Aglycosylated IgG variants expressed in bacteria that selectively bind FcγRI potentiate tumor cell killing by monocyte-dendritic cells

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The N-linked glycan of immunoglobulin G (IgG) is indispensable for the interaction of the Fc domain with Fcγ receptors on effector cells and the clearance of target cells via antibody dependent cell-mediated cytotoxicity (ADCC). *Escherichia coli* expressed, aglycosylated Fc domains bind effector FcγRs poorly and cannot elicit ADCC. Using a novel bacterial display/flow cytometric library screening system we isolated Fc variants that bind to FcγRI (CD64) with nanomolar affinity. Binding was critically dependent on an amino acid substitutions (E382V, and to a lesser extent, M428I) distal to the putative FcγRI binding epitope within the CH3 domain. These mutations did not adversely affect its pH-dependent interaction with FcRn in vitro nor its serum persistence in vivo. Remarkably, the anti-Her2 IgG trastuzumab containing the E382V, M428I substitutions and expressed in *E. coli* exhibited highly selective binding to FcγRI but not to the other activating receptors (FcγRIIA, FcγRIIB) nor to the inhibitory receptor, FcγRIIB. In contrast, the glycosylated version of trastuzumab (E382V, M428E) purified from HEK293T cells bound to all Fcγ receptors in a manner similar to that of clinical grade trastuzumab. *E. coli*-purified trastuzumab (E382V, M428I), but not glycosylated trastuzumab (E382V, M428E) or clinical grade trastuzumab, was capable of potentiating the killing of Her2 overexpressing tumor cells with dendritic cells (DCs) as effectors. These results indicate that aglycosylated IgGs can be engineered to display unique Fc/R selectivity profiles that, in turn, mediate ADCC via mechanisms that are not normally displayed by glycosylated monoclonal antibodies.

antibody engineering | bacterial display | bacterial expression | directed evolution | effector function

Fc receptors (FcγRs) play a crucial role in linking the adaptive immune system to the effector functions of innate cells such as antigen presentation and antibody homeostasis (1). Human immune cells express six immunoglobulin (IgG) binding FcγRs (FcγRI, FcγRIIA, FcγRIIB, FcγRIIC, FcγRIIIA, FcγRIIIB) that enable various functions, including phagocytosis, cytokine production, superoxide production, release of serotonin, and inhibition of B cell activation (1, 2). An additional IgG binding receptor, the neonatal Fc receptor (FcRn), plays a critical role in IgG homeostasis by mediating antibody recycling via intracellular trafficking, thus preventing degradation in lysosomes (3). With the exception of FcγRIIB, human FcγRs are type I transmembrane glycoproteins. Activating FcγRs such as FcγRI (CD64), FcγRIIA (CD32a), and FcγRIIIA (CD16a) have an immunoreceptor tyrosine-based activation motif in their cytoplasmic tail and trigger activation of immune responses. The inhibitory receptor FcγRIIB (CD32b) processes an immunoreceptor tyrosine-based inhibitory motif in its cytoplasmic domain that downregulates immune responses (4). The affinity of FcγRII and FcγRIII to monomeric IgG is very low ($K_d = 10^{-6} - 10^{-7}$ M), whereas FcγRI exhibits high affinity to monomeric IgG ($K_d = 10^{-8} - 10^{-9}$ M).

The effector FcγRs interact with the upper CH2 and hinge region of the IgG-Fc domain (Fig. 1A) in a manner that is critically dependent on the presence and composition of the single N-linked glycan attached to N297. In contrast, FcRn, which binds to the interface of CH2 and CH3 (5), is not significantly affected by the glycosylation status of the antibody constant region. The glycosylation state of IgG determines its affinity for effector FcγRs and thus, modulates the activation of cytotoxic immune cells and their ability to participate in antibody-dependent cell-mediated cytotoxicity (ADCC) (6, 7). ADCC is important for the clinical efficacy and outcomes of many important antibody therapeutics (8). Glycan composition and glycoform heterogeneity, which is intrinsic to the expression of antibodies in mammalian cells, has a well-documented effect on the response to widely used therapeutics such as Rituxan (rituximab, anti-CD20) and Herceptin (trastuzumab, anti-Her2) (8, 9). This has led to intense efforts to generate improved antibodies via glycoengineering (7). Wild-type aglycosylated antibodies can be expressed at a high yield in bacterial cells and exhibit normal serum persistence and binding to FcRn (10). However, their lack of glycosylation renders them completely unable to bind to FcγRs, and therefore they do not induce FcγR-mediated effector functions (9). Recently, structure-based mutagenesis and, in a separate study screening of small saturation libraries by yeast display, were used to isolate aglycosylated IgG1 variants lacking N297 and carrying mutations in residues S298 and T299 that conferred binding to FcγRIIIa or FcγRIIB, respectively (11, 12). However, the respective antibody variants, following expression in HEK293 cells, exhibited significant binding to the inhibitory FcγRIIB receptor (12). This result was not surprising given the high degree of homology between FcγRIIa and FcγRIIB.

We have developed a system that relies on bacterial expression and flow cytometric screening of very large libraries of Fc mutants for the isolation of variants that display a high degree of selectivity and receptor affinity (Fig. 1B). In this study, we focused on the isolation of aglycosylated IgG that bound to FcγRI, as its role in ADCC is far less understood than that of other activating receptors (1, 2). It has been suggested that because of its high affinity, FcγRI on effector cells is occupied by circulating IgG and thus is involved in ADCC only during the early stage of the immune response when antibody concentration is low (13). However, the avidity associated with the formation of immune complexes must be capable of displacing soluble IgG from FcγRI

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and consistent with this hypothesis there is increasing evidence that the activation of this receptor plays an important role in the resolution of certain infections and in cancer (14–16).

We report here the engineering of aglycosylated IgG antibodies that contain amino acid substitutions in the CH3 domain that are distant from the FcγRI-binding epitope located in the CH2 domain yet confer low nanomolar affinity to FcγRI but not to the inhibitory FcγRIIB or to other effector FcγRs. Engineered, bacterially expressed trastuzumab IgGs potentiates the lysis of Her2 overexpressing SKBr3 breast cancer cells by effector monocyte-derived dendritic cells (mDCs). Strikingly, glycosylated IgGs, clinical grade trastuzumab (Herceptin) or mammalian cell-expressed trastuzumab that contained the same CH3 domain mutations failed to activate the cytotoxic ability of mDCs. Our results indicate that aglycosylated IgGs engineered to display unique FcγR-binding characteristics may be useful both for elucidating the function of FcγRI and for the selective recruitment of mDCs in cancer therapy.

Results

Bacterial Display of Aglycosylated Fc Domains. Simmons (10) previously reported that aglycosylated, correctly folded IgG1 antibodies purified from E. coli exhibit negligible binding to the extracellular domain of FcγRI or Clq. To identify amino acid substitutions that enable aglycosylated IgG to bind to the extracellular domain of FcγRs, it was first necessary to develop an appropriate system for E. coli library screening. The e-clonal technology we developed previously (17) is well suited for the isolation of IgGs with desired antigen specificities by fluorescence-activated cell sorting (FACS). However, in the e-clonal system, the Fc domain of IgG secreted into the periplasm is captured by an inner-membrane anchored ZZ domain from Protein A and, therefore, is not suitable for engineering Fc domains. We noticed that, under certain conditions, soluble Fc secreted into the periplasm via a posttranslational signal peptide (such as the widely used PelB leader) (Fig. S1A) is retained on the surface of spheroplasted cells following treatment with Tris-EDTA-lysozyme (Fig. 1B). Thus, spheroplasts grown in terrific broth media and expressing secreted, soluble Fc exhibited 2-fold higher fluorescence upon labeling with Protein A-fluorescein isothiocyanate (FITC) relative to spheroplasts expressing an unrelated scFv (Fig. 1C). The retention of secreted Fc by spheroplasted cells was highly dependent on growth conditions. Optimal labeling with Protein A-FITC was obtained with cells grown at 25 °C in the presence of 0.5 M trehalose but not with other nonmetabolizable sugars such as sucrose or sorbitol. Under these conditions spheroplasts expressing the Fc and labeled with Protein A-FITC exhibited 10-fold higher signal than controls (Fig. 1C). Western blot analysis revealed that a significant fraction of the Fc protein was retained in spheroplasted cells after growth with 0.5 M trehalose (Fig. S1B). Secretion of Fc by the signal recognition particle dependent DsbA signal sequence (18) did not result in significant protein retention following spheroplasting, even in the presence of 0.5 M trehalose (Fig. S1C).

High Throughput Screening of Aglycosylated Fc Libraries for Increased FcγRI Binding. The retention of ligand-accessible Fc in spheroplasted cells was exploited for the flow cytometric screening of large libraries for FcγRI binding. We initially constructed libraries composed of (i) random mutants by error-prone PCR (−9.2 × 10⁵ transformants) containing 0.5% substitutions per gene (as determined by the sequencing of 20 random clones) and (ii) 10-a.a. random insertions encoded by NNS randomization scheme (where N = equimolar A, T, C, G; S = equimolar C, G) (−2.8 × 10⁴ transformants representing a miniscule fraction of the theoretical diversity) between N297 and S298 with the intent of replacing the glycan that is normally attached to N297 with a peptide epitope. Transformants from the two libraries were pooled and, following induction of protein synthesis, spheroplasts were prepared and labeled with the appropriate FcγR conjugated to FITC (Fig. 1B). Highly fluorescent clones were isolated by FACS. The respective genes were rescued by PCR, subcloned, and transformants were subjected to additional rounds of FACS sorting. Positive populations were enriched following iterative screening with fluorescently labeled extracellular domain of FcγRI (CD64) (Fig. S1D). After the fourth round, 50 clones were selected at random and their individual fluorescence was determined. The six clones displaying the highest fluorescence (>5x above background, Fig. 1D) upon labeling with FcγRI-FITC were isolated and sequenced (Fig. S1E). All six clones were derived from the error-prone PCR Fc library (Fig. S1E) and contained two consensus amino acid substitutions, E382V and M428I that fall within the CH3 C and F disulfide bridge (Fig. 2A). Published crystal structures (19) of dimeric Fc protein (Fig. 2C) suggest that the activation of this receptor plays an important role in cancer therapy.
Fig. 2. Characterization of isolated aglycosylated Fc variants. (A) Amino acid substitutions in Fc5 (a.a. 382 and 428) represented on the 3D structure of glycosylated IgG1 Fc (PDB Code: 1FC1). (B) Two beta sheets including 382E and 428M in β-sheet C and 482M in β-sheet F of CH3 domain represented on the crystal structure of wild-type full glycosylated IgG. (C) Nonreducing SDS-PAGE showing the purified wt Fc and engineered Fc variants. Lane 1: M.W. standards; Lane 2: wt Fc; Lane 3: Fc5; Lane 4: Fc11; Lane 5: Fc49. (D) Binding of wt Fc, Fc5, Fc11, and Fc49 to FcRI extracellular domain detected by ELISA. Errors bars indicate the standard deviations from duplicate run on the same sample.

(SPR) analysis revealed that wild-type aglycosylated Fc does not bind to FcγRI (K_D > 30 μM), whereas Fc5 and Fc49 exhibited high affinities with K_D values of 31 and 92 nM, respectively (k_on,Fc5 = 4.5×10^4 M^-1 sec^-1, k_off,Fc5 = 1.4×10^-3 sec^-1; k_on,Fc49 = 2.5×10^4 M^-1 sec^-1, k_off,Fc49 = 2.3×10^{-3} sec^{-1}).

We next performed additional saturation mutagenesis of residues 380, 382, and 384 that lie in the F-contacting C strand and contained two additional mutations: K338R and G341V of the region of the CH3 (Fig. S4). Two beta sheets including 382E and 428M interfere with the G and A strands of CH2 and CH3, respectively (Fig. S4E).

Engineered, E. coli-Expressed Aglycosylated Trastuzumab Exhibits Highly Selective FcγRI Binding and pH-Dependent FcRn Binding.

Herceptin (glycosylated trastuzumab produced in Chinese hamster ovary (CHO) cells) is a highly effective therapeutic antibody specific for Her2/neu (Erbb2) that is overexpressed in ~30% of breast carcinomas (8). Extensive evidence indicates that recruitment of innate immune cells via interactions with Fcγ receptors plays an important role in the therapeutic action of Herceptin (8). For preparative production of aglycosylated trastuzumab (AglycoT), aglycosylated trastuzumab-Fc5 (AglycoT-Fc5) and aglycosylated trastuzumab-Fc601 (AglycoT-Fc601) in E. coli, heavy and light chains were fused to the PelB signal peptide and placed downstream from the lac promoter in a dicistronic operon (Fig. S5A). E. coli BL21(DE3) cells were grown at 30°C to an OD_600 of ~100 by fed-batch fermentation in pH-stat control mode, protein synthesis was induced, the fermentor was cooled to 25°C, and cells were harvested 7 hr later at an OD_600 of ~130–140 (Fig. S5B). Under these conditions, the yield of aglycosylated tetrameric IgG1 was 40–50 mg/L with >70% retained in the periplasmic space. IgG1 protein was released from the cell pellet by incubation with lysozyme and EDTA for 16 hr at 30°C and was purified to >80% homogeneity by protein A and size exclusion chromatography (Fig. 3A and Fig. S5C). SPR analysis (Fig. 3B and Fig. S5D) revealed that the E. coli expressed AglycoT-Fc5 and AglycoT-Fc601 IgG1s showed dramatically improved affinity toward FcγRI relative to AglycoT. For example, AglycoT-Fc601 bound to FcγRI with an equilibrium dissociation constant (K_D) nearly identical to that of commercial-grade glycosylated trastuzumab (Herceptin) and >130-fold greater than wild-type bacterially expressed AglycoT. Both AglycoT and AglycoT-Fc5 displayed higher affinity for FcγRI than the isolated Fc domains. Notably and unlike Herceptin, which binds to all effector human Fcγ receptors with physiologically relevant affinities, AglycoT-Fc5, and AglycoT-Fc601 (as well as AglycoT) exhibited poor binding to FcγRIIa, FcγRIIb (EC50 > 1000-fold and 100-fold higher for GST fused FcγRIIa and FcγRIIb, respectively, FcγRIIa and FcγRIIb (Fig. 3E)).

Because the mutations that impart the specific interaction of AglycoT-Fc5 to FcγRI are distal to the biochemically defined binding epitope that encompasses the glycosylated N297 residue, we sought to examine whether glycosylation might have an effect on binding affinity and specificity. Glycosylated antibodies against Her2/neu were produced in HEK293T cells, and served as glycosylated counterparts to AglycoT-Fc5 and AglycoT, which we labeled as glycosylated trastuzumab-Fc5 (GlycoT-Fc5) and glycosylated trastuzumab (GlycoT) (Fig. S6A). The affinities of GlycoT-Fc5 and GlycoT for the extracellular domains of effector FcγRs were determined by ELISA. GlycoT-Fc5 showed almost identical affinity for FcγRI as wild-type GlycoT and clinical grade Herceptin (trastuzumab produced in CHO rather than HEK293T cells) (Fig. 3F). Surprisingly, and in contrast to its aglycosylated counterpart, GlycoT-Fc5 did not exhibit selectivity for FcγRI and showed binding to FcγRI as well as FcγRIIa (Fig. S6B). FcγRIIb (Fig. S6C), and FcγRIIa (Fig. S6D) similar to GlycoT and typical of other IgG1 human antibodies. These results reveal that glycosylation at N297 has a dominant effect relative to that of E382V and M428I mutations.

The mutations in AglycoT-Fc5 and AglycoT-Fc601 reside close to the epitope recognized by the neonatal Fc receptor (FcRn) and therefore might affect their ability to escape endosomal degradation—a consequence that could be detrimental for human therapeutic purposes (21). AglycoT-Fc5 showed pH-dependent binding to human FcRn at pH 5.5 and pH 6.0 and significantly reduced binding at pH 7.4 typical of human IgG, including Herceptin. On the other hand, AglycoT-Fc601 showed reduced affinity to FcRn binding at pH 6.0, indicating that the additional two substitutions in the CH2-CH3 interface of Fc601 interfere with binding to the human FcRn (Fig. 3G).

For a preliminary evaluation of the effect of the E382V and M428I mutations on IgG homeostasis, the serum persistences of GlycoTand AglycoT-Fc5 in Balb/c mice were compared to that of Herceptin. Antibodies were administered by intraperitoneal
injections at dosages of 2.0 mg/kg and serum antibody concentration was examined by ELISA 132 hr later. As shown in Fig. 3H, the concentration of AglycoT-Fc5 in the serum was 16.6 μg/ml, a value that is ~80% of the injected dose and slightly higher than that of Herceptin (13.1 μg/ml) and significantly higher than AglycoT (5.7 μg/ml). This was presumably due to the documented favorable effect of the M428I mutation on pH-dependent FcRn binding (22). Although the mouse FcRn binds to human IgG1 with higher affinity relative to its murine counterpart IgG2a (23), the mouse FcRn system provides useful information for predicting the rank-order of IgG variants in humans (24).

**E. coli Expressed, Aglycosylated Trastuzumab Potentiates the Killing of Her-2 Overexpressing Cells by mDCs.** Macrophages and immature DCs greatly outnumber the classical killer cells (NK and T cells) in tumors (25). In recent years, the cytotoxic properties of various subpopulations of DCs toward cancer cells has attracted significant interest (26). Human circulating DCs express FcγRI, FcγRIIA, and FcγRIIB, but not FcγRIIIa (27).

Human CD11c+ mDCs were differentiated from peripheral blood mononuclear cells (PBMCs) by incubation for 7 d in the presence of interleukin-4 (IL-4) and granulocyte macrophage colony stimulating factor. These mDCs activated by lipopolysaccharide (LPS) expressed high levels of FcγRI (Fig. S7A). SkBr3 breast carcinoma epithelial cells overexpressing Her-2 were incubated either with Herceptin or with AglycoT-Fc5 or AglycoT-Fc601 at 10 μg/ml. AglycoT and media without effector cells were used as negative controls. Activated mDCs were then added at either 25:1 or 100:1 ratios and the lysis of the SkBr3 target cells was determined by monitoring the release of radioactive isotope chromium 51 (51Cr) 24 h later. No cell lysis above background was detected with Herceptin (Fig. 4A) or with GlycoT (Fig. S7B). In contrast, AglycoT-Fc5 induced ~40% (25:1; E:T ratio) and ~70% (100:1; E:T ratio) tumor cell killing. A comparable degree of cell lysis was observed with AglycoT-Fc601 (Fig. 4A). Incubation of AglycoT-Fc5 with Herceptin markedly reduced ADCC. Similar to Herceptin and to GlycoT, only background lysis of SkBr3 cells was observed with GlycoT-Fc5 that binds nonspecifically to all effector FcγRs (Fig. S7B). In contrast to these results and in agreement with earlier studies (28, 29), when PBMCs were used as effector cells, Herceptin potentiated efficient lysis of SkBr3 cells (Fig. 4B). PBMCs are composed of several cell types that express various activating FcγRs. Most notably, NK cells that express only FcγRIIIa (11), constitute the largest innate immune cell population in PBMCs. Therefore, glycosylated Herceptin, which displays FcγRIIIa (Fig. S7E), was able to induce significant ADCC when PBMCs were used as effector cells. In contrast, AglycoT-Fc5 and AglycoT-Fc601 were unable to induce statistically significant tumor cell lysis above background levels with PBMCs, consistent with the fact that they do not bind FcγRIIIa (Fig. 3E).

**Discussion**

We developed a facile system for the screening of large combinatorial libraries of aglycosylated Fc domains expressed in E. coli by FACS selection for binding to Fcγ receptors. In the absence of the single glycan at N297, IgG antibodies display low to negligible binding to receptors on effector cells or to Clq and thus cannot mediate ADCC and complement dependent cytotoxicity (CDC). In this report we engineered glycosylated IgG1 mutants that selectively bind to FcγRI with a similar affinity to that of CHO-derived, glycosylated antibodies (Herceptin).

Two amino acid substitutions in CH3 conferred highly selective binding to FcγRI and not to other effector FcγRs. The high-binding selectivity of aglycosylated IgGs containing the E382V...
Aglycosylated antibodies engineered for ADCC bypass the need for glycoengineering, mammalian expression, and problems related to glycan heterogeneity. Together with technologies for IgG isolation (17) and commercial level expression (10, 34), it is feasible to carry out the complete development of therapeutic antibodies in bacteria. The therapeutic utility of aglycosylated antibodies will further be determined by pharmacological parameters, including stability, biodistribution, and immunogenicity in humans.

Materials and Methods
(For additional materials and methods see SI Materials and Methods)

Library Screening. For library screening, the extracellular domain of glycosylated FcγR (R&D Systems) was labeled with FITC according to the manufacturer’s instruction (Invitrogen). Binding of FITC-labeled soluble FcγRI to human IgG-Fc was confirmed by fluorescent ELISA on plates coated with glycosylated human IgG-Fc (Bethyl Laboratories). Prepared spheroplasts were sorted on a MoFlo droplet deflection flow cytometry (Dako Cytomation) using a 488 nm Argon laser for excitation and detection through a 530/40 band pass filter. Sorted cells were resorted immediately after initial sorting. The Fc mutant genes in the resorted spheroplasts were cloned into the pEBlFLAG vector (Table S1) after PCR amplification using two specific primers STJ#16 and STJ#220 (Table S2), and transformed in E. coli.

**Fig. 4.** Antibody-dependent cellular cytotoxicity (ADCC) toward SkBr3 with monocyte-derived dendritic cells (mDCs) as effector cells. (A) Target (T) Her2 overexpressing tumor cells, SkBr3, were incubated with either E. coli-purified AglycoT, AglycoT-Fc5, AglycoT-Fc601, or Herceptin at 10 μg/ml for 1 hr prior to incubation with LPS-activated mDCs. Cell lysis was measured by 51Cr release at 24 hr. (B) SkBr3 cells were incubated with IgGs as in (A), peripheral blood mononuclear cells (PBMCs) were added 1 hr later and cell lysis was determined as above. Standard deviations calculated from triplicate samples are represented by error bars. **: P < 0.01; *: P < 0.05; unpaired Student’s t-test relative to controls with AglycoT antibody and with no IgG, respectively.

and M428I mutation was abolished when this antibody was produced as a glycoprotein in HEK293T cells. We are not aware of any other instances where the presence of a naturally occurring glycan in a protein abolishes ligand selectivity. The human FcγRs for which crystal structures are available show that IgG binding occurs at an epitope that comprises residues in the hinge and CH2 regions. Biochemical data indicate that FcγRII also binds to the same region (30). The differences in the binding specificity of the GlycoT-Fc5 and AglycoT-Fc5 and also the fact that the E382V and M428I mutations are distal to the FcγRII binding epitope suggest that these amino acid substitutions likely induce a conformational change in the protein. Conceivably, this conformational change might cause the CH2 domain to slightly bulge out, thus mediating a transition from the “closed” form of IgG with truncated glycans to the “open” structure of fully glycosylated IgG (13). This effect is not manifest in GlycoT-Fc5 in which the two heavy chain polypeptides assume the open conformation as a result of the glycan appended to N297. Unfortunately, a detailed structural interpretation is not possible at the moment, as neither the structure of aglycosylated IgGs nor that of FcγRII is yet available. IgG homeostasis is regulated by pH-dependent binding to FcRn that recognizes an epitope comprised of a.a. in the CH3 and the CH3-CH2 hinge (5). Although the two critical mutations for high affinity binding of aglycosylated antibodies to FcγRI are located in the CH3, they do not adversely affect pH-dependent binding to human FcRn. In fact mutations in M428I have been shown to favorably affect FcRn affinity at pH 6.0 (22). AglycoT-Fc5 exhibited slightly higher serum persistence in mice relative to glycosylated Herceptin. Mutations that further increase the affinity of aglycosylated antibodies for FcγRII can interfere with FcRn binding as was the case of AglycoT-Fc601. However, this is not a general phenomenon and we have isolated other AglycoT-Fc5 variants that display increased affinity for FcγRII without affecting proper interactions with FcRn.

FcγRII is mainly expressed on monocytes, macrophages, DCs, and at lower levels on neutrophils (31). DCs, which are professional antigen presentation cells, are also able to mediate tumor cell killing directly by a variety of apoptotic and necrotic pathways (26) or indirectly by stimulating tumor cell specific cytotoxic T cells (32) and NK cells (33). We showed that mature, LPS-activated mDCs elicit potent cytotoxicity of Her2 overexpressing cells in the presence of AglycoT-Fc5 or AglycoT-Fc601. By contrast, Herceptin, and significantly glycosylated antibodies containing the E382V and M428I mutations were not able to stimulate the lysis of the target cells. Unlike AglycoT-Fc5 and AglycoT-Fc601, immune complexes formed by Herceptin can trigger signaling via the inhibitory FcγRIIB. Earlier studies have shown that selective engagement of FcγRIIa or FcγRIIB mediate opposing effects on mDC maturation and function (34). Additionally, antitumor effect in mice was demonstrated by abrogation of inhibitory FcγRIIB signaling on DCs (35). Thus, the most straightforward explanation for our findings is that immune complexes formed by AglycoT-Fc5 or AglycoT-Fc601 selectively engage FcγRII to mediate cytotoxicity by mDCs, the interaction of Herceptin (and also GlycoTor GlycoT-Fc5) with FcγRIIB may be the reason for the attenuation of this function. With PBMCs that are comprised by a large fraction of FcγRIIIa expressing NK cells but relatively low numbers of circulating immune cells expressing FcγRI, the situation is reversed and Herceptin exhibited strong ADCC in contrast to AglycoT-Fc5 or AglycoT-Fc601. Activated mDCs sensitized with the engineered aglycosylated antibodies described above were found to also phagocyte the target cells. It is possible that in addition to their cytotoxic activity the mDCs may also be able to effectively present antigen and activate tumor specific cytotoxic T cells that in turn could further enhance tumor destruction. Future studies will explore if aglycosylated FcγRII-binding antibodies are able to enhance other relevant DC-mediated functions (i.e., antigen presentation and activation of tumor specific T cells).
electrocompetent _E. coli_ Jude-1 cells. The resulting transformants were subjected to the next round sorting and resorting.

**Preparative Expression of Aglycosylated IgG.** Preparative expression was performed by fed-batch fermentation using a 3.3 L jar fermentor (New Brunswick Scientific) with 1.2 working volume. Cells were grown at 30 °C in R/2 medium (36) consisting of 2 g of (NH₄)₂HPO₄, 6.75 g of KH₂PO₄, 0.93 g of citric acid·H₂O, 0.34 g of MgSO₄·7 g of glucose, 0.05 g of ampicillin and 5 ml of trace metal solution dissolved in 2 N HCl (10 g of FeSO₄·7H₂O, 2.25 g of ZnSO₄·7H₂O, 1 g of CuSO₄·5H₂O, 0.35 g of MnSO₄·H₂O, 0.23 g of Na₂B₄O₇·10H₂O, 1.5 g of CaCl₂, and 0.1 g of (NH₄)₆Mo₇O₂₄ per L). _E. coli_ BL21(DE3) (EMD Chemicals) harboring pST4-AgGlcyO, pST4-AgGlcyO-Tcs, or pST4-AgGlcyO-Tcs/601 were cultured in 500 ml baffled flask with 120 ml R/2 media at 30 °C at 250 rpm for 8 h and used to inoculate the fermentor. The dissolved oxygen concentration was maintained at 40% of the air saturation using automatic cascaded control by increasing agitation speed from 100–1000 rpm, air flow rate from 1–3 SLPM (standard liquid per minute) and pure oxygen flow rate from 0–1.5 SLPM when required. Initial pH was adjusted to 6.8 and controlled by the addition of 30% (w/v) ammonium hydroxide when pH decreased to <6.75, and by the supply of either growth feeding solution (700 g/L of glucose and 10 g/L of MgSO₄·7H₂O) before induction or production feeding solution (500 g/L glucose, 10 g/L of MgSO₄·7H₂O, and 100 g/L of yeast extract) after induction when the pH increased above 6.9. When _OD₅₆₅_ reached 100, the culture temperature was reduced to 25 °C and 30 min later, protein expression was induced with 1 mM of IPTG. The culture broth was harvested 7 h after induction.

**ADCC Assays.** Target Skbr3 tumor cells were labeled with Na⁺¹¹⁰⁴ CrO₄⁻ (Perkin Elmer Life Sciences) at 100 uCi/10⁵ cells for 1 h at 37 °C. Cells were then washed twice with PBS and resuspended in RPMI and added to a 96-well plate at 10⁴ cells/well. Engineered Fc antibodies with trastuzumab and relevant controls were added to the target cells in triplicate wells and incubated at 37 °C for 1 h. The plate was then centrifuged at 2000 rpm for 1 min and washed twice with PBS. Effector cells, either fully differentiated mDCs (day 7) or freshly isolated PBMCs, were resuspended in RPMI 1640, 2% low IgG FBS (Invitrogen), LPS (250 ng/10⁶ cells) and added to the wells at various ratios. Target cells and mDCs were incubated at 37 °C for 24 h. The isolation levels present in cell media were then measured in a liquid scintillation counter. Incubation of target cells with 0.5 SDs was used as a positive control for maximum lysis and incubation with no effector cells was used as background lysis.

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