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Homogeneous antibody and CAR-T cell with improved effector functions targeting SSEA-4 glycan on pancreatic cancer

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Supplementary Materials

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Fig. S1 to S11

Tables S1

Materials and Methods

Cell culture. The pancreatic cancer cell lines used in this study, including PL45, BxPC3, Pan 0203, MIA PaCa-2, AsPC-1, Pan1005, Capan 2, HPAF-2 and CFPAC-1 were purchased from American Type Culture Collection (ATCC); PANC-1 and HPAC were purchased from Bioresource Collection and Research Center (BCRC, Taiwan); KP-4 was purchased from Japanese Collection of Research Bioresources (JCRB, Japan); PK1 and VGHPa8 cells were derived from patients[1] and provided by Dr. Chia-Ning Shen. PANC-1, AsPC-1, and MIAPaCa-2 are partially resistant to three conventional chemotherapy drugs, gemcitabine, 5-fluorouracil (5-FU) or cisplatin[2]. The breast cancer and brain tumor cell lines used in the study were from ATCC. The lung cancer cell lines CL-1[3], PC9, PC13 were kindly provided by Prof. Pan-Chyr Yang. Lung cancer cell lines H1299, A549 were obtained from ATCC or National Cancer Institute (NCI). All these cancer cell lines were routinely maintained in high-glucose DMEM or RPMI 1640 (Thermo Fisher Scientific) supplemented with 10% (vol/vol) FBS (Biological Industries).

Flow cytometry. Cells (5×10^5) were stained with 1 μg Alexa Flour 488-conjugated anti-SSEA-3 mAb (MC-631, BioLegend), anti-SSEA-4 mAb (MC-813-70, BioLegend), or anti-Globo H mAb (VK9, a gift from Philip O. Livingston, Memorial Sloan–Kettering Cancer Center, New York) in 100 μL FACS buffer (PBS solution with 1% FBS) on ice for 30 min before analysis on FACS Calibur system (BD Biosciences).

Cell staining. Anti-SSEA-4 antibody was conjugated with APC (Nova Biosciences, 705-0030), anti-Globo H antibody (VK9) was conjugated with FITC (Abcam, ab188285). MDA-MB-231 and HPAC were fixed and then stained 1-2 h with 1 μg/mL of MC-813-70-APC and 25 μg/mL of VK9-FITC. Hoechst 33342 was used to stain the nucleus with dilution to 1:500. Cells were incubated at 37 °C for 5-15 min, then washed with PBS and imaged.

Xenograft mouse model. Tumor xenografts were generated by s.c. injection of 1×10^6 BxPC3 cells in athymic-nu/nu mice (N>5). Intraperitoneal (i.p.) antibody injection was started when tumors reached 40 mm^3. mAb MC48 was administered at a dose of 25 μg/mouse (1.25mg/kg) or 10 μg/mosue (0.5mg/kg), twice per week through i.p. injection for 1 month. PBS and mAb MC-813-70 were separately injected in parallel for comparison. Tumor size was determined by Vernier Caliper Measurements, and the tumor volume was calculated (in mm^3) as length × width × height. All of the animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Academia Sinica.

Generation and characterization of SSEA-4 mAb. The hybridoma technology was employed for the development of mAbs specific to SSEA-4. Female BALB/c mice, aged 6-8 weeks old, were immunized three times subcutaneously with the SSEA-4-conjugate synthesized in our laboratory. Three immunizations were given at 2-wk intervals. Each vaccination contained 2 μg of SSEA-4 vaccine. All of the sera were obtained by centrifugation at 4,000 × g for 10 min. The serologic responses were analyzed by ELISA and glycan array. A final boost was given intraperitoneally with 2 μg of SSEA-4 vaccine, and 3 days later, the spleen cells from immunized mice were used for generating hybridomas. The hybridoma supernatants were screened against SSEA-4 using ELISA or glycan array analysis. For mouse monoclonal isotyping, the IsoQuick Strips was used (Sigma, I9535). The specificity of antibodies was confirmed with glycan array analysis and cell-based binding assay using cells with and without the target glycan SSEA-4.

Glycan array fabrication. Glycan microarrays were printed (BioDot; Cartesian Technologies, Irvine, CA) by robotic pin (SMP3; TeleChem International Inc., Sunnyvale, CA) with deposition of ~0.6 nL glycan-containing solution per spot. The amine-containing glycans[4] in printing buffer (300 mM sodium phosphate, pH 8.5, 0.01% Triton X-100) were spotted onto *N*-hydroxysuccinimide (NHS)-activated glass slides[4,5]. Each glycan was printed at 100 μM and

replicate of twelve for Kd determination. Printed slides were allowed to incubate in 80% humidity for 30 min, followed by desiccation for overnight. The remaining NHS groups were blocked by immersing the slides for 1 h in SuperBlock (PBS) Blocking Buffer (Pierce, Appleton, WI).

Determination of the surface dissociation constant of SSEA-4 binding to antibodies. SSEA-4 was printed in duplicate with 100 μM concentrations on each sub-array. The images were obtained from the array incubated with different concentrations of MC48 and MC-813-70 Ab conjugated with a fluorochrome for signal detection. Binding curves were generated from the analysis of antibodies binding to SSEA-4 glycan. All the curves were fitted to a standard one-site binding model by nonlinear regression analysis using Prism 5 (GraphPad) and the Kd values were determined from Langmuir isotherm[5].

Complement-dependent cytotoxicity assay. SSEA-4 antigen high and low cell lines were used for cytotoxicity assay. The complement-dependent cytotoxicity activity of anti–SSEA-4 mAbs was measured by lactate dehydrogenase (LDH)-release assay using CytoTox96 Non-Radioactive Cytotoxicity Assay kit (Promega). Cells (1×10^4) were plated in each well of 96-well plates and were washed with PBS twice after overnight growth. The cells were then incubated with 1-2 μg SSEA-4 mAbs or PBS control in 50 μL phenol red-free DMEM or RPMI 1640 with rabbit complement or human complement (10% or 20%) (Thermo Fisher Scientific). After incubation in a 5% CO2 incubator at 37 °C for 2-4 h, the degree of cell lysis was determined by measuring the amount of LDH released into the culture supernatant. Maximum LDH release was determined by lysing the cells with the lysis solution provided by the commercial source. Percentage of specific lysis was calculated according to the equation: % lysis = (experimental release - spontaneous release) / (maximum release - spontaneous release) × 100.

Isolation of antibody DNA from hybridoma. 5×10^6 hybridoma (MC48) cells were used. Total RNA and mRNA extractions were handled according to the protocol described by the

manufacturer's protocols (Qiagen). cDNA synthesis was carried out using SuperScript™ First-Strand Synthesis System and random primers, according to manufacturers' protocols (Thermo Fisher Scientific) Primer mixtures were used for PCR amplification of the variable regions of heavy chain and light chain, and the PCR product was cloned to pGEM-T and sequenced. The immunoglobulin sequences and complementarity determining regions (CDRs) were obtained from the NCBI/IGBLAST bank.

Construction, expression and purification of humanized MC48 antibody. Antibody humanization began with the analysis of variable region of mouse antibody. The most similar consensus and germline sequences were determined through alignment with sequences in the ImMunoGeneTics database[6.] Humanized heavy chain and light chain were designed by CDR grafting method, and phage display was used for screening the good binder. All humanized genes were synthesized and cloned to human IgG1 antibody expression vector. The constructed plasmids were transfected into the 293F cells using transfection reagent as described by the manufacturer's protocols (Thermo Fisher Scientific). Recombinant antibody was purified by protein A Sepharose CL-4B as described by manufacturers' protocols (GE Healthcare Bioscience).

Antibody-dependent cell-mediated cytotoxicity (ADCC) assay. HPAC, BxPC3, and PL45 (5×10^3 cells) pancreatic cancer cell lines were seeded on a 96-well plate and cultured until ~80% confluent. Then, these cells were incubated with antibodies (e.g. hMC48, chMC813-70, or nonspecific human IgG (NHIgG) 10 μg/ml and PBMCs (effectors, E) at 37°C for 16 h. The effector functions of antibody are affected by the glycan attached to the Asn-297 position of the Fc region which interact with different Fc receptors expressed on various immune cells. 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 while the α2,3-linked SCT or FSCT glycoforms showed a 4-fold reduction in binding to the receptor. After treatment, the LDH

expressed level was detected by CytoTox-ONE™ Homogeneous Membrane Integrity Assay Kit (Promega)[7]. The fluorescence intensity of the reaction was read with an excitation wavelength of 560 nm and an emission wavelength of 590 nm (Molecular Device, SpectraMax M5).

ELISA screening of selected phage clones. To detect antigen recognition, microwell plates (Nunc) were coated with 0.2 μg/mL of SSEA-4-BSA, Globo H-BSA, SSEA-3-BSA, or BSA. The selected phage clones were diluted (1:2) in PBS containing 3% BSA and added to each well. The plates were incubated at room temperature for 1 h, washed with $PBST_{0.1}$, and incubated with horseradish peroxidase (HRP)-conjugated mouse anti-M13 phage antibody (GE Healthcare). The plates were washed again, and OPD and H_2O_2 were added. After termination of reaction by 3 N HCl, the absorbance was measured at 490 nm using a microplate reader (Model 680, BioRad). The phagemids from ELISA-positive phage clones were extracted to identity scFv coding regions by auto-sequencing.

Humanization of MC48. Two human genes, GenBank accession Q9UL73 and AY577298, were most similar to MC48 V_H and V_L, respectively. We humanized three sequences of MC48, including the 1st humanized MC48 (hMC48) V_H consisted of framework (FR) 1 modified to FR4 of Q9UL73 gene, the 1st hMC48 V_L consisted of four FRs from the accession AY577298, the 2nd hMC48 FRs of V_H followed by 1YY8 from PDB, while the 2nd hMC48 V_L was the same as 1st sequence, and the 3rd hMC48 V_H sequence had FR1, 2 and 4 of Q9UL73 gene modified and the 3rd hMC48 V_L only had FR2 and FR4 changed to human AY577298 gene.

Generation of humanized MC48 scFv phage clones. hMC48 variant phagemids were transformed to TG1 *E-coli* and recovered in 2 × YT medium (BD Pharmingen) containing 100 μg/mL ampicillin and 2% glucose and rescued by M13KO7 helper phage (NEB) for 1 h at 37°C. After centrifugation at 1,500 × g for 10 min, these pellets were resuspended in 2 × YT medium containing 100 μg/mL ampicillin and 50 μg/mL kanamycin overnight to generate scFv-phages.

Expression of enzymes. The endo-glycosidases Endo-S, Endo-S2 and Endo-S2 mutant (D233Q), and the α-L-fucosidase from Bacteroides fragilis NCTC9343(BfFucH) were expressed in E. coli and purification of enzyme was performed with Ni-NTA agarose beads. The yields of enzymes: Endo-S, 63 mg/200 mL; Endo-S2, 215 mg/200 mL; Endo-S2 mutant (D233Q), 26.7 mg/200 mL; BfFucH, 19.4 mg/2L.

Preparation of α2,6-SCT-oxazoline. Following the procedures described previously[8], sialylglycopeptide (SGP) was digested by Endo-S2 at 37°C for 48 h, and purified by gel filtration chromatography, and the product was analyzed by LC-MS. The mixture of α2,6-SCT, CDMBI, and NEt3 was incubated at 4°C for 1 h to generate α2,6-SCT-oxazoline which was purified by gel filtration chromatography with 0.05% aqueous NEt3 and characterized by LC-MS.

Preparation of chMC813-70 and mono-GlcNAc-chMC813-70. Anti-SSEA-4 antibody (chMC813-70) produced by Expi293F™ GnTI$^-$ cells contained high mannose glycoforms which were purified by protein G agarose resin and characterized by Nanospray LC-MS to confirm that the glycans are mainly Man5, so the monoglycosylated antibody was easily prepared with endo-H cleavage. The procedures for transglycosylation were similar to that reported previously. Briefly, mono-GlcNAc-chMC813-70 (2 mg) and Endo-S2 (D233Q) (200 μg) were added to a solution of glycan oxazoline (α2,6-SCT-oxazoline) (6 mg) in 50 mM Tris buffer (pH 7.4). The solution was incubated for 2 h at 37°C, followed by centrifugation at 700 rpm. Nanospray LC-MS was used to confirm the transglycosylation product: 96% yield, chMC813-70-SCT (2h). Then, the reaction mixture was purified with a protein-A affinity column to collect the desired product. For the transglycosylation of mono-GlcNAc-chMC813-70 with α2,6-FSCT-oxazoline, mono-GlcNAc-chMC813-70 (25 μg) and Endo-S2 (D233Q) (2.5 μg) were added to a solution of glycan oxazoline (α2,6-FSCT-oxazoline) (25 μg) in 50 mM Tris buffer (pH 7.4). The solution was incubated for 3.5

h at 37°C, followed by centrifugation at 700 rpm. Nanospray LC-MS was used to confirm the transglycosylation product: 97% yield, chMC813-70-FSCT (3 h).

ADCC of chMC813-70-SCT toward pancreatic cancer cell lines. The ADCC activity on pancreatic cancer cells with different expression levels of SSEA-4 was evaluated with anti-SSEA-4 antibody chMC81370 and its homogeneous glycoforms chMC813-70-SCT and chMC813-70-FSCT using the procedures described previously[7].

Primary human NK cell culture and *ex vivo* expansion of a subpopulation of NK cells isolated by chMC813070-SCT. Cryopreserved primary human NK cells were purchased from Cellero, which were enriched by negative selection. NK cells were cultured in MACS Medium (Miltenyi Biotec) with 5% human AB serum (Sigma-Aldrich) and 1000 U/ml Interleukin 2 (IL-2). DNase I (10 U/ml) treatment is necessary to prevent cell aggregation and death after thawing. Afterward, we utilized the NK killing assay to measure the NK cytotoxicity. NK cells were treated with Activation/Expansion Kit to expand the primary NK cells. Anti-Biotin MACSi-Beads™ conjugated with CD335 (NKp46)-Biotin and CD2-Biotin (Miltenyi Biotec) was used to detect NK-cell markers. NK cells were stained with chMC81370-SCT-conjugated FITC and sorted by flow cytometry. The sorted specific NK cells were resuspended in NK MACS medium with 5% human AB serum and then incubated at 37°C, 5% CO2 for five days until analysis and expansion. The expanded NK cells exhibited high cell viability and cell proliferation rate.

Analysis of primary NK-cell cytotoxicity via NK-cell killing assay. NK killing assay was performed using the LDH-Glo™ Cytotoxicity Assay. The target cells (10,000 cells/100 μL) were seeded into a 96-well flat-bottom microplate overnight. Then the effector cells (NK cells) in a 5:1 E/T ratio were added to the 96-well flat-bottom microplate. The cells were incubated for an additional 4 h at 37°C, 5% CO2. After incubation, the reaction mixture was transferred to the other 96-well microplate and 100x diluted by LDH storage buffer. The diluted mixture of 50 μL and

LDH-detection buffer of 50 μL were mixed. The white microplate was incubated at room temperature for 1 h, and the luminescence was read by CLARIOstar. The percentage of target cell lysis was calculated according to the CDC assay formula: the percentage of cell lysis = (luminescence of the experimental – luminescence of the spontaneous) / (luminescence of the maximum – luminescence of the spontaneous) x 100.

Gene expression profiling and survival analysis in tumors. Cancer gene expression for each type of cancers was evaluated using GEPIA database[9] which that visualizes pre-computed data from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx). The overall survival in pancreatic cancer patients was evaluated using the Kaplan-Meier (KM) plotter[10] which generated gene expression data and survival information of patients from Gene Expression Omnibus (GEO).

Generation of anti-SSEA-4 human CAR-T cells. To expand the repertoire of monoclonal antibodies suitable for SSEA-4-targeted immunotherapy, we used mouse hybridoma and B cell cloning to identify and characterize novel monoclonal antibodies specific to SSEA-4. Here we chose one of such antibodies for CAR-T recognizing SSEA-4-positive human carcinoma cell lines but not that of the negative. This mouse antibody undergoes extensive humanization processes and protein engineering. The final single-chain antibody suitable for the construction of CAR-T contains an N-terminal light chain variable region followed by a 3x linker, and ended with the variable region of the heavy chain. This anti-SSEA-4 variable region (scFv sequence) was subcloned into a lentiviral vector containing different CAR cassettes widely used for lentivirus-based transduction of human CD4+/CD8+ T cells.

Real time cytotoxicity assay (RTCA) of anti-SSEA-4 CAR-T cells. One day before the addition of CAR-T cells, target cells (HPAC, human pancreatic adenocarcinoma cell line) were plated onto sample wells to form a monolayer suitable for RTCA monitoring. Percent cytotoxicity was plotted

at 4, 24, and 48 h post addition of CAR-T cells. RTCA, via xCELLigence machine, counts Cell Index by measuring cell impedance. Killed target cells produce a significant difference in impedance value than that of living cells.

Efficacy and toxicity of Anti-SSEA-4 CAR-T cells in HPAC tumor-bearing NSG mice. About 100-million freshly purified CD4+/CD8+ human T cells were transduced with lentivirus bearing PMC300 to generate nearly 80-million SSEA-4-recognizing CAR-T cells. These samples were de-identified prior to use for the study. A varying number of such T cells, 5-million, 10-million, and 20-million, were i.v. injected into NSG mice bearing luminescently tagged HPAC tumors (formed in 12 days before CAR-T cell injections) on Day 12, along with appropriate controls (PBS and untransduced T cells). Luminescence was imaged twice a week. The remaining mice were removed for autopsy and pathological studies.

Demonstration of efficacy without significant toxicity in HPAC tumor cells. Mice were randomly divided into six groups with ten mice per group. Twice per week, all mice were photographed for luminescence. On day 11 when tumors were clearly formed in all mice, 0.5-million, 1-million, 2.5-million, and 5-million anti-SSEA-4 CAR-T cells were injected into four groups of NSG mice, along with one group with PBS and another group with 5-million untransduced human T cells as negative controls. The experiment ended on day 74 when no mouse died nor loss of body weight. Enigmatically, upon the second injection of the same number of CAR-T cells on Day 59, these mice significantly regained the body weight. The second injection of CAR-T cells on Day 59 did not seem to impact the overall anti-tumor efficacy of CAR-T cells. It is noted that SSEA-4 has been studied as a target for therapeutic development against various cancers[11-13], however, the toxicity and efficacy of therapeutic agents have not been fully evaluated, and in certain cases some undesirable toxicity has been reported. This study represents a

comprehensive evaluation of therapeutic antibodies and CAR-T cells targeting SSEA-4 positive pancreatic cancer.

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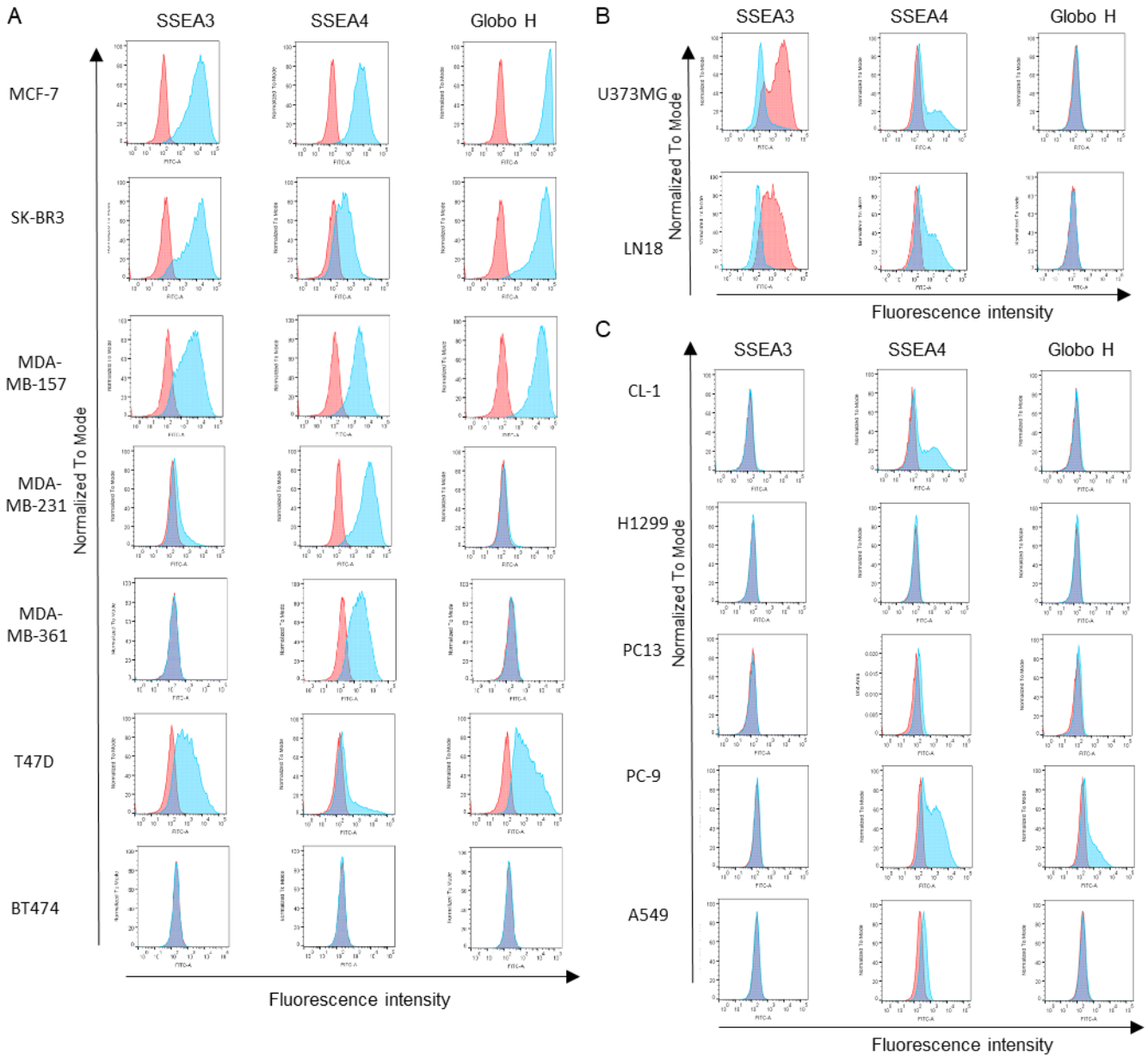
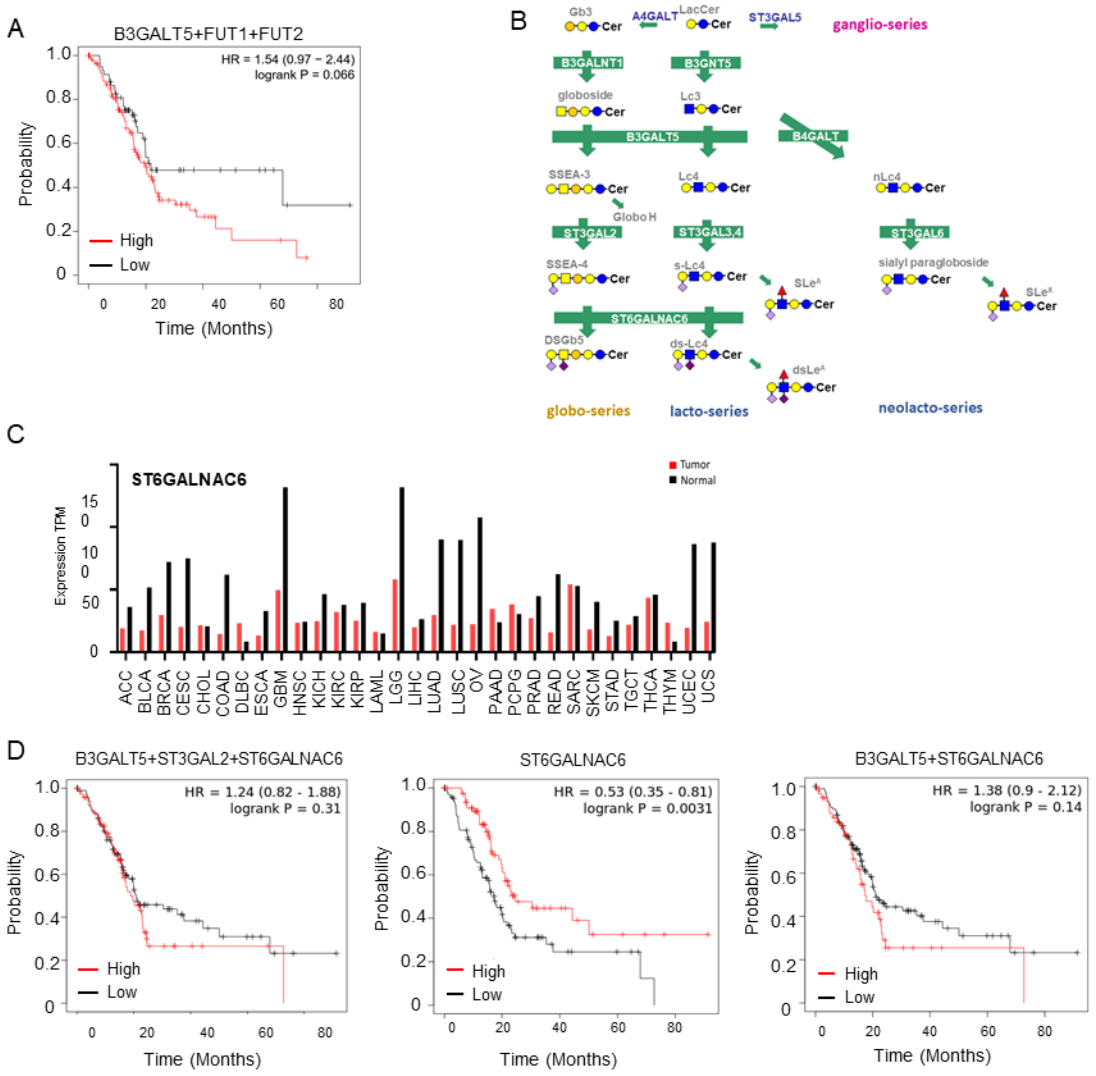
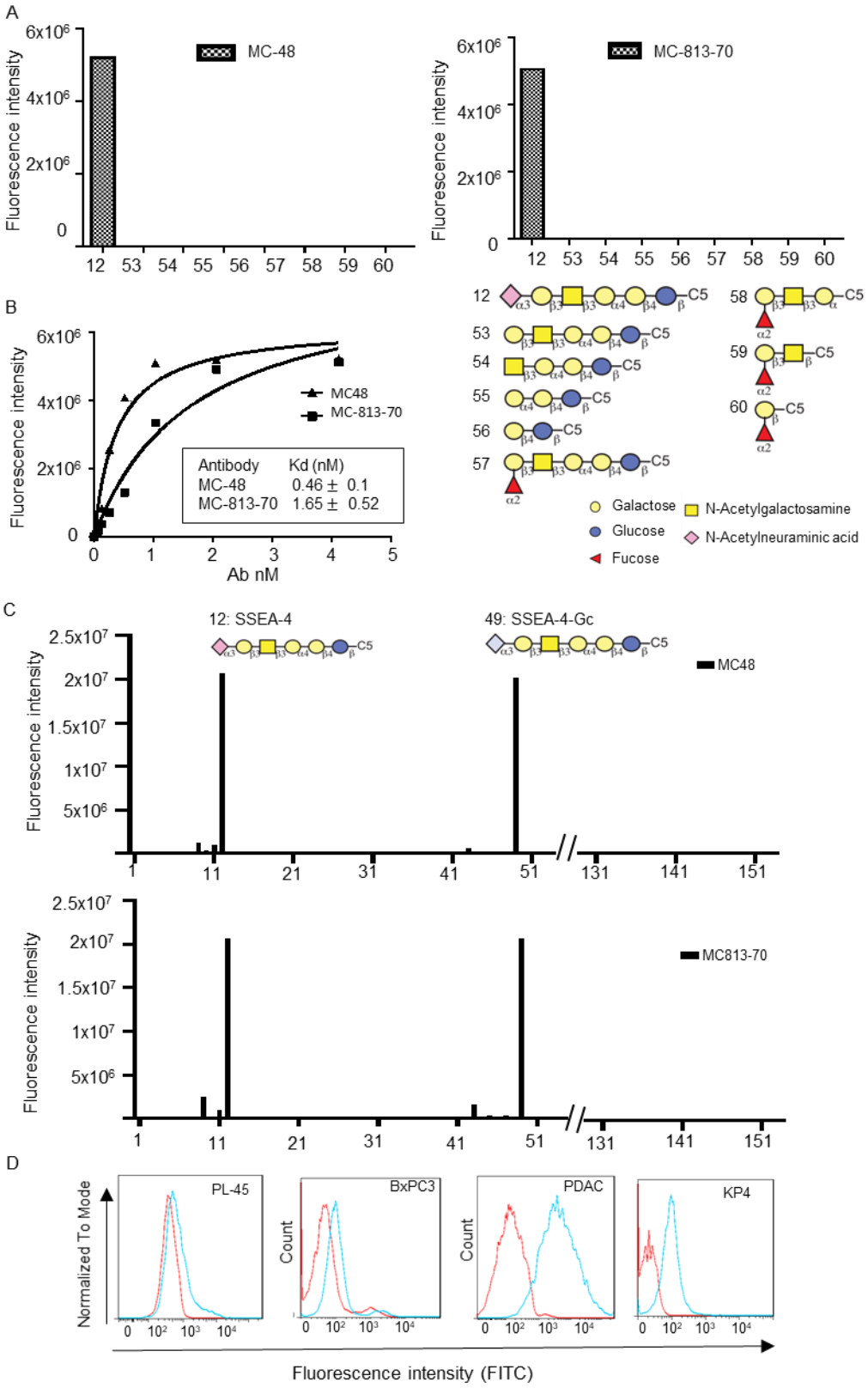


Supplementary Fig. 1. Expression of globo-series glycolipids on cancer cell lines. SSEA-4, SSEA-3 and Globo-H on breast (**A**), brain (**B**), and lung (**C**) cancer cell lines were stained by MC-813-70, MC-631 and VK9 antibodies (blue histograms) and the corresponding isotype control (red histograms). Representative data are shown (n=3~5 for each cell line).

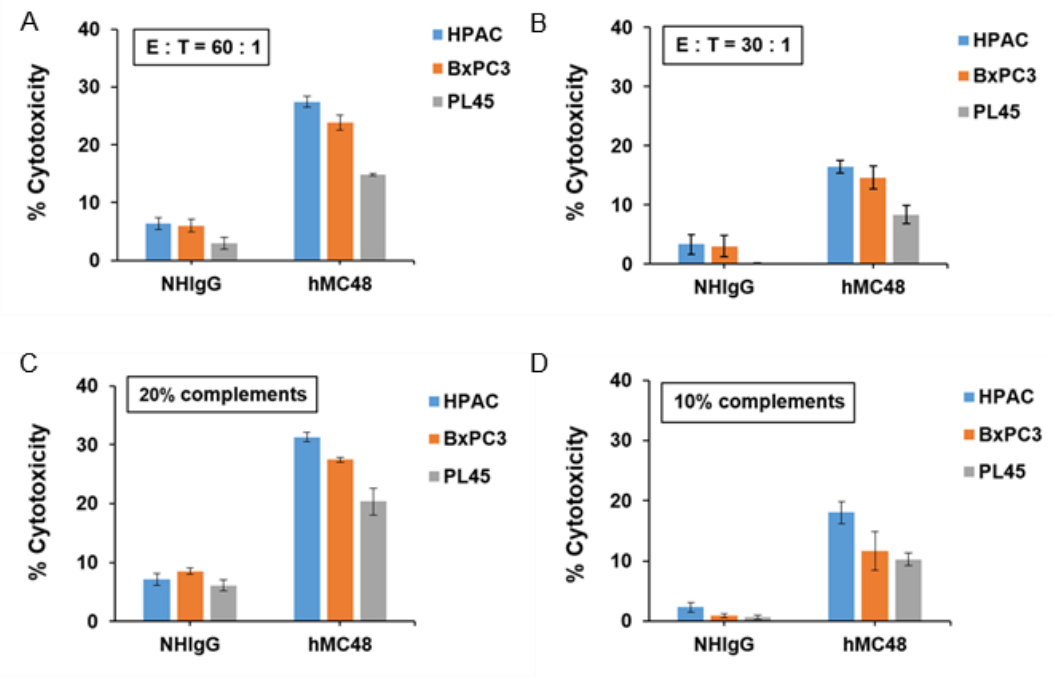


Supplementary Fig. 2. Expression of ST6GALNAC6 in cancer patients and its relationship to patient survival. A, , Kaplan-Meier plots of overall survival for patients with pancreatic cancer in relation to the expression levels of *B3GALT5*, *FUT1+FUT2*. **B**, Schematic diagram of the biosynthesis of SSEA-3, SSEA-4, Globo-H, disialyl Gb5 (DSGb5), and sialyl Le^a through B3GALT5 and ST3GAL2. The enzyme ST6GALNAC6 catalyzes the transfer of a sialic acid to the GalNAc residue in SSEA-4 to form DSGb5. **C**, The ST6GALNAC6 gene expression profile across all tumor samples and paired normal tissues. (Bar plot). **D**, Kaplan-Meier plots of overall survival for patients with pancreatic cancer in relation to the expression levels of B3GALT5+ST3GAL2+ ST6GALNAC6, ST6GALNAC6 only or B3GALT5+ST6GALNAC6. ST6GALNAC6 expression is found in normal cells and cancer cells associated with SSEA-4 biosynthesis, and is downregulated in cancer to reduce the reaction product DSGb5 and result in the accumulation of SSEA-4 and reduction of patients' survival.

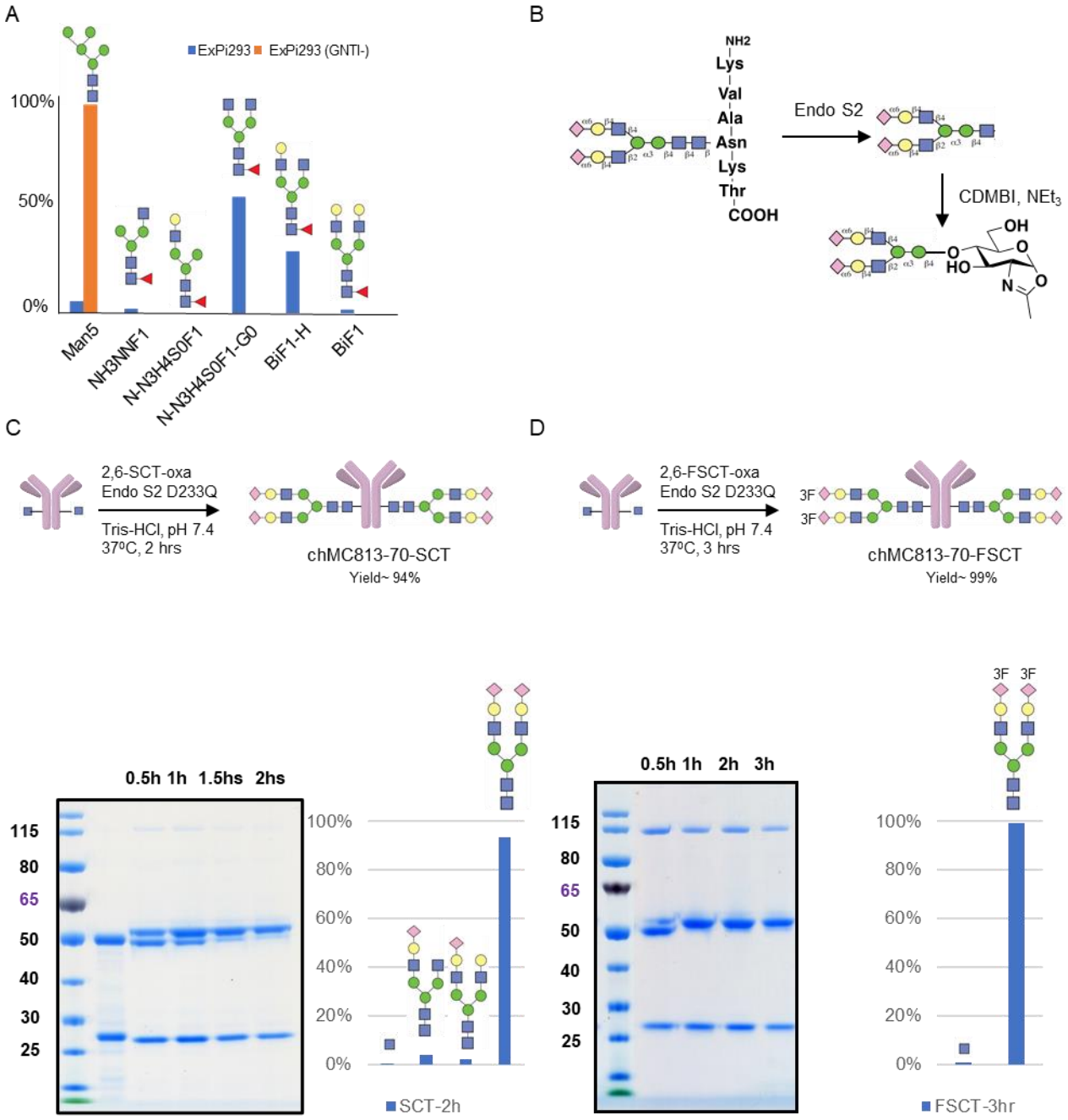

A
MC-48
MC-813-70
Fluorescence intensity
B
MC48
MC-813-70
Antibody Kd (nM)
MC-48 0.46 ± 0.1
MC-813-70 1.65 ± 0.52
Ab nM
Galactose
Glucose
Fucose
N-Acetylgalactosamine
N-Acetylneuraminic acid
C
12: SSEA-4
49: SSEA-4-Gc
MC48
MC813-70
D
PL-45
BxPC3
PDAC
KP4
Normalized To Mode
Count
Fluorescence intensity (FITC)

Supplementary Fig. 3. Characterization of SSEA-4 antibodies. A, The binding profiles of mAbs MC48 and MC-813-70 to SSEA-4 and its analogs on glycan array. **B**, The dissociation constant (Kd) of mAbs toward SSEA-4 was determined with a glass slide printed with SSEA-4 glycan. **C**, The binding profiles of mAbs MC48 and MC-813-70 to SSEA-4 and its N-glycolyl analog on the total glycan array (152-glycan). The glycan microarrays on glass slides were incubated with mAbs MC48 (10 μg/mL), then probed with a secondary antibody conjugated with Alexa Flour 647 and read with an array scanner at 635 nm. The result showed that mAb MC48 is capable of binding specifically to Neu5Acα2,3Galβ1,3GalNAcβ1,3Galα1,4Galβ1,4Glcβ1 (#12 on the glycan array), and Neu5Gcα2,3Galβ1,3GalNAcβ1,3Galα1,4Galβ1,4Glcβ1 (#49 on the glycan array). Data are presented as mean ± SD. C5, C5H10NH2. **D**, Validation of MC48 binding to pancreatic cancer cells. Pancreatic cancer cell lines PL-45, BxPC3, HPAC, and KP4, were stained with mAb MC48 (blue) or an isotype control mAb (red) plus IgG-specific secondary antibody.

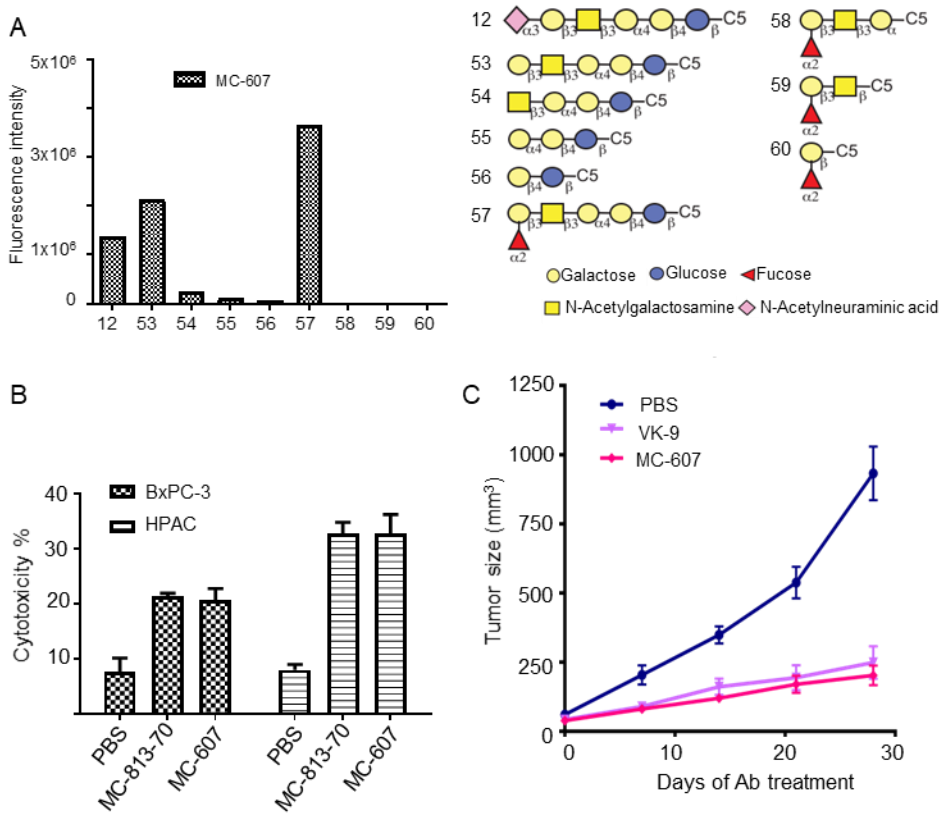
Supplementary Fig. 4. Chemical structures of 152 glycans arrayed on glass slides. The graphical notation of glycan structures in this figure is based on the symbols proposed by the Consortium for Functional Glycomics. Enantiomeric ratios are indicated for the glycans containing enantiomers. C5, C5H10NH2; C6, C6H.



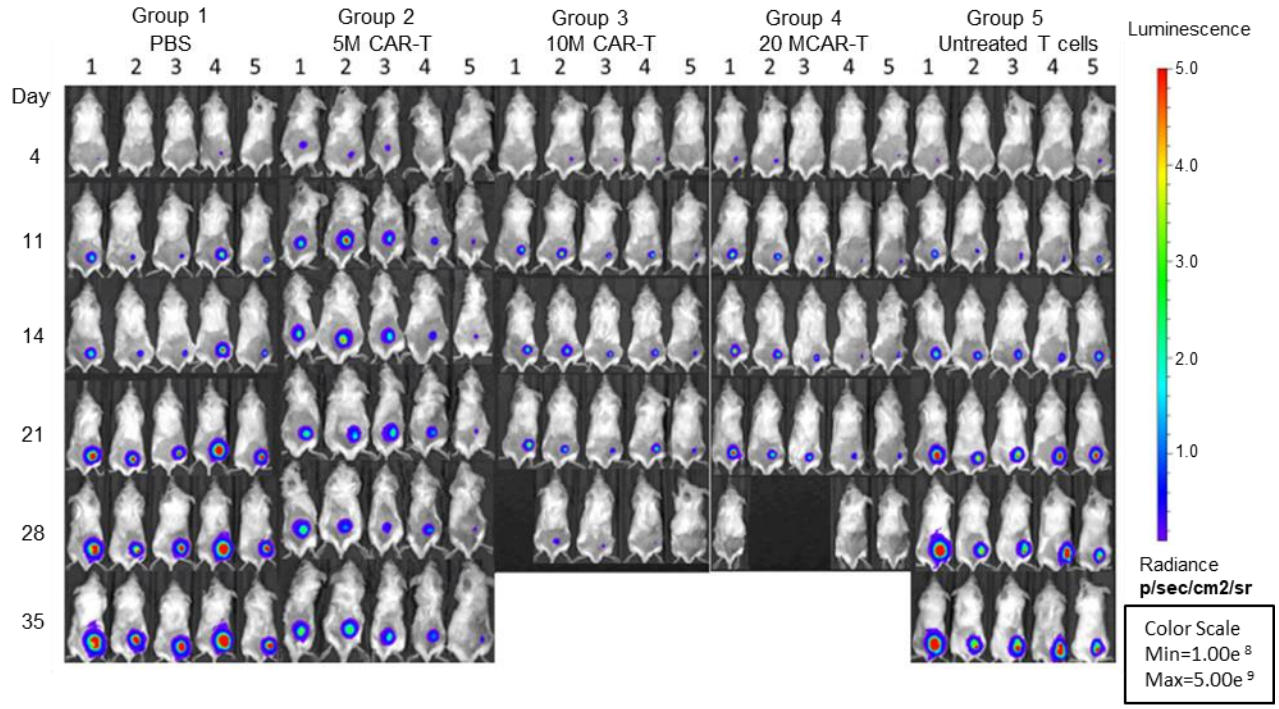
Supplementary Fig. 5. ADCC and CDC assays in pancreatic cancer cell lines with anti-SSEA-4 antibodies. Humanized hMC48 triggered ADCC (**A** and **B**) or CDC (**C** and **D**) in pancreatic cancer cell lines, HPAC, BxPC3 and PL45, at the concentration of 10 μg/mL. Normal human IgG (NHIgG) was used as a negative control. The ratio of effector: target (E:T) cell was 60:1 or 30:1. The data are shown as mean ± SD.



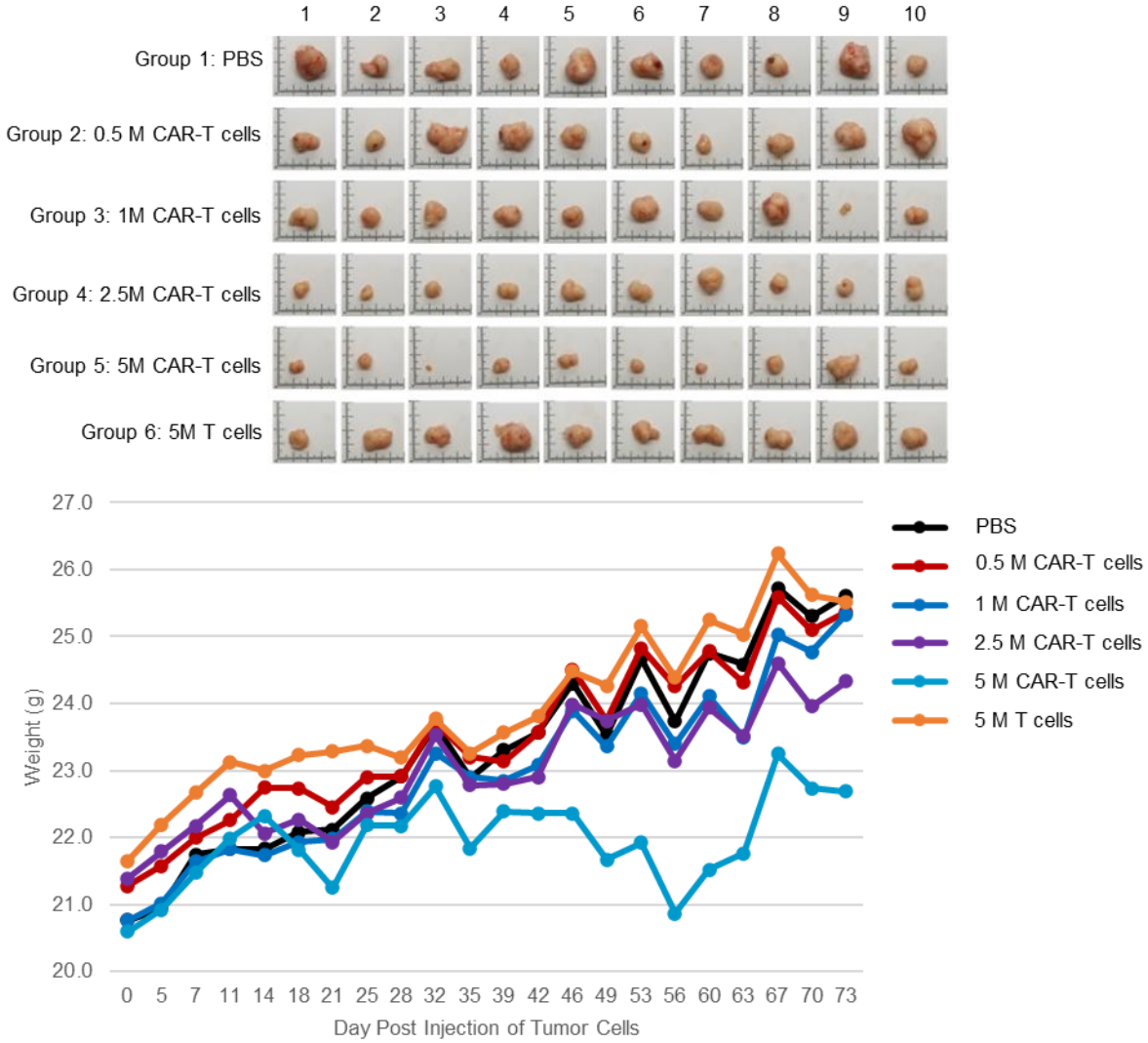
Supplementary Fig. 6. Characterization of homogeneous antibodies chMC813-70-SCT and chMC813-70-FSCT. A, The use of nanospray LC-MS to confirm the glycosylation pattern of chMC813-70 expressed from Expi293F™ Cells as multiple glycoforms and from Expi293F™ GnTI$^-$ Cells as high mannose glycoforms. **B**. Preparation of α2,6-SCT-oxazoline. **C**, SDS-PAGE and nanospray LC-MS of chMC813-70-SCT and **D**. chMC813-70-FSCT.



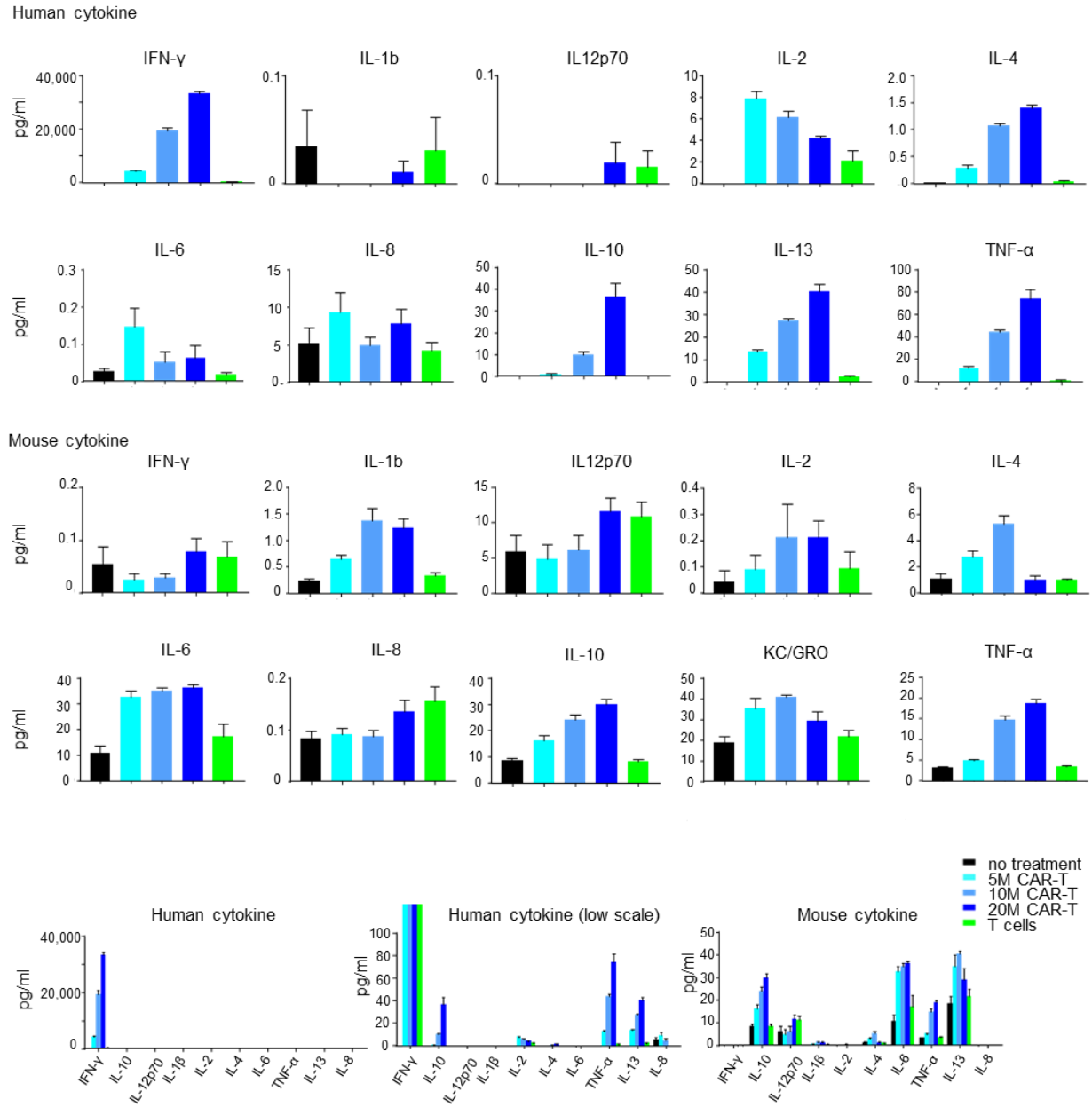
Supplementary Fig. 7. Characterization of antibody MC607 and its *in vitro* and *in vivo* efficacy on pancreatic cancer cells. A, The binding profiles of mAb MC607 to SSEA-4 and its analogs on glycan array. **B**, mAb MC607 mediates complement-dependent cytotoxicity against pancreatic cancer cell lines. Pancreatic cancer cell lines (BxPC-3 and HPAC) were treated with 20 μg/mL mAb MC607 and rabbit complement to observe Ab-induced cell lysis. The CDC activity was measured by the LDH release assay as described in Materials and Methods. Data are shown as mean ± SD. **C**, Growth inhibition of pancreatic cancer cell line BxPC-3 in nude mice treated with SSEA-4 (MC-607) or Globo H (VK-9) mAb. All mAbs were administered at a dose of 1.25mg/kg twice per week i.p. injection for one month. The tumor volume in each group was measured at different time points and shown as mean ± SD.



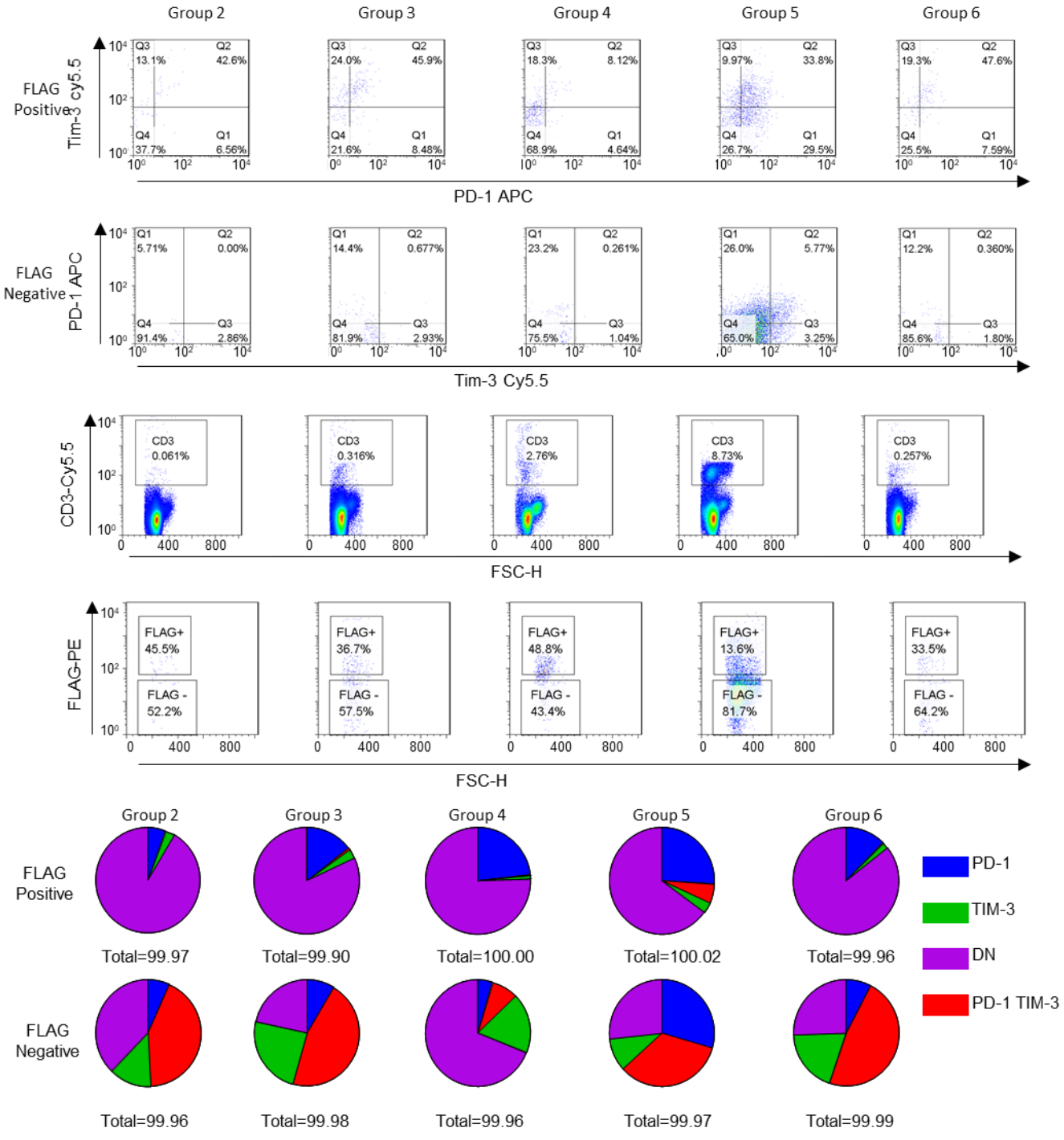
Supplementary Fig. 8. Efficacy and toxicity of anti-SSEA-4 CAR-T cells in HPAC tumor-bearing NSG mice. As described in Fig. 4, about 100-million freshly purified CD4+/CD8+ human T cells were transduced with lentivirus bearing PMC300 to generate about 80-million SSEA-4-recognizing CAR-T cells. The CAR-T cells, 5-million (2nd group), 10-million (3rd group), and 20-million (4th group), were i.v. injected into 5 NSG mice bearing luminescence tagged HPAC tumors on day 12, along with appropriate controls (PBS (1st group) and untransduced T cells (5th group)). Luminescence was imaged twice a week for all mice in different groups. On day 28, one mouse in the group receiving 10-million CAR-T cells and two mice in the group receiving 20-million CAR-T cells died probably due to cytokine storms. The remaining mice were removed for autopsy and pathological studies at the University of California, Davis.



Supplementary Fig. 9. Demonstration of efficacy without significant toxicity in HPAC tumor-bearing mice with lower numbers of anti-SSEA-4 CAR-T cells Sixty NSG mice were implanted s.c. with 2-million luminescently tagged HPAC cells. Mice were randomly divided into six groups with ten mice per group. Twice per week, all mice were photographed for luminescence. On day 11 when tumors were clearly formed in all mice, 0.5-million, 1-million, 2.5-million, and 5-million anti-SSEA-4 CAR-T cells were injected into four groups of NSG mice, along with one group with PBS and another group with 5-million untransduced human T cells as negative controls. The experiment ended on day 74 when no mouse died, nor loss of weight except the group receiving 5-million CAR-T cells. Enigmatically, upon the second injection of the same number of CAR-T cells on day 59, these mice significantly regained the body weight.



Supplementary Fig 10. Cytokine profiles from mouse plasma. Blood bled on day 24 from each and all mice in this experiment was analyzed for various cytokines from human and mouse. The most significant mouse cytokines detected include IL-10, IL12p70, IL-6, TNF-α, and KC/GR), whereas the most significant human cytokines detected consist of IFN-γ, IL-10, TNF-α, and IL-13. The elevation of IL-6 and IL-10 supports the notion of the presence of cytokine storms in dying mice. The presence of IFN-γ, TNF-α, and IL-2 may be consistent with the anti-tumor activity of anti-SSEA-4 CAR-T cells. The concentration of human IFN-γ, IL-10, TNF-α, and IL-13 in mouse plasma correlates with the number of anti-SSEA-4 CAR-T cells injected.



Supplementary Fig. 11. Analyses of cells from the terminal bleed on day 74. The presence of FLAG, CD3, PD-1 and TIM-3 expression in cells from blood of terminal bleed (PD-1 and TIM-3 are exhaustion markers) was shown with the frequency of expression in each group. FLAG-

negative cells (except Group 4) overwhelmingly expressed PD-1 and TIM-3 (red) as compared to the FLAG-positive cells. Most FLAG-positive cells are negative for both PD-1 and TIM-1 (DN, double negative). Consistent with the findings on the tumor status in the mice, Groups 2, 3, and 6 also exhibited the greatest number of double-positive (red) markers for exhaustion.

Supplementary Table 1. A, Expression profiles of globo-series GSLs in other cancer cell lines. Expression of globo-series GSLs was determined by flow cytometry. Cell lines with less than 5% of total cells stained positively were labelled as " –", with 5-20% as "+", with more than 20% as "++". **B,** Expression percentage of globo-series GSLs in fourteen pancreatic cancer cell lines.

A

Antigen	Pancreatic cancer cell lines
SSEA-3+	4/14
SSEA-4+	14/14
Globo H+	7/14
SSEA-3+ and SSEA-4+	4/14
SSEA-3+ and Globo H+	2/14
SSEA-4+ and Globo H+	7/14
SSEA-3+, SSEA-4+, and Globo H+	4/14

B

Cell type	FACS analysis		
	SSEA-4	SSEA-3	Globo H
Breast cancer cell lines			
MCF7	++	++	++
SK-BR3	++	++	++
MDA-MB-157	++	++	++
MDA-MB-231	++	+	-
MDA-MB-361	++	-	-
T47D	+	++	++
BT474	-	-	-
Lung cancer cell lines			
CL-1	+	-	-
H1299	-	-	-
PC-13	-	-	-
PC-9	++	-	+
A549	-	-	-
Brain cancer cell lines			
U373MG	+	++	-
LN18	+	++	-