable ADCP activity (supplemental Fig. S4).

The LALA-PG Variant Demonstrates an Improved Safety Profile in Vivo—Bispecific antibodies targeting the murine TfR have been shown recently to evoke complement-dependent reticulocyte depletion even in the presence of the N297G effector-reducing substitution (21). In this work, we have grafted this anti-TfR domain (anti-TfRD) onto bispecific frameworks for human IgG1 and murine IgG2a. An additional murine IgG2a-bispecific antibody containing murinized complement-determining regions from the anti-TfRD variable domain was also generated (noted as anti-TfRm). Based on the in vitro data, the LALA-PG variant should be as effective as the DANG and NG variants in reduction of Fc-γ/H9253-mediated effector function and superior to DANG in reduction of CDC and ADCP effector functions in vivo. As expected, the bispecific anti-TfRαβ/control and the anti-TfRαβ/control bispecific antibodies with the LALA-PG variants eliminated the residual reticulocyte reduction observed with the DANG variant (Fig. 6A). This effect was not a result of any change in clearance of the anti-TfRαβ/control LALA-PG bispecific antibody, as confirmed by similar serum levels of all antibodies evaluated (Fig. 6B). Finally, as would be predicted based on the locations of the LALA-PG variants, the PK profile is similar to values for historic control antibodies (Fig. 6C).

Discussion

Using both in vitro and in vivo experiments, we have demonstrated that the LALA-PG variant is highly effective in eliminating both Fc-γ- and complement-mediated antibody effector functions for both human IgG1 and murine IgG2a antibodies. In particular, we show a notable reduction in ADCP and/or CDC activity for murine IgG2a antibodies in a highly sensitive in vivo context using anti-TfR antibodies with a known safety liability (21) (e.g. reduction in reticulocytes via multiple effector-mediated mechanisms). This assay can reveal even small levels of effector function masked in less sensitive assays. Furthermore, we have shown that murine IgG2a antibodies with the LALA-PG substitutions retain favorable biophysical and manufacturing properties. Additionally, the LALA-PG variant is compatible with the knobs-into-holes technology for
Stable, Reduced Effector Function Murine IgG2a Antibodies

In this study, we characterize the LALA-PG variant in murine IgG2a antibodies. Although the murine IgG2a-DANG variant increases the effective antibody concentration for some complement reactions, the maximum activity remains almost the same as the wild-type antibody for both the serum C3 fixation assay and isolated C1q binding (Fig. 4). In contrast, the LALA-PG variant in murine IgG2a antibodies has the dramatic effect of reducing C1q binding and C3 fixation in serum to just above background levels and completely eliminating antibody binding by isolated C1q.

It has been shown previously that anti-TfR/BACE1 bispecific antibodies containing the effector function-attenuating NG variant deplete reticulocytes in mice, and this reticulocyte depletion is eliminated in C3 knockout mice (21). The reduced in vitro complement activation of the murine IgG2a-LALA-PG variant was confirmed by our in vivo experiments and resulted in an improved safety profile for the anti-TfR LALA-PG bispecific antibody relative to the DANG bispecific antibody. Further work will be necessary to understand why the reduction in complement activity by the LALA-PG variant is more pronounced for the murine IgG2a antibodies than for human IgG1 antibodies, and it is possible that the effect is a function of the species-specific variation in the strength of complement response, as has been noted previously (38). However, it is clear from the data in supplemental Fig. S4 that ADCP activity in the human IgG1 is completely eliminated by the LALA-PG variant, something not accomplished by the antibody N297G aglycosylation variant.

In this study, we characterize the LALA-PG variant in murine IgG2a and human IgG1 antibodies. Additional work will be necessary to determine whether the results translate to other antibody isotypes and species. However, as glycosylation has a structural role in the antibody C1q2, the LALA-PG substitutions will be always preferred over aglycosylation for antibody stability. The impact of aglycosylation on expression and assembly of murine IgG2a half-antibodies is independent of the antibody variable domains over all antibodies evaluated.

We have not evaluated the effect of the LALA-PG substitutions on the posttranslational modification of antibodies. It was shown recently that expression of glycosylated half-antibodies results in production of heterogeneous glycosylation patterns.
(39). This altered glycosylation could potentially complicate analytical characterization, although this heterogeneity did not present any obstacles to this study.

In summary, we have identified the LALA-PG variant as a solution to efficiently attenuate both Fc-γ-dependent ADCC and C1q/C3-dependent CDC activity in murine antibodies. The amino acid changes do not impact the thermal stability of the molecule, making them attractive for use in a regular monoclonal antibody as well as engineered antibody formats that suffer from reduced stability. The lack of detectable interaction of murine IgG2a LALA-PG with murine Fc-γ receptors and C1q now enables a safe path for murine in vivo studies with antibodies to targets that do not tolerate any cytotoxic activity, thus improving the translation of preclinical animal models to the clinic.

**Experimental Procedures**

*Research Ethics*—All animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (Publication 8523, revised 1985). The Institutional Animal Care and Use Committee at Genentech reviewed and approved all animal protocols.

*Plasmid Construction and Antibody Expression*—Antibodies and antibody Fc regions were cloned by standard molecular biology techniques into mammalian expression vectors (40) as described previously. IgGs were expressed as 1 liter of transient transfection cultures of CHO (41). All antibody positions are according to the Eu numbering convention.

*Half-antibody and Bispecific Antibody Production*—The bispecific antibodies, including the anti-TfR/control (29), were generated using the knobs-into-holes technology described previously (21). The half-antibodies were expressed in CHO cells and affinity-purified over a GE mAb Select SuRe column (GE Healthcare). The purified half-antibodies were assembled under reducing conditions with reduced glutathione and were allowed to assemble over 3 days at room temperature at pH 8.5 for human IgG1 and pH 9.5 for murine IgG2a. The bispecific antibodies were purified with a hydrophobic interaction column (ProPac HIC-10, Thermo Scientific, Sunnyvale, CA). Assembly efficiency was calculated based on the theoretic maximum yield using the input amounts of the half-antibodies and then normalized to the recovery from wild-type bispecific antibody.

*Melting Temperature and Enthalpy Calculations*—The thermostability of the murine IgG2a variants was determined by measuring the melting temperatures ($T_m$) and enthalpies ($\Delta H_m$) of the antibodies via differential scanning calorimetry (MicroCal VP-DSC, Northampton, MA). Raw melt data were fit to a non-two-state model to calculate $T_m$ and $\Delta H_m$.

*Serum C1q Binding and C3 Fixation*—ELISA for C1q binding and C3 fixation were adapted from Hessell et al. (25). Briefly, purified bispecific antibodies were serially diluted in 1× PBS and coated onto microtiter plates overnight at 4 °C. The plates were blocked with 5% gelatin/PBST (0.5× PBS and 1% (v/v) Tween 20), followed by incubation with murine or human serum (10% (v/v) in PBST). C1q binding was detected by HRP-conjugated rabbit anti-C1q (Bioss Inc.) at a 1:500 dilution in PBST. To test C3 fixation, rabbit anti-C3 (Abcam) was added at a 1:1000 dilution, followed by HRP-conjugated chicken anti-rabbit IgG (Abcam) at a 1:2000 dilution. The plates were developed with TMB (KPL, Gaithersburg, MD). The reaction was stopped with 1 M H₃PO₄ and read at 450 nm using 620 nm for background reading on a Tecan M1000 fluorescence reader (Tecan, Mannedort, Switzerland). EC₅₀ values were calculated by fitting the data to a log (agonist) versus response variable slope (four-parameter) model using GraphPad Prism (Sunnyvale, CA).

*Isolated C1q Binding ELISA—MaxiSorp* 384-well plates (Thermo Scientific, Nunc, Roskilde, Denmark) were coated with serially diluted antibodies (0.039–40 μg/ml mouse IgG2a or 0.049–50 μg/ml human IgG1) in 50 mM carbonate buffer (pH 9.6) (coat buffer) at 4 °C overnight. Plates were washed with PBS containing 0.05% polysorbate 20 (pH 7.4) and blocked with PBS containing 0.5% BSA, 0.05% polysorbate 20, 15 ppm Proclin, and 10% blocker casein (Thermo Scientific) (pH 7.4). After 1-h incubation at room temperature, plates were washed. Mouse C1q (0.5 μg/ml, Genentech) or human C1q (1 μg/ml, Quidel, San Diego, CA) in the same buffer was added and incubated for 1.5 h. Bound C1q was detected by adding biotinylated mouse anti-mouse C1q (20 ng/ml, Hycult Biotech, Plymouth Meeting, PA; cross-reacting with human C1q) for 1.5 h, followed by HRP-conjugated streptavidin (GE Healthcare Life Sciences) for 1 h. To check for coating efficiency, some coated wells received buffer only for the first two incubation steps and received goat anti-mouse F(ab’)₂-HRP (for murine IgG2a-coated wells) or anti-human F(ab’)₂-HRP (for human IgG1-coated wells) when the wells were used for measuring C1q binding received streptavidin-HRP. Plates were washed after each incubation step. Peroxidase activity was detected with the substrate 3,3’,5,5’-tetramethyl benzidine (TMB) (Kirkegaard and Perry Laboratories). The reaction was stopped with 1 M H₃PO₄, and absorbance was measured at 450 nm using a Multiskan Ascent reader (Thermo Scientific, Hudson, NH). Dose-response binding curves were fitted with a four-parameter model, and EC₅₀ values were calculated using KaleidaGraph (Synergy Software, Reading, PA).

*Isolation of Murine C1q*—Murine C1q was purified directly from mouse serum similar to methods described previously (42). Briefly, 10 mM EDTA and 1 mM PMSF, final concentrations, were added to raw mouse serum and 0.2-μm-filtered. The serum was then applied to immobilized mouse and/or rabbit IgG-Sepharose (Sigma-Aldrich), and C1q was eluted in PBS with an additional 1 M NaCl and 10 mM EDTA. Fractions containing C1q, as determined by immunoblot against C1q-A, were pooled and dialyzed to 100 mM NaCl for cation exchange chromatography (Mono S, GE Healthcare), washed with 50 mM HEPES (pH 7.2), 100 mM NaCl, 0.1% Triton X-114, and 0.02% Triton X-100, followed by 50 mM HEPES (pH 7.2), 100 mM NaCl to remove detergent. C1q was eluted with a 25 column volumes gradient to 500 mM NaCl. Peak fractions containing C1q were pooled from the eluate. To remove any IgG contaminants, the eluate from the cation exchange step was applied directly onto MabSelect SuRe (GE Healthcare), and the IgG-free C1q was collected from the flow-through. Finally, the material was applied to a gel filtration column (Superdex 200,
GE Healthcare) in PBS supplemented with 1 M NaCl. C1q peak fractions were pooled and dialyzed into 50 mM HEPES (pH 7.2), 150 mM NaCl, and 20% glycerol, and then 0.2-μm sterile-filtered. The identity of the purified material was confirmed by immunoblotting as well as mass spectrometric analysis. The native molecular weight was verified by size exclusion chromatography-multi-angle laser light scatter (MALS).

**Soluble Fc-γ Receptor Binding ELISAs**—For mouse FcyR binding, soluble mouse Fc-γRI, Fc-γRIIb, and Fc-γRIII were expressed as recombinant fusion proteins with Gly-His6-GST at the C terminus of the extracellular domain of the receptor α chains (Fc-γR His-GST) (Genentech). Soluble mouse Fc-γRII was expressed as Fc-γR-His. MaxiSorp 384-well plates were coated with mouse Fc-γRs (1 μg/ml) in coat buffer. Plates were washed and blocked with PBS containing 0.5% BSA and 15 ppm Procion (pH 7.4). After a 1-h incubation, plates were washed, and mouse IgG2a (0.42–25,000 ng/ml in 3-fold serial dilution in duplicate) in PBS containing 0.5% BSA, 0.05% polysorbate 20, and 15 ppm Procion (pH 7.4) was added to the plates and incubated for 2 h. Bound IgG2a was detected with goat F(ab′)2, anti-mouse F(ab′)2-HRP (Jackson ImmunoResearch Laboratories) using TMB as a substrate. The reaction was stopped, and the plate was read as described above. The dose-dependent binding curve of the wild-type antibody was fitted with a four-parameter curve-fitting program (KaleidaGraph, Synergy Software). The relative affinity of the variant versus the wild type was estimated by dividing the equivalent nanograms per milliliter wild type concentration at 25,000 ng/ml variant by 25,000 ng/ml.

Human Fc-γR binding ELISAs were carried out similarly using plates coated with human Fc-γR-His-GST molecules (43). For measuring binding to human Fc-γRII and Fc-γRIII, human IgG1 was preincubated with anti-human κ antibody to form a complex to increase binding avidity. Serially diluted FcRIII were expressed as recombinant fusion proteins with Gly-His6-GST at the C terminus of the extracellular domain of the receptor α chains (Fc-γR His-GST) (Genentech). Soluble mouse Fc-γRIII was expressed as Fc-γR-His. MaxiSorp 384-well plates were coated with mouse Fc-γRs (1 μg/ml) in coat buffer. Plates were washed and blocked with PBS containing 0.5% BSA and 15 ppm Procion (pH 7.4). After a 1-h incubation, plates were washed, and mouse IgG2a (0.42–25,000 ng/ml in 3-fold serial dilution in duplicate) in PBS containing 0.5% BSA, 0.05% polysorbate 20, and 15 ppm Procion (pH 7.4) was added to the plates and incubated for 2 h. Bound IgG2a was detected with goat F(ab′)2, anti-mouse F(ab′)2-HRP (Jackson ImmunoResearch Laboratories) using TMB as a substrate. The reaction was stopped, and the plate was read as described above. The dose-dependent binding curve of the wild-type antibody was fitted with a four-parameter curve-fitting program (KaleidaGraph, Synergy Software). The relative affinity of the variant versus the wild type was estimated as described above, except that, for Fc-γRII binding, the equivalent nanograms per milliliter wild type concentration at 500 ng/ml variant was divided by 500 ng/ml.

**Antibody Affinity Measurements**—The anti-TfR competition ELISA was performed in MaxiSorp plates (Nunc) coated with purified muTfR-His (2.5 μg/ml) in PBS at 4 °C overnight. Plates were washed with PBS/0.05% Tween 20 and blocked with SuperBlock blocking buffer in PBS (Thermo Scientific). A 1:3 serially titrated IgG was combined with 1 nM biotinylated anti-TfR and added to the plate for 1 h at room temperature. Plates were washed with PBS/0.05% Tween 20, and HRP-streptavidin (SouthernBiotech) was added to the plate and incubated for 1 h at room temperature. Plates were washed with PBS/0.05% Tween 20, and biotinylated anti-TfR bound to the plate was detected with TMB substrate (BioFX Laboratories).

**Hematology and Chemistry Analysis**—Reticulocyte counts were determined on K-EDTA blood with a Sysmex XT-2000iV. The Sysmex detects and classifies reticulocytes by flow cytometry with a fluorescent polymethylene dye to bind cellular RNA and cell light scatter characteristics.

**Measuring Antibody Concentrations in Mouse Plasma**—Total antibody concentrations in mouse plasma were measured with a generic human Fc ELISA. Nunc 384-well MaxiSorp immunoplates were coated with an F(ab′)2 fragment of donkey anti-human IgG and Fc fragment-specific polyclonal antibody (Jackson ImmunoResearch Laboratories) overnight at 4 °C. Plates were blocked with PBS and 0.5% BSA for 1 h at 25 °C. Each antibody (control IgG and anti-TfR/control-bispecific) was used as a standard to quantify respective antibody concentrations. Plates were washed with PBS and 0.05% Tween 20 using a microplate washer (Bio-Tek Instruments Inc.), and standards and samples were diluted in PBS containing 0.5% BSA, 0.35 M NaCl, 0.25% CHAPS, 5 μM EDTA, and 0.05% Tween 20, and 15 ppm was added for 2 h at 25 °C. Bound antibody was detected with HRP-conjugated F(ab′)2 goat anti-human IgG and Fc-specific polyclonal antibody (Jackson ImmunoResearch Laboratories) and developed with TMB (KPL Inc.), and absorbance was measured at 450 nm on a Multiskan Ascent reader (Thermo Scientific). Concentrations were determined from the standard curve with a four-parameter nonlinear regression program. Wild-type female C57B/6 mice aged 6–8 weeks were used for all studies.

**Author Contributions**—J. A. E. and C. S. conceived the project. M. L., H. S. K., R. K. T., T. W. B., J. M. V., Y. G. M., Y. Z., J. Y. Y. Z., R. J. B., C. S., and J. A. E. designed and performed the assays. M. L., R. K. T., T. W. B., and J. A. E. contributed to the purification of recombinant proteins. Y. J. Y. Z. performed the mouse experiments. Y. J. Y. Z., J. A. C., R. J. W., and J. L. designed and analyzed the murine reticulocyte and pharmacokinetic analyses. M. L., H. S. K., R. K. T., T. W. B., J. M. V., Y. G. M., Y. Z., Y. J. Y. Z., R. J. W., M. S. D., Y. L. L., S. C., R. J. W., and J. A. E. analyzed the data. M. L., H. S. K., R. T., T. W. B., Y. G. M., C. S., and J. A. E. drafted the manuscript. All authors reviewed the results and approved the final version of the manuscript.

**Acknowledgments**—We thank our Genentech colleagues in Protein Chemistry, Antibody Engineering, Neuroscience, and Safety Assessment, the Biomolecular Engineering Group, and Laboratory Animal Resources for technical assistance.

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Stable, Reduced Effector Function Murine IgG2a Antibodies

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