Selective Deactivation of Serum IgG: A General Strategy for the Enhancement of Monoclonal Antibody Receptor Interactions

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Serum IgG is a potent inhibitor of monoclonal antibody (mAb) binding to the cell-surface Fcγ receptors (FcγRs), which mediate cytotoxic and phagocytic effector functions. Here, we show that this competition can be eliminated, selectively, by the introduction to serum of (i) an enzyme that displaces Fc from FcγRs and (ii) a modification present in the therapeutic mAb that renders it resistant to that enzyme. Specifically, we show that (i) EndoS (endoglycosidase S) cleaves only complex-type glycans of the type found on IgG but (ii) is inactive against an engineered IgG Fc with oligomannose-type glycans. EndoS thus reduces FcγR binding of serum IgG, but not that of engineered mAb. Introduction of both the engineered mAb and endoglycosidase in serum leads to a dramatic increase in FcγR binding compared to the introduction of mAb in serum alone. Antibody receptor refocusing is a general technique for boosting the effector signal of therapeutic antibodies.

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of the activating FcγRIIIa receptor found on natural killer cells.\textsuperscript{9–11} One well-explored approach to enhance effect or function is therefore to increase the intrinsic affinity for FcγR by engineering the Fc structure of a given mAb.\textsuperscript{12} However, an additional factor also impacts Fc:FcγR interactions: the presence of competing serum IgG.

Serum antibody (sAb) (50–100 μM) is present in significant excess of the dissociation constant ($K_d$) for IgG Fc:FcγR interactions (0.1–10 μM).\textsuperscript{13,4} The majority of cellular FcγRs are therefore bound to IgG Fc under physiological conditions. For example, FcγRIIIa (Val158 variant) exhibits a $K_d$ of around 0.1 μM for IgG1 Fc.\textsuperscript{14} Therefore, regardless of the affinity of an Fc for an FcγR, the limited availability of unbound FcγR imposes an external constraint on antibody effect or potency. While Fc engineering can help overcome this effect for antigens expressed at high levels on the cell surface,\textsuperscript{15} low-affinity or low-copy epitopes on infected or cancerous cells are efficiently protected from ADCC by serum

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**Fig. 1.** Endoglycosidase-mediated deactivation of serum IgG. (a) Binding of human IgG1 Fc to immobilized FcγRIIIa was determined, by ELISA, in the presence of PBS (phosphate-buffered saline) or increasing concentrations of human serum and detected using a secondary antibody specific for the monoclonal Fab domain (Supplementary Methods). (b) The crystal structure of Fc\textsubscript{GlcNAc} (blue ribbon) overlaid with structures of glycosylated human IgG Fcs using SHP17 by superposition of C\textalpha\  residues from one protomer while leaving the second protomer free (pink ribbons; PDB IDs 1FC1, 1HST, 1H3U, 1H3V, 1H3W, 1H3X, 1H3Y, 2DTQ, 2DTS, 3DNK, 3D03 and 3HKF). Broken lines are drawn between equivalent C\textalpha\ atoms (Tyr296) in Fc\textsubscript{GlcNAc} and naturally glycosylated structures and indicate a displacement of approximately 8 Å. For crystallographic analysis, Fc\textsubscript{GlcNAc} (Supplementary Methods) was concentrated to 7.0 mg/mL and was crystallized after 42 days with the use of the sitting-drop vapor diffusion method\textsuperscript{18} using 100 nL protein plus 100 nL precipitant equilibrated against 95 μL reservoirs. Crystals grew at room temperature in a precipitant containing 25% (w/v) polyethylene glycol 1500 and 0.100 M SPG System buffer (pH 4). Crystals were flash frozen by immersion in a cryoprotectant containing the mother liquor diluted in 25% (v/v) glycerol and then rapidly transferred to a gaseous nitrogen stream. X-ray diffraction data were recorded at beamline I03 at Diamond Light Source, Oxfordshire, England. Data were processed and scaled using DENZO and SCALEPACK,\textsuperscript{19} and the structure was solved using Phaser\textsuperscript{20} with native Fc (PDB accession number 3AVE) as a search model. Model building was performed with Coot\textsuperscript{21} and iteratively refined using restrained refinement with TLS in the CCP4 supported program REFMAC5.\textsuperscript{22} (c) Binding of EndoS-treated or mock-treated human sera to immobilized FcγRIIIa determined using an anti-human IgG secondary antibody (Supplementary Methods).
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immunoglobulins. This leads to dosages of clinical therapeutic antibody several orders of magnitude greater than would be predicted by serum-free assays. We designed an assay to replicate how mAb binding to the FcγRIIIa receptor is affected by human serum. In order to allow selective detection of the engineered antibody Fc within an excess of serum Fc domains, we constructed a chimeric mAb containing the full human IgG1 Fc and a murine Fab region (Supplementary Methods) allowing detection with an anti-murine Fab secondary antibody (Fig. 1a). Consistent with previous reports, even at extensive serum dilutions, mAb binding was significantly reduced, and at serum concentrations approaching physiological levels, mAb binding was barely detectable. Therefore, the elimination of competing sAb represents a route to the enhancement of mAb:FcγR interactions.

One solution in circumventing the inhibitory effect of sAb is to selectively eliminate serum IgG binding to FcγRs while leaving therapeutic mAb function unperturbed. A number of bacterial enzymes are able to interrupt Fc:FcγR interactions. Notably, secreted endoglycosidases are able to cleave the core GlcNAcβ1→4GlcNAc linkage of the Fc glycan, release IgG from cellular FcγRs and abrogate Fc-mediated effector function. To elucidate the molecular basis of IgG deactivation by endoglycosidases, we deglycosylated IgG1 Fc to a single GlcNAc moiety and determined its structure by X-ray crystallographic analysis to a resolution of 2.5 Å (Fig. 1b and Table 1 and Supplementary Methods and Fig. S1). Comparison of this FcGlcNAc with known glycosylated IgG Fc structures reveals that deglycosidase cleavage induces an inward movement of the Cγ2 domains coupled with a rotation around the central axis (Fig. 1b). This structural transformation displaces equivalent Cα atoms by up to 8 Å. By analogy with the conformation observed in aglycosylated murine IgG Fc (Supplementary Fig. 1), the fully closed quaternary structure observed here is incompatible with known Fc:FcγR interactions. This structure is consistent with solution-phase NMR data that reveal significant changes in chemical shifts from the Cγ2 domain residues following endoglycosidase cleavage. Similarly, displacement of the Cγ loop in our FcGlcNAc structure provides a plausible explanation for the altered hydrogen/deuterium exchange kinetics reported in this region following deglycosylation. We also note that mutations able to restore functionality to aglycosylated antibodies include residues in the Cγ loop.

The change in conformation seen in our FcGlcNAc structure also offers a structural basis for natural immune evasion by a common human pathogen. EndoS (endoglycosidase S), from Streptomyces pyogenes, deglycosylates human IgG and decreases FcγR binding of antibacterial antibodies, an observation confirmed here for FcγRIIIa (Fig. 1c). The activity of this enzyme has also been employed for therapeutic applications: EndoS is under preclinical development as an immunosuppressive agent to diminish antibody-mediated pathology via elimination of Fc:FcγR interactions of autoimmune antibodies. However, we hypothesized that EndoS could also be used to enhance binding of mAbs to FcγR provided deactivation was focused to bulk serum IgG and not to recombinant mAb. This would require an engineered antibody that maintains productive FcγR binding with a carbohydrate component unaffected by EndoS.

The carbohydrate specificity of EndoS is not known. However, consideration of its evolved function suggested that commonly occurring IgG Fc glycoforms would be efficiently hydrolyzed. By contrast, it seemed less likely that EndoS would have acquired activity against glycans not normally
found on human IgG. For example, oligomannose-type structures are devoid of the terminal carbohydrate motifs typically present on antibody glycans (principally, NeuNAc\(\alpha_2\rightarrow6\)Gal, Gal\(\beta_1\rightarrow4\)GlcNAc and GlcNAc\(\beta_1\rightarrow2\)Man). Fortuitously, however, oligomannose Fc glycoforms exhibit high-affinity binding to all human FcγRs\(^{14,36,37}\) and serum clearance equivalent or slightly reduced\(^{38}\) compared to complex-type glycoforms.

The ability of EndoS to hydrolyze either naturally glycosylated IgG1 Fc or an engineered Fc bearing oligomannose-type glycans was therefore determined. An advantage of the particular Man\(_5\)GlcNAc\(_2\) Fc glycoforms is that chemically homogenous glycoproteins can be readily manufactured at high yields through manipulation of the mammalian glycan biosynthetic pathway.\(^{37,39,40}\) Mass spectrometric analysis of complex-type N-glycans, released from IgG1 Fc and subsequently exposed to EndoS, showed complete cleavage of the core GlcNAc\(\beta_1\rightarrow4\)GlcNAc linkage (Fig. 2a and b). In contrast, the oligomannose-specific EndoH (endoglycosidase H) showed no detectable hydrolysis (Fig. 2c). A reciprocal pattern of specificities was observed for an engineered oligomannose Fc glycoform that displayed complete resistance to EndoS (Fig. 2d–f).

The enzymatic resistance of oligomannose-type mAb provides a route to selective elimination of natural antibody glycoforms from cellular FcγRs. We therefore repeated our FcγR binding assay using an oligomannose mAb glycoform, in the presence of serum and EndoS, EndoH, both EndoS and EndoH or no enzyme (Fig. 3a). Consistent with the data from Fig. 1, the enzyme-free serum efficiently blocked the binding of the oligomannose-type mAb to FcγRIIIa. However, the addition of EndoS led to a dramatic increase in apparent affinity of oligomannose mAb for FcγRIIIa. Moreover, the addition of EndoH led to a striking increase in apparent affinity of oligomannose mAb for FcγRIIIa with 50% receptor saturation achieved at approximately 0.05 \(\mu\)M mAb, a level approaching that of mAb: FcγR determined for IgG in the complete absence of serum and consistent with the reported value (0.08 \(\mu\)M) of this interaction.\(^{4}\) This enhancement

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**Fig. 2.** Resistance of oligomannose containing Fc glycoforms to EndoS-mediated hydrolysis. Matrix-assisted laser desorption/ionization mass spectrometry spectra of PNGase-F released N-glycans from IgG1 Fc expressed in GnT-I-deficient HEK (human embryo kidney) 293S cells (Supplementary Methods). These expression systems yielded, respectively, Fc with complex-type (a–c) or oligomannose-type (d–f) glycans, which were exposed to no enzyme (a and d), EndoS (b and e) or EndoH (c and f). The spectra of the oligomannose glycans reveal the presence of GnT-I-independent fucosylation.\(^{31}\) The cleavage of the core GlcNAc\(\beta_1\rightarrow4\)GlcNAc bond by endoglycosidases results in the removal of a single GlcNAc (predicted \(\Delta m/z=203.1\)) or Fuc\(\alpha_1\rightarrow6\)GlcNAc (predicted \(\Delta m/z=349.1\)). Symbolic representation of glycan structures follows that of Harvey et al.\(^{42}\) \(\bigcirc\), Gal; ■, GlcNAc; ○, Man; ◊, Fuc. The linkage position is shown by the angle of the lines linking the sugar residues (vertical line, 2-link; forward slash, 3-link; horizontal line, 4-link; back slash, 6-link). Anomericity is indicated by continuous lines for \(\beta\)-bonds and by broken lines for \(\alpha\)-bonds.
was a direct consequence of the differential glycosylation of the engineered mAb and natural sAb. This glycoform dependence was confirmed by the addition of EndoH, which led to loss of detectable FcγRIIIa binding regardless of whether or not the competing sera had also been treated with EndoS. These data indicate that it is possible to selectively target Fc receptors to IgGs with specific glycoforms despite a large excess of competing serum Fc (Fig. 3b). The well-documented biological and pharmacological properties of both the oligomannose glycoforms and the endoglycosidase enzymes point toward the in vivo development of this approach for almost any Fc:FcγR-dependent process. Similarly, any EndoS-resistant antibodies including aglycosylated mAbs engineered to exhibit functional FcyR interactions could be employed. An obvious additional application of mAbs, resistant to IgG deactivating enzymes, would be in the treatment of infections by bacteria, such as S. pyogenes, which secrete these immune evasion factors.

While mammalian glycosylation is heterogeneous, manipulation of cellular glycan biosynthesis can yield chemically homogenous, precisely defined protein glycoforms. Similarly, despite cleaving the same core GlcNAcβ1→4GlcNAc linkage conserved in all N-linked glycans, endoglycosidases have evolved a remarkable selectivity for terminal carbohydrate motifs. In combination, these tools allow for precise and independent control of natural and engineered glycoproteins, even in the complex biochemical environment of human serum. Our strategy, which we term receptor refocusing, provides a general approach for boosting the immunological signal provided by mAbs: by redirecting the cellular immune system to a single antibody glycoform, which is in turn directed to a single target antigen.

Accession codes

Coordinates and structure factors of FcGlcNAc have been deposited in the Protein Data Bank (PDB accession number 4ACP).

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Fig. 3. EndoS-mediated deactivation of serum leads to enhancement of mAb binding to FcyRIIIa. (a) ELISA showing the interaction between monoclonal IgG1 containing oligomannose (Man9GlcNAc2) glycans and immobilized FcyRIIIa in the presence of PBS, serum, serum and EndoS, serum and EndoH or serum and EndoS and EndoH (Supplementary Methods). Binding was detected using a secondary antibody specific for the monoclonal Fab domain as in Fig. 1a. Data points represent the calculated mean of three independent measurements from a total of four experiments. (b) Schematic illustration of the differential binding of FcyRIIIa to oligomannose and natural Fc glycoforms in the presence of EndoS. Deactivated FcGlcNAc is shown in blue, and activated Fc in complex with FcyRIIIa (gray surface; PDB ID 1T83) is shown in pink. Glycans are shown as yellow spheres.
**Author Contributions.** K.B., M.C., R.A.D. and C.N.S. designed the experiments. K.B. and T.A.B. designed and performed the crystallographic experiments. K.B., B.A.K. and C.N.S. performed glycan analysis and ELISA experiments. K.B., T.A.B., M.C. and C.N.S. wrote the manuscript.

**Conflict of Interest.** The authors declare no competing financial interests.

**Supplementary Data**

Supplementary data to this article can be found online at doi:10.1016/j.jmb.2012.04.002

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