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Effective Phagocytosis of Low Her2 Tumor Cell Lines with Engineered, Aglycosylated IgG Displaying High FcγRIIa Affinity and Selectivity

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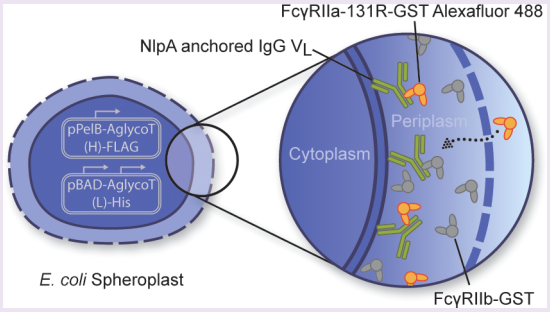
Supporting Information

ABSTRACT: Glycans anchored to residue N297 of the antibody IgG Fc domain are critical in mediating binding toward FcγRs to direct both adaptive and innate immune responses. However, using a full length bacterial IgG display system, we have isolated aglycosylated Fc domains with mutations that confer up to a 160-fold increase in the affinity toward the low affinity FcγRIIa-R131 allele as well as high selectivity against binding to the remarkably homologous human inhibitory receptor, FcγRIIb. The mutant Fc domain (AglycoT-Fc1004) contained a total of 5 amino acid substitutions that conferred an activating to inhibitory ratio of 25 (A/I ratio; FcγRIIa-R131:FcγRIIb). Incorporation of this engineered Fc into trastuzumab, an anti-Her2 antibody, resulted in a 75% increase in tumor cell phagocytosis by macrophages compared to that of the parental glycosylated trastuzumab with both medium and low Her2-expressing cancer cells. A mathematical model has been developed to help explain how receptor affinity and the A/I ratio relate to improved antibody dependent cell-mediated phagocytosis. Our model provides guidelines for the future engineering of Fc domains with enhanced effector function.



Antibodies trigger a wide array of responses in leukocytes by linking cellular targets on pathogens with Fc γ receptors (FcγRs) expressed on the surface of macrophages, granulocytes, dendritic cells, natural killer (NK) cells, and B cells. In humans, the binding of immune complexes (ICs) to activating receptors FcγRI, FcγRIIa, and FcγRIIIa initiates signaling through intracellular immunoreceptor tyrosine-based activation motifs (ITAMs) to effect potent antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP), degranulation, T cell proliferation, and cytokine release responses.[1] Activation is moderated by the binding of ICs to the sole inhibitory receptor, FcγRIIb, inducing immunoreceptor tyrosine-based inhibitory motif (ITIM) signaling that leads to anti-inflammatory responses.[2] The affinity of IgG antibodies for activating and inhibitory FcγRs depends on subclass, glycosylation state, and other more subtle effects such as Fc allotype.[3] IgG1 is the most commonly used isotype for therapeutic applications and engages all of the activating receptors, as well as the inhibitory FcγRIIb.[3] There is evidence that the ratio of binding affinities of IgG1 in solution for activating and inhibitory FcγRs (A/I ratio) plays a significant role in determining the extent of effector functions displayed by immune cells.[4−6] However, since signaling by FcγR receptors requires interactions with multivalent ICs, other parameters such as avidity of the ICs and expression level and cell surface distribution of the FcγRs on effector cells likely impact the nature of the response in addition to the A/I ratio.

There are two common polymorphisms in each of the FcγRIIIa and FcγRIIa subclasses that also affect the binding affinity to IgG1 antibodies.[3] Engagement of FcγRIIIa is considered important for the recruitment of NK effector cells that do not express the inhibitory receptor FcγRIIb.[7] Lymphoma patients expressing the high affinity FcγRIIIa allotype, FcγRIIIa-V158, show improved clinical outcomes when treated with anti-CD20 (rituximab) compared to patients homozygous for the low affinity FcγRIIIa-F158 allele.[8,9] Amino

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acid substitutions and engineering of the glycan appended to N297 of the Fc have been employed successfully to enhance affinity toward both FcγRIIIa alleles.[10,11] MGAH22, an Fc engineered Her2-specific antibody that shows improved clearance of medium and low Her2-expressing cancer cells, is under phase I clinical evaluation.[12] Likewise, the defucosylated antibody GA201 (anti-EGFR) that exhibits about 50-fold higher FcγRIIIa-V158 affinity is in phase I/II studies for high EGFR expressing solid tumors.[13]

Expression of the high affinity FcγRIIa-H131 allele correlates with improved clinical response rates following treatment with trastuzumab, rituximab, or cetuximab.[9,14,15] Conversely, individuals homozygous for FcγRIIa-R131 have an increased risk of bacterial infection and autoimmunity due to the low affinity interaction with IgGs impairing immune complex clearance.[16−19] Because the lower affinity FcγRIIa-R131 allele occurs at a frequency between 0.47−0.57 in African Americans, 0.45−0.53 in Caucasians, and 0.19−0.45 in Asians, the engineering of antibodies that can enhance responses in patients bearing the FcγRIIa-R131 allele is of significant mechanistic and clinical interest.[19−21] Engagement of FcγRIIa is particularly important for phagocytosis of tumor cells by macrophages, which were recently shown to comprise almost 20% of infiltrating leukocytes in breast cancer biopsies.[22]

Attempts to engineer IgG1 antibodies with FcγRIIa selectivity have led either to increased affinity for both FcγRIIa and FcγRIIb and/or relatively modest improvements in the A/I ratio (<6-fold change compared to wild-type).[10,23,24] Two predominant issues complicate the engineering of antibodies that display optimal FcγRIIa/FcγRIIb selectivity and FcγRIIa affinity for enhanced phagocytic potential: First, FcγRIIa shares 96% amino acid identity with FcγRIIb, and therefore the generation of Fc ligands that discriminate between the two is challenging.[7] Second, because of the avidity effects involved in the interaction of antibody-decorated tumors with effector cells it is not easy to determine *a priori* whether high affinity to FcγRIIa, or a higher A/I ratio, or both are needed to enhance phagocytosis.

Typically, the interaction of antibodies with cognate FcγRs is critically dependent on N-linked glycosylation at residue N297 in the Fc.[25] Aglycosylated antibodies show nearly complete loss of FcγR binding and effector functions.[24,26,27] Contrary to previous findings,[28] recent analysis of aglycosylated, bacterially expressed human Fc domains suggested that the absence of the glycan increases the conformational flexibility around the CH2-CH3 hinge and that the "closed conformations" (reduced CH2 distances) observed in prior aglycosylated crystal structures are likely due to crystal packing effects.[29] Screening of very large combinatorial libraries by bacterial display led to the isolation of the aglycosylated Fc5 variant, which has high affinity toward FcγRI[26] and displays a CH2 distance intermediate between those of fully glycosylated and aglycosylated Fc domains. Collectively, these findings suggest that the high flexibility of aglycosylated Fcs might allow for the identification of amino acid substitutions that stabilize conformers not accessible to glycosylated antibodies. We hypothesized that such substitutions might confer unusually high FcγRIIa affinity and selectivity with respect to binding to the highly homologous FcγRIIb for improved phagocytic potentiation. Accordingly, we report here that using a new library screening system we succeeded in isolating engineered trastuzumab variants displaying >160-fold increased affinity for the FcγRIIa-R131 allele and a >25 increase in A/I ratio compared to clinical grade glycosylated trastuzumab (Herceptin). The engineered trastuzumab was shown to elicit markedly increased ADCP with human macrophages as effector cells relative to Herceptin for both +3 (SKOV-3) and +2 (MDA-MB-453) Her2 expressing cell lines.[30] A mathematical model was developed to provide design criteria for further improving the efficacy of Fc variants by establishing the relationship between IgG binding to FcγRIIa/FcγRIIb and the activation of phagocytosis.

RESULTS AND DISCUSSION

So far, aglycosylated antibodies have been used for applications where inflammatory engagement of FcγRs or C1q complement proteins is undesirable.[31] Yet the generation of aglycosylated antibodies with unique affinities and selectivities may open the possibility for a new class of therapeutics with intriguing biological properties. To discover new aglycosylated Fc mutants, a bacterial display system has been developed in which full length IgG1 antibodies are tethered to the inner membrane of *E. coli*, leaving the Fc domain free to interact with fluorescently labeled FcγRs. By screening very large libraries (>10^8 variants) of IgGs containing randomly mutated Fc domains we isolated an aglycosylated antibody, AglycoT-Fc1004, that displays remarkably high affinity toward FcγRIIa-R131 as well as selectivity over FcγRIIb binding. To the best of our knowledge, this is the first report of an aglycosylated antibody that binds to FcγRs with affinity and selectivity that far exceed those of clinically used glycosylated antibodies or of any engineered glycosylated mutants reported thus far, engineered either through mutagenesis programs or *via* glycoengineering.[10,11,23,32]

Development of a Bacterial Display System for IgG Having a Free Fc Domain. In earlier work we showed that *E. coli* spheroplasts (bacteria in which the outer membrane has been removed by chemical treatment to render periplasmic and inner membrane proteins accessible to exogenously added proteins), expressing the human Fc domain and hinge, could bind FcγRI with K_D's comparable to those determined *in vitro* using SPR analysis of binding kinetics.[26] However, we found that the binding of the aglycosylated Fc2a mutant, which had been engineered to bind FcγRIIa/FcγRIIb,[24] was impaired when displayed in the homodimeric Fc display system.[26] Therefore, it was necessary to develop a full length IgG1 display system to accurately capture the conformational and steric effects present when antibodies interact with Fc binding ligands (Figure 1). For this purpose, the light chain (L chain) was fused at the N-terminal with the PelB leader peptide and the first 6 amino acids of the *E. coli* inner membrane lipoprotein NlpA, which anchor it to the periplasmic side of the bacterial inner membrane.[33] To avoid steric constrains that might arise if both L chains of an IgG molecule are membrane-anchored, we constructed the dicistronic pBAD-AglycoT(L)-His plasmid, which expresses NlpA anchored L chain (V_L-C_κ) as well as unanchored L chain from the same promoter (Supplementary Figure S1A). Heavy chain polypeptides expressed by pPelB-AglycoT(H)-FLAG and secreted into the periplasm assemble either with (i) two unanchored L chains, (ii) a membrane-anchored and an unanchored L chain, or (iii) two membrane-anchored L chains. The unanchored IgG in the periplasm is preferentially released from the bacteria following spheroplasting, whereas anchored IgG comprising one membrane-anchored and one unanchored L chain (or two membrane-anchored L chains) is retained on the surface of the spheroplasts. The Fc domain in the membrane-anchored IgG

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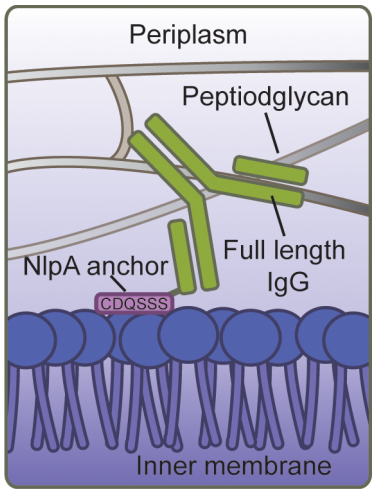

Figure 1. Development of a covalently anchored full length IgG1 display system for Fc engineering. Schematic diagram showing the display of full length IgG1 by NlpA tethering to inner membrane *via* a single membrane-anchored Light chain. Soluble fluorescent FcγRIIa-R131-GST and non-fluorescent FcγRIIb-GST can then compete for binding to full length library variants on the surface of bacterial spheroplasts before FACS sorting.

is free to interact with exogenously added, fluorescently labeled FcγRs for fluorescence activated cell sorting analysis (FACS) and library screening. To validate the IgG display strategy, the H and L chains of AglycoT-Fc5 (aglycosylated trastuzumab with E382V/M428I[26] and AglycoT-Fc2a (aglycosylated trastuzumab with S298G/T299A[24] were cloned separately into pPelB-AglycoT(H)-FLAG vector and expressed in *E. coli*. Under optimized culture conditions, AglycoT-Fc5 expressing spheroplasts displayed high fluorescence following labeling with FcγRI-FITC (Supplementary Figure S1B), whereas spheroplasts expressing AglycoT-Fc2a could be labeled selectively with FcγRIIa-GST/anti-GST-FITC (Supplementary Figure S1C). In both instances, a large increase in fluorescent signal was observed relative to the negative control, indicating that inner membrane-anchored IgG display is well suited for the engineering of aglycosylated antibody Fc variants that bind to other Fc receptors (FcγRI, FcγRIIa, FcγRIIb, FcγRIIIa/b, or FcRn).

Isolation and Characterization of Aglycosylated IgG Fc Variants Displaying High Affinity and Binding Selectivity toward the FcγRIIa-R131 Allele. An IgG that combined mutations from both Fc5 and Fc2a (E382V/M428I/S298G/T299A) was constructed and shown to display binding affinity to both FcγRI and FcγRIIa/FcγRIIb comparable to that of Fc5 and Fc2a, respectively, when expressed as individual variants (Supplementary Figure S1D). SPR analysis of the purified AglycoT-Fc5-2a revealed that binding to FcγRI was significantly reduced relative to Herceptin (11-fold decrease or a 3-fold decrease relative to AglycoT-Fc5). Interestingly, this antibody showed a small (40%) but reproducible increase in the selectivity for FcγRIIa-R131 allele relative to FcγRIIb ($A_{IIa\text{-}R131}$/I ratio) when compared to Herceptin and an even more significant increase in $A_{IIa\text{-}R131}$/I relative to AglycoT-Fc2a (Table 1). Therefore, AglycoT-Fc5-2a was used as a template for random mutagenesis and the isolation of variants that exhibit improved binding to the low affinity FcγRIIa-R131 allele and simultaneously increased $A_{IIa\text{-}R131}$/I ratios. A library of random mutations in the Fc domain of AglycoT-Fc5-2a was created by error prone PCR to yield 4.8×10^9 transformants. Cells were incubated with a progressively increasing excess of unlabeled FcγRIIb-GST (extracellular domain of FcγRIIb fused with glutathione-*S*-transferase (GST) from *Schistosoma japonicum*), over highly homologous fluorescent FcγRIIa-GST-Alexa488 conjugate to help enrich variants displaying improved FcγRIIa/FcγRIIb selectivity. After five rounds of sorting, four individual clones showing markedly improved fluorescence upon incubation with 20 nM FcγRIIa-GST-Alexa-488 and 100 nM of FcγRIIb-GST relative to spheroplasted cells expressing AglycoT-Fc5-2a were isolated (Supplementary Figures S2 and S3A). Cells expressing the clone termed AglycoT-Fc1001 (S298G, T299A, H268P, E294K, N361S and E382V, M428L (Figure 2A)) exhibited the highest fluorescence under these conditions. Interestingly, the best performing variant in ADCP assays, AglycoT-Fc1004 (S298G, T299A, N390D, E382V,

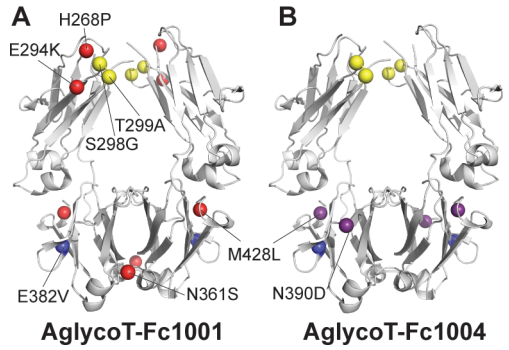

Figure 2. Mutations isolated in AglycoT-Fc1001 (A) and AglycoT-Fc1004 (B) are shown on the 3D structure of an aglycosylated IgG1 Fc (PDB code: 3S7G). Yellow = AglycoT-Fc2a base mutations, blue = AglycoT-Fc5 base mutations, red = AglycoT-Fc1001 mutations, and purple = AglycoT-Fc1004.

Table 1. SPR Analysis Showing the Ratio of Variant Affinity of Aglycosylated Trastuzumab Fc Variants to That of Glycosylated Herceptin for FcγRIIa-R131, FcγRIIa-H131 FcγRIIb, and FcγRI

	ratio of variant affinity relative to Herceptin				A/I ratio	
variant	FcγRI	FcγRIIa (H131)	FcγRIIa (R131)	FcγRIIb	H131	R131
Herceptin	1[a]	1	1	1	1	1
AglycoT-Fc5-2a	0.096	0.32	1.1	0.81	0.40	1.4
AglycoT-Fc1001	0.020	0.60	21	2.8	0.22	7.5
AglycoT-Fc1002	N/A	0.63	1.4	0.68	0.92	2.1
AglycoT-Fc1003	N/A	0.67	2.6	1.3	0.51	2.0
AglycoT-Fc1004	0.024	5.7	163	6.5	0.88	25

[a]Affinity reported in a previous study,[26] using the same method.

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M428L (Figure 2B)) showed the lowest fluorescent signal. Expression differences between the Fc1001 and Fc1004 variants are likely to be responsible for the qualitative difference between FACS signal and FcγRIIa binding; this can be addressed by two color sorting that also monitors antibody expression. Note that in AglycoT-Fc5-2a, residue M428 had been mutated to Ile, which was further changed to a Leu in AglycoT-Fc1004 and the three other selected clones pointing to the functional significance of this residue for effector FcγR binding (Supplementary Figure S2).

The four IgG variants corresponding to the sequences encoded by the highest fluorescence clones isolated by FACS were expressed in HEK293F cells to limit endotoxin contamination and purified (Supplementary Figure S3B). All IgGs contained the S298G/T299A mutations that block glycosylation at N297 and thus do not bind Concanavalin A. Binding of dimeric purified GST fusions of FcγRIIa-H131, FcγRIIa-R131, and FcγRIIb to each IgG variant was determined by SPR analysis (Table 1). A bivalent binding model was used to fit the sensorgram curves and obtain monomeric binding constants in accordance with earlier studies (Supplementary Figure S4).[34] This methodology resulted in equilibrium dissociation values, k_{on} and k_{off} rate constants for the binding of Herceptin and the various mutant Fcs to FcγRs that were in excellent agreement with the recent comprehensive binding data of all human IgG subclasses to effector FcγRs.[3] For easy comparison in Table 1, the binding data is reported as $K_{D,mutant}/K_{D,Herceptin}$ ratios and also as $(A/I)_{mutant}/(A/I)_{Herceptin}$ values. AglycoT-Fc1004 IgG displayed the highest affinity for both FcγRIIa polymorphisms: a remarkable 163-fold improvement in K_D for the low affinity FcγRIIa-R131 relative to Herceptin and a more modest 5.7-fold affinity improvement for the high affinity FcγRIIa-H131 allele (Figure 2A). The large increase in affinity toward FcγRIIa-R131 is consistent with the use of this receptor polymorphism for FACS screening. While AglycoT-Fc1004 showed somewhat higher affinity toward the inhibitory FcγRIIb, the much greater increase in FcγRIIa-R131 affinity resulted in 25-fold improvement in $A_{IIa\text{-}R131}/I$ selectivity compared to Herceptin and a 16-fold increase in selectivity relative to its parental variant, AglycoT-Fc5-2a. AglycoT-Fc1001 displayed 21-fold higher FcγRIIa-R131 affinity than Herceptin and an $A_{IIa\text{-}R131}/I$ of 7.5 (5.3-fold increase relative to AglycoFc5-2a). The two other IgGs isolated from the screening displayed only marginal increases in FcγRIIa-R131 binding and lower affinity toward the FcγRIIa-H131 allele.

The binding kinetics of the two highest affinity clones AglycoT-Fc1004 and AglycoT-Fc1001 and their parental AglcycoT-Fc5-2a toward FcγRI were evaluated by SPR, and the data were fit with a 1:1 Langmuir binding model.[34] AglycoT-Fc5-2a displayed a 10-fold decrease in FcγRI binding compared to Herceptin, which was further reduced in AglycoT-Fc1004 and AglycoT-Fc1001 to almost a 50-fold lower level. Evidently, the S298G/T299A mutations that conferred binding to FcγRIIa and FcγRIIb to aglycosylated IgGs suppressed the ability of the Fc5 amino acid substitutions to bind FcγRI. AglycoT-Fc1004 has only two amino acid substitutions in the CH3 domain (N390D, M428L) yet has 5-fold lower K_D for FcγRI relative to its parental AglycoT-Fc5-2a template. Our data show that the reason for the decreased FcγRI affinity of Fc1004 results from the N390D mutation and the subtle change I428L (compared to I428M in the parental antibody Fc5-2a). Both of these mutations are in the CH3 domain, which is distal to the FcγR binding epitope, underscoring the significance of conformational flexibility in these interactions. This finding is consistent with the recent structural data that indicates in the absence of glycosylation the Fc domain displays a high degree of conformational flexibility and our hypothesis that mutations within the CH3 domain can stabilize particular conformers with unusual FcγR binding properties.[29] In particular, M428L is located near the hinge region of the CH2 and CH3 domains where this mutation likely influences the local conformation and favorably impacts the binding of antibodies to FcRn.[35]

The isolated IgGs displayed no binding to either of the two FcγRIIIa polymorphisms (F158 and V158) as determined by ELISA (Supplementary Figure S3C). Interestingly, both AglycoT-Fc1001 and AglycoT-Fc1004 showed higher binding to the neonatal Fc receptor, FcRn, at endosomal pH 6.0 but not at physiological pH (Supplementary Figure S3D). This was most likely the consequence of the M428L mutation, which had been shown earlier to improve binding affinity for FcRn at pH 6.0 by 11-fold and thus confer extended IgG serum persistence and pharmacokinetics in animal models.[35]

Enhanced ADCP of Low and Medium Her2$^+$ Cell Lines. Surface expression of Her2 on SKBR-3, SKOV-3, and MDA-MB-453 Her2$^+$ cells was evaluated by FACS following incubation with Herceptin and a fluorescently labeled secondary antibody. SKBR-3 cells showed a 2-fold higher FACS signal relative to SKOV-3, which in turn had a 2-fold greater Her2 binding signal compared to that of MDA-MB-453 cells (Supplementary Figure S5A). These results are consistent with the total Her2 expression in these cell lines as determined by Western blotting.[36]

CD14$^+$ CD11b$^+$ macrophages were prepared from pooled PBMCs (peripheral blood mononuclear cells) following cultivation with GM-CSF cytokine.[23] The surface densities of FcγRI, FcγRIIa, FcγRIIb, and FcγRIII on macrophages were determined by FACS. We observed high FcγRI, FcγRIIa, and FcγRIIb levels in macrophages prepared at different times and from different pooled PBMC fractions (Supplementary Figure S5B). Notably, the level of FcγRI (CD64) was comparable to that of FcγRIIa and FcγRIIb, in contrast to an earlier report that showed low level FcγRI expression in human macrophages.[23]

ADCP was determined by incubating PKH67 labeled, IgG opsonized tumor cells with macrophages at a low (1:5 MDA-MB-453 or 1:10 SKOV-3) ratio of target to effector cells. The numbers of CD11b$^+$ CD14$^+$ macrophages that stained with PKH67 (arising from associated/ingested tumor cells) were determined by FACS [23] (Supplementary Figure S6) and fluorescence microscopy (Figure 3C–E). As a positive control we used glycosylated trastuzumab with the mutation G236A (GlycoT-G236A), which has been reported to confer the highest $A_{IIa\text{-}R131}/I$ and $A_{IIa\text{-}H131}/I$ ratios reported in the literature (5.5 and 5.7, respectively, relative to wild-type IgG) and to mediate increased macrophage mediated ADCP of EpCAM$^+$ LS180 cells.[23] We found that AglycoT-Fc1004 internalized approximately 75% more tumor cells ($P < 0.01$) relative to Herceptin and 40% more relative to its glycosylated, Fc engineered GlycoT-G236A variant (Figure 3A and B, Supplementary Figure S6). Equally importantly, AglycoT-Fc1004 displayed the same high level of ADCP with both the SKOV-3 and with the even lower Her2 expressing MDA-MB-453 cell line. These two cell lines correspond to approximately +3 and +2 Her2$^+$ tumor cells, respectively, which have been shown to be recalcitrant to Herceptin treatment.[37,38] Consequently, AglycoT-Fc1004 may provide the opportunity

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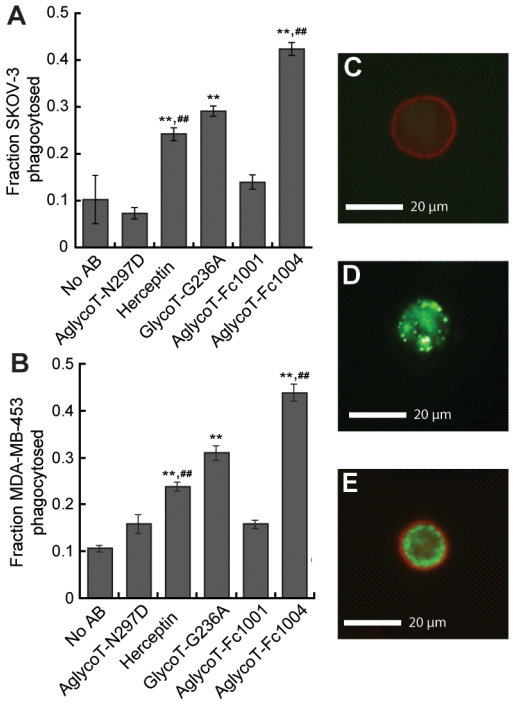

Figure 3. ADCP mediated by human monocyte derived macrophages using AglycoT-Fc1001 and AglycoT-Fc1004 with +3 Her2$^+$ SKOV-3 ovarian cancer (A) and +2 Her2$^+$ MDA-MB-453 breast cancer (B) tumor cell lines as target cells. ** Welch's t test $P < 0.01$ for samples compared to No Ab negative control, $^{\#\#}$ Welch's t test $P < 0.01$ for samples relative to G236A positive control. (C−E) Fluorescent microscopy images showing macrophages labeled with 10 μg/mL anti-CD14-APC and 10 μg/mL anti-CD11b-APC (C), PKH67 membrane labeled MDA-MB-453 tumor cells (D), and phagocytosed tumor cells (E).

to treat patients having tumors with Her2 moderate or low expression that do not respond well to Herceptin. We expect binding to the low affinity FcγRIIa-R131 polymorphism to be particularly desirable since therapeutic responses in patients that are homozygotic or heterozygotic for this allele show poorer responses to anti-neoplastic antibodies.[9,14,15] On the other hand, AglycoT-Fc1001 could not induce phagocytosis of tumor cells above background for either cell line. Likewise, the addition of 2B6, an antagonistic FcγRIIb antibody, had no significant effect on ADCP with any of the engineered antibodies tested or with Herceptin, suggesting that tumor cell surface-bound ICs likely displace 2B6 from FcγRIIb allowing ITIM mediated signaling to occur.

Mathematical Modeling of ADCP. To gain insights on the puzzling lack of phagocytosis of Her2 expressing tumor cells opsonized with the AglycoT-Fc1001 antibody, we developed a quantitative model to relate the formation IgG:FcγRIIa and IgG:FcγRIIb ICs to phagocytosis (see Figure 4A and Supporting Information for model details). Our simulations suggest that antigen-bound IgG, but not monomeric IgG, can elicit a significant phagocytic response because the clustered presentation of antigen-bound IgGs in the contact region results in an increased local concentration of Fc domains that leads to the displacement of monomeric IgG and promotes a sufficiently high level of FcγR cross-links to elicit effector responses. This result is in complete agreement with *in vivo* observations that serum IgG alone, in the absence of ICs, cannot elicit FcγR signaling.[1] To link biomolecular interactions at the cell surface to ADCP efficacy, we assigned an "intrinsic signaling potency" parameter to each receptor subunit and assumed that the signaling potency of each dimeric IgG:FcγR (used as a proxy for higher-order IgG:FcγR clusters) was equal to the sum of its constituents. The net signal generated by all FcγR complexes on the macrophage surface constituted the level of ADCP response.[3] With the exception of the intrinsic signaling potencies for FcγRIIa-H131 and FcγRIIa-R131 (relative to FcγRIIb), all other parameters were obtained from experimental data, literature values, or simulations (Supplementary Table S4). To parametrize the two relative signaling potencies for FcγRIIa-H131 and FcγRIIa-R131, the model was fit only to ADCP data for Herceptin and AglycoT-Fc1001 and then used to predict the expected ADCP response of AglycoT-Fc1004 (Figure 4B and C). Similarly, parametrization of the model using the data for any two antibodies within the Herceptin, AglycoT-Fc1001, and AglycoT-Fc1004 set accurately predicted the ADCP response for the third antibody (Supplementary Figure S7A−E).

The model provides a framework for understanding the molecular basis for enhanced ADCP response. Figure 4D−F show heat maps of the predicted phagocytic responses for different $K_{D\_IIa\text{-}H131}$, $K_{D\_IIa\text{-}R131}$, and K_{D_IIb} values. The IgG variants examined in this work were overlaid in Figure 4D on the basis of their $K_{D\_IIa\text{-}H131}$ and $K_{D\_IIa\text{-}R131}$ values to show their approximate expected ADCP responses (see Supplementary Figure S7E for actual predictions). The simulations show that enhanced ADCP most dominantly correlates with increased affinity to FcγRIIa-H131, which is predicted to have a greater intrinsic signaling potency than FcγRIIa-R131 (Supplementary Table S5). This prediction is also consistent with clinical observations that FcγRIIa-R131 homozygous individuals are more susceptible to bacterial infections and autoimmune diseases,[16−18] perhaps due to inefficient generation of activation signals in the absence of FcγRIIa-H131 subunits.

It has been suggested that effector response can be predicted by the A/I ratio, defined as the ratio of IgG Fc affinity for FcγRIIa to that for FcγRIIb.[6] To investigate the utility of A/I ratio in predicting phagocytosis, lines of constant $A_{IIa\text{-}H131}/I$ or $A_{IIa\text{-}R131}/I$ were plotted for fixed $K_{D\_IIa\text{-}R131}$ and $K_{D\_IIa\text{-}H131}$, respectively in Figure 4D (*i.e.*, $K_{D\_IIb}/K_{D\_IIa\text{-}H131}$ in Figure 4E and $K_{D\_IIb}/K_{D\_IIa\text{-}R131}$ in Figure 4F). Even though a higher A/I ratio tends to elicit a higher effector response, phagocytic activity can vary significantly even for a fixed A/I ratio, indicating that absolute receptor affinities also play a significant, if not dominant, role in shaping cellular response. Interestingly, for a fixed A/I ratio, increasing FcγRIIa-H131 affinity generally leads to increased phagocytosis (Figure 4E), while increasing FcγRIIa-R131 affinity leads to decreased phagocytosis (Figure 4F). This can be explained by the relative intrinsic signaling potencies of the receptor subunits (Supplementary Table S5). At a fixed $A_{IIa\text{-}H131}/I$, increasing the affinity for FcγRIIa-H131 (potency >1) would also increase the affinity for FcγRIIb (potency = −1), but the net effect would be increased activation due to the differential potencies. By contrast, at a fixed $A_{IIa\text{-}R131}/I$, increasing affinities for both subunits would reduce ADCP response because FcγRIIb is more potent at signaling than FcγRIIa-R131 (potency <1). Our findings agree with recent crystal structure data that suggest the FcγRIIa-R131 polymorphism adopts a conformation that maybe less favorable for multimerization than FcγRIIa-H131, therefore limiting the proximity of ITAM domains required to transduce activation signals.[39] The model suggests that efforts to improve the design

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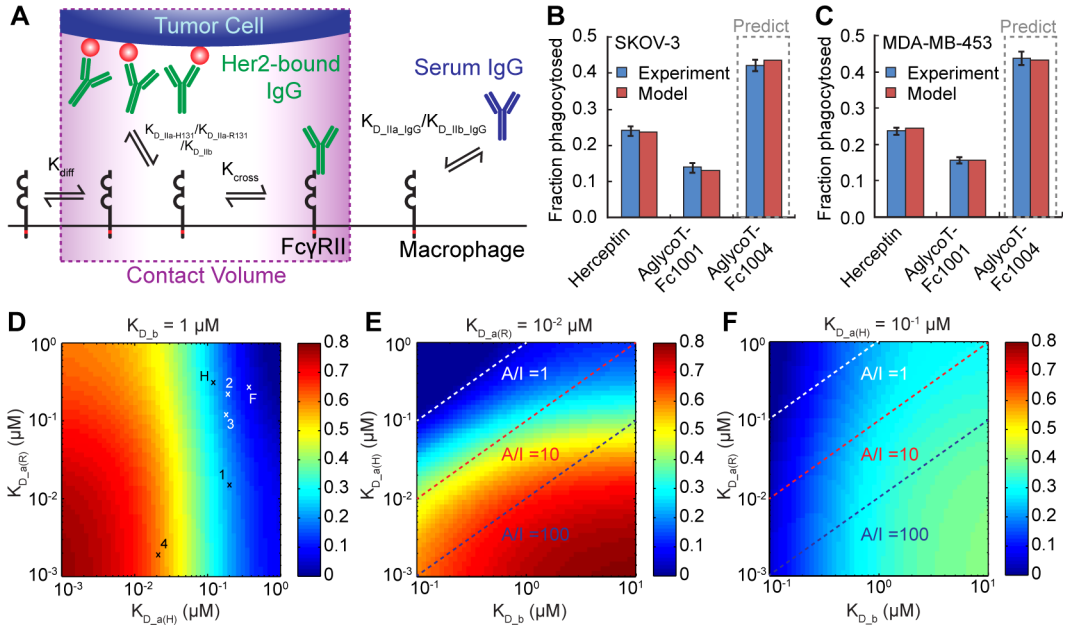

Figure 4. Mathematical modeling provides insights into design rules governing phagocytic potency of Fc variants. (A) Schematic depicting the binding reactions considered in the mathematical model. (B, C) Validation of the mathematical model (red bars) with ADCP data (blue bars). ADCP data for trastuzumab and AglycoT-Fc1001 were used to fit intrinsic signaling potencies of the receptor subunits (Supplementary Table S5). This model was then directly used to predict ADCP responses for AglycoT-Fc1004 with both SKOV-3 cells (B) and MDA-MB-453 cells (C). (D) Predicted fraction of SKOV-3 cells phagocytosed for a range of $K_{D\_IIa\text{-}H131}$, $K_{D\_IIa\text{-}R131}$, and K_{D_IIb} values. K_{D_IIb} was held constant at 1 μM, and $K_{D\_IIa\text{-}H131}$ and $K_{D\_IIa\text{-}R131}$ values were varied. Phagocytic responses for Herceptin (H), AglycoT-Fc5-2a (F), AglycoT-Fc1001 (1), AglycoT-Fc1002 (2), AglycoT-Fc1003 (3), and AglycoT-Fc1004 (4) were predicted on the basis of their experimentally determined equilibrium binding constants to FcγRIIa-H131 and FcγRIIa-R131 (see Table 1). (E) $K_{D\_IIa\text{-}R131}$ was held constant at 0.01 μM, and K_{D_IIb} and $K_{D\_IIa\text{-}H131}$ values were varied: fixed $K_{D\_IIb}/K_{D\_IIa\text{-}H131}$ ratios of 1 (white dash), 10 (red dash), and 100 (blue dash) are shown. (F) $K_{D\_IIa\text{-}H131}$ was held constant at 0.1 μM, and K_{D_IIb} and $K_{D\_IIa\text{-}R131}$ values were varied. The same ratios of A(R)/I affinity as in panel E are shown.

of IgG Fc variants for enhanced ADCP should first focus on increasing affinity for FcγRIIa-H131 (Figure 4D and E) and, if not possible or insufficient, then on decreasing affinity for FcγRIIb (Figure 4E). If binding to the low affinity FcγRIIa-R131 allele is required, then both improved R131 affinity and A/I ratio are necessary for more potent ADCP (Figure 4F). While FcγRIIa and IIb have been established to be by far the most important receptors for phagocytosis by tumor cells,[23,40] the inclusion of FcγRI and FcγRIIIa in the model could be used to determine design criteria for glycosylated antibodies or future aglycosylated variants with more complex FcγR mediated effector function.

The generation of highly FcγR selective Fc domains might allow for directed recruitment of certain immune effector cells, such as macrophages as shown here (because AglycoT-Fc1004 does not engage FcγRIIIa and has 50-fold lower affinity for FcγRI). So far, aglycosylated antibodies have been used for applications where inflammatory engagement of FcγRs or C1q complement proteins is undesirable.[31] The generation of aglycosylated antibodies with unique affinities and selectivities may open the possibility for a new class of therapeutics with intriguing biological properties. Fc engineered aglycosylated IgGs offer additional advantages by bypassing the need for N-linked glycosylation; eliminating glycan heterogeneity and broadening the range of expression systems that can be used to produce large quantities of antibody. In several recent clinical trials, no evidence of aglycosylated antibody immunogenicity has been reported.[31] However, at this point it is unclear whether mutations to an aglycosylated structure will induce immunogenicity either through residue change or the generation of non-native conformational epitopes. The results from these studies will be reported in the future.

METHODS

(For additional materials and methods see Supporting Information)

ADCP Assays. Tumor cells were recovered from culture flasks by tryspinization, labeled with PKH67 (Sigma-Aldrich) according to the manufacturer's instructions, and resuspended in RPMI with 10% human AB serum. These cells were seeded at 2×10^4 per well in 96-well V-bottom plates (Corning) followed by addition of macrophages at the appropriate effector: tumor cell ratio (1:5 MDA-MB-453, 1:10 SKOV-3) and antibodies, to a final concentration of 0.5 μg/mL. The plate was briefly spun at 2,000 rpm for 1 min and incubated at 37 °C with 5% CO_2 for 2 h (MDA-MB-453) or 4 h (SKOV-3). Cells were detached from the plate by HyQTase treatment for 15 min and stained with 10 μg/mL anti-CD11b-APC and 10 μg/mL anti-CD14-APC on ice for 1 h. Phagocytosis was evaluated by FACS on an LSRFortessa (BD Bioscience) and reported as the fraction of double positive cells over the total number of tumor cells in the sample (see Supplementary Figure S5C for process overview). Fluorescent microscopy was performed on an Axiovert 200M (Carl Zeiss MicroImaging GmbH).

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): The authors have filed a provisional patent application that relates to this work.

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