

Aglycosylated immunoglobulin G₁ variants productively engage activating Fc receptors

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Immunoglobulin G plays a vital role in adaptive immunity and antibody-based therapy through engagement of its Fc region by the Fc γ receptors (Fc γ Rs) on immune cells. In addition to specific protein-protein contacts, N-linked glycosylation of the IgG Fc has been thought to be essential for the recognition of Fc by Fc γ R. This requirement for the N-linked glycan has limited biomanufacture of therapeutic antibodies by restricting it to mammalian expression systems. We report here aglycosylated Fc domain variants that maintain engagement to Fc γ Rs, both *in vitro* and *in vivo*, demonstrating that Fc glycosylation is not strictly required for the activation of immune cells by IgG. These variants provide insight into how the N-linked glycan is used biologically in the recognition of Fc by Fc γ Rs, as well as represent a step toward the production in alternative expression systems of antibody-based therapeutics capable of eliciting immune effector functions.

surface plasmon resonance | antibody engineering | directed evolution | yeast display | antibody biomanufacture

Fc gamma receptor (Fc γ R) engagement is essential to the function of IgG in both immunity (1) and in antibody-based therapy (2, 3). IgGs act as the adaptor between a pathogen and the immune response by simultaneously binding antigen through their variable regions and activating an immune response through interaction of conserved Fc regions with Fc γ Rs on cells of the immune system. The human Fc γ R (hFc γ R) family consists of the activating receptors Fc γ RI, Fc γ RIIA, and Fc γ RIIIA, and the inhibitory receptor Fc γ RIIB. While Fc γ RI binds IgG with high affinity (nanomolar binding constants), Fc γ RIIA, Fc γ RIIB, and Fc γ RIIIA bind IgG with micromolar affinity, becoming activated only via avid multivalent interactions with opsonized antigen (1). The binding of IgG to Fc γ R is highly sensitive to the presence of glycosylation at a single N-linked glycosylation site at asparagine 297 (N297) in its CH2 domain (4, 5), with a loss of binding to the low-affinity Fc γ Rs observed in N297 point mutants (6, 7), enzymatic Fc deglycosylation (8), recombinant IgG expression in the presence of the N-linked glycosylation-inhibitor tunicamycin (9), or expression in bacteria (10, 11). In addition, the nature of the carbohydrate attached to N297 modulates the affinity of the Fc γ R interaction (12, 13). The sensitivity of Fc γ R binding to specific glycoforms has limited therapeutic antibody biomanufacture to mammalian expression systems, and has led to the development of glycosylation-engineered mammalian cell lines (13, 14) and microbial strains with humanized glycosylation (15) as methods of enhancing antibody cytotoxicity.

In crystal structures of the complex, Fc γ R/Fc contact is mediated not only by protein-protein contacts, but also by specific interactions with the glycan on the Fc that are proposed to contribute to binding affinity (16, 17). Additional intramolecular contacts are made between the Fc-linked glycan and residues on the IgG CH2 domain, and it is thought that these interactions stabilize an open Fc conformation capable of being engaged by Fc γ R (4). Successive truncation of an IgG₁ glycan

results in an incremental loss of binding affinity (8) and concomitant incremental collapse of the open Fc conformation (18). However, glycosylation is not strictly required for engagement of all Ig receptors with their corresponding Fc ligands, notably in the binding of IgE Fc to IgE γ R (19). Interestingly, the IgE Fc adopts a similar mode of binding to Fc γ R as the IgG₁ Fc in the IgG₁ Fc:Fc γ RIII complex and both receptors and Fcs share structural similarity (20). In the IgG₁ Fc:Fc γ RIII complex, extensive contacts are made by both chains of the IgG₁ hinge region, with additional receptor contacts made by the B/C loop, F/G loop, and both side chains and glycosylation of the C'/E loop of the CH2 domain (16, 17) [Fig. 1A, [supporting information \(SI\) Fig. S1](#)]. It is particularly striking that this loop plays a part in receptor recognition through both direct side chain contacts as well as in encoding information for a critical posttranslational modification.

In the present study, we reasoned that by optimizing the protein-protein interactions about the C'/E loop:Fc γ R interface at the expense of glycosylation, we could identify aglycosylated IgG₁ variants that maintain engagement to Fc γ Rs. Here, we demonstrate that a small subset of substitutions at both N297 and T299 of the glycosylation motif lead to aglycosylated Fcs that maintain engagement of Fc γ Rs, and in a particular example are active *in vivo*. Such aglycosylated antibodies would be facile templates for further efforts to engineer Fc-effector functions and potentially enable a far wider range of options for therapeutic antibody biomanufacture.

Results

Screening for Aglycosylated Fc Variants that Bind Fc γ RIIA. To determine if glycosylation of the Fc was an absolute requirement for Fc γ R engagement by hIgG₁, we constructed saturation mutagenesis libraries at the Fc C'/E loop and screened them by displaying the full-length IgG variants on the yeast cell surface (Fig. S2). In this display system, the femtomolar affinity fluorescein-binding 4m5.3 single-chain antibody (21) was reformatting as a hIgG₁, allowing 4m5.3 hIgG₁ library variants to be captured on fluorescein-labeled yeast from which they are secreted, by using a cell-surface secretion capture assay (22) in conjunction with an engineered leader sequence that allows for the improved secretion of fully-assembled hIgG₁ from *S. cerevisiae* (Rakestraw JA, S.L.S., Piatasi A, Antipov E, K.D.W., unpublished data). Three saturation libraries centered about the

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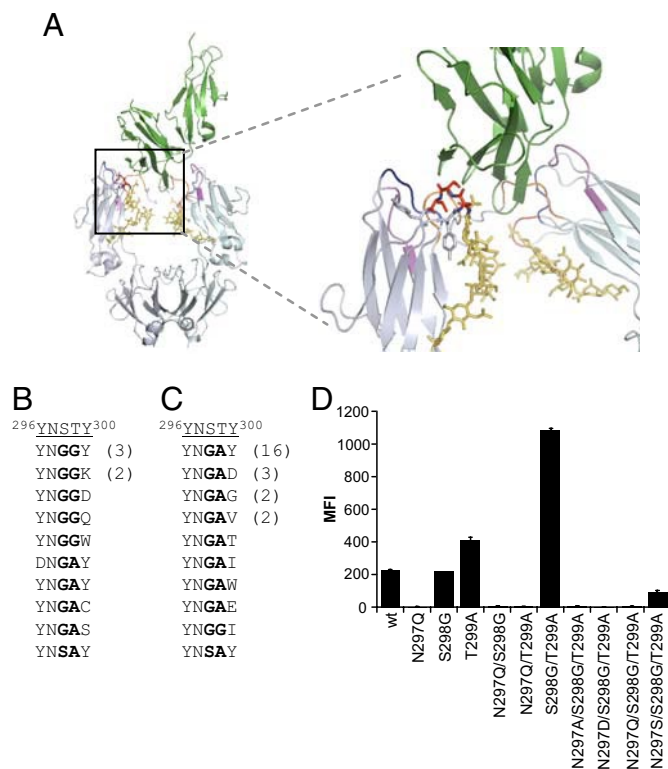


Fig. 1. Aglycosylated C/E loop variants with FcγRIIA binding. (A) Cartoon representation of the crystal structure of the hIgG₁ Fc complex with FcγRIII (PDB ID 1E4K). FcγRIII is shown in green and (both chains of) the Fc in pale blue. Fc contact surfaces are colored: lower hinge (orange), B/C loop (blue), C/E loop (red), and F/G loop (purple). Fc glycosylation is shown in yellow. The highlighted area shows an enlarged view of the C/E loop interaction with FcγRIII. Fc residues 296–300 are shown as sticks. (B) Unique sequences of aglycosylated Fc variants isolated for FcγRIIA binding after two rounds of FACS. Displayed sequences represent the residues randomized in the saturation libraries, positions 296–300, with the WT sequence underlined. Numbers in parentheses denote number of times a particular mutant was isolated. In some cases, identical protein sequences were isolated from multiple unique clones at the DNA level. Sequences of glycosylated variants enriched from the screen have been omitted. (C) Unique sequences of aglycosylated Fc variants isolated for FcγRIIA binding after a third round of FACS, using a more stringent screening strategy. (D) Binding of yeast-produced 4m5.3 hIgG₁ variants to 10 nM FcγRII^{131R} streptavidin-Alexa 647 tetramers. IgG from yeast culture supernatants was loaded onto fluorescein-conjugated yeast and median fluorescence intensity (MFI) of receptor labeling was measured by flow cytometry. Protein A Alexa 647 labeling of all samples was assessed to determine similar IgG loading (data not shown). All data represent the average of two trials.

C/E loop—theoretically encoding all amino acid combinations at residues 296–299, 297–299, and 297–300—were pooled and screened for binding to fluorophore-labeled tetramers of a soluble form of the FcγRIIA^{131R} allele by multiple rounds of FACS.

Using this screening strategy, in addition to glycosylated variants and the wild type (WT) clone, mutants lacking the canonical Asn-X-Ser/Thr N-linked glycosylation motif were enriched from the C'/E loop libraries for binding to FcγRIIA (Fig. 1B). After two rounds of screening, three aglycosylated motifs were identified: the double mutants S298G/T299A and S298G/T299G, and the single mutant T299A. After a third round of screening at increased stringency, the sublibrary was dominated by the S298G/T299A variant, suggesting it as the highest-affinity FcγRIIA binding motif in the library (Fig. 1C).

To study the contributions of the S298G and T299A mutations to receptor binding, a series of point mutants were constructed,

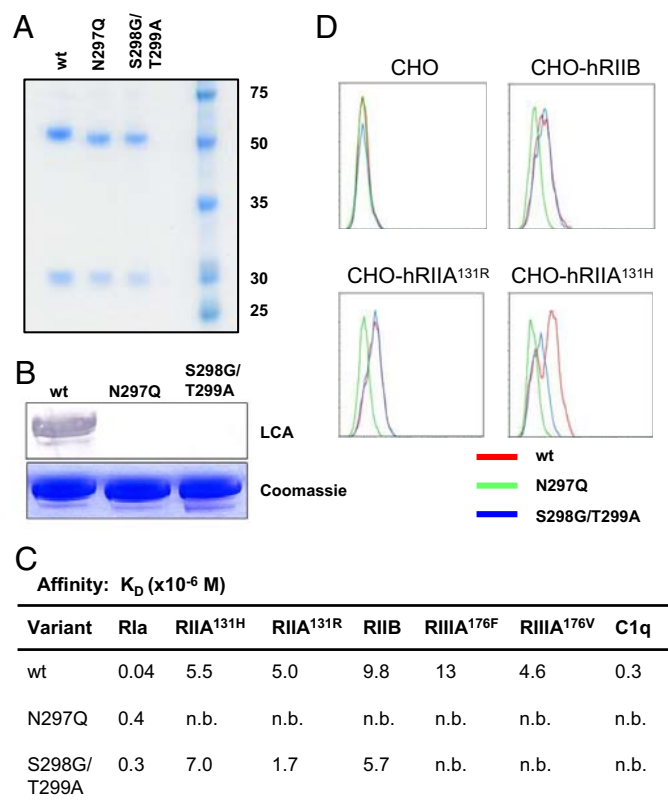
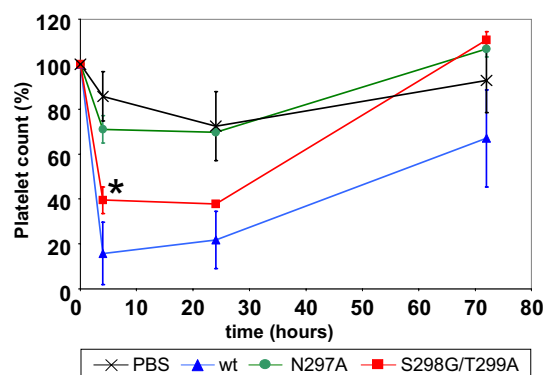


Fig. 2. S298G/T299A binds to soluble and cell surface Fc γ RIIA and Fc γ RIIB. (A) Reducing SDS/PAGE of HEK-produced WT 4m5.3 hlgG₁ and the aglycosylated variants S298G/T299A and N297Q. (B) Glycan blotting with the mannose-specific lectin LCA (*Upper*). Coomassie staining of SDS/PAGE was assessed to demonstrate similar protein loading (*Lower*). (C) Dissociation constants (K_d) for binding of Fc γ Rs and C1q to WT 4m5.3 hlgG₁ and aglycosylated variants; n.b., no binding detected. (D) Labeling of CHO cell lines stably transfected with Fc γ Rs with 4m5.3 IgG immune complexes. Cells were incubated with 1 μ g of ICs and analyzed by flow cytometry.

secreted from yeast, and assayed for their ability to bind to Fc γ RIIA^{131R} (Fig. 1D). Both the S298G and T299A mutations alone retain binding to Fc γ RIIA at comparable or increased levels to WT IgG₁, and the S298G mutation in the aglycosylated T299A background yields a variant capable of binding Fc γ RIIA to a much greater extent. The S298G/T299A double mutant is incapable of rescuing binding in the N297Q, N297D, and N297A backgrounds, suggesting there is a strong requirement for asparagine at position 297 for Fc γ RIIA binding, even in the absence of conjugated carbohydrate, a finding consistent with its conservation in the initial library screen.

S298G/T299A Is Aglycosylated and Binds to FcγRs. To confirm that the potential aglycosylated motifs identified from the yeast-based screen indeed lacked the N-linked glycan and were capable of engaging FcγRs, WT, S298G/T299A, and the nonreceptor binding N297Q aglycosylated control were expressed and purified from HEK 293 cells. Both N297Q and S298G/T299A exhibit increased mobility by reducing SDS/PAGE (Fig. 2A) and, critically, are not recognized by the mannose-specific lectin LCA (Fig. 2B), consistent with the expected absence of N-linked glycosylation. Surface plasmon resonance measurements show that S298G/T299A binds to both FcγRIIA alleles, FcγRIIA^{131R} and FcγRIIA^{131H}, and to FcγRIIB; however, this mutant does not bind to FcγRIIA nor the complement component C1q, and binding to FcγRI was weakened by 10-fold. (Fig. 2C). S298G/T299A binds FcγRIIA^{131R} with a dissociation constant (K_d) of



1.7 μM , approximately threefold stronger than WT Fc, and binds Fc γ RIIA^{131H} with a K_d of 7.0 μM , slightly weaker than WT. A small increase in affinity compared to WT for Fc γ RIIB was also observed, suggesting a preferential binding of Fc γ R with an arginine at position 131 (see Fig. 2C and Fig. S3).

To determine whether S298G/T299A can engage native FcγRs as expressed on the cell surface, CHO cell lines stably transfected with FcγRIIA^{131R}, FcγRIIA^{131H}, and FcγRIIB were labeled with 4m5.3 hIgG₁ immune complexes (ICs) (Fig. 2D and Fig. S4). Both WT and S298G/T299A IgG ICs label all receptor-expressing CHO cells in a concentration-dependent manner, demonstrating that S298G/T299A binds FcγRs in this context as well. Consistent with the soluble binding measurements, S298G/T299A IgG ICs label FcγRIIA^{131R}- and FcγRIIB-expressing CHO cells at similar levels as WT; however, S298G/T299A shows only intermediate labeling of the FcγRIIA^{131H} allele, compared to WT and the aglycosylated control.

S298G/T299A Activates FcγRIIA *in Vivo*. To determine whether this aglycosylated IgG is functional *in vivo*, a murine platelet clearance model was used to test the extent of S298G/T299A activity. The platelet integrin antigen-binding antibody 6A6 was reformatted as a mouse-human IgG₁ chimera and the S298G/T299A mutations subsequently introduced into the human Fc domain. The antibody was tested in a transgenic mouse model, in which the endogenous murine FcγRs have been deleted by gene targeting and the human activation FcγR, FcγRIIA^{131R}, is expressed as a transgene, thus maintaining cell-type expression appropriate for the human transgene (23). Mice with this genotype were treated with WT, N297A, and S298G/T299A 6A6 hIgG₁ purified from HEK 293 cells and the extent of platelet clearance measured over time (Fig. 3). After 4 hours, S298G/T299A-6A6-treated mice (*n* = 3) showed a statistically significant drop in platelet count when compared to those treated with N297A-6A6 or PBS, exhibiting a response that was comparable to WT-6A6 and demonstrating the ability of S298G/T299A to productively engage FcγRIIA *in vivo* and result in platelet clearance.

Model of S298G/T299A-Fc γ RIIA Interaction. To explore the structural basis for Fc γ R binding of this aglycosylated Fc domain variant, we constructed homology models of Fc:Fc γ RIIA com-

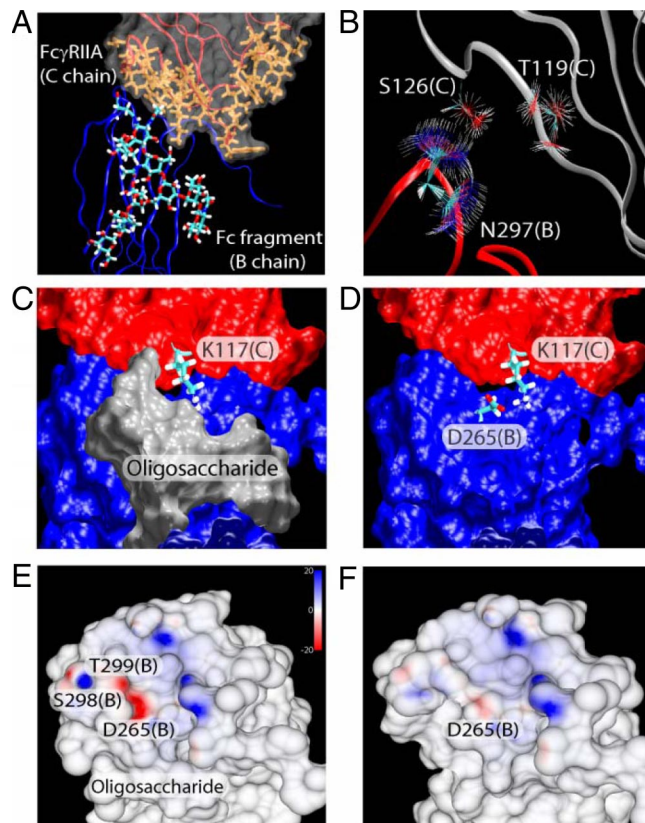


Fig. 4. Homology model of WT and S298G/T299A Fc interactions with hFcγRIIA. (A) Structure of the WT B-chain Fc fragment bound to the constructed FcγRIIA^{131R} homology model. The portion of FcγRIIA (chain C) highlighted as orange sticks shows those side chains that were conformationally relaxed, through rotamerization, during model construction. (B) Structure of the aglycosylated N297(B) interactions with a superposition of the possible discrete side chain conformations (rotameric states) of N297(B), T119(C), and S126(C) in the S298G/T299A mutant form of the Fc fragment. (C and D) Structure of the K117(C)-D265(B) salt bridge in the WT structure (C) enclosed within the binding cavity in the presence of the oligosaccharide, and the same salt bridge as seen in the aglycosylated S298G/T299A mutant (D). (E and F) Residual potential after binding mapped onto the interaction face of the B chain of the Fc fragment in the WT (E) and S298G/T299A mutant (F) structures. White regions indicate areas of ideal complementarity between the Fc fragment and FcγRIIA^{131R}, while deep red or blue regions indicate areas of poor complementarity because of ligand desolvation costs uncompensated by interactions made upon binding. Red corresponds to negative residual potentials and blue to positive residual potentials. The coloring scale, identical in both panels, is indicated in units of *kT/e*.

further engineering can also lead to aglycosylated variants with WT or improved binding to Fc γ RIIIA. In particular, we anticipate that introducing mutations into the T299A background, which weakly binds both Fc γ RIIA and Fc γ RIIIA, will lead to fully Fc γ R competent aglycosylated antibody variants. Building upon these aglycosylated Fc γ RIIIA-binding variants will be essential for their potential use as cytotoxic antibodies, which have emerged as a promising class of therapeutics for treatment of human cancer in recent years (28). Support for a critical role for Fc γ R engagement in the mechanism of antitumor activity, and specifically for Fc γ RIIIA, has come from three independent studies, which found a strong, positive correlation between patient response and the presence of specific alleles of the activating Fc γ R Fc γ RIIIA that conferred enhanced binding for the IgG1 Fc domain of the antibody (29–31). While the S298G/T299A variant does not bind complement, the above studies, as well as murine models that demonstrate a dominant role for Fc γ R engagement in therapeutic antibody activity (3), suggest that restoration of complement binding would be unnecessary for engineered Fc variants. In addition to their ability to bind Fc γ R, it will also be important to assess the stability of these variants, as previously characterized Fc variants (32) and deglycosylated WT Fc (33) have displayed reduced thermal stability.

Given the small number of mutations required to achieve N-linked glycosylation-independent Fc γ R binding, it is striking that all naturally occurring IgGs nevertheless use this posttranslational modification. Among different antibodies there is variation in the fucose and galactose-sialic acid attached to the core glycan structure (Fig. S6), and it has been reported that these variations dramatically influence the antibody activity. The absence of fucose in the glycan was reported to enhance the affinity of Fc γ RIIIA for IgG up to 50-fold (13), thereby switching the antibody into an inflammatory mode. This is required, for example, for cytotoxic antibodies, but also occurs when autoantibodies generate pathogenic immune complexes and activate autoimmune cascades. In contrast to fucose, the presence of terminal sialic acid was demonstrated to be the critical factor for the anti-inflammatory action of high-dose intravenous Ig (12, 34). Sialic acid reduces the affinity of Fc γ Rs to IgG by 5- to 10-fold (12) and, in addition, marks IgGs and subsequently allows them to bind to non-FcR lectins (34) and mediate downstream actions through these unique interactions, resulting in anti-inflammatory responses, including the up-regulation of Fc γ RIIB on effector macrophages (35). The conservation of the N-S/G-T glycosylation motif among different species (Fig. S1) at the expense of this posttranslational variability supports the view that the glycan, although not necessarily required for Fc γ R binding, serves as a platform for further modulation of the IgG's activity, enabling posttranslational switching or tuning of the IgG function between an anti-inflammatory or inflammatory mode.

Finally, our demonstration that IgG variants that have uncoupled Fc γ R binding from N-linked glycosylation can be generated opens up unique possibilities for protein engineering and biomanufacture. Our results suggest that receptor binding affinity and specificity can be engineered on the simpler template of an unmodified polypeptide chain, and these properties can be selected by yeast surface display of aglycosylated Fc mutant libraries. Such mutants could then be produced in essentially any recombinant expression system without loss of the desired altered effector functions.

Materials and Methods

Library Construction. Libraries were constructed by homologous recombination of a mutated heavy-chain constant-region insert into the 4m5.3 heavy-chain yeast-secretion vector template according to previously published methods (36). The 4m5.3 heavy-chain secretion vector was previously constructed from the pRS316 shuttle vector by insertion of the GAL10 promoter and alpha terminator, signal peptide, and 4m5.3 variable heavy-chain domain upstream

of the hIgG1 CH1 to CH3 constant domains (Rakestraw JA, S.L.S., Piatasi A, Antipov E, K.D.W., unpublished data). Preparation of mutagenic C'/E loop gene inserts and digested template vector are detailed in *SI Materials and Methods*. Gene inserts were transformed with digested template vector by electroporation into the yeast strain YVH10/LC, a derivative of the yeast strain YVH10, containing a chromosomally integrated copy of the 4m5.3 light-chain yeast-secretion vector. The 296–299 and 297–300 saturation libraries had $\approx 6 \times 10^7$ transformants, 60-fold greater than their theoretical diversity at the DNA level ($32^4 \approx 1.0 \times 10^6$); the 297–299 library had $\approx 4 \times 10^7$ transformants.

Oligonucleotides. Oligonucleotides are detailed in *SI Materials and Methods*.

Library Screening. Library screening was performed using the cell-surface secretion assay (CeSSA) (22). Briefly, libraries were grown in SD-CAA (2% glucose, 0.67% yeast nitrogen base, 0.54% Na₂HPO₄, 0.86% NaH₂PO₄·H₂O, 0.5% casein amino acids) at 30 °C to an OD₆₀₀ of ≈ 5 , and then induced in YPG (2% galactose, 2% peptone, 1% yeast extract, 0.54% Na₂HPO₄, 0.86% NaH₂PO₄·H₂O) for 12 h at 20 °C. Following this preinduction phase, yeast were labeled with fluorescein-PEG-NHS (Nektar) and reinduced in YPG containing 15% PEG (wt/vol) at 20 °C for 36 h. Cells were washed with PBS containing 0.1% (wt/vol) BSA (PBS/BSA) and labeled with biotinylated hFc γ RIIA^{131R} preloaded onto streptavidin-Alexa 647 (Invitrogen). The library was sorted on a BD FACSAria (Becton Dickinson) and collected cells grown in SD-CAA supplemented with penicillin/streptomycin (Invitrogen), for a total of three rounds of screening. Library populations were labeled at increasingly stringent concentrations of Fc γ RIIA tetramer as follows: round one (50-nM Fc γ RIIA tetramer), round two (2-nM Fc γ RIIA tetramer), and round three (80-pM Fc γ RIIA tetramer). All clones isolated from screening were retransformed into YVH10/LC and individually assayed for Fc γ RIIA binding.

Site Directed Mutagenesis. Site-directed mutagenesis is detailed in *SI Materials and Methods*.

Characterization of Yeast-Secreted Fc Mutants. Fc mutants freshly transformed into YVH10/LC were grown in 5-ml SD-CAA at 30 °C until an OD₆₀₀ ≈ 5 , then induced in 5-ml YPG at 20 °C for 72 h. Cell-culture supernatants were loaded onto fluorescein-conjugated yeast overnight at 4 °C; yeast were then washed with PBS/BSA, labeled with 10 nM of biotinylated Fc γ R preloaded onto streptavidin-Alexa 647 at 4 °C for >2 h, and analyzed by flow cytometry. Labeling with 10- μ g/ml Protein A-Alexa 647 (Invitrogen) was performed as a separate IgG loading control for all samples.

Mice. $\gamma^{-/-}$ Fc γ RIIB^{-/-} mice were generated in the Ravetch laboratory, backcrossed for 12 generations to the C57BL/6 background, and crossed to hFc γ RIIA^{tg} mice (The Jackson Laboratory). Female mice at 2 to 4 months of age were used for the experiments and maintained at the Rockefeller University animal facility. All experiments were performed in compliance with federal laws and institutional guidelines and have been approved by the Rockefeller University.

Cell Culture. CHO cells were cultured according to the American Type Culture Collection guidelines. CHO-hFc γ RIIA^{131H}, CHO-hFc γ RIIA^{131R}, and hFc γ RIIB were obtained by transfection of the pCMV-Script-hFc γ RIIA^{131H}, CHO-hFc γ RIIA^{131R}, and hFc γ RIIB plasmids and subsequent selection with 1-mg/ml geneticin (Invitrogen).

Antibodies and Recombinant Proteins. The 6A6-human Fc chimeric variants and soluble hFc γ -receptors were produced by transient transfection of 293T cells and subsequent purification from culture supernatants. For protein production, cells were cultured in DMEM supplemented with 1% Nutridoma SP (Roche). Cell-culture supernatants were harvested 6 days after transfection, and protein was precipitated by ammonium sulfate precipitation. The 4m5.3-human Fc chimeric variants were produced by transient transfection of 293F cells (Invitrogen) and subsequent purification from cell-culture supernatants. For protein production, cells were cultured in Freestyle 293F Expression Medium (Invitrogen). Recombinant receptors were purified with Ni-NTA (Qiagen) and recombinant antibodies were purified with protein G Sepharose (GE Healthcare) or immobilized protein A (Pierce) by affinity chromatography. All proteins were dialyzed against PBS. Purity was assessed by SDS/PAGE followed by Coomassie Blue staining.

Immune Complex Binding Assay. For studying immune complex binding to surface Fc γ Rs, ICs were generated by incubating 10 μ g of the respective 4m5.3 (anti-FITC) chimera with 10 μ g of BSA-FITC (Sigma) in 1-ml PBS for 2 h at 37 °C

while shaking gently. CHO cells were stained for 2 h at 4 °C with 1 μ g, 0.5 μ g, 0.2 μ g, or 0.1 μ g of ICs, washed with PBS and analyzed by FACS analysis.

Surface Plasmon Resonance Analysis. To determine the interaction between soluble hFc γ -receptors RIa (R&D Systems), Fc γ RIIA^{131H}, Fc γ RIIA^{131R}, Fc γ RIIB, Fc γ RIIIa, Clq (Calbiochem), and 4m5.3 antibody chimera, steady-state affinity measurements on a Biacore T100 biosensor were performed. Antibodies were immobilized at high densities to CM5 sensor chips (Biacore) by standard amine coupling. Soluble hFc γ -receptors were injected in five different concentrations through flow cells at room temperature in HBS-EP running buffer (Biacore) for 3 min at a flow rate of 30 μ l/min and dissociation was observed for 10 min. K_d values were calculated after subtraction of background binding to a control flow cell using Biacore T100 Evaluation software.

Lectin Blot. Lectin blotting is detailed in [SI Materials and Methods](#).

In Vivo Model Systems. Mice were injected intravenously with 50- μ g 6A6-hFc1 WT, N297A, or S298G/T299A in 100- μ l PBS. Platelet counts were determined

before injection and at 4, 24, and 72 h after injection by blood collection of 50 μ l from the retro-orbital plexus and measuring platelet counts of a 1:10 dilution in PBS/5% BSA in an Advia 120 hematology system (Bayer). Platelet clearance for mice treated with each 6A6-hIgG₁ variant was analyzed 4 h after injection by a one-way ANOVA test using SIGMASTAT. Error bars represent the standard deviation of three mice per group.

Computational Modeling. Computational modeling is detailed in [SI Materials and Methods](#).

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- Nimmerjahn F, Ravetch JV (2008) Fc γ receptors as regulators of immune responses. *Nat Rev Immunol* 8:34–47.
- Desjarlais JR, Lazar GA, Zhukovsky EA, ChuSY (2007) Optimizing engagement of the immune system by anti-tumor antibodies: an engineer's perspective. *Drug Discov Today* 12:898–910.
- Nimmerjahn F, Ravetch JV (2007) Antibodies, Fc receptors and cancer. *Curr Opin Immunol* 19:239–245.
- Jefferis R, Lund J (2002) Interaction sites on human IgG-Fc for Fc γ RIIIa: current models. *Immunol Lett* 82:57–65.
- Arnold JN, Wormald MR, Sim RB, Rudd PM, Dwek RA (2007) The impact of glycosylation on the biological function and structure of human immunoglobulins. *Annu Rev Immunol* 25:21–50.
- Shields RL, et al. (2001) High resolution mapping of the binding site on human IgG1 for Fc γ RI, Fc γ RII, Fc γ RIII, and Fc γ Rn and design of IgG1 variants with improved binding to the Fc γ RI. *J Biol Chem* 276:6591–6604.
- Tao MH, Morrison SL (1989) Studies of aglycosylated chimeric mouse-human IgG. Role of carbohydrate in the structure and effector functions mediated by the human IgG constant region. *J Immunol* 143:2595–2601.
- Mimura Y, et al. (2001) Role of oligosaccharide residues of IgG1-Fc in Fc γ RIIb binding. *J Biol Chem* 276:45539–45547.
- Walker MR, Lund J, Thompson KM, Jefferis R (1989) Aglycosylation of human IgG1 and IgG3 monoclonal antibodies can eliminate recognition by human cells expressing Fc γ RI and/or Fc γ RII receptors. *Biochem J* 259:347–353.
- Mazor Y, Van Blarcom T, Mabry R, Iverson BL, Georgiou G (2007) Isolation of engineered, full-length antibodies from libraries expressed in *Escherichia coli*. *Nat Biotechnol* 25:563–565.
- Simmons LC, et al. (2002) Expression of full-length immunoglobulins in *Escherichia coli*: rapid and efficient production of aglycosylated antibodies. *J Immunol Methods* 263:133–147.
- Kaneko Y, Nimmerjahn F, Ravetch JV (2006) Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science* 313:670–673.
- Shields RL, et al. (2002) Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fc γ RIII and antibody-dependent cellular toxicity. *J Biol Chem* 277:26733–26740.
- Yamane-Ohnuki N, et al. (2004) Establishment of FUT8 knockout Chinese hamster ovary cells: an ideal host cell line for producing completely defucosylated antibodies with enhanced antibody-dependent cellular cytotoxicity. *Biotechnol Bioeng* 87:614–622.
- Li H, et al. (2006) Optimization of humanized IgGs in glycoengineered *Pichia pastoris*. *Nat Biotechnol* 24:210–215.
- Radaev S, Motyka S, Fridman WH, Sautes-Fridman C, Sun PD (2001) The structure of a human type III Fc γ receptor in complex with Fc. *J Biol Chem* 276:16469–16477.
- Sondermann P, Huber R, Oosthuizen V, Jacob U (2000) The 3.2-Å crystal structure of the human IgG1 Fc fragment-Fc γ RIII complex. *Nature* 406:267–273.
- Krapp S, Mimura Y, Jefferis R, Huber R, Sondermann P (2003) Structural analysis of human IgG-Fc glycoforms reveals a correlation between glycosylation and structural integrity. *J Mol Biol* 325:979–989.
- Basu M, et al. (1993) Purification and characterization of human recombinant IgE-Fc fragments that bind to the human high affinity IgE receptor. *J Biol Chem* 268:13118–13127.
- Garman SC, Wurzburg BA, Tarchevskaya SS, Kinet JP, Jardetzky TS (2000) Structure of the Fc fragment of human IgE bound to its high-affinity receptor Fc ϵ RI α . *Nature* 406:259–266.
- Boder ET, Midelfort KS, Wittrup KD (2000) Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity. *Proc Natl Acad Sci USA* 97:10701–10705.
- Rakestraw JA, Baskaran AR, Wittrup KD (2006) A flow cytometric assay for screening improved heterologous protein secretion in yeast. *Biotechnol Prog* 22:1200–1208.
- McKenzie SE, et al. (1999) The role of the human Fc receptor Fc γ RIIIa in the immune clearance of platelets: a transgenic mouse model. *J Immunol* 162:4311–4318.
- Maxwell KF, et al. (1999) Crystal structure of the human leukocyte Fc receptor, Fc γ RIIIa. *Nat Struct Biol* 6:437–442.
- Lee LP, Tidor B (2001) Optimization of binding electrostatics: charge complementarity in the barnase-barstar protein complex. *Protein Sci* 10:362–377.
- Stavenhagen JB, et al. (2007) Fc optimization of therapeutic antibodies enhances their ability to kill tumor cells *in vitro* and controls tumor expansion *in vivo* via low-affinity activating Fc γ receptors. *Cancer Res* 67:8882–8890.
- Idusogie EE, et al. (2001) Engineered antibodies with increased activity to recruit complement. *J Immunol* 166:2571–2575.
- Waldmann TA (2003) Immunotherapy: past, present and future. *Nat Med* 9:269–277.
- Cartron G, et al. (2002) Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor Fc γ RIIIa gene. *Blood* 99:754–758.
- Weng WK, Czerwinski D, Timmerman J, Hsu FJ, Levy R (2004) Clinical outcome of lymphoma patients after idiotype vaccination is correlated with humoral immune response and immunoglobulin G Fc receptor genotype. *J Clin Oncol* 22:4717–4724.
- Weng WK, Levy R (2003) Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. *J Clin Oncol* 21:3940–3947.
- Oganesyan V, Damschroder MM, Leach W, Wu H, Dall'Acqua WF (2008) Structural characterization of a mutated, ADCC-enhanced human Fc fragment. *Mol Immunol* 45:1872–1882.
- Mimura Y, et al. (2000) The influence of glycosylation on the thermal stability and effector function expression of human IgG1-Fc: Properties of a series of truncated glycoforms. *Mol Immunol* 37:697–706.
- Anthony RM, et al. (2008) Recapitulation of IVIG anti-inflammatory activity with a recombinant IgG Fc. *Science* 320:373–376.
- Nandakumar KS, et al. (2007) Endoglycosidase treatment abrogates IgG arthritogenicity: importance of IgG glycosylation in arthritis. *Eur J Immunol* 37:2973–2982.
- Chao G, et al. (2006) Isolating and engineering human antibodies using yeast surface display. *Nat Protoc* 1:755–768.

Supporting Information

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SI Materials and Methods

Library Construction: Preparation of Digested Template Vector and Mutagenic Gene Inserts. The 4m5.3 heavy-chain template vector was prepared by digestion with *NheI* (New England Biolabs) and *XhoI* (New England Biolabs), which flank the 5' region of the hIgG₁ CH1 domain (*NheI*) and 3' region of the CH3 domain (*XhoI*). Saturation mutagenesis of the C'/E loop was performed by gene reconstruction with the oligonucleotides 297–299NNK (all oligos from Integrated DNA Technologies), 296–299NNK, and 297–300NNK for each of the three libraries, respectively. In a first PCR step, the mutagenic oligo and reverse primer 4m-CH3-epPCR-rev were used to amplify the region 5' of the C'/E loop through the 3' region of the CH3 domain, using the WT vector as a template. In a second PCR step, this PCR product was used along with the forward primer 4m-CH1-epPCR-for to amplify the 3' region of the 4m5.3 variable heavy chain to the 3' end of the CH3 domain, reconstructing the heavy chain CH1 to CH3 gene insert with ≈ 50 base pairs of overlap with the digested template vector for efficient yeast homologous recombination.

Oligonucleotides. 297–299NNK (5'-AGCCGCGGGAGGAG-CAGTACNNKNNKNTACCGTGTGGTCAGCGTCCT)

296–299NNK (5'-CAAAGCCGCGGGAGGAGCAGNN-KNNKNNKNNKTACCGTGTGGTCAGCGTCCT)

297–300NNK (5'-AGCCGCGGGAGGAGCAGTACNN-KNNKNNKNNKCGTGTGGTCAGCGTCCTCAC)

4m-CH1-epPCR-for (5'-ATGGAATACTTGGGTCAAG-GAACCTCAGTCACCGTCTCCGCTAGC)

4m-CH3-epPCR-rev (5'-ATTTTGTACATCTACACTGT-TGTTATCAGATTTCGCTCGAGTCA)

297NNK (5'-CCGCGGGAGGAGCAGTACNNKAG-CACGTACCGTGTGGTCAG)

298NNK (5'-GCGGGAGGAGCAGTACAACNNKACG-TACCGTGTGGTCAGCG)

299NNK (5'-GGAGGAGCAGTACAACAGCANNKTAC-CGTGTGGTCAGCGTC)

297NHC (5'-CCGCGGGAGGAGCAGTACNHCAG-CACGTACCGTGTGGTCAG)

298NHC (5'-GCGGGAGGAGCAGTACAACNHCAG-TACCGTGTGGTCAGCG)

299NHC (5'-GGAGGAGCAGTACAACAGCNHCTAC-CGTGTGGTCAGCGTC)

N, H, and K encode the following groups of nucleotide bases: N encodes all four nucleotides; K encodes G and T; H encodes A, C, and T.

Site Directed Mutagenesis. For side chain scanning of positions 297, 298, and 299, mutagenesis of the 4m5.3 heavy-chain yeast-secretion vector was performed using the Quikchange Multi Site-Directed Mutagenesis Kit (Stratagene) and the degenerate oligos 297NNK, 298NNK, 299NNK, 297NHC, 298NHC, and 299NHC. Clones were identified and confirmed by subsequent sequencing and resequencing. All other point mutants were constructed by PCR-amplification of the entire vector, using complementary primers containing the desired point mutations.

Lectin Blot. 10 μ g of 4m5.3 WT, N297Q, and S298G/T299A antibody chimera were resolved by SDS/PAGE using a polyacrylamide gel (NuPAGE, Invitrogen) under nonreducing conditions. Proteins were transferred to a PVDF membrane (Millipore), blocked with Western Blocking Reagent (Roche), and followed by incubation with biotinylated LCA lectin (2 μ g/ml, Vector Laboratories) and alkaline phosphatase-conjugated goat anti-biotin antibody (Sigma). Bound antibody was visualized with 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (Roche).

Computational Modeling. Beginning from the crystal structure of the extracellular portion of the Fc γ -RIIB receptor bound to the Fc region of the human IgG₁ (PDB ID 1E4K) (1), structures were prepared using methods from Lippow *et al.* (2). Hydrogen atoms were placed and the side chains of H116(C) and H131(C) on the receptor were flipped by 180° around χ_2 and treated in their neutral, ϵ -protonated form. In the Fc fragment, all histidine side chains were neutral and protonated as indicated, to maximize hydrogen bonding potential: 268(A)- δ , 268(B)- ϵ , 285(A)- δ , 285(B)- δ , 310(A)- δ , 310(B)- ϵ , 429(A)- δ , 429(B)- δ , 433(A)- δ , 433(B)- δ , 435(A)- δ , and 435(B)- δ . A preliminary homology model of the corresponding Fc γ RIIA complex was constructed on this backbone as follows: all non-alanine, non-glycine residues further than 4.75 Å from an interface residue were replaced by alanine; both glycosylated and aglycosylated forms of the structure were prepared; and in the glycosylated structure, a sliding, restrained harmonic minimization was performed on the side chain of the N-glycosylated N297(B). Partial atomic charges for the N-glycosylated N297(C) residues were derived by fitting to the electrostatic potential using the restrained fitting methods of Bayly *et al.* (3) for each monosaccharide. The charges associated with hydrogens missing in the polysaccharide were added to their parent atoms to ensure charge conservation. To generate the Fc γ RIIA receptor structure, all Fc γ RIIB interfacial residues were mutated to their Fc γ RIIA^{R131} counterparts using the dead-end elimination and A* protocol described by Lippow *et al.* (2) in the presence of WT or mutant Fc region. For each mutant sequence, the global minimum energy conformation, as well as a collection of progressively higher energy conformations, was identified in the context of discrete rotameric conformational freedom of all placed side chains, except for the glycosylated form of N297. All of the Fc mutants examined were generated in the presence of the receptor during this conformational search. Note that one interfacial residue in the linker region of the Fc γ R structure (E86 in the Fc γ RIIA sequence) was left as a glycine, as all glutamate rotamers searched had a van der Waals clash with the receptor backbone. In the unbound Fc γ RIIA crystal structure (4), the two domains of the receptor separate slightly to accommodate this larger residue. The solvent-screened electrostatic interactions and the residual electrostatic potential upon binding for these structural models were computed by solving the linearized Poisson–Boltzmann equation as described by Lee and Tidor (5). PARSE radii and charges were used for all examined complexes (6).

1. Sondermann P, Huber R, Oosthuizen V, Jacob U (2000) The 3.2-Å crystal structure of the human IgG1 Fc fragment-Fc gammaRIII complex. *Nature* 406:267–273.
2. Lippow SM, Wittrup KD, Tidor B (2007) Computational design of antibody-affinity improvement beyond *in vivo* maturation. *Nat Biotechnol* 25:1171–1176.

3. Bayly CI, Cieplak P, Cornell WD, Kollman PA (1993) A well-behaved electrostatic potential based method using charge restraints for deriving atomic charges—the RESP Model. *J Phys Chem* 97:10269–10280.
4. Maxwell KF, *et al.* (1999) Crystal structure of the human leukocyte Fc receptor, Fc gammaRIIIa. *Nat Struct Biol* 6:437–442.

5. Lee LP, Tidor B (2001) Optimization of binding electrostatics: charge complementarity in the barnase-barstar protein complex. *Protein Sci* 10:362–377.
6. Sitkoff D, Sharp KA, Honig B (1994) Accurate calculation of hydration free-energies using macroscopic solvent models. *J Phys Chem* 98:1978–1988.
7. Lefranc MP et al. (2005) IMGT, the international ImMunoGeneTics information system((R)). *Nucl Acids Res* 33:D593–D597.
8. Lefranc MP et al. (2005) IMGT unique numbering for immunoglobulin and T cell receptor constant domains and Ig superfamily C-like domains. *Dev Comp Immunol* 29(3):185–203.
9. Rakestraw JA, Baskaran AR, Wittrup KD (2006) A flow cytometric assay for screening improved heterologous protein secretion in yeast. *Biotechnol Prog* 22:1200–1208.
10. Sondermann P, Kaiser J, Jacob U (2001) Molecular basis for immune complex recognition: a comparison of Fc-receptor structures. *J Mol Biol* 309:737–749.

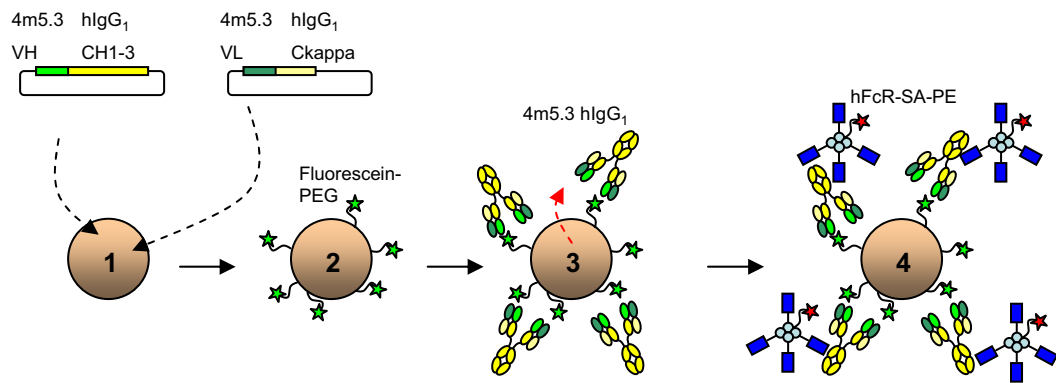


Fig. S2. Full-length hIgG₁ Fc display on the yeast cell surface. Yeast transformed with 4m5.3 heavy-chain and light-chain secretion vectors are conjugated with NHS-PEG-fluorescein, then induced for secretion in PEG-containing medium. IgG variants are preferentially captured on the fluorescein-labeled yeast cell from which they are secreted (9). The displayed library is subsequently screened with preformed tetramers consisting of biotinylated-soluble hFcγRs and fluorophore-conjugated streptavidin (SA).

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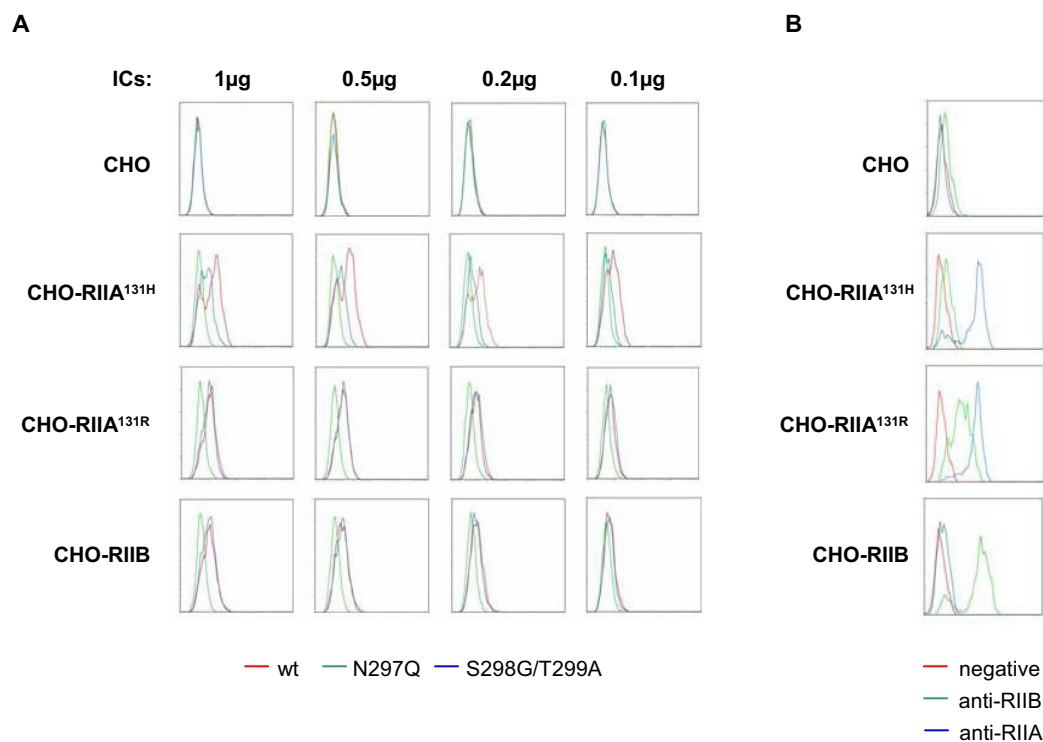


Fig. S4. S298G/T299A labels Fc γ Rs expressed on CHO cells. (A) Labeling of CHO cell lines stably transfected with Fc γ Rs with 4m5.3 IgG ICs. Cells were incubated with 0.1 to 1 μ g of ICs and analyzed by flow cytometry. (B) Control staining of CHO cell lines with anti-Fc γ RIIA and anti-Fc γ RIIB antibodies. The anti-Fc γ RIIB antibody was used at a concentration that results in a detectable cross reactivity with Fc γ RIIA^{131R}. Labeling at this concentration distinguishes between Fc γ RIIA^{131R} and Fc γ RIIA^{131H}.

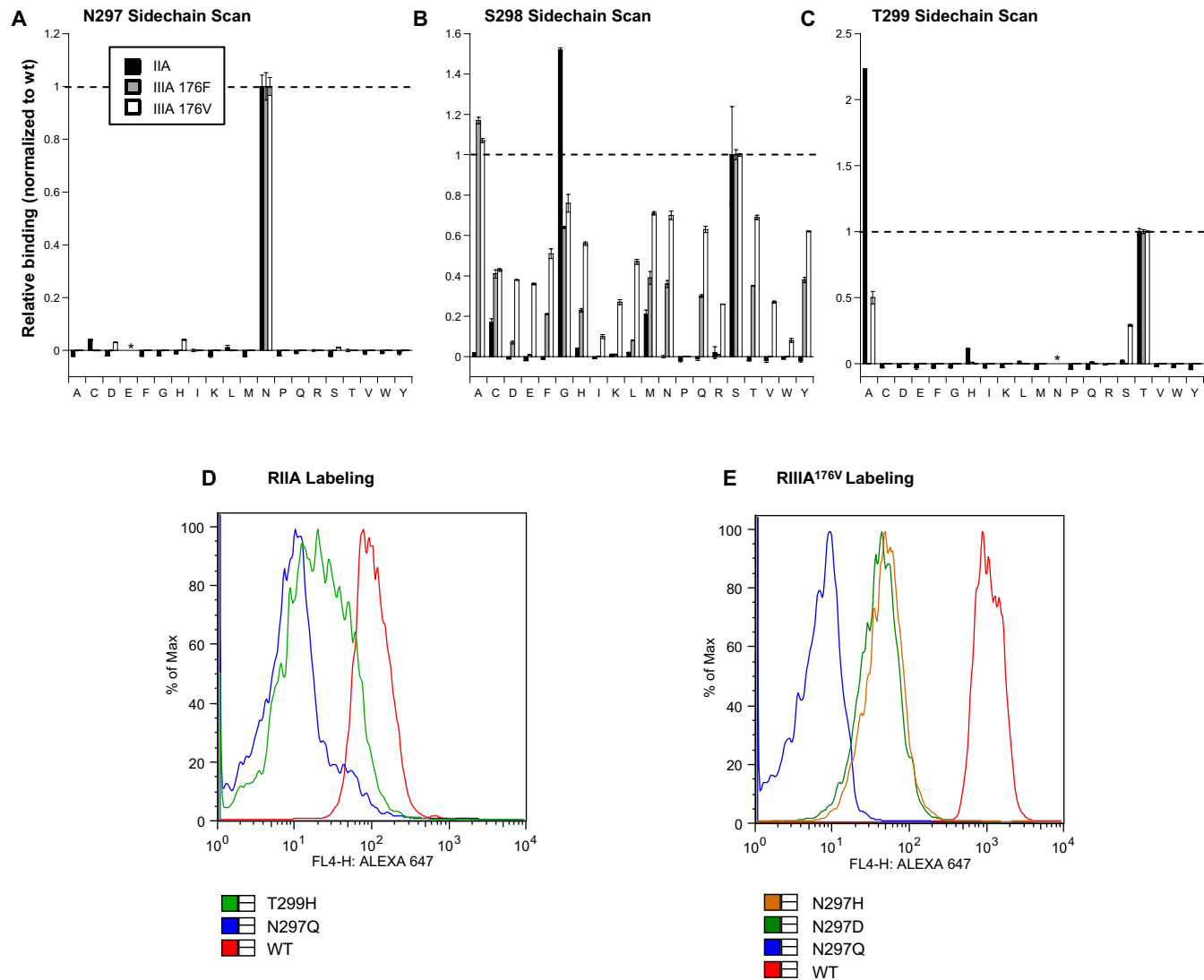


Fig. S6. Composition of the N-linked glycan attached to Asn-297. Gal, galactose; GlcNAc, N-acetylglucosamine; Man, mannose; Sial, sialic acid. Dark lines represent core glycosylation, dotted lines represent glycoforms variably attached to the core structure.