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Mapping Antibody Fc-glycosylation for Optimal Effector Functions

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Materials and Methods

Expression and purification of Anti-SSEA-4 antibody: The chMC813-70 antibody expression plasmid was received from Dr. Han-Chung Wu at Academia Sinica, Taiwan. Then the constructed plasmids were transfected into the Expi293 and Expi293GnTI$^-$ cells using transfection reagent described by the manufacturer's protocols (Thermo Fisher Scientific). The recombinant antibody was purified by protein A-Sepharose 100 CL-4B as described by the manufacturer's protocols.

Expression and purification of enzymes

a) EndoS2-WT (*S. pyogenes*), Neuraminidase nanH (*C. perfringens*), β-galactosidase (*A. muciniphila*), α2,6-Sialyltransferase, (*Photobacterium sp*. JT-ISH-224), α2,3-Sialyltransferase, (*Vibrio sp.* JT-FAJ-16), α-fucosidase AlfC (*L. casei*) and β-1,4 galactosyl transferases (*Bovine*) were obtained from *E. coli* expression system using the following procedure: a gene sequence of the respective enzyme was cloned into pET28a vector. Plasmid-bearing BL21 cell was cultured in TB medium with 100 μg/ml kanamycin at 37^{o}C with shaking until OD_{600} reached 0.8. The enzyme expression was induced by 0.1 mM IPTG at 16 ^{o}C overnight with shaking. The BL21 cell was harvested by centrifugation, and the pellet was disrupted via ultrasonics in a working buffer (50 mM MOPS, 300 mM NaCl, 10 mM imidazole). The supernatant collected by centrifugation was passed through Ni-charged resin, which was washed with working buffer and then eluted with elution buffer (50 mM MOPS, 300 mM NaCl, 250 mM imidazole). The eluted protein was buffer-exchanged to 50 mM Tris-HCl pH 7.4, and the purity was confirmed by Coomassie blue staining.

b) Human N-acetylglucosaminyltransferase V (GnTV) was obtained from the Expi293F™ cells expression system using the following procedure: The soluble form of GnTV was cloned into a pCMV3 vector, and the plasmid was transfected in Expi293F™ cell via ExpiFectamine™ 293 transfection kit. After incubating cells for 6 days, the supernatant was harvested by centrifugation, then passed through an Anti-FLAG® M2 affinity gel, which was washed with TBS buffer (50 mM Tris-HCl, with 150 mM NaCl pH 7.4) and eluted with 0.1 M glycine (pH 3.0). The resulting fraction was neutralized by adding 1/20 volume of 1 M Tris-HCl (pH 8.2) and buffer-exchanged to 50 mM Tris-HCl pH 7.4. The protein purity was confirmed by Coomassie blue staining.

Enzymatic synthesis of glycans from sialylglycopeptide (SGP):

The synthesis of bi- and tri-antennary glycoforms has been described in supporting information, the sialylglycopeptide (SGP) isolated from egg yolk was treated with a EndoS2WT (S. pyogenes) to afford glycan SCT S1 (Scheme S1, ESI†). Desialylation of

S1 with neuraminidase (C. perfringens) provided glycan CT (S2) which was further reacted with α 2,3 sialyltransferases to afford bi-antennary complex type glycans with terminal α 2,3-sialic acid residues S7. The terminal galactose residues of S2 were removed by bacterial β-galactosidase (A. muciniphila) to afford G0 glycoform (S3), an intermediate to prepare tri-antennary complex type glycans. Tri-antennary glycoform S4 was prepared using human N-acetylglucosaminyltransferase V (GnTV) in the presence of uridine diphosphate N-acetylglucosamine (UDP-GlcNAc). Galactosylation of S4 using bovine β-1,4 galactosyl transferases was performed in the presence of UDP-galactose to afford glycan S5. Finally, terminal α-2,6 sialylation of S5 was afforded using α 2,6 sialyltransferases JT-ISH-224 (photobacterium sp.) to give fully sialylated tri-antennary glycoform S6 (Scheme S1).

Synthesis of 9-azido sialic acid modified glycoform (S8) was commenced with preparation of 9-azido sialic acid intermediate 4, which was then transferred on the terminal galactose residues of bi-antennary complex type glycan (CT), under the catalytic action of cytidine 5'-monophosphate (CMP)-sialic acid synthetases (CSSs) and α 2,6-sialyltransferases from marine bacteria in the presence of cytidine triphosphate (CTP) and $MgCl_2$ to afford glycoform S8 (Scheme S2).

1. Synthesis of 2,6SCT glycan (compound S1) As shown in Scheme 1, a solution of sialylglycopeptide (BioChemSyn.com) (50 mg) in a Tris buffer (50mM, pH 7.4) was incubated with Endo S2WT (100 µg). The reaction was incubated at 37 °C for 16h. and monitored by TLC. The reaction was quenched by heating the solution at 95 °C for 5 min., the denatured enzymes were removed by filtration, and the crude compound **S1** was purified by Bio-Gel P-2 column chromatography (eluent water, 90%).

ESI-MS: *m/z* calcd for $C_{76}H_{125}N_5O_{57}$; 2020.81 found 1008.8433 $(M-H)^{2-}$

2. Synthesis of CT glycan (compound S2): Compound S1 (30 mg) in a Tris buffer (50mM, pH 7.4, 50mM) was incubated with sialidase (60ug). The reaction was incubated at 37 °C for 16h. and monitored by TLC. The reaction was quenched by heating the solution at 95 °C for 5 min., the denatured enzymes were removed by filtration, and the crude compound **S2** was purified by Bio-Gel P-2 column chromatography (eluent water, 90%).

ESI-MS: *m/z* calcd for $C_{54}H_{91}N_3O_{41}$;1438.30 found 1460.4991$(M+Na)^+$.

3. Synthesis of G0 glycan (compound S3): A mixture of Compound S2 (10 mg) and galactosidase (100 µg) in Tris buffer (50 mM, pH 7.5) was incubated at 37°C for 12 h and monitored by TLC. The reaction was quenched by heating the reaction mixture at 95 °C for 5 min. The reaction mixture was centrifuged, and the supernatant was subjected to gel filtration over P2-Bio-gel (eluent water). The product's fractions were combined and lyophilized to give the product **S3** as amorphous white solids. **ESI-MS:** *m/z* calcd for $C_{42}H_{71}N_3O_{31}$;1113.41 found 1136.3947 $(M+Na)^+$.

4. Synthesis of Tri antennary glycan (compound S4): Compound **S3** (8 mg, 7.18 µmol) and UDP-GlcNAc (11 mg, 16.89 µmol) were dissolved in Tris buffer (50 mM, pH 7.5) and $MnCl_2$ (20mM) and MGAT5 (90ug) were added. The resulting reaction mixture was incubated at 37°C for 12 h. The reaction was quenched by heating the reaction mixture at 95 °C for 5 min. The reaction mixture was centrifuged, and the supernatant was subjected to gel filtration over P2-Bio-gel (eluent water). The product's fractions were combined and lyophilized to give the product **S4** (7.5 mg, 93%) as amorphous white solids. **ESI-MS**: *m/z* calcd for $C_{50}H_{84}N_4O_{36}$;1317.21 found 1339.4739 $(M+Na)^+$.

5. β1,4-galactosylation of tri-antennary glycan (S5): A solution of glycan **S4** (4mg, 1equiv), UDP-galactose (7-10 mg, 4-5 eq.), B4GalT1(40-50ug), and 20mM $MnCl_2$ in Tris buffer (50 mM, pH 7.5) was incubated at 37°C for 12h. After checking TLC, B4GalT1 was deactivated by heating the reaction mixture at 95 °C for 5 min. The reaction mixture was subject to gel filtration chromatography on a P2-Bio-gel (eluent water). The fractions containing the products were combined and lyophilized. **ESI-MS**: *m/z* calcd for $C_{68}H_{114}N_4O_{51}$; 1803.64 found 1825.6374 $(M+Na)^+$.

6. α2,6-sialylation glycan (S6): A solution of glycan (**S5)** (4-5 mg), CMP–Neu5Ac (7-10 mg), α2,6SialT (40-50ug), and 20mM $MgCl_2$ in Tris buffer (50 mM, pH 8.4) was incubated at 37 °C for 2-3h. After checking TLC, the enzyme was deactivated by heating the reaction mixture at 95 °C for 5 min. The reaction mixture was subject to gel filtration chromatography on a P2-Bio-gel (eluent water). The fractions containing the products were combined and lyophilized. **ESI-MS**: *m/z* calcd for $C_{101}H_{165}N_7O_{75}$; 2675. found 1338.9653 $(M+2H)^{2+}$

7. Preparation of α 2,3-SCT glycan (S7): A solution of glycan (**S2)** (3 mg), Neu5Ac (10-12 mg), CTP (15mg), CMP–Neu5Ac synthase (100ug), α2,3SialT (30ug), and 20mM $MgCl_2$ in Tris buffer (50 mM, pH 8.4) was incubated at 37°C for 2-3h. After checking TLC, the enzyme was deactivated by heating the reaction mixture at 95°C for 5 min. The reaction mixture was subject to gel filtration

chromatography on a P2-Bio-gel (eluent water). The fractions containing the products were combined and lyophilized.

ESI-MS: *m/z* calcd for $C_{76}H_{125}N_5O_{57}$; 2020.81 found 1008.8433 $(M-H)^{2-}$.

8. Preparation of α2,6-9N$_3$SCT glycan (S8):

9-azido sialic acid is prepared as follows. To a stirred solution of Neu5Ac (compound **1**, 20 g, 64.7mmol, 1 eq.) in MeOH (600 mL) was added trifluoroacetic acid (4.95 mL, 64.7 mmol, 1 equiv.). The reaction mixture was allowed to stir at 60 °C for 16 hrs. The solvent was removed by rotary evaporation under reduced pressure and co-evaporated with toluene twice to remove traces of water. Neu5Ac methyl ester (**2**) (250mg; 0.808 mmol) was dissolved in pyridine (5mL) and cooled to 0°C. p-toluensulfonyl chloride (308.3 mg, 1.62 mmol) was added to the reaction mixture and stirred overnight. After TLC analysis showing complete consumption of starting material, pyridine was removed by rotary evaporation under reduced pressure; and compound **(3)** was purified by silica gel column chromatography. Then compound **3** (100mg; 0.22 mmol) and sodium azide (54 mg; 0.83 mmol) were refluxed in water: acetone (12 mL; 1:3) for 12 h. The final product was purified by P2 bio gel column chromatography by using water as an eluent to get a white solid.

The 9-azido sialic acid was transferred to the terminal galactose of CT glycan (Scheme 2).

Protocol: In the solution of CT (S2) and 9-azido sialic acid **(4)** in a 50mM Tris–HCl buffer (pH 8.4) containing $MgCl_2$, CTP, CMP–Neu5Ac synthase, α 2,6 SiaT was added, and the reaction mixture was incubated at 37°C for 2-4 hr. The reaction was monitored by TLC (1:1:1, n-BuOH: AcOH: H_2O by volume). After heating the solution at 95°C for 5 min., the denatured enzyme was removed by filtration, and then the solution was concentrated in vacuo. The product was purified by Bio-Gel P-2 chromatography (eluent water), followed by lyophilization to obtain the desired product S8 as a white solid, which was then changed to oxazoline form (Scheme 2).

Synthesis of glycan oxazolines: A solution of respective glycans (5-10 mg), 2-chloro-1, 3-dimethyl imidazolinium chloride (DMC) (6-10 mg), and Et_3N (10-20 µL) in water (300-500 µL) was stirred at 4° C. for 2-4 h. The reaction mixture was subject to gel filtration chromatography on a Sephadex G-25 column eluted by 0.05% aqueous Et_3N. The fractions containing the products were combined and lyophilized.

Preparation of GlcNAc- chMC813-70:

A chMC813-70 expressed from the Expi293F cell line was incubated with a mixture of EndoS2-WT and α-fucosidase AlfC at 37 °C for 22 hrs.[1] Whereas the chMC813-70 expressed from Expi293F™ GnTI$^-$ cell line was incubated with EndoH to generate GlcNAc-chMC813-70.[2] SDS-

PAGE and Intact protein mass analyses indicated the complete cleavage of the N-glycans on the heavy chain. The reaction mixture was subjected to affinity chromatography using protein A-agarose resin (5 mL; GE Healthcare). The first column was pre-equilibrated with a binding buffer of 50 mM Tris-HCl buffer (pH 7.4, 5CV). Then bound antibody was released with 100 mM glycine-HCl (pH 3.0), and the elution fractions were immediately neutralized with 1.0 M Tris-HCl buffer (pH 8.2). The antibody fractions were concentrated using a 10k Amicon Ultra centrifugal filter (Millipore, Billerica, MA) to give GlcNAc-chMC813-70.

Transglycosylation of GlcNAc-chMC813-70 with glycan oxazolines: A glycan oxazoline was added to the mixture of an EndoS2 mutant and GlcNAc-chMC813-70 in 50 mM Tris buffer (pH 7.4). The solution was incubated for 60 to 120 min at 37 ℃. Then, the reaction mixture was purified by protein A affinity column (GE Healthcare). The transglycosylation reaction was evaluated by both the SDS PAGE and the intact protein mass analysis (Figure S3).

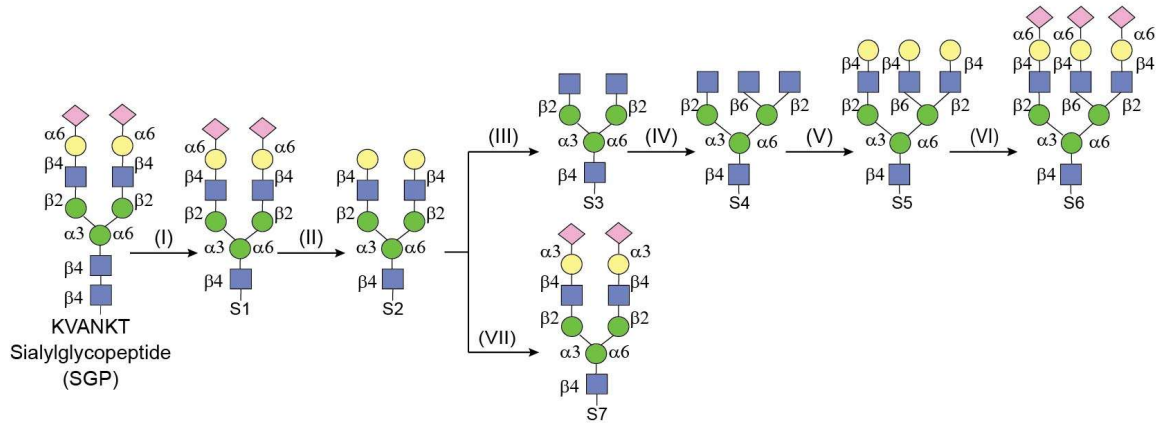
Mass spectrometric analysis of glycoengineered mAb. For the intact protein mass spectrometric analysis, an experiment was performed on a reverse phase 6230 TOF LC/MS with a Dual AJS ESI ion source system (Agilent Technologies). Agilent PLRP-S 1000Å 5μm column was used for IgG analysis: mobile phases, buffer A: 0.1% formic acid in H_2O and buffer B: 0.1 formic acid in acetonitrile at 0.3 ml/min flow rate.

The binding affinity of glycoengineered chMC813 antibodies to FcγRIIIa, FcγRIIa and Fcγ RIa by ELISA:

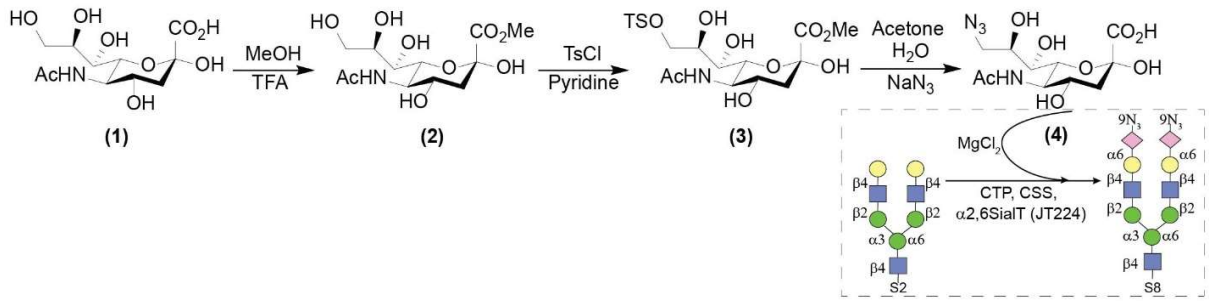
The affinity of the remodeled glycoforms of chMC813-70 for FcγIIIA FcγIIa and FcγIa receptors was examined by ELISA. Microtiter plate (Corning® 96 Well Clear Flat Bottom Polystyrene High Bind, #9018) was coated with 50 ng/well of recombinant soluble FcγRIIIA diluted in 50 mM bicarbonate/carbonate coating buffer (pH 10) overnight at 4°C. The plate was washed 3 times with PBST (0.05% Tween 20 in PBS) and blocked with 5% BSA in PBST for 1 hr at room temperature. The binding activity of glycoengineered antibodies (chMC813 G8, G11, G17, G18, G20, G21-G4) was determined for serial eight dilutions, starting with a concentration of 50μg/ml in 2% BSA/PBST in duplicates. The plate was incubated for 1 hr at room temperature and washed 3 times with PBST. Next, 100 μL of goat anti-human IgG conjugated to horseradish peroxidase (Jackson immune, #109-035-088) in 2% BSA/PBST was added per well and incubated for 40 min at room temperature. The plate was washed 5 times with PBST, then 100 μL per well of TMB substrate (eBioscience, #00-4201-56) was added, and the resulting plate was incubated in the

dark for 15 min at room temperature. The absorbance value was determined at 450 nm by an ELISA reader (Molecular Devices Corporation, Sunnyvale, CA, US A). The binding assay towards FcγRIIa and Fcγ Ia (150 ng/ml and 10 ng/ml, respectively; Sino Biological Inc.) were performed by using similar coating methods, blocking procedures, and developments of the substrate,

ADCC of chMC813-70: ADCC reporter bioassay (Promega)[3] was performed using engineered Jurkat cells expressing the human FcγRIIIa-V158 receptor. The ADCC activity on HPAC pancreatic cancer cells with higher expression levels of SSEA-4 was evaluated with anti-SSEA4 antibody chMC81370, and its homogeneous glycoforms G18, G20, G23, and G24 using the procedures described previously. A pancreatic cancer cell line HPAC (5×10^3 cells) was seeded on a 96-well plate. Then, these cells were incubated with antibodies chMC813-70, chMC813-70-GlcNAc, G18, G20, G23, and G24) 10 µg/ml and Jurkat cells expressing human FcγRIIIa-V158 receptor (effectors, E) at 37 °C for 6 hrs. Multiple cross-linking of target cells with Jurkat cells by antibodies leads to Jurkat cell luciferase production, which can be quantified to determine Jurkat cell activation. We have shown that either α2,6-SCT or α2,6-FSCT glycan attached to Asn-297 was able to maximize the ADCC effector function through binding to the FcγIIIA receptor. The effector cell: target cell (E: T) ratio was 6:1. After 6-hour incubation at 37°C, 5% CO_2; the plate was allowed to cool to room temperature for 15 min, followed by the addition of a luciferase substrate. Five minutes after luciferase substrate addition, luminescence was measured using an Elisa reader (Molecular Device, SpectraMax M5). The induction fold was calculated by dividing RLU (induced – background) by RLU (no antibody control – background) and was analyzed using GraphPad Prism 9 software.



Scheme S1: Enzymatic synthesis of tri-antennary glycan from sialylglycopeptide (SGP): Reagents and conditions: (I) Endoglycosidase (EndoS2-WT), (II) Neuraminidase; (III) Galactosidase; (IV) GnTV, UDP-GlcNAc; (V) β4GalT, UDP-Gal; (VI), CMP-Neu5Ac, α-2,6-SiaT; (VII) CTP, CSS, Neu5Ac, α-2,3-SiaT.



Scheme S2: Synthesis of 9-azido-sialic acid (**4**) and $9N_3$SCT glycan (S8)

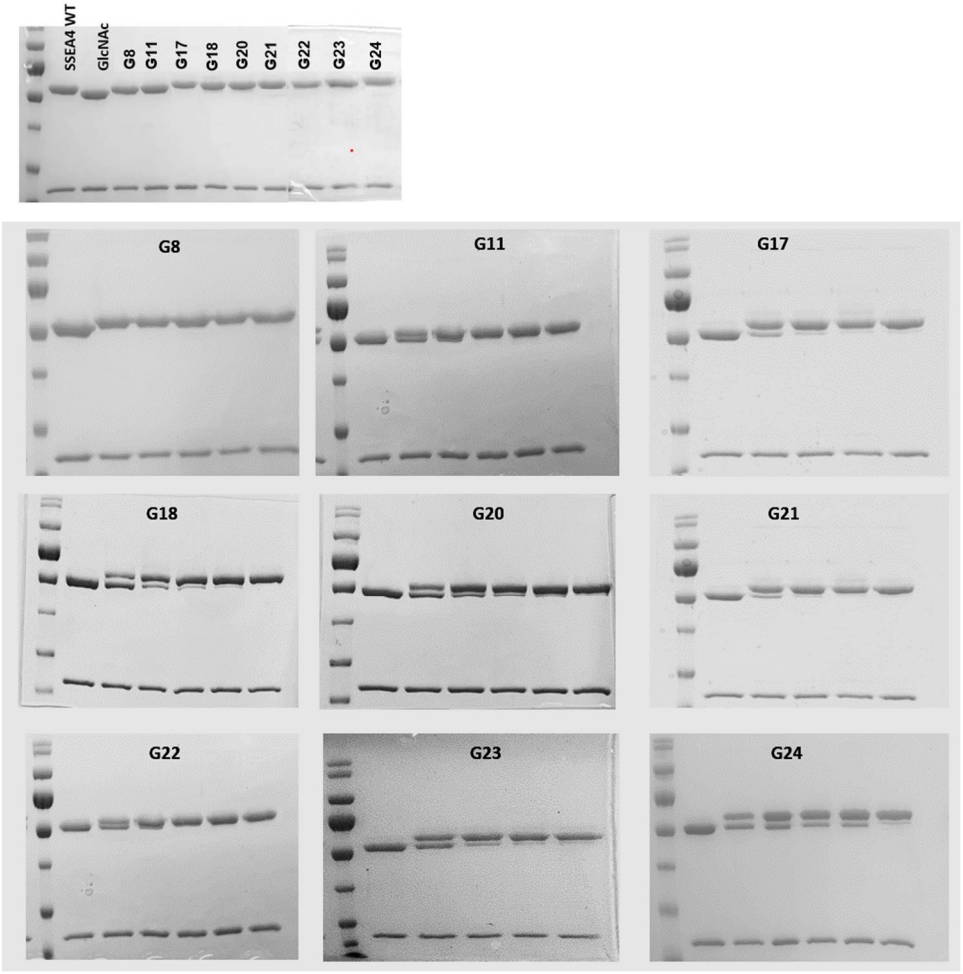


Figure S1: The SDS PAGE of transglycosylation reactions.

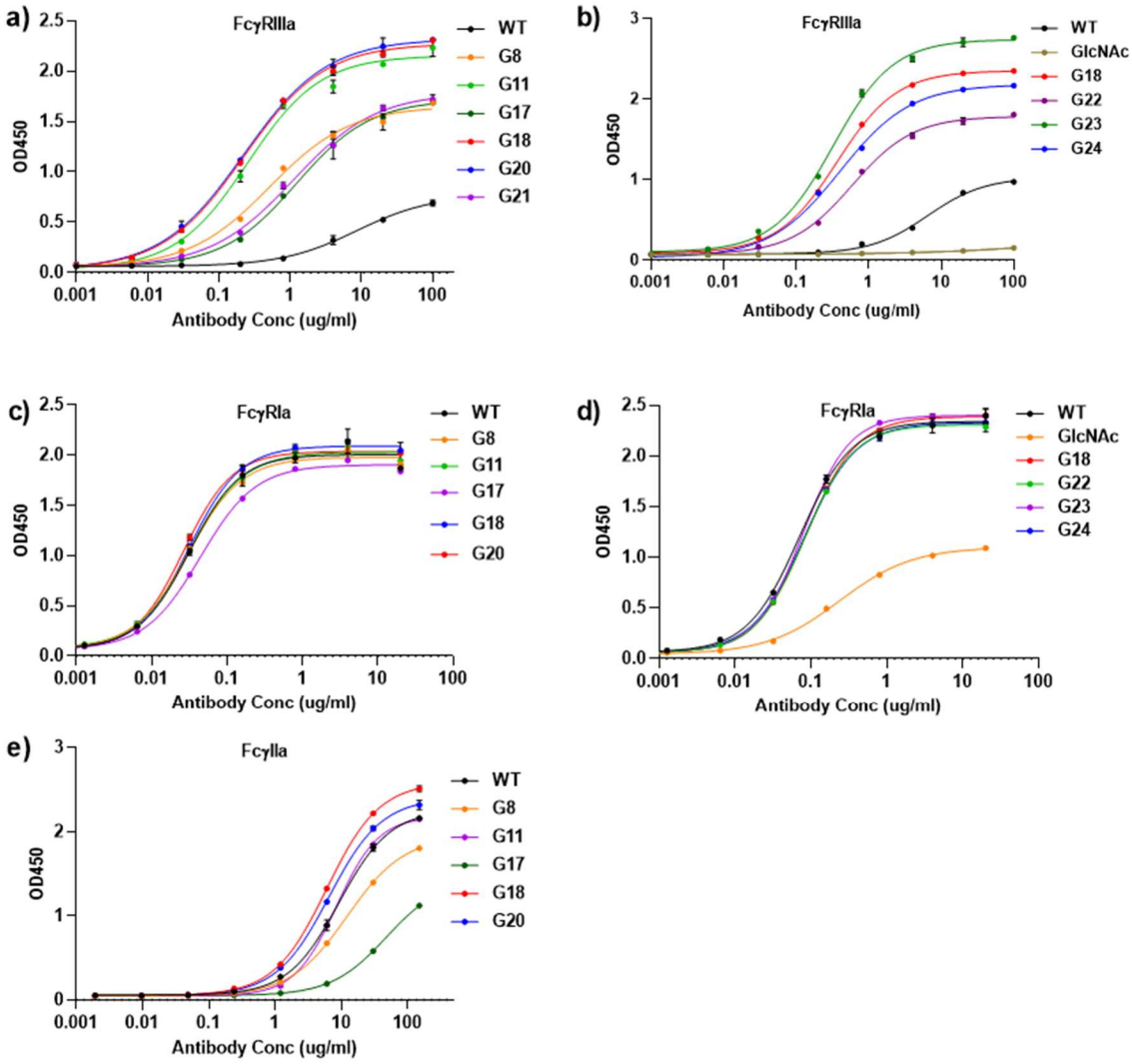


Figure S2: FcRn receptor bindings of chMC813-70 and glycoengineered antibodies. a) and b) FCγIIIa receptor; c) and d) FCγIa receptor; e) FCγIIa receptor.

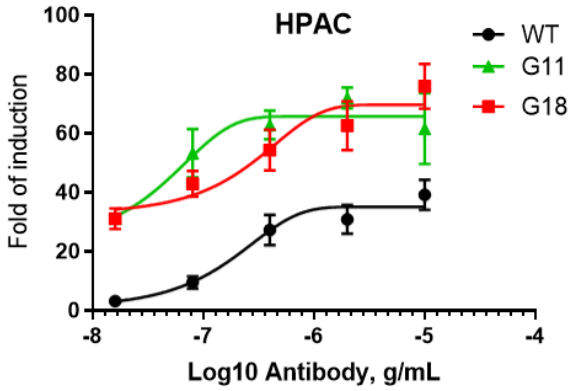
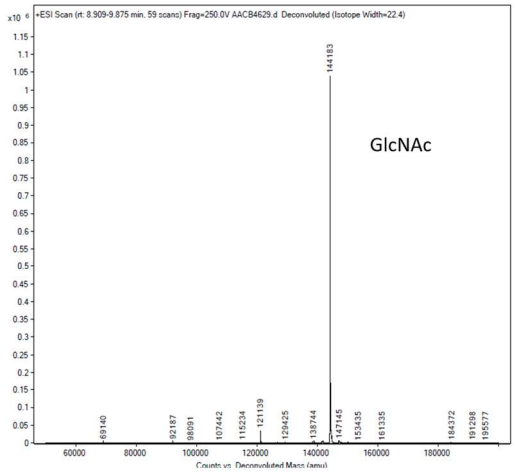
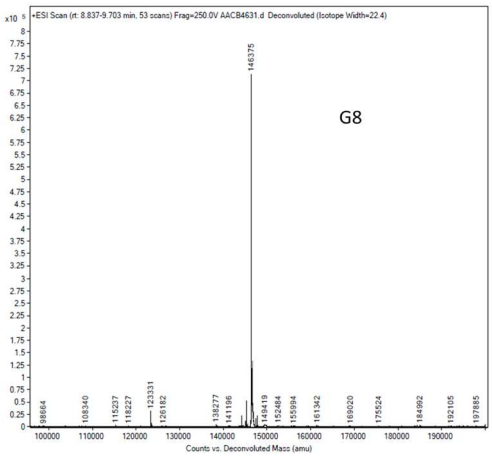
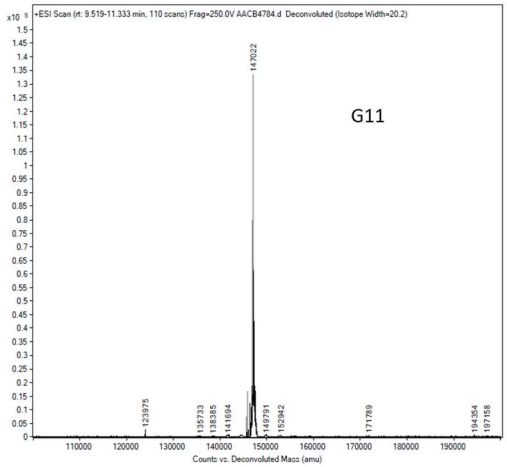
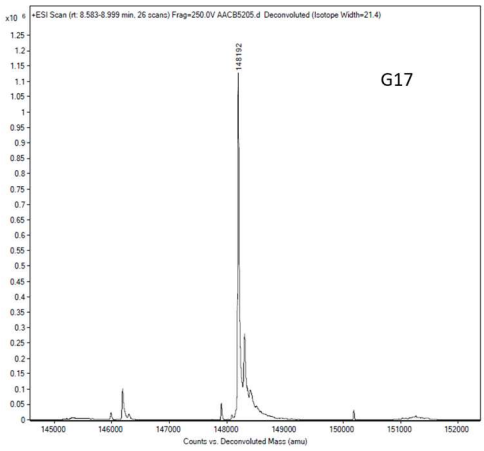


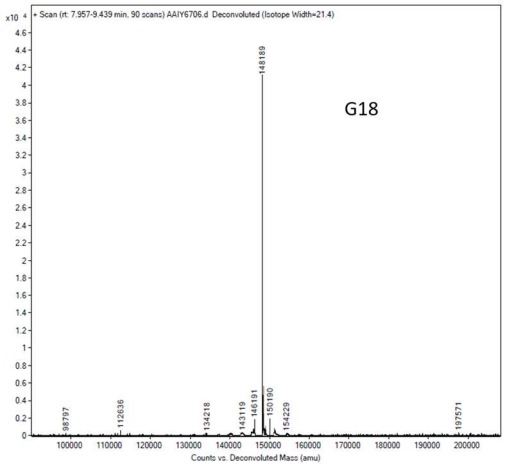
Figure S3: The ADCC activity of G11 vs G18 was evaluated using pancreatic adenocarcinoma epithelial cell line (HPAC).

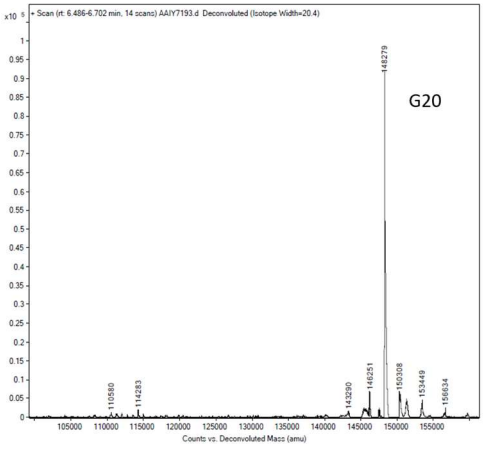

+ESI Scan (rt: 8.909-9.875 min, 59 scans) Frag=250.0V AACB4629.d Deconvoluted (Isotope Width=22.4)
GlcNAc
144183
Counts vs. Deconvoluted Mass (amu)


+ESI Scan (rt: 8.837-9.703 min, 53 scans) Frag=250.0V AACB4631.d Deconvoluted (Isotope Width=22.4)
G8
146375
Counts vs. Deconvoluted Mass (amu)


+ESI Scan (rt: 9.519-11.333 min, 110 scans) Frag=250.0V AACB4784.d Deconvoluted (Isotope Width=20.2)
G11
147022
Counts vs. Deconvoluted Mass (amu)


+ESI Scan (rt: 8.583-8.999 min, 26 scans) Frag=250.0V AACB5205.d Deconvoluted (Isotope Width=21.4)
G17
148192
Counts vs. Deconvoluted Mass (amu)


+ Scan (rt: 7.957-9.439 min, 90 scans) AAIY6706.d Deconvoluted (Isotope Width=21.4)
G18
148189
Counts vs. Deconvoluted Mass (amu)


+ Scan (rt: 6.486-6.702 min, 14 scans) AAIY7193.d Deconvoluted (Isotope Width=20.4)
G20
148279
Counts vs. Deconvoluted Mass (amu)

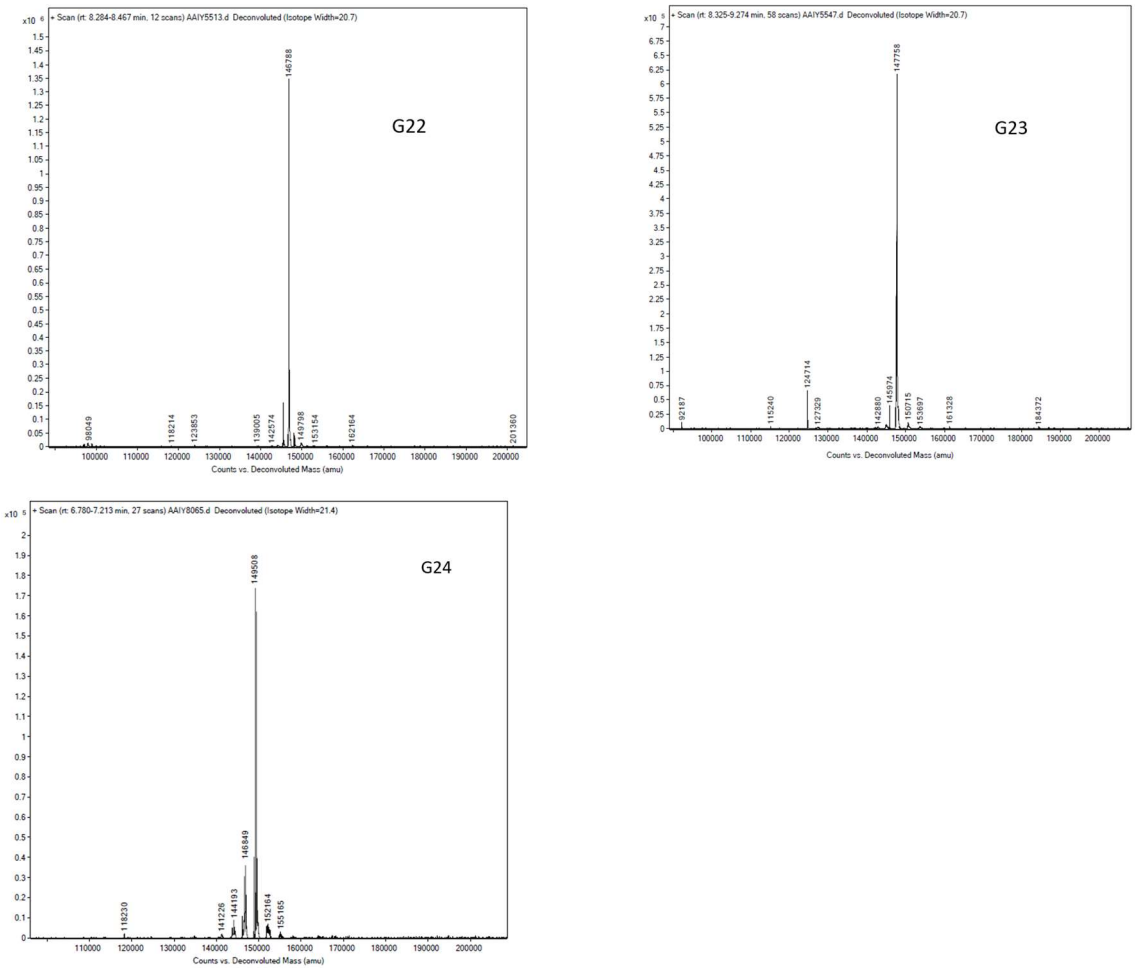
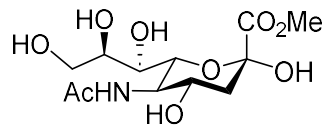
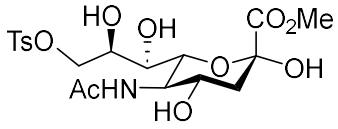
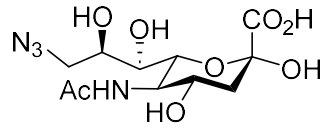


Figure S4: Intact mass analysis of glycoengineered antibodies.

Code	1H NMR
S1	1H NMR (500 MHz, D_2O) δ 5.21 (bs, 1H), 5.14 (bs, 1H), 4.45 (d, J = 7.9 Hz, 4H), 4.27 (bs, 2H), 4.20 (bs, 2H), 4.12 (bs, 3H), 4.01 – 3.96 (m, 6H), 3.95 – 3.85 (m, 13H), 3.85 – 3.69 (m, 19H), 3.69 – 3.59 (m, 13H), 3.59 – 3.41 (m, 10H), 2.67 (d, J = 12.5 Hz, 2H), 2.23 – 1.97 (m, 15H), 1.73 (t, J = 12.3 Hz, 2H).
S2	1H NMR (500 MHz, D_2O) δ 5.13 (d, J = 3.1 Hz, 1H), 5.04 (s, 1H), 4.85 (s, 2H), 4.51 (d, J = 7.6 Hz, 2H), 4.39 (dd, J = 8.0, 2.1 Hz, 2H), 4.18 (t, J = 4.1 Hz, 1H), 4.12 (d, J = 3.4 Hz, 1H), 4.05 – 4.01 (m, 1H), 3.94 (d, J = 4.4 Hz, 1H), 3.87 (dp, J = 25.4, 5.4 Hz, 10H), 3.77 (ddd, J = 12.0, 8.4, 4.6 Hz, 3H), 3.74 – 3.62 (m, 16H), 3.59 (ddd, J = 10.0, 3.3, 1.7 Hz, 2H), 3.57 – 3.39 (m, 10H), 2.29 (d, J = 1.2 Hz, 1H), 2.13 (dd, J = 13.0, 4.8 Hz, 1H), 2.05 (d, J = 6.8 Hz, 1H), 2.00 – 1.95 (m, 9H), 1.75 (t, J = 12.2 Hz, 1H).
S3	1H NMR (600 MHz, D_2O) δ 5.12 (d, J = 3.3 Hz, 1H), 5.03 (s, 1H), 4.83 (s, 2H), 4.63 (d, J = 8.0 Hz, 1H), 4.47 (d, J = 8.5 Hz, 2H), 4.20-4.15 (m, 1H), 4.10 (dd, J = 3.5, 1.6 Hz, 1H), 4.03-4.00 (m, 1H), 3.90-3.77 (m, 10H), 3.73-3.58 (m, 13H), 3.57-3.50 (m, 5H), 3.50-3.45 (m, 2H), 3.44-3.31 (m, 7H), 1.96 (d, J = 2.4 Hz, 9H).
S4	1H NMR (600 MHz, D_2O) δ 4.99 (s, 5H), 4.91 (s, 3H), 4.84 – 4.44 (m, 16H), 4.33 (d, J = 16.8 Hz, 4H), 4.11 – 3.91 (m, 5H), 3.88 (s, 3H), 3.70 (d, J = 13.0 Hz, 6H), 3.55 (dd, J = 26.4, 12.1 Hz, 9H), 3.38 (d, J = 33.4 Hz, 6H), 3.23 (s, 7H), 1.84 (s, 12H).
S5	1H NMR (500 MHz, D_2O) δ 5.99 (d, J = 3.5 Hz, 2H), 5.20 (d, J = 3.3 Hz, 1H), 5.13 (bs, 1H), 4.87 (bs, 1H), 4.63 – 4.52 (m, 4H), 4.47 (t, J = 7.3 Hz, 2H), 4.41 – 4.35 (m, 1H), 4.31 – 4.14 (m, 6H), 4.10 (d, J = 3.6 Hz, 1H), 4.02 (d, J = 8.2 Hz, 1H), 3.99 – 3.89 (m, 12H), 3.85 (td, J = 12.0, 6.4 Hz, 5H), 3.81 – 3.69 (m, 25H), 3.69 – 3.65 (m, 3H), 3.63 – 3.48 (m, 14H), 3.45 – 3.38 (m, 2H), 2.05 – 2.03 (m, 12H).
S6	1H NMR (600 MHz, D_2O) δ 5.12 (d, J = 3.3 Hz, 1H), 5.04 (bs, 1H), 4.79 (bs, 2H), 4.48 (dd, J = 21.8, 7.6 Hz, 6H), 4.39 (t, J = 7.6 Hz, 2H), 4.14 (m, 4H), 4.03 (m, 4H), 3.95-3.74 (m, 26H), 3.71-3.39 (m, 53H), 3.33 (s, 2H), 2.67 (dd, J = 12.5, 4.8 Hz, 3H), 2.01-1.89 (m, 19H), 1.72 (t, J = 11.4 Hz, 2H).
S7	1H NMR (500 MHz, D_2O) δ 4.41 (d, J = 5.0 Hz, 1H), 4.27 – 4.01 (m, 6H), 4.01 – 3.87 (m, 9H), 3.87 – 3.71 (m, 10H), 3.68 (t, J = 7.4 Hz, 1H), 3.65 – 3.47 (m, 5H), 3.47 – 3.33 (m, 4H), 2.86 (d, J = 6.6 Hz, 1H), 2.38 (s, 3H), 2.19 – 2.06 (m, 3H), 2.05 – 1.89 (m, 15H), 1.84 – 1.67 (m, 3H).
S8	1H NMR (500 MHz, D_2O) δ 5.19 (d, J = 2.5 Hz, 1H), 5.11 (bs, 1H), 4.25 – 4.24 (m, 2H), 4.19 – 4.18 (m, 2H), 4.10 (d, J = 11.8 Hz, 4H), 4.06 – 3.93 (m, 8H), 3.93 – 3.82 (m, 10H), 3.80 – 3.65 (m, 25H), 3.64 – 3.54 (m, 12H), 3.52 – 3.46 (m, 5H), 2.98 (s, 3H), 2.81 (s,

	1H), 2.78 – 2.67 (m, 3H), 2.10 – 2.00 (m, 15H), 1.79 (t, J = 12.1 Hz, 2H).
3F-SCT	1H NMR (500 MHz, D_2O) δ 6.01 (d, J = 7.2 Hz, 1H), 5.28 – 4.92 (m, 4H), 4.86 (d, J = 6.0 Hz, 1H), 4.51 (d, J = 7.1 Hz, 2H), 4.43 – 4.26 (m, 3H), 4.26 – 4.04 (m, 6H), 3.95 – 3.80 (m, 15H), 3.80 – 3.61 (m, 26H), 3.61 – 3.46 (m, 15H), 3.46 – 3.40 (m, 3H), 2.07 – 1.81 (m, 15H).
	**SA-Me-Ester** ^{1}H NMR (500 MHz, D_2O) δ 3.98 (dq, *J* = 9.7, 5.4 Hz, 2H), 3.84 (t, *J* = 10.4 Hz, 1H), 3.81-3.72 (m, 4H), 3.65 (ddd, *J* = 9.1, 6.4, 2.3 Hz, 1H), 3.61-3.49 (m, 1H), 3.47 (d, *J* = 9.2 Hz, 1H), 2.28-2.18 (m, 1H), 1.97 (d, *J* = 1.8 Hz, 3H), 1.84 (t, *J* = 12.5 Hz, 1H).
	**SA-Me-Ester-PTS** ^{1}H NMR (500 MHz, D_2O) δ 7.76 (d, *J* = 8.1 Hz, 2H), 7.43 (d, *J* = 8.0 Hz, 2H), 4.23 (dd, *J* = 10.5, 2.4 Hz, 1H), 4.13 (dd, *J* = 10.6, 5.0 Hz, 1H), 4.02-3.84 (m, 3H), 3.84-3.68 (m, 5H), 3.48 (d, *J* = 9.1 Hz, 1H), 3.27 (s, 1H), 2.38 (s, 3H), 2.29-2.20 (m, 1H), 1.97 (d, *J* = 1.6 Hz, 3H), 1.88-1.73 (m, 1H).
	**Azide SA** ^{1}H NMR (500 MHz, D_2O) δ 4.05-3.64 (m, 6H), 3.53 (dd, *J* = 11.9, 6.5 Hz, 2H), 3.43 (d, *J* = 9.3 Hz, 1H), 2.17-2.09 (m, 1H), 2.09-1.91 (m, 3H), 1.88-1.71 (m, 2H).

References

1. C. W. Lin, M. H. Tsai, S. T. Li, T. I. Tsai, K. C. Chu, Y. C. Liu, M. Y. Lai, C. Y. Wu, Y. C. Tseng, S. S. Shivatare, C. H. Wang, P. Chao, S. Y. Wang, H. W. Shih, Y. F. Zeng, T. H. You, J. Y. Liao, Y. C. Tu, Y. S. Lin, H. Y. Chuang, C. L. Chen, C. S. Tsai, C. C. Huang, N. H. Lin, C. Ma, C. Y. Wu and C. H. Wong, *Proc Natl Acad Sci U S A*, 2015, **112**, 10611-10616.
2. C. W. Lin, Y. J. Wang, T. Y. Lai, T. L. Hsu, S. Y. Han, H. C. Wu, C. N. Shen, V. Dang, M. W. Chen, L. B. Chen and C. H. Wong, *Proc Natl Acad Sci U S A*, 2021, **118**.
3. B. S. Parekh, E. Berger, S. Sibley, S. Cahya, L. Xiao, M. A. LaCerte, P. Vaillancourt, S. Wooden and D. Gately, *MAbs*, 2012, **4**, 310-318.