Online Methods

Mice. The *Cmah* null mice used for this study have been described previously[18], and were backcrossed to C57Bl/6 mice for >10 generations. All experiments were approved by the UC San Diego IRBC committee responsible for approving animal experiments.

Sialidase treatment of Therapeutic Antibodies. One mg each of Cetuximab or Panitumumab (obtained from the UCSD pharmacy or the manufacturer) were treated with 50 mU of active or heat-inactivated *Arthrobacter ureafaciens* sialidase (EY Laboratories) in 100 mM sodium acetate, pH 5.5, at 37˚C for 24 h. Samples were used for ELISA or Western Blots**.**

Periodate treatment of Therapeutic Antibodies on ELISA plate. Untreated Cetuximab and Panitumumab (1 μg/well) were used for coating, then blocked with PBST for 2 h and incubated with freshly made 2 mM sodium metaperiodate in PBS for 20 min at 4˚C in the dark. The reaction was stopped using 200 mM sodium borohydride to a final concentration of 20 mM. As a control, periodate and borohydride were pre-mixed and then added to the wells (the borohydride inactivates the periodate). To remove resulting borates, wells were then washed 3 times with 100 mM sodium acetate, 100 mM NaCl, pH 5.5 before further analysis.

ELISA detection of Neu5Gc on Therapeutic Antibodies. For the ELISA, wells were coated with 1 μg of Cetuximab or Panitumumab (either pre-sialidase treated or post-periodate treated), blocked with TBST for 2 h, and then incubated with affinity-purified chicken anti-Neu5Gc IgY or control IgY for 1 h (1:20,000 in TBST). Binding of IgY was detected using HRP-conjugated donkey anti-chicken IgY antibody (1:50,000 in TBST) and development with *O*-phenylenediamine in citrate- phosphate buffer, pH 5.5, with absorbance being measured at 495 nm. ELISA samples were studied at least in triplicate. Similar to the ELISA with the anti-Neu5Gc chicken IgY, human anti-Neu5Gc IgG that had been purified from the serum of healthy

humans[11] and biotinylated was also used as the primary antibody (1:100 in TBST). Binding of the human antibodies to the therapeutic antibodies was detected using HRP-conjugated Streptavidin (1:10,000) followed by development as described above. Samples were studied in triplicate.

Western Blot detection of Neu5Gc on Therapeutic Antibodies. For Western Blot detection Cetuximab or Panitumumab (1 µg per lane) were separated by 12.5% SDS-PAGE, and Coomassie stained or blotted on nitrocellulose membranes. Blotted membranes were blocked with TBST containing 0.5% cold water fish skin gelatin overnight at 4˚C and subsequently incubated with affinity-purified chicken anti-Neu5Gc IgY for 4 h at room temperature (1:100,000 in TBST). Binding of the chicken anti-Neu5Gc IgY was detected using an HRP-conjugated donkey anti-chicken IgY antibody for 1 h (1:50,000 in TBST), followed by incubation with SuperSignal West Pico Substrate (Pierce) as per manufacturer's recommendation, exposed to X-ray film and the film developed. Similar to the Western blot with the chicken anti-Neu5Gc IgY, purified biotinylated human anti-Neu5Gc IgG was also used as the primary antibody (1:100 in TBST). Binding of the human antibodies to the therapeutic antibodies was detected using HRP-conjugated Streptavidin (1:10,000 in TBST) followed by development as described above.

CIC-C1q Binding Assay. Immune complex formation was detected by using the CIC (C1Q) ELISA Kit (Buehlmann) as described in the manufacturers's guidelines[34]. Briefly, 100 µl of human serum with low or high anti-Neu5Gc antibodies (S30 and S34, respectively, from Ref. 11) was incubated with 40 µg of Cetuximab or Panitumumab for 14 h at 4˚C. 1:50 dilutions of the mix were applied to human C1q coated ELISA wells, and incubated for 1 h at 25˚C. Binding was detected using alkaline phosphatase conjugated Protein A. After another washing step, the

enzyme substrate (para-nitrophenyl-phosphate) was added followed by a stopping step. The absorbance was measured at 405 nm. Samples were studied in triplicate.

Generation of murine anti-Neu5Gc antibodies. *Haemophilus influenzae* strain 2019[35] was a generous gift from Michael Apicella, Department of Microbiology, University of Iowa. Bacteria were grown to mid-log in sialic acid-free media[36] with or without addition of 1 mM Neu5Gc, heat-killed, and injected intraperitoneally (200 µl of OD_{600nm} = 0.4) into *Cmah* null mice.

Kinetics of therapeutic antibodies in mice with a human-like Neu5Gc-deficiency, in the absence or presence of anti-Neu5Gc antibodies. Cetuximab or Panitumumab in PBS (0.24 µg per gram mouse body weight) were injected i.v. and 14 h later, mouse serum pooled from syngeneic *Cmah* null mice containing anti-Neu5Gc antibodies (or pooled serum from syngeneic naïve or control immunized mice) was passively transferred via IP injection into syngeneic *Cmah* null mice. Mice were bled 0, 2, 8, 32, 56 and 80 h after the passive transfer of mouse serum. For quantification of therapeutic antibodies concentration in the sera, wells of ELISA plates were coated with 1 µg of anti-human IgG (Biorad), then blocked with TBST for 2 h and incubated with 1:500 dilutions of the sera per well. Captured therapeutic antibodies were detected by HRP-conjugated anti-human Fc antibody (1:10,000, Jackson), with development by *O*-phenylenediamine in citrate-phosphate buffer, pH 5.5, and absorbance measured at 495 nm (n=5 for injections of both control sera groups, n=10 for injections of anti-Neu5Gc serum groups) .

Quantification of anti-Neu5Gc IgG antibodies in Neu5Gc-immunized mice.

A Neu5Gcα2-6Galβ1-4Glc-conjugate[11] (1 µg/well) and serial dilutions of mouse IgG as standards (0.625–20 ng/well) were used for coating overnight, then blocked with PBST for 2 h and incubated with pooled serum from Neu5Gc immunized mice (1:250 dilution) for 2 h at 25˚C.

Binding of mouse IgG was detected by using HRP-conjugated goat anti-mouse IgG-Fc (Jackson, 1:10,000 in PBST) and development with *O*-phenylenediamine in citrate-phosphate buffer, pH 5.5, with absorbance being measured at 490 nm. ELISA samples were studied in triplicate.

Levels of anti-Neu5Gc IgG in mice with a human-like Neu5Gc-deficiency after injections of the therapeutic antibodies, or murine IgG. *Cmah* null mice were injected i.v. with 4 μg antibody/gram mouse body weight in PBS weekly for three weeks. Mice were bled initially, and again one week after the third i.v. injection. Wells of ELISA plates were coated with 1/1000 dilutions of human (Neu5Gc-deficient) or chimpanzee (Neu5Gc-positive) serum glycoproteins (Note that the only major difference between human and chimp serum glycosylation is the absence or presence of Neu5Gc[37]). Alternatively, wells were coated with human or bovine fibrinogen, which carry Neu5Ac or Neu5Gc on otherwise identical N-glycans[38]. Wells were then blocked with TBST for 2 h followed by incubation with 1:100 dilutions of the mouse sera. Binding of the mouse antibodies was detected by using HRP-conjugated goat anti-mouse IgG Fc fragment antibody (1:10000 in TBST). Neu5Gc-specific binding (change in OD_{495nm}) was determined by subtracting the background signal of the wells coated with Human serum or Human fibrinogen (no Neu5Gc) from the signal with Chimpanzee serum or Bovine fibrinogen (Neu5Gc-containing) coated wells. Data were obtained in triplicate (n=5 for injection of mIgG, n=4 for injection of Panitumumab, n=6 for injection of Cetuximab).

An Approach to Reduce Neu5Gc Contamination in Biotherapeutic Products. 293T human kidney cells were grown in DME supplemented with 10% FCS. Cells were lifted from culture plate using 20 mM EDTA in PBS and allowed to grow to 50% confluence. At this point buffered 100 mM Neu5Gc was added to the culture in duplicate for a final 5 mM concentration, and the cells were grown in this supplemented media for 3 days. At the end of this Neu5Gc pulse, the

cells were once again lifted using 20 mM EDTA in PBS, pelleted, washed once with PBS to remove any excess Neu5Gc and then suspended in 30 ml of growth medium. Five ml of this cell suspension was added to each of 5 P-100 dishes. The last aliquot of cell suspension, time "0", was immediately harvested by pelleting the cells, washing once with PBS, followed by suspending the cells in 1 ml of PBS and transferring to a 1.5 ml microcentrifuge tube. The cells were re-pelleted and frozen until all time points were collected. Buffered 100 mM Neu5Ac was added to each of the other 5 plates for the "Neu5Ac chase", and an equivalent amount of media added to the "minus chase" samples. The cells were harvested at day 1, 2, 3, 4 and 5 by scraping into the culture media, collecting by pelleting, washing once with PBS, transferring to a 1.5 ml microcentrifuge tube, pelleting and freezing the cell pellet. At the end of the 5 days of chase, all collected cell pellets were homogenized in 300 µl of ice-cold 20 mM potassium phosphate pH 7 using 3–20 sec burst with a Fisher Sonicator. Glycoconjugate-bound Sias were precipitated by adding 700µl of 100% ice-cold ethanol (final 70% ethanol) and incubating at -20˚C overnight. The samples were spun at 20000 X g for 15 min and the supernatants transferred to clean tubes and dried on a speed vac. The precipitated glycoconjugates and dried ethanol supernatants were each suspended in 100 µl of 20 mM potassium phosphate pH 7 by sonication. Sias were released from both fractions by acid hydrolysis with 2 M acetic acid (final) and incubating at 80˚C for 3 h. Samples were passed through a Microcon-10 filter and the filtrate derivatized with DMB reagent, for analysis of Sias by HPLC.

A similar approach was taken to CHO cells stably expressing a Siglec-Fc protein in the medium, except that the Neu5Gc pulse was omitted, and the secreted glycoproteins were captured on Protein-A Sepharose beads. The cells were also processed similarly, except that total cell membranes were pelleted by centrifugation. The Sia content of the secreted proteins

and cell membranes was determined by acid hydrolysis, DMB derivatization and HPLC. The cell membranes were also studied by Western-blotting with the chicken anti-Neu5Gc IgY, as described above.