Supporting Information

Modulation of Siglec-7 Signaling via i*n situ* Newly Created *cis*-Ligands

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Contents:

Supplementary Figures

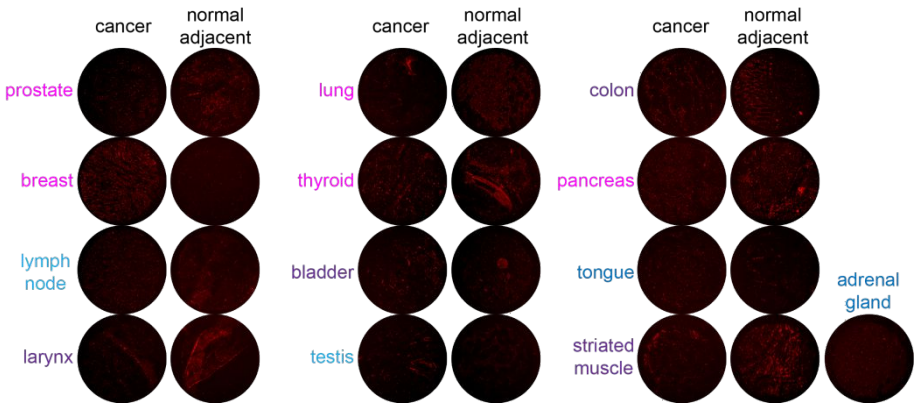

Figure S1. Profiling the expression of Siglec-7 ligands on tumor tissues and their related adjacent normal tissues using recombinant Siglec-7-Fc (red).

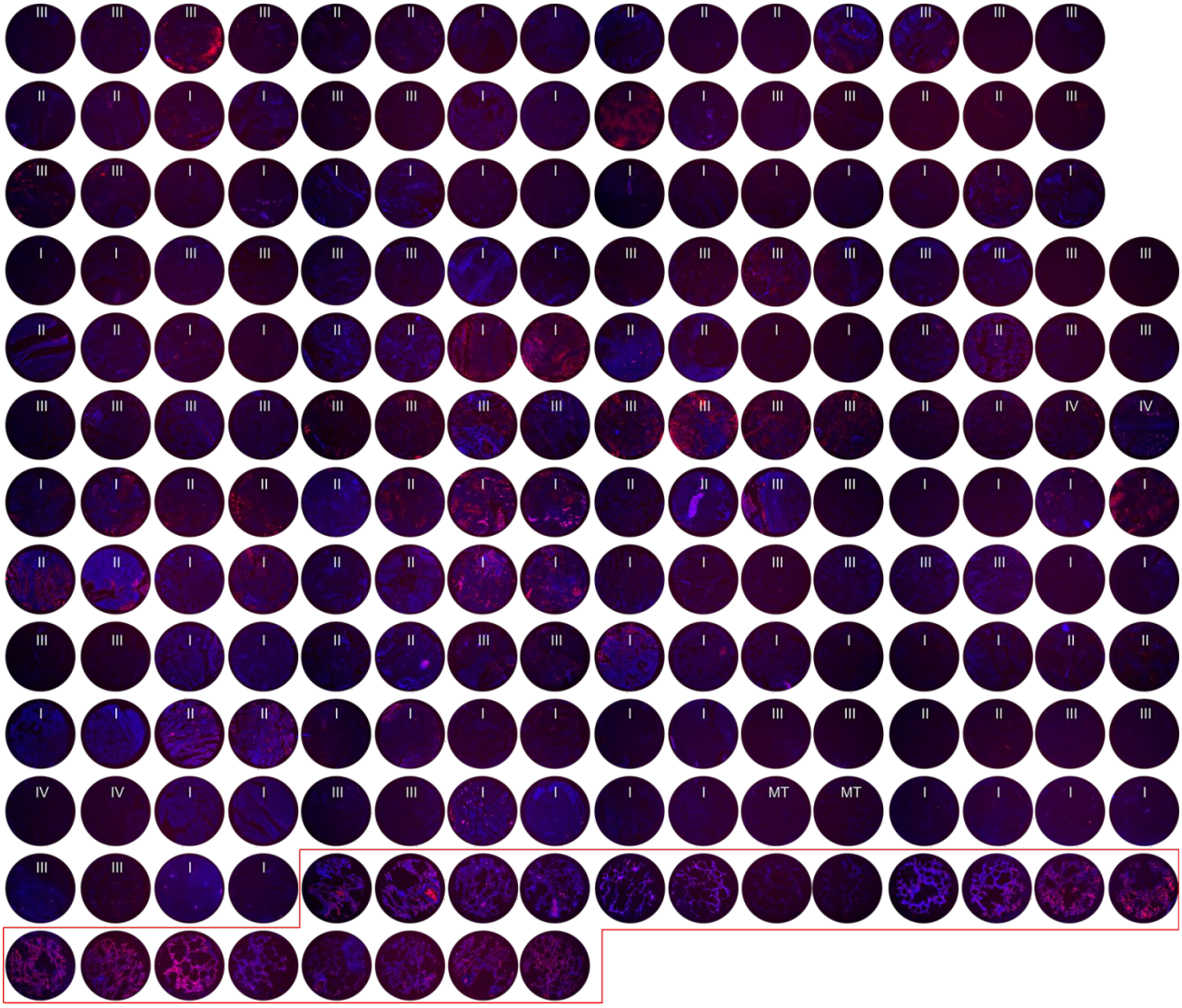

Figure S2. Siglec-7-Fc-based detection of Siglec-7 ligands on lung samples of cancer and healthy tissue (shown in the red box). The cells were stained with DAPI (blue) and Siglec-7-Fc (red). The stage I malignant (n=75), stage II

malignant (n=38), stage III malignant (n=58), stage IV malignant (n=4) were indicated according to the resources from the supplier (US Biomax, white number). Note: MT, presents the malignant tumor.

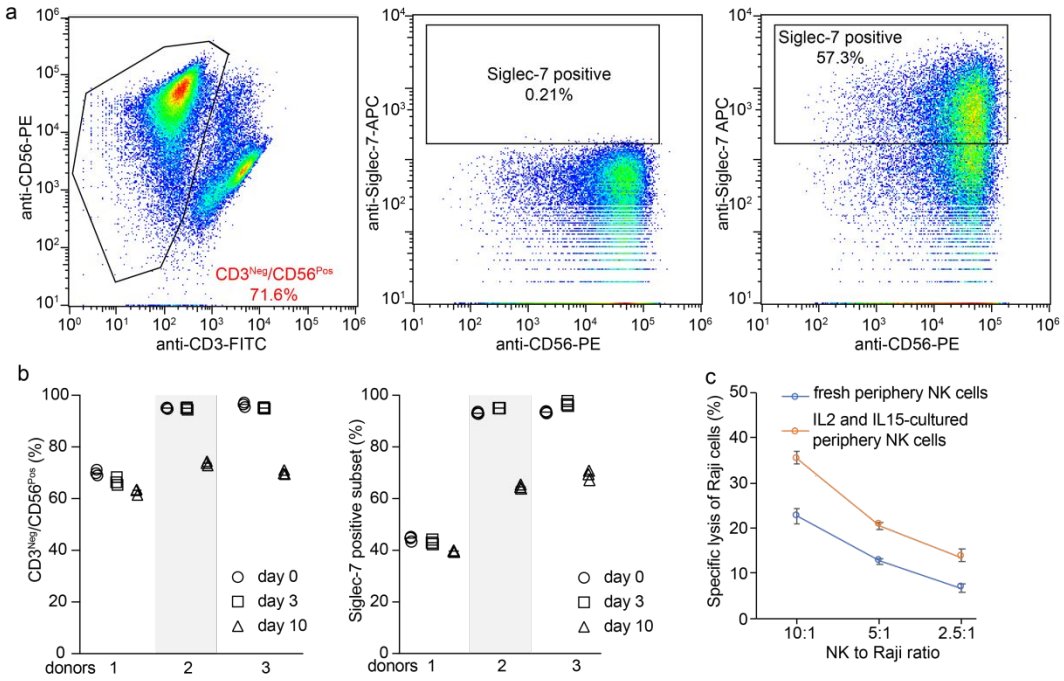

Figure S3.The isolated human peripheral NK cells were cultured for 10 days in the medium supplemented with IL-2 and IL-15. (a) The NK cells were identified by anti-CD3 antibody staining (negative) and anti-CD56 antibody staining (positive), and the Siglec-7 expression was probed by anti-Siglec-7-APC (clone 6-434) antibody staining. (b) The $CD3^{Neg}/CD56^{Pos}$ and Siglec-7 positive subsets of peripheral NK cells isolated from different donors (n=5) were monitored by flow cytometry on day 0, 3 and 10. (c) The LDH release assay for quantifying the cytotoxicity of the peripheral NK cells against Raji cells. The number of peripheral NK cells was determined via $CD3^{Neg}/CD56^{Pos}$ population via flow cytometry before incubation with Raji cells at different NK to Raji ratios. Error bars present the standard deviation of three biological repeats.

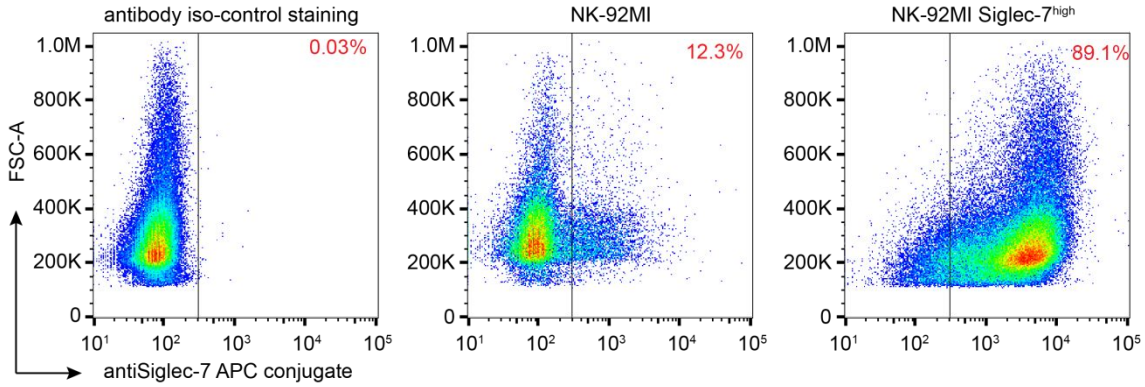

Figure S4. The Siglec-7 expression in NK-92MI cells or the long-term cultured (˜120 days) NK-92MI-$S7^{high}$ cells were stained with anti-Siglec-7-APC (clone 6-434) antibody and analyzed with flow cytometry. The red number shown in the figure indicates the ratio of the Siglec-7 positive population.

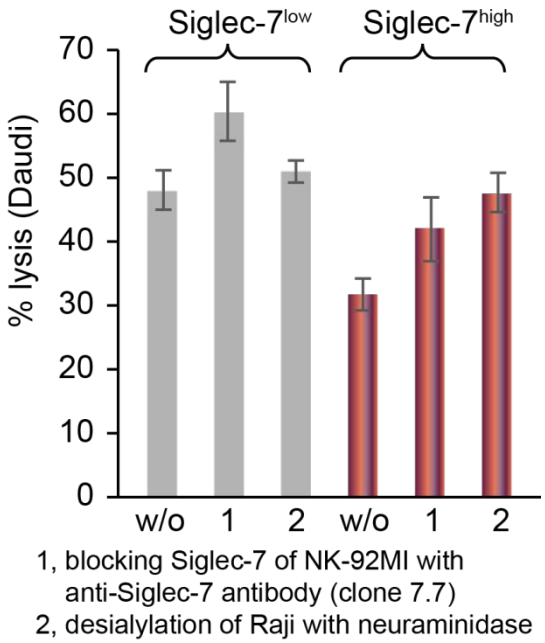

Figure S5. LDH release assay for quantifying the cytotoxicity of NK-92MI (Siglec-7^{low}) or NK-92MI-S7^{high} cells against Daudi cells, a B-lymphoma cell line with a moderate level of Siglec-7 ligands on the cell surface. The neuraminidase-assisted desialylation, ST6Gal1-mediated glycan editing, and anti-Siglec-7 antibody (clone s7.7) blocking were performed before the co-incubation of NK-92MI and Daudi cells at an effector to target ratio of 5:1. Error bars present the standard deviation of three biological repeats.

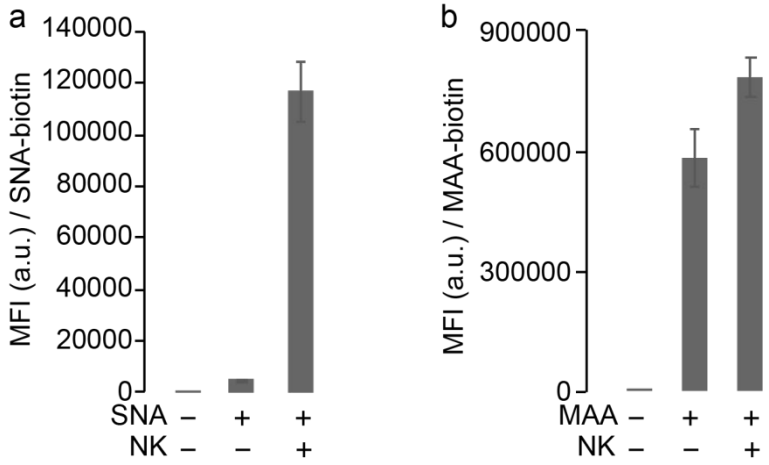

Figure S6. Co-incubation of Raji cells with peripheral NK cells significantly up-regulated the level of Raji cell-surface sialylation. Raji cells were washed and co-incubated with or without NK cells for 2 hrs, the sialic acids on live Raji cells was probed and quantified to reveal the increase of Raji cell-surface sialylated glycans. The sialosides on Raji cells were probed by SNA (a) and MAA (b) lectin staining. Error bars present the standard deviation of three biological repeats.

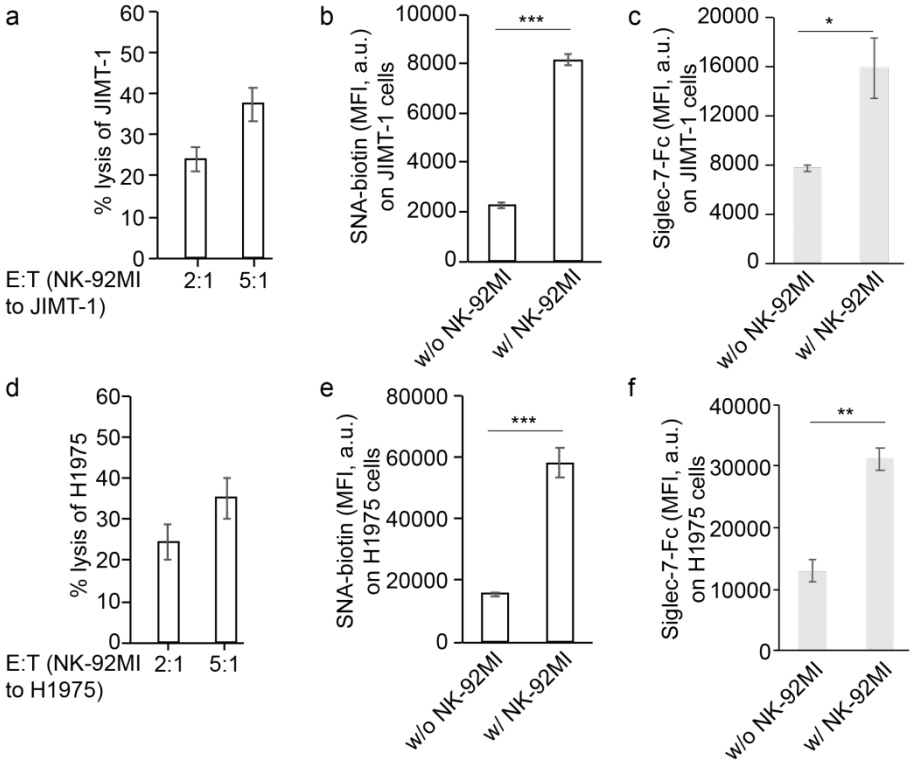

Figure S7. Co-incubation with NK-92MI cells significantly up-regulated the level of cell-surface Siglec-7 ligands on JIMT-1-eGFP-Luc cells (a-b) and H1975-eGFP-Luc cells (d-f). NK-92MI-induced specific lysis of JIMT-1 (a) and H1975 (d) was quantified via an LDH cytotoxicity assay. After co-incubation of NK-92MI cells with JIMT-1-eGFP-Luc (b and c) and H1975-eGFP-Luc cells (e and f) at an E/T ratio of 5:1 for 1.5 h, the sialosides on target cells were stained with SNA-biotin, following by Streptavidin AF647 staining, and analyzed by flow cytometry. The Siglec-7 ligands on JIMT-1-eGFP-Luc (c) and H1975-eGFP-Luc cells (f) were probed by the premixture of Siglec-7-Fc and anti-human Fc APC conjugated antibody. NK-92MI cells in the co-culture system were probed with an anti-CD56 antibody and target cells were marked by the endogenous GFP fluorescence. Error bars present the standard deviation of three repeats. The significance was analyzed with the two-sided Student's t-test. Note, *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

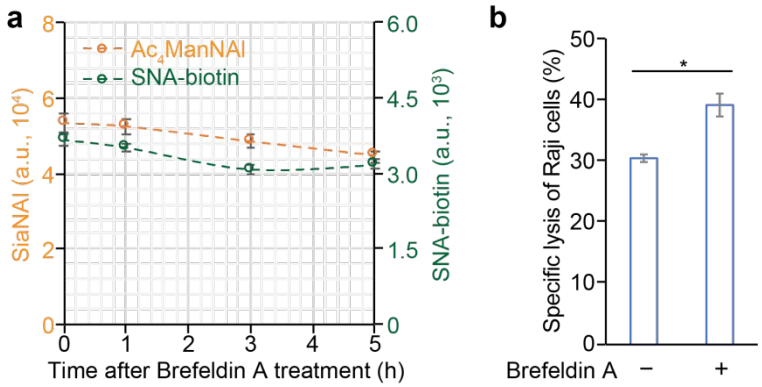

Figure S8. Pretreating Raji cells with Brefeldin A blocked temporally the transportation of *de novo* sialosides onto the plasma membrane and enhanced NK-based killing of target cells. (a) Monitoring Raji cell-surface sialylation with chemical reporter strategy ($Ac_4ManNAl$ labeling) and lectin (SNA-biotin) staining. The Raji cells were maintained in the medium containing 10 μg/mL Brefeldin A for 2hrs. After the pulse of Brefeidin A-treatment, the cells were chased with fresh medium and cell-surface sialylation was quantified via flow cytometry. (b) Specific lysis of Raji cells pretreated with Brefeldin A or not by NK-92MI-S7^{high} cells at an E/T ratio of 5:1 by LDH assay. The significance was analyzed with the two-sided Student's t-test. Note, *, $p<0.05$.

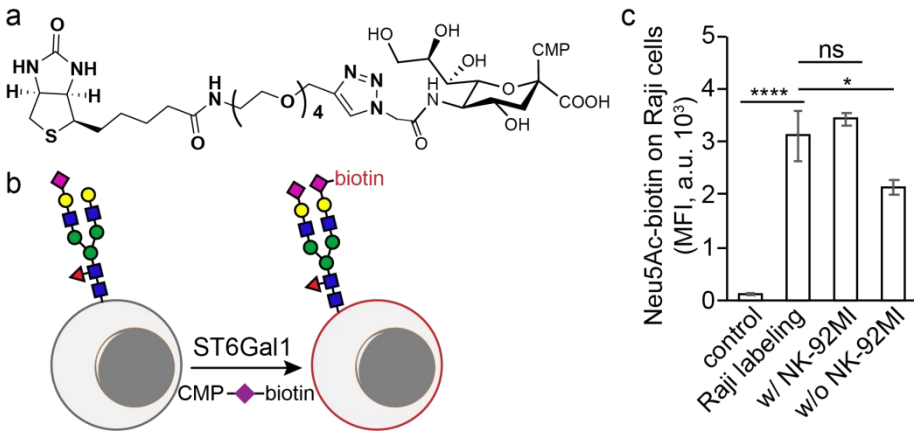

Figure S9. Assessing the changes of cell-surface glycans on Raji cells upon co-incubation with NK-92MI. Raji cells were modified with ST6Gal1 and biotin-Neu5Ac, washed and co-incubated with NK-92MI. After 2 hrs, the biotin on live Raji cells was probed and quantified to reveal the endocytosis of Raji cell-surface glycans. (a) Chemical structure of biotinylated CMP-Neu5Ac. (b) Scheme of the ST6Gal1-assisted in situ adding of biotin-Neu5Ac onto live Raji cells. The N-linked glycans on Raji cells were pre-labeled via ST6Gal1-assisted (40 μg/mL) chemoenzymatic labeling with biotinylated CMP-Neu5Ac (50 μM) as substrate. After co-incubation with NK-92MI cells for 2-hrs or not, cell-surface biotin was directly probed with streptavidin-AF488. (c) The cell-surface biotin signal was quantified with flow cytometry. Raji cells were probed with anti-CD19 antibody APC conjugate, and NK-92MI cells were differentiated with anti-CD56 antibody PE conjugate. The significance was analyzed with the two-sided Student's t-test. Note, ns, not significant; *, $p<0.05$; ****, $p<0.001$.

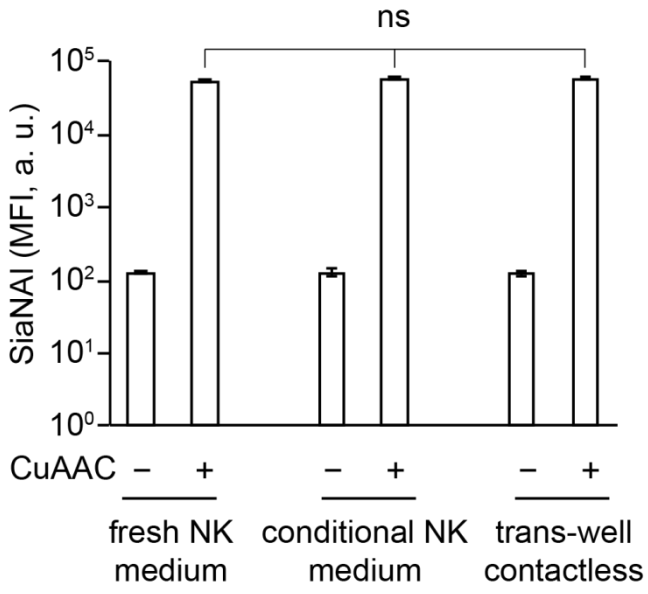

Figure S10.Trans-well-based contactless co-culture of NK-92MI cells with Raji cells did not trigger the increase of Raji cell-surface sialosides. Raji cells were cultured with $Ac_4ManNAl$ for 2 days, washed and further cultured with fresh NK-92MI complete medium, or conditioned medium of NK-92MI, or co-incubation with NK-92MI cells separated via trans-well insertions. After 1.5h, Raji cells were collected for CuAAC-labeling of the metabolically incorporated SiaNAl. Error bars present the standard deviation of three repeats. The significance was analyzed with the two-sided Student's t-test. Note, ns, not significant.

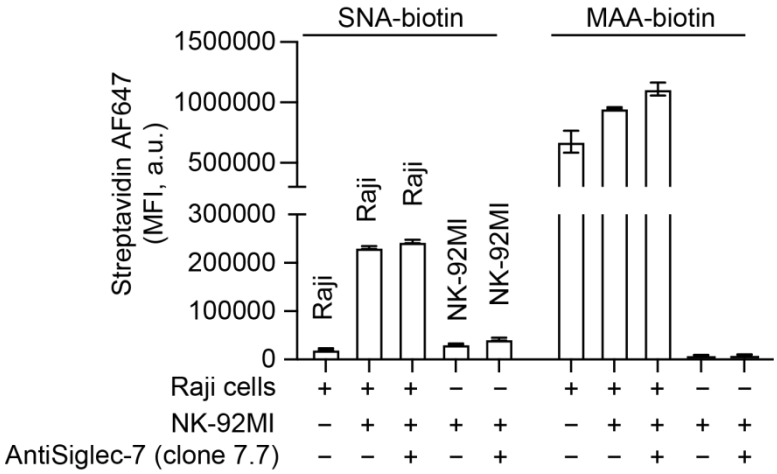

Figure S11. The cell-surface sialylation on Raji cells was increased after co-culturing with NK-92MI-S7^{high} cells as revealed by lectin staining. NK-92MI and Raji cells cultured alone were stained as controls. NK-92MI-S7^{high} cells in the co-culture system were probed with anti-CD56 antibody and Raji cells were probed by the anti-CD19 antibody. Error bars present the standard deviation of three repeats.

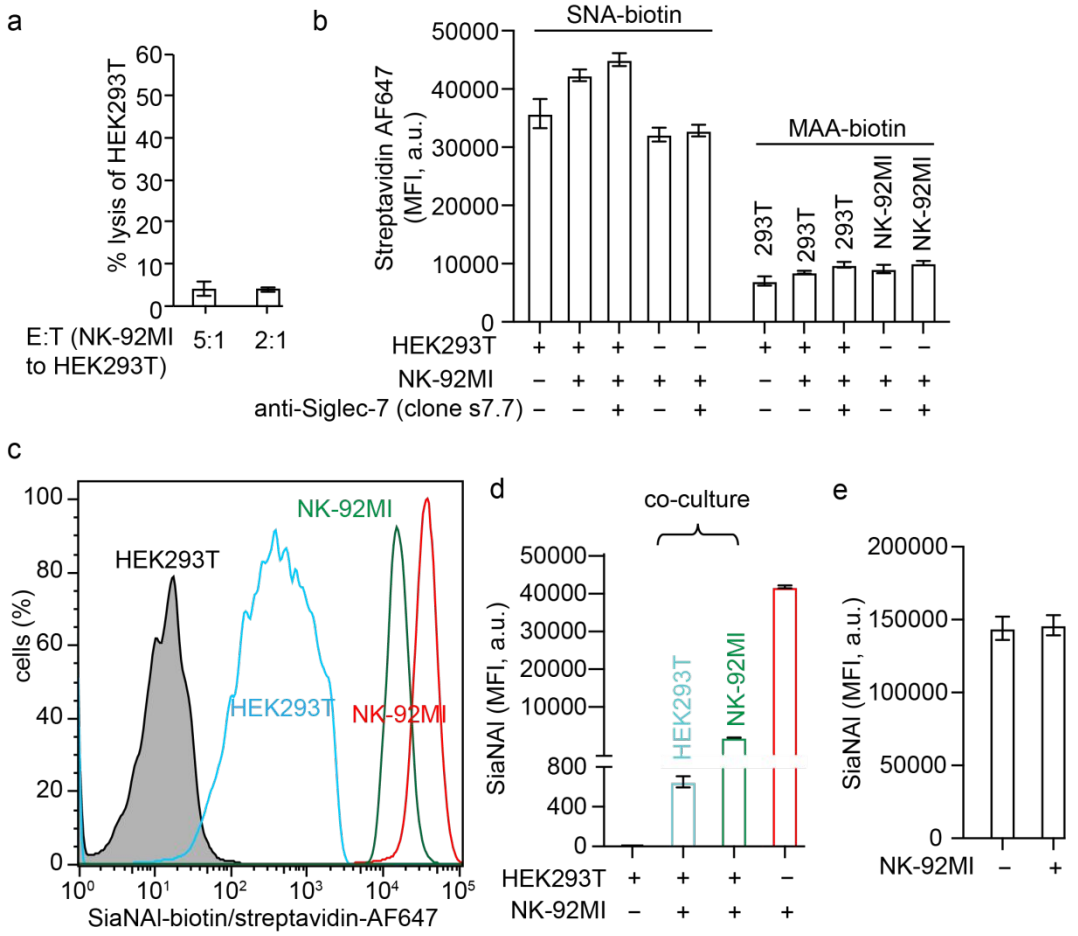

Figure S12. The dynamics of sialylation on HEK293T cells after encountering NK-92MI-S7^{high} cells. (a) LDH release assay for quantifying NK-92MI-S7^{high} cytotoxicity against HEK293T cells. (b) Probing sialylation on HEK293T cells with SNA or MAA lectin. The NK-92MI-S7^{high} cells (CD56 Positive) in co-culture with HEK293T cells (CD56 Negative) were probed with the anti-CD56 antibody. (c and d) Probing the transfer of NK-92MI-S7^{high} cell-surface sialosides to HEK293T cells via Ac_4ManNAl-based metabolic glycan labeling. NK-92MI-S7^{high} cell-surface sialosides were labeled by metabolic glycan labeling after pretreating with Ac_4ManNAl for 2 days. Then, HEK293T cells were coincubated with NK-92MI-S7^{high} cells for 1 h. The resultant SiaNAl-labeled glycoconjugates on HEK293T cells and NK-92MI-S7^{high} cells were further labeled with biotin in DPBS buffer (pH 7.4) containing 2% FBS, 50 μM azide-PEG4-biotin, 300 μM BTTPS and 50 μM Cu^{2+} premixture, and 2.5 mM freshly prepared sodium ascorbate for 5 mins. Finally, cell-surface biotin was further probed by 2 μg/mL streptavidin-Alexa Fluor 647 conjugate and quantified by flow cytometry. NK-92MI-S7^{high} and HEK293T cells without co-culture were stained as controls. (e) Probe sialylation biosynthesis in HEK293T cells via Ac_4ManNAl metabolic labeling. Error bars present the standard deviation of three repeats.

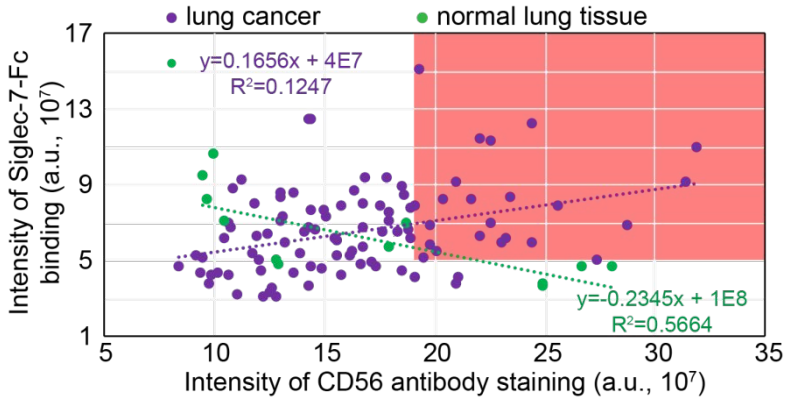

Figure S13. Immunofluorescence analysis of NK marker CD56 and Siglec-7 ligand expression in human lung cancer (n=96) and normal lung (n=12) tissues. Results were obtained using human tissue microarray LC1201a (USBiomax)

containing multiple types of lung carcinoma and probed with anti-CD56 and Siglec-7-Fc, quantification of immunofluorescence via ImageJ, and linear correlation analysis in Excel.

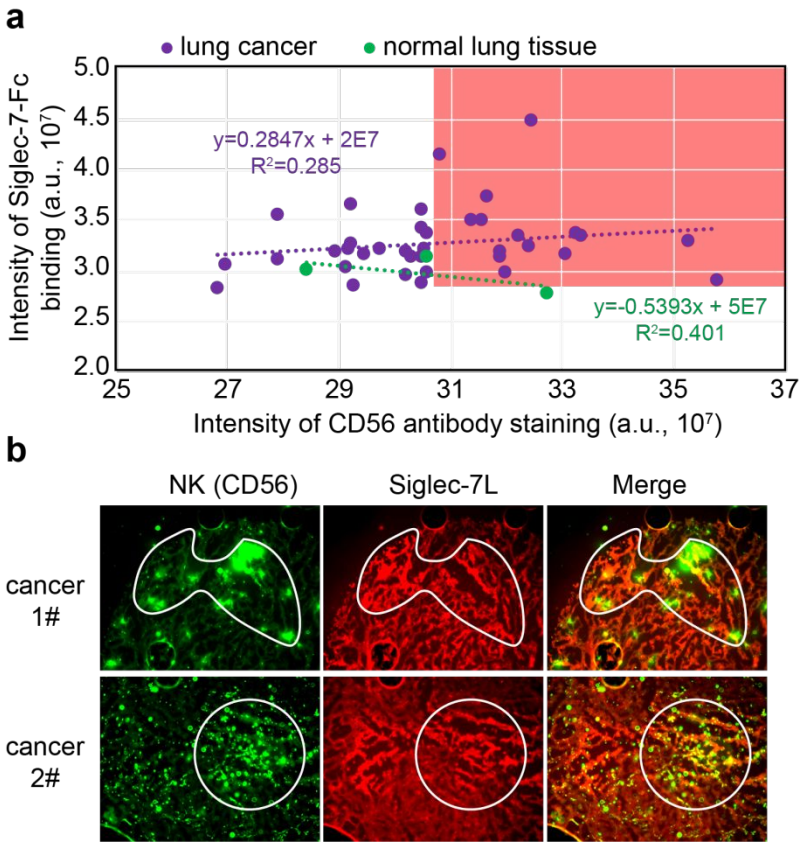

Figure S14. Immunofluorescence analysis of NK marker CD56 and Siglec-7 ligand expression in frozen human lung cancer (n=39) and normal lung (n=3) tissues. (a) Correlation between NK tissue infiltration into lung tumor tissue and the in situ expression of Siglec-7 ligands. Results were obtained using human tissue microarray FLU401C G035 (USBiomax) containing multiple types of lung carcinoma and probed with anti-CD56 and Siglec-7-Fc, quantification of immunofluorescence via ImageJ, and linear correlation analysis in Excel. (b) Representative images of tumor tissues.

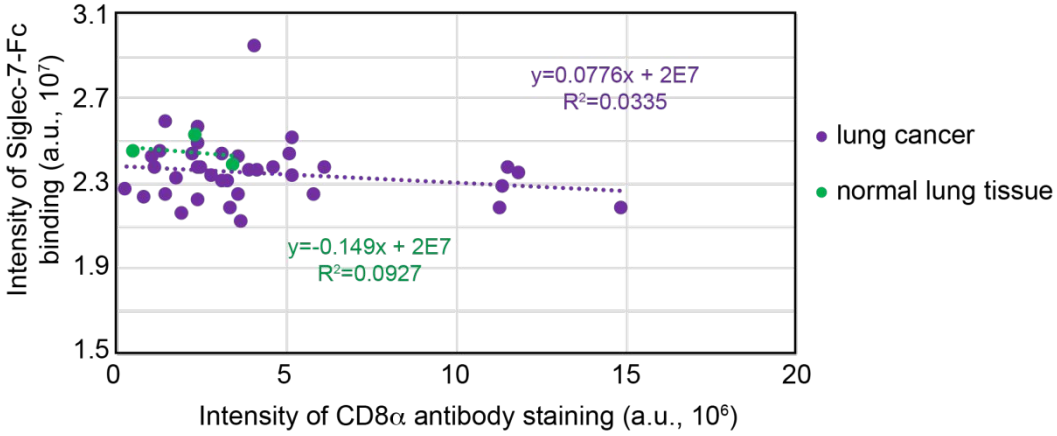

Figure S15. Immunofluorescence analysis of CD8α and Siglec-7 ligand expression in human lung cancer (n=39) and normal lung (n=3) tissues. Correlation between CD8α and the in situ expression of Siglec-7 ligands in both normal or tumoral lung tissues. Results were obtained using human tissue microarray FLU401C G036 (USBiomax, consecutive sections of G035 used in Figure s14) containing multiple types of lung carcinoma, and probed with anti-human CD8α antibody and Siglec-7-Fc. Quantification of immunofluorescence via ImageJ and linear correlation analysis in Excel.

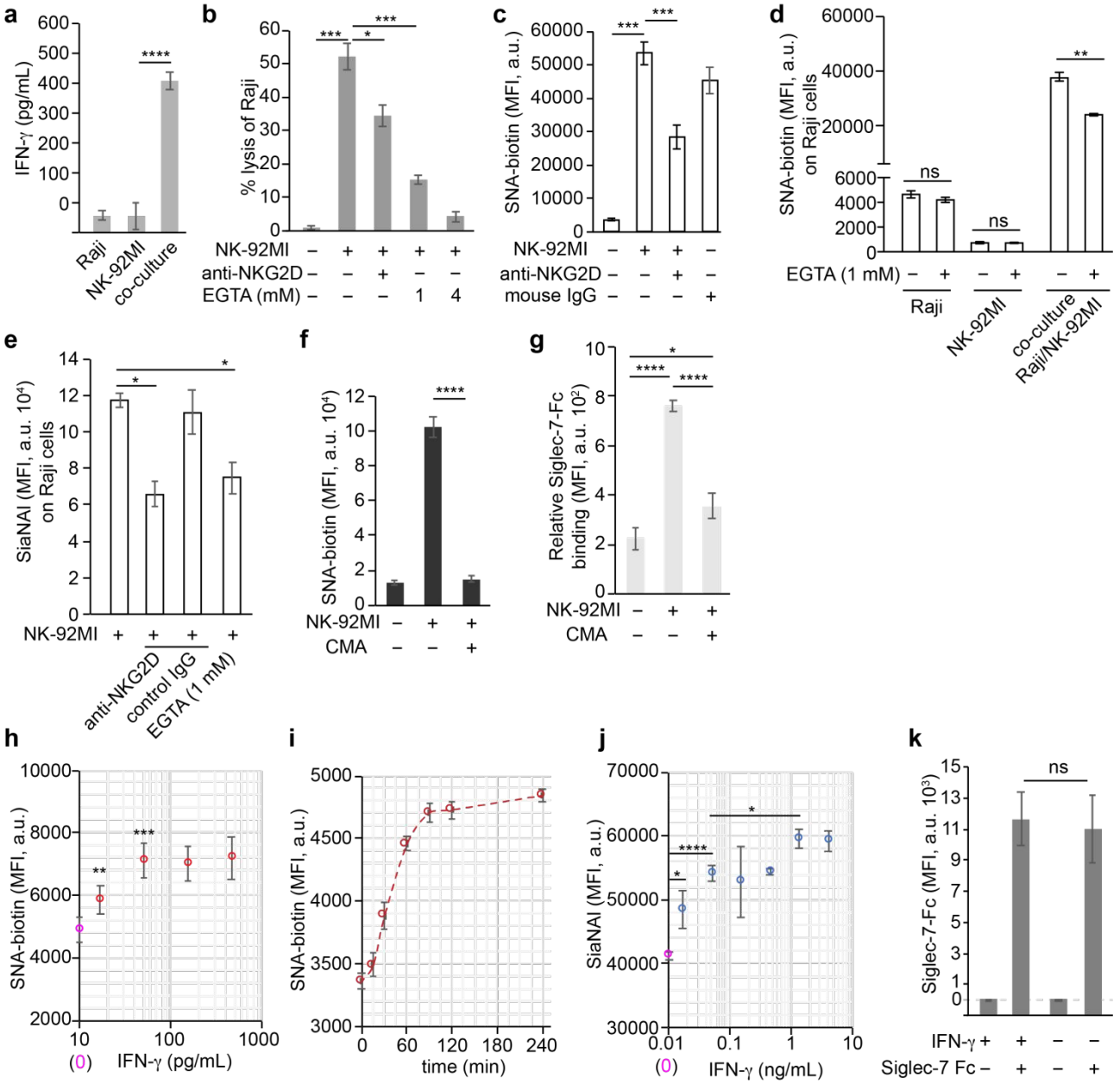

Figure S16. Upon NK-92MI encountering, Raji cell-surface sialylation level was up-regulated dominantly via the successful formation of killing synapse between NK-92MI and Raji cells. (a) Secretion of INF-γ during NK-92MI killing of Raji cells. (b) NK-92MI-induced specific lysis of Raji cells upon the presence of NKG2D blocking antibody (clone 1D11 and the mouse IgG was introduced as a control) or calcium chelator EGTA, was quantified via an LDH cytotoxicity assay. (c-e) Comparison of Raji cell-surface sialosides via SNA lectin staining (c and d) and Ac4ManNAl metabolic labeling (e) after 1.5h-incubation with NK-92MI cells that were pretreated with NKG2D blocking antibody or in the presence of EGTA. (f and g) Comparison of Raji cell-surface sialosides via SNA lectin staining (f) and Siglec-7-Fc staining (g) after 1.5h-incubation with NK-92MI cells that were pretreated with 50 ng/mL concanamycin A (CMA, the vacuolar-type H^+-ATPase inhibitors) or not for 3h. (h-j) IFN-γ dependent increase of Raji cell-surface sialylation was probed SNA lectin staining (h and i) or $Ac_4ManNAl$-based metabolic glycan labeling (j). (k) Siglec-7 ligands on Raji cells remain unchanged upon IFN-γ (50 ng/mL) treatment. The IFN-γ-supported medium was prepared by docking IFN-γ directly to the Raji culture medium at indicated concentrations (c and e) or 10 ng/mL (d). In the co-culture of Raji and NK-92MI cells, the Raji cells were probed with anti-CD19 antibody and NK-92MI cells were probed with an anti-CD56 antibody. The bars represent the standard error of three biological repeats of samples. The significance was analyzed with the two-sided Student's t-test. Note, ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$; ****, $p<0.001$.

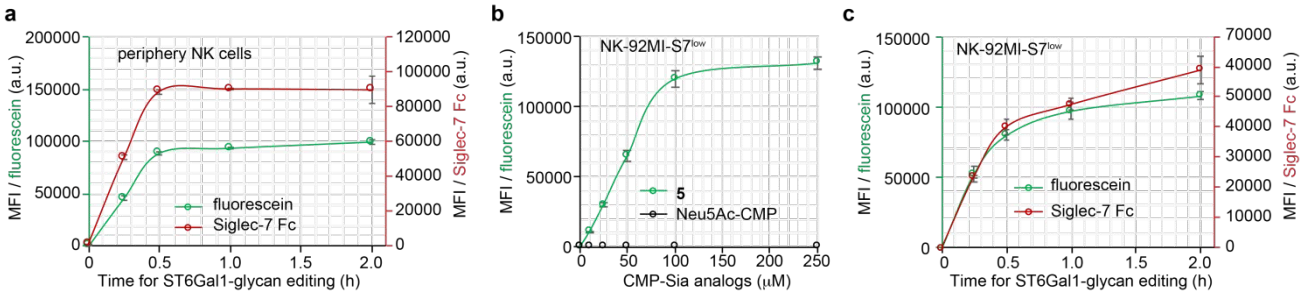

Figure S17. The dose and time-dependent incorporation of CMP-Sia analogs onto peripheral NK cells (a) and NK-92MI-S7^{low} cells (b and c) via ST6Gal1-glycan editing. For the time-course study (a and c), 200 μM ^{FTMC}Neu5Ac-CMP (**5**) and 40 μg/mL ST6Gal1 were used. The error bars present the standard deviation of three repeats.

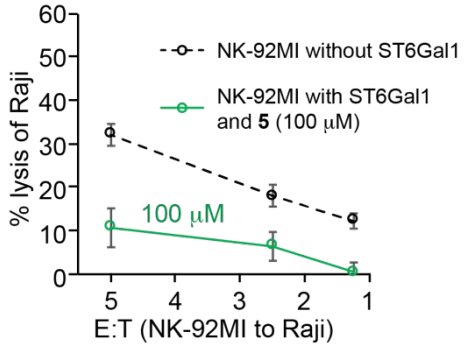

Figure S18. LDH release assay for quantifying cytotoxicity of NK-92MI-S7^{high} cells with ^{FTMC}Neu5Ac or not against Raji cells. Before co-incubation with Raji cells for 4 h, the NK-92MI-S7^{high} cells were pre-treated with ST6Gal1and ^{FTMC}Neu5Ac-CMP (**5**, 100 μM), or without ST6Gal1 as control. The error bars present the standard deviation of three repeats.

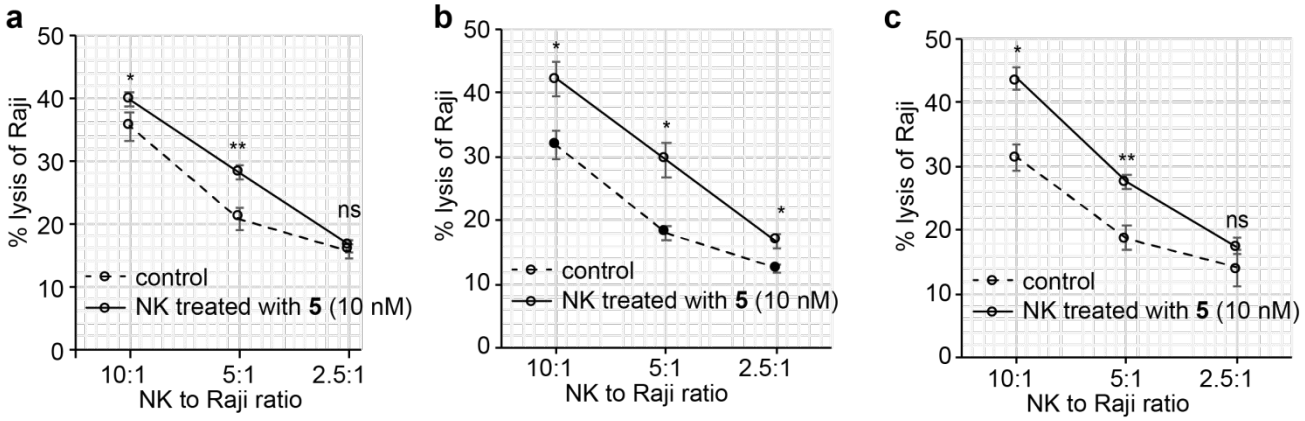

Figure S19. The specific lysis of Raji using the peripheral NK cells with or without ^{FTMC}Neu5Ac modification. Raji cells were incubated with peripheral NK cells from four different healthy donors at indicated E/T ratios for 4h. The specific lysis of Raji cells was measured via the LDH release assay. The bars represent the standard error of three biological repeats of samples. The significance was analyzed with the two-sided Student's t-test. Note, ns, not significant; *, $p<0.05$; **, $p<0.01$.

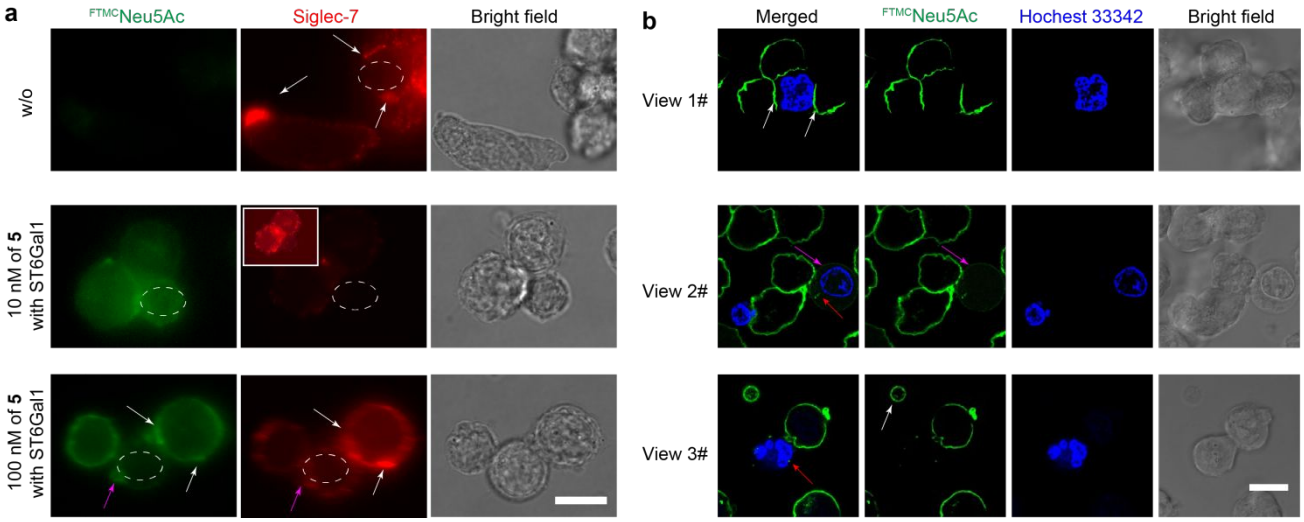

Figure S20. (a) Fluorescence microscopy images of Siglec-7 on NK-92MI-S7^{high} cells with ST6Gal1-mediated glycan editing. The white dash circles indicate the presence of target cells, the white arrows indicate the identified clusters of Siglec-7 and the purple arrows indicate the transfer of ligands (green) to target cells from NK cells. White rectangles present the brightened pictures of the same views. Scale bar: 20 μm. (b) Confocal images of ^{FIMC}Neu5Ac-modified NK-92MI-S7^{high} cells coincubation with Raji cells. NK-92MI-S7^{high} cells were treated with ST6Gal1 and CMP- ^{FIMC}Neu5Ac before coincubation with Hochest 33342 labeled Raji cells (blue) for 1 hr. Then the cells were gently moved to ConA pretreated bottom-glass dishes for confocal imaging. The white arrows indicate the cluster of Siglec-7 ligands (green) on E/T interfaces. The ligands that originally loaded on NK cells were observed to present on the plasma membrane of target Raji cells (purple arrows) and inside Raji cells (red arrows). Three presentative views were presented and the scale bar is 20 μm.

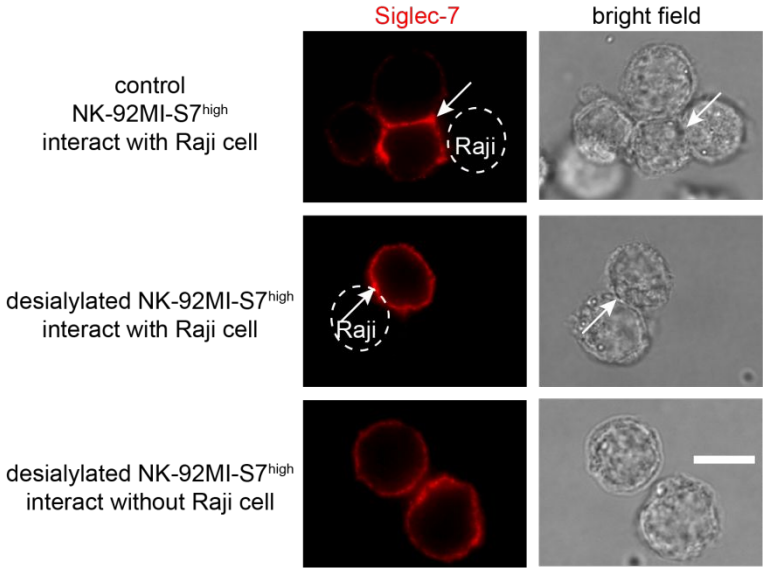

Figure S21. Fluorescent images of NK-92MI-S7^{high} cells with or without 'self' sialosides after neuraminidase treatment. NK-92MI-S7^{high} cells co-incubated with or without Raji cells were stained with anti-Siglec-7-PE antibody (clone 6-434, in red) and imaged by fluorescent microscopy. NK-92MI-S7^{high} cells desialylated by neuraminidase before coincubating with target Raji cells at an effector to target ratio of 5:1 for 1 h. The scale bar is 20 μm.

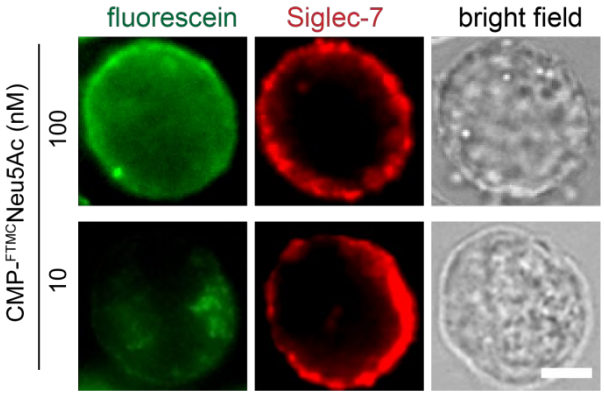

Figure S22. Representative images of NK-92MI-S7^{high} cells modified with ^{FTMC}Neu5Ac without interaction with other NK cells or in contact with Raji target cells in the co-culture system. The NK-92MI-S7^{high} cells were treated with ST6Gal1 and CMP- ^{FTMC}Neu5Ac, and coincubated with Raji cells at an E/T ratio of 5:1. The fluorescein groups on ^{FTMC}Neu5Ac were directly imaged (green), and Siglec-7 was probed with anti-Siglec-7-PE. The fluorescein and PE were imaged with a fluorescence microscope. Scale bar: 10 μm.

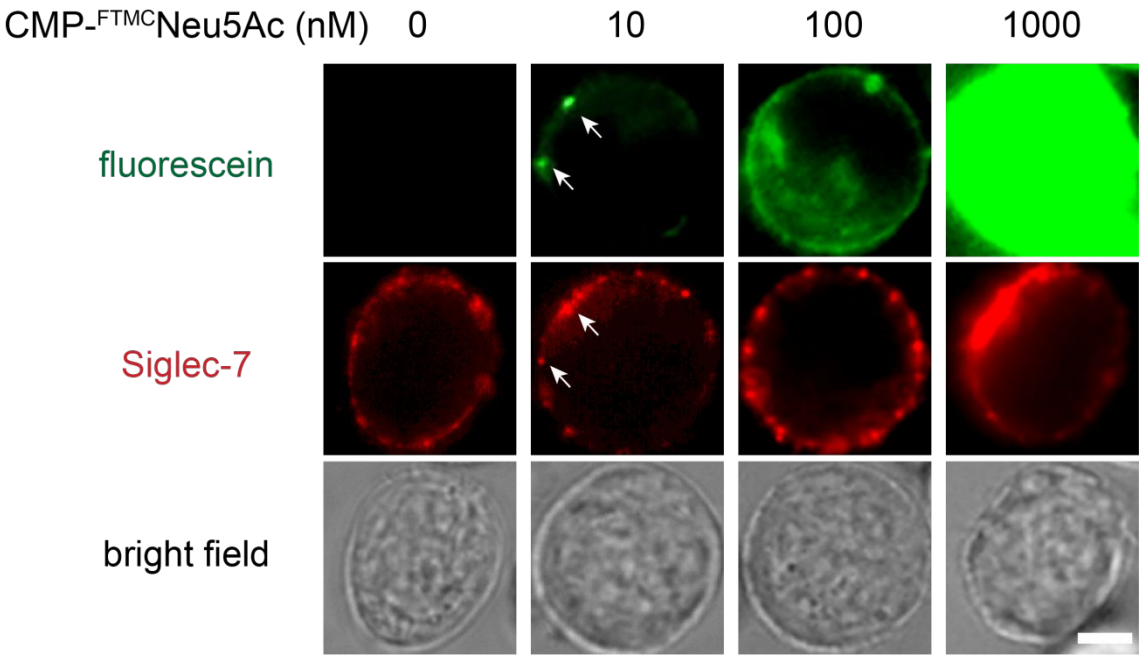

Figure S23. Immunoimaging Siglec-7 on NK-92MI-S7^{high} cells. The NK-92MI-S7^{high} cells were treated with ST6Gal1 and indicated concentrations of CMP- ^{FTMC}Neu5Ac for 30 min, before the NK cells were washed and stained with the anti-Siglec-7-PE antibody. The fluorescein and PE were imaged with a fluorescence microscope. Scale bar: 10 μm.

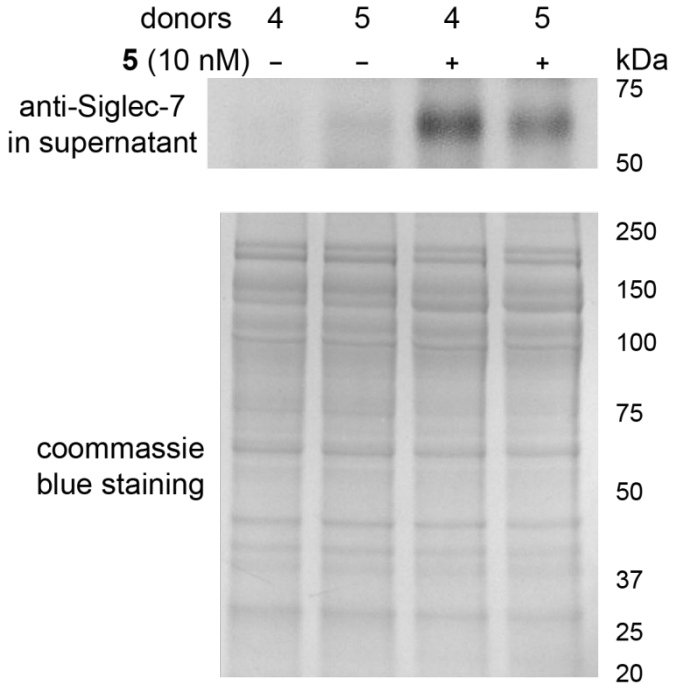

Figure S24. Western blot analysis of Siglec-7 released into the supernatant of the coculture of peripheral NK cells with Raji cells at an E/T ratio of 10:1. The sorted Siglec-7^{+} peripheral NK cells were expanded with IL-2 and IL-15 supported the NK culture medium. After incubation with ST6Gal1 and substrate **5** (10 nM) or not, The peripheral NK cells were cocultured with Raji cells for 2 hours, and the supernatant was collected and resolved with SDS-PAGE for anti-Siglec-7 Western blot analysis. The equal cell loading in each group was assessed by co-culture cell lysate via coommassie blue staining (lower panel).

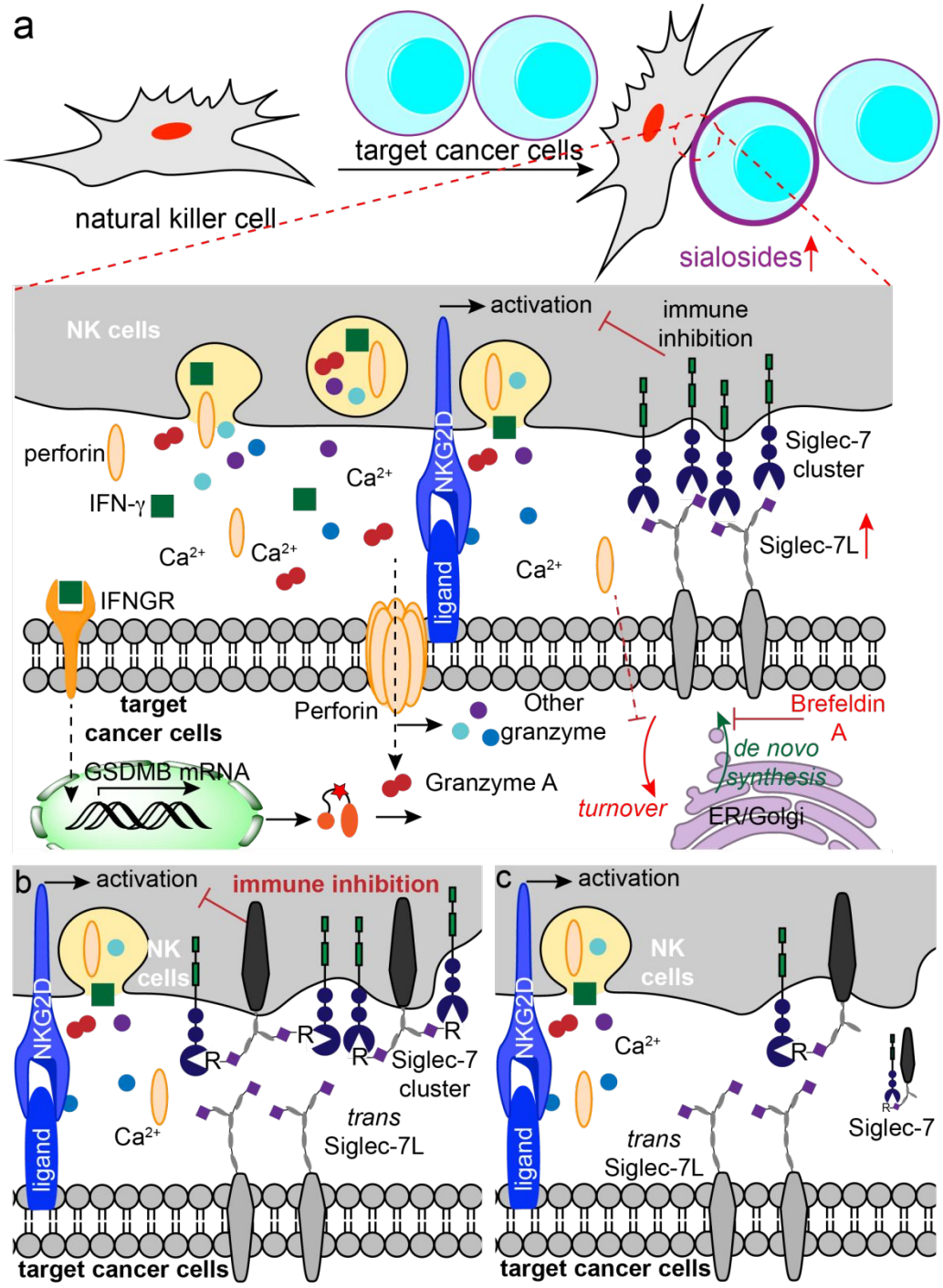

Figure S25. Schematic model of the modulation of Siglec-7 inhibitory signaling in NK cells upon target cell encountering. (a) During the NK-based killing of target cancer cells, the *de novo* synthesized Siglec-7 ligands were transported onto the plasma membrane, while the turnover of cell-surface sialsosides was blocked on target cancer cells that could be triggered by the secretion of maturated perforin from NK cells. In turn, the increased Siglec-7 ligands on target cells further recruited Siglec-7 inhibitory receptors for immune suppression. (b) *in situ* addition of a high-dose of high-affinity Siglec-7 ligands could generate new clustering centers of Siglec-7 to facilitate Siglec-9 inhibitory signaling; (c) the incorporation of a low-dose of high-affinity Siglec-7 ligands (10 nM) triggered the releasing of Siglec-7 into medium upon target cell encountering, and thus block the Siglec-7 inhibitory signaling.

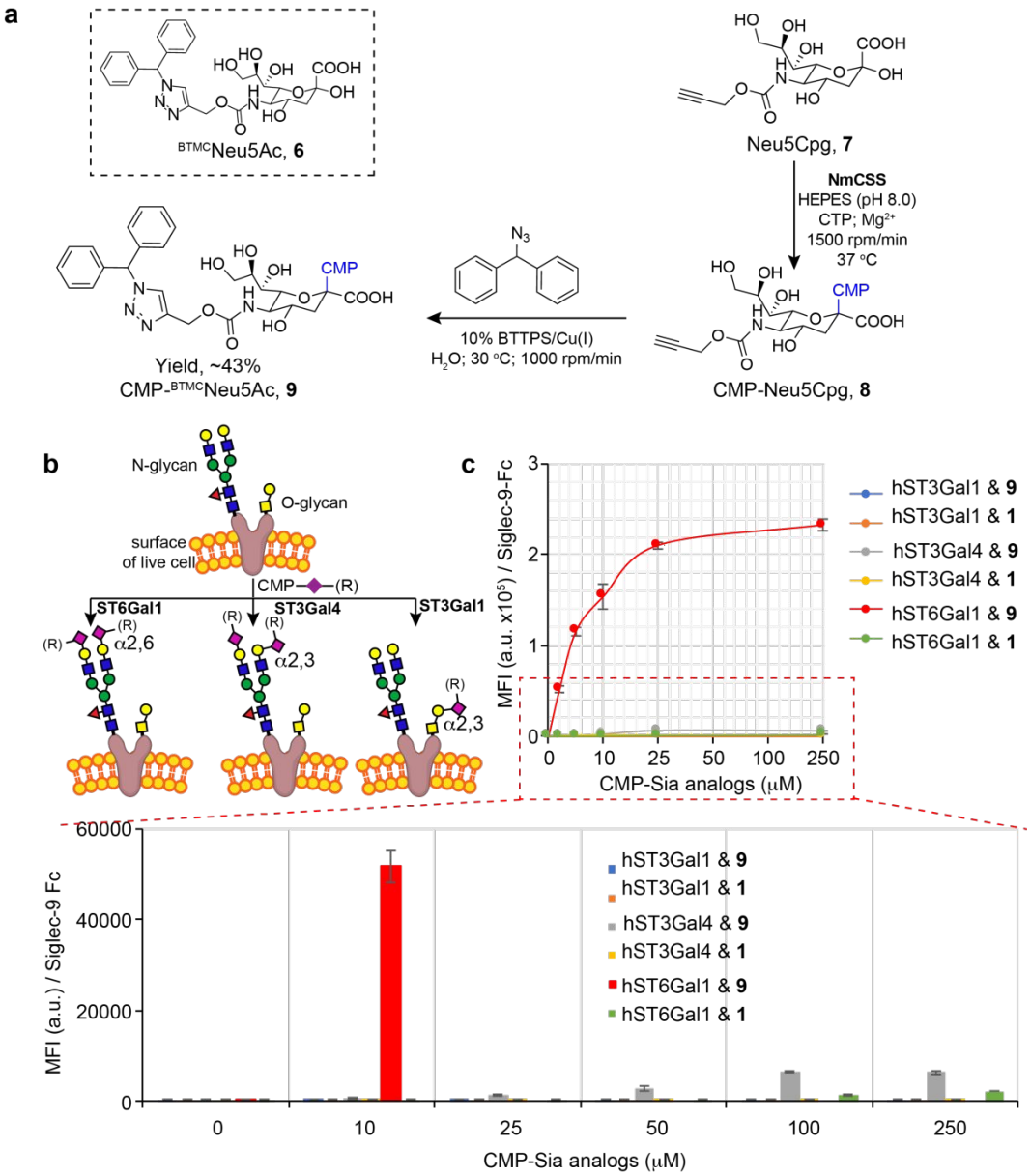

Figure S26. Using *in situ* creation of Siglec-9 high-affinity ligands to target Siglec-9 signaling. (a) Chemical structure of ^{BTMC}Neu5Ac (**6**) and route for one-pot synthesis of CMP-^{BTMC}Neu5Ac (**9**). CMP-^{BTMC}Neu5Ac (**9**) was prepared from C5-N-propargyloxycarbonyl Neu5Ac (Neu5CPg) (**7**) by conjugating CMP-Neu5CPg (**8**) with diphenylmethyl azide with a yield of ~43%. (b) Workflow for STs-assisted incorporation of ^{BTMC}Neu5Ac onto Chinese hamster ovary (CHO) Lec2 mutant cells. Lec2 cells possessing relatively homogeneous peripheral N-glycans terminated with LacNAc that are capable of being modified by STs to add 2-6- and 2-3-linked sialic acid. (c) Lec2 cells treated with STs (ST3Gal1, ST3Gal4, or ST6Gal1) under the presence of **9**, CMP-Neu5Ac (**1**) or not, were stained with Siglec-9-Fc and quantified via flow cytometry. The lower panel figure presents the magnified details of the figure upper panel labeled by a red box. Error bars present the standard deviation of three biological repeats.

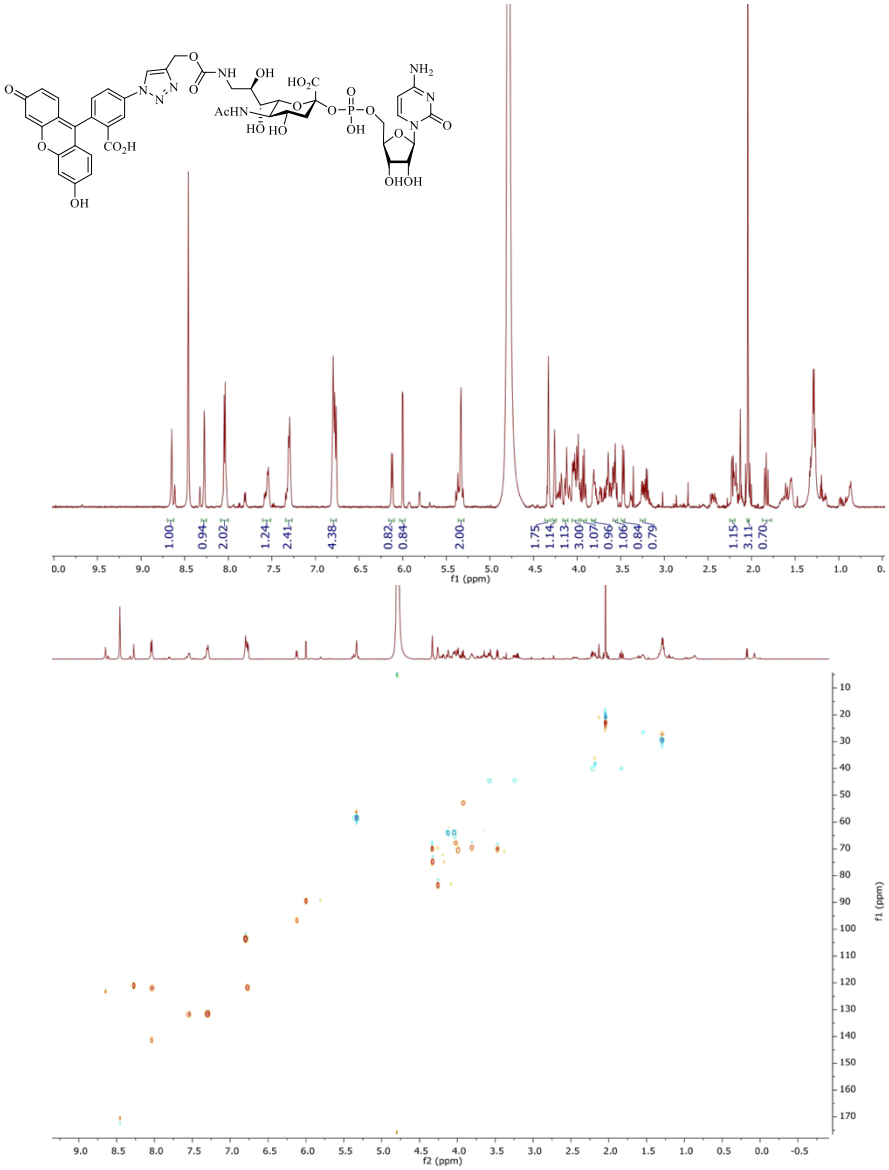

Figure S27. 1H and 13C NMR spectra of CMP-^{FTMC}Neu5Ac.

^{1}HNMR (600 MHz, D_2O): δ = 1.84 (t, 1H, J = 12.0 Hz), 2.04 (s, 3H), 2.22 (dd, 1H, J = 4.8, 12.6 Hz), 3.24 (dd, 1H, J = 7.8, 13.8 Hz), 3.47 (d, 1H, J = 8.0 Hz), 3.53-3.58 (m, 1H), 3.78-3.84 (m, 1H), 3.89-3.96 (m, 1H), 3.97-4.06 (m, 3H), 4.10-4.15 (m, 1H), 4.24-4.28 (m, 1H), 4.30-4.37 (m, 2H), 5.29-5.37 (m, 2H), 6.00 (d, 1H, J = 3.6 Hz), 6.12 (d, 1H, J = 7.8 Hz), 6.74-6.83 (m, 4H), 7.25-7.35 (m, 2H), 7.51-7.61 (m, 1H), 7.99-8.08 (m, 2H), 8.27 (s, 1H), 8.65 (s, 1H). ^{13}CNMR (150 MHz, D_2O): δ = 22.82, 40.02, 44.42, 52.6, 58.29, 63.91, 67.75, 69.58, 69.86, 69.95, 70.49, 74.68, 83.47, 89.42, 96.66, 103.46, 121.04, 121.7, 122.08, 123.38, 131.61, 131.78, 141.4 (Observed from HSQC). HRMS: m/z calc. for $C_{44}H_{46}N_8O_{22}P$: 1069.2464; found: 1069.2487 $[M + H]^+$

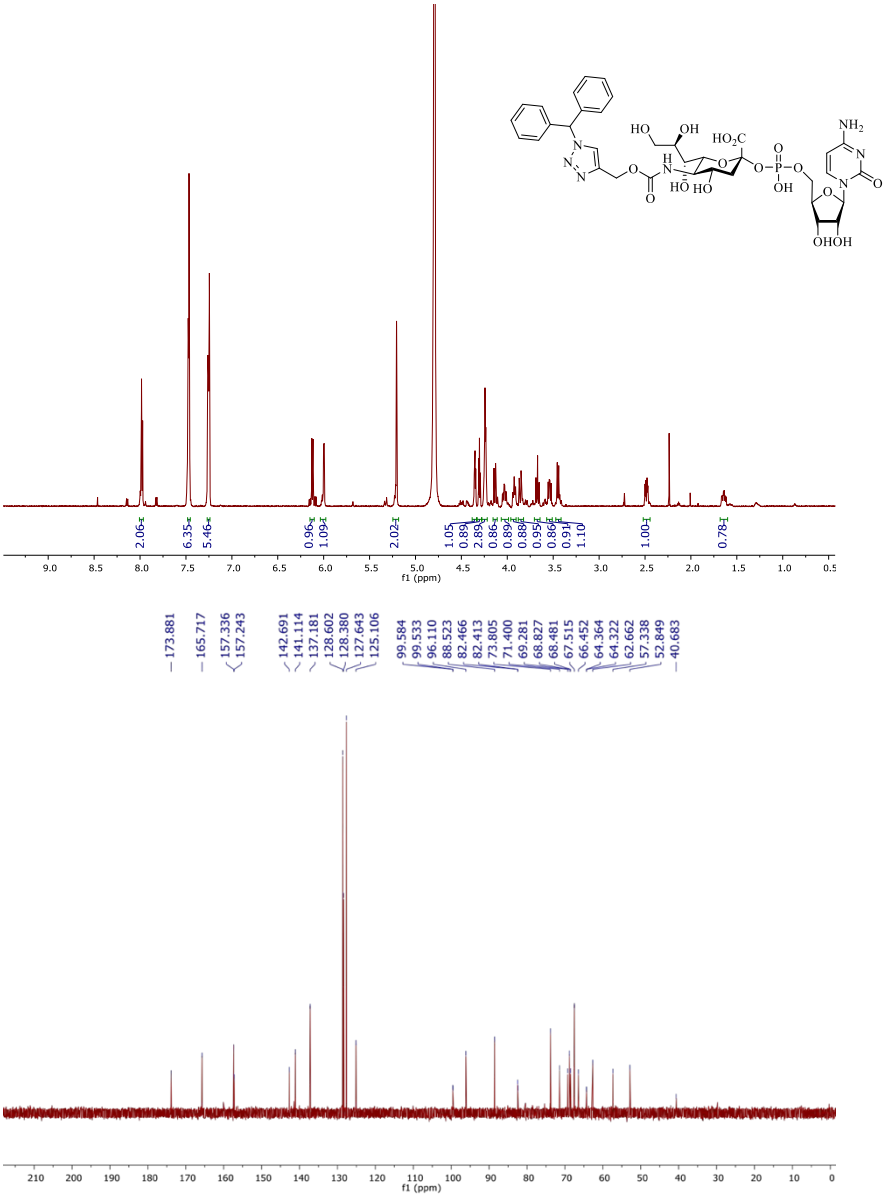

Figure S28 1H and 13C NMR spectra of CMP-^{BTMC}Neu5Ac.

^{1}HNMR (600 MHz, D_2O): δ = 1.64 (dt, 1H, J = 6.0, 12.0 Hz), 2.49 (dd, 1H, J = 4.8, 13.2 Hz), 3.45 (d, 1H, J = 9.6 Hz), 3.54 (dd, 1H, J = 7.2, 12.0 Hz), 3.67 (t, 1H, J = 10.8 Hz), 3.86 (dd, 1H, J = 2.4, 12.0 Hz), 3.93 (ddd, 1H, J = 2.4, 7.2, 9.6 Hz), 4.03 (dt, 1H, J = 4.8, 10.8 Hz), 4.14 (d, 1H, J = 10.8 Hz), 4.22-4.28 (m, 3H), 4.31 (t, 1H, J = 4.8 Hz), 4.35 (t, 1H, J = 4.8 Hz), 5.21 (s, 2H), 6.00 (d, 1H, J = 4.2 Hz), 6.12 (d, 1H, J = 7.8 Hz), 7.22-7.28 (m, 5H), 7.44-7.50 (m, 6H), 7.95-8.01 (m, 2H). ^{13}CNMR (150 MHz, D_2O): δ = 40.68, 52.85, 57.34, 62.66, 64.32, 64.36, 66.45, 67.51, 68.48, 68.83, 69.28, 71.4, 73.81, 82.41, 82.47, 88.52, 96.11, 99.53, 99.58, 125.11, 127.64, 128.38, 128.6, 137.18, 141.11, 142.69, 157.24, 157.34, 165.72, 173.88. HRMS: m/z calc. for $C_{35}H_{43}N_7O_{17}P$: 864.2453; found 864.2479$[M + H]^+$

Supplementary Information

Experimental Procedures

General methods and materials: All chemical compounds were purchased from commercialized suppliers, and used directly without further purification unless indicated. Az-*PEG*$_4$-biotin was obtained from Click Chemistry Tools. Streptavidin-Alexa Fluor 647 (AF647) and streptavidin-Alexa Fluor 488 (AF488) were obtained from Invitrogen. The recombinant human IFN-γ was purchased from SinoBiological Inc. Dynasore, brefeldin A, folic acid, inosite, 2-mercaptoethanol, $MgSO_4$, ATP, and CTP were purchased from Sigma-Aldrich. Cell culture reagents including media, fetal bovine serum, horse serum, penicillin G, and streptomycin were purchased from GIBCO. The egtazic acid (EGTA provided by Research Products International co.) was a gift from Chi-Huey Wong lab at Scripps. Concanamycin A (CMA) was bought from APExBio (A8633). The NKG2D specific blocking antibody (clone 1D11) was purchased from Biolegend (320802). The 12 mm Corning® Transwell® with a 0.4 m pore polycarbonate membrane insert was bought from Sigma-Aldrich. The plate reader used in this study is Synergy^{TM} H4 multi-mode microplate reader (Bio-Tek). Images of Coomassie blue SDS-PAGE protein gels and Western blotting membrane were collected on ChemiDoc XRS+ (Bio-Rad). The fluorescent and bright-field images were taken by a Keyence BZ-X700 microscope. The flow cytometry studies were performed on an Attune NxT flow cytometer. Cell Sorting was performed on a Sony multi-application cell sorter MA900. The confocal images were collected using a Zeiss LSM880 microscope maintained in State Key Laboratory of Natural and Biomimetic Drugs, Peking University. No unexpected or unusually high safety hazards were encountered.

Synthesis of chemical compounds: CMP-Neu5Ac, 9AzSia[1], C9-^{CPg}Neu5Ac (**3**)[2], Neu5CPg (**7**)[3], CMP-SiaNAl[4], BTTPS[5], Ac_4ManNAl[6], and biotinylated CMP-Neu5Ac[7] were synthesized and purified following former reports. Enzymes for chemical synthesis, including CMP-Sialic acid synthase (CSS)[1] and inorganic pyrophosphatase (iPPA)[8] were purified from *E. coli* following previously reported procedures.

General Procedure for preparation of Sia-CMP. The corresponding unnatural sialic acid (0.1 mmol), along with cytidine triphosphate (0.12 mmol, 1.2 eq) were dissolved in 100 mM Tris and 20 mM $MgCl_2$ (3 mL), and the pH was adjusted to 8.5. 10 U of CMP-NeuAc Synthetase was then added to the mixture. The reaction was kept at 37 ℃ for 2-4 hr until TLC (ethyl acetate: methanol: H_2O = 5 : 4 : 1 vol/vol) indicated the reaction was complete. The reaction was then quenched with 5 mL methanol for 20 min and centrifuged at 4000 rpm to remove insoluble precipitates. The supernatant was evaporated to remove methanol and the remaining aqueous solution was frozen and lyophilized. After lyophilization, the residue was re-dissolved in H_2O (1 mL). The solution was then subjected to BTTPS-assisted copper-catalyzed alkyne-azide [3+2] cycloaddition reaction[5] at 30 ^oC for 4 hr. The solution was then filtered through a 0.22 μm filter and purified through HPLC.

Compound **3** was subjected to the general procedures for the preparation of Sia-CMP and for copper-catalyzed azide-alkyne cycloaddition, which gave compound **5** in a yield of 26% over 2 steps.^{1}HNMR (600 MHz, D_2O): δ = 1.84 (t, 1H, J = 12.0 Hz), 2.04 (s, 3H), 2.22 (dd, 1H, J = 4.8, 12.6 Hz), 3.24 (dd, 1H, J = 7.8, 13.8 Hz), 3.47 (d, 1H, J = 8.0 Hz), 3.53-3.58 (m, 1H), 3.78-3.84 (m, 1H), 3.89-3.96 (m, 1H), 3.97-4.06 (m, 3H), 4.10-4.15 (m, 1H), 4.24-4.28 (m, 1H), 4.30-4.37 (m, 2H), 5.29-5.37 (m, 2H), 6.00 (d, 1H, J = 3.6 Hz), 6.12 (d, 1H, J = 7.8 Hz), 6.74-6.83 (m, 4H), 7.25-7.35 (m, 2H), 7.51-7.61 (m, 1H), 7.99-8.08 (m, 2H), 8.27 (s, 1H), 8.65 (s, 1H). ^{13}CNMR (150 MHz, D_2O): δ = 22.82, 40.02, 44.42, 52.6, 58.29, 63.91, 67.75, 69.58, 69.86, 69.95, 70.49, 74.68, 83.47, 89.42, 96.66, 103.46, 121.04, 121.7, 122.08, 123.38, 131.61, 131.78, 141.4 (Observed from HSQC). HRMS: m/z calc. for $C_{44}H_{46}N_8O_{22}P$: 1069.2464; found: 1069.2487 $[M + H]^+$

Compound **7** was subjected to the general procedures for preparation of Sia-CMP and for copper-catalyzed azide-alkyne cycloaddition, which gave compound **9** in a yield of 43% over 2 steps.^{1}HNMR (600 MHz, D_2O): δ = 1.64 (dt, 1H, J = 6.0, 12.0 Hz), 2.49 (dd, 1H, J = 4.8, 13.2 Hz), 3.45 (d, 1H, J = 9.6 Hz), 3.54 (dd, 1H, J = 7.2, 12.0 Hz), 3.67 (t, 1H, J = 10.8 Hz), 3.86 (dd, 1H, J = 2.4, 12.0 Hz), 3.93 (ddd, 1H, J = 2.4, 7.2, 9.6 Hz), 4.03 (dt, 1H, J = 4.8, 10.8 Hz), 4.14 (d, 1H, J = 10.8 Hz), 4.22-4.28 (m, 3H), 4.31 (t, 1H, J = 4.8 Hz), 4.35 (t, 1H, J = 4.8 Hz), 5.21 (s, 2H), 6.00 (d, 1H, J = 4.2 Hz), 6.12 (d, 1H, J = 7.8 Hz), 7.22-7.28 (m, 5H), 7.44-7.50 (m, 6H), 7.95-8.01 (m, 2H). ^{13}CNMR (150 MHz, D_2O): δ = 40.68, 52.85, 57.34, 62.66, 64.32, 64.36, 66.45, 67.51, 68.48, 68.83, 69.28, 71.4, 73.81, 82.41, 82.47, 88.52, 96.11, 99.53, 99.58, 125.11, 127.64, 128.38, 128.6, 137.18, 141.11, 142.69, 157.24, 157.34, 165.72, 173.88. HRMS: m/z calc. for $C_{35}H_{43}N_7O_{17}P$: 864.2453; found 864.2479$[M + H]^+$

Cell culture: Chinese Hamster Ovary (CHO) glycosylation mutant Lec2 cells, NK-92MI cells, Raji cells, Daudi cells, JIMT-1 cells, H1975 cells, and HEK293T cells were obtained from ATCC and cultured as suggested. Lec2 cells, JIMT-1 cells, and HEK293T cells were routinely kept in the high-glucose DMEM medium supported with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin G, and 100 mg/mL streptomycin. Raji cells, Daudi cells and H1975 cells were cultured in RPMI 1640 media supported with 10% (vol/vol) FBS, 10

mM HEPES, 1% (vol/vol) MEM non-essential amino acids, 100 U/mL penicillin G, and 100 mg/mL streptomycin. NK-92MI cells were cultured in αMEM supported with 12.5% FBS, 12.5% horse serum, 200 μM inositol, 20 μM folic acid, 50 μM 2-mercaptoethanol, 100 U/mL penicillin G, and 100 mg/mL streptomycin.

Preparation of PBMC Cells and the activated T cells: Human PBMC cells were isolated from the blood samples of healthy donors using Ficoll (Ficoll® Paque Plus, GE Healthcare, 17-1440-02) gradient separation in SepMate^{TM}-50 tubes (StemCell, 85450), following supplier instructions. Briefly, the blood samples were diluted 2times in DPBS containing 2 mM EDTA and subjected to Ficoll gradient separation. The resultant cells were collected and washed with DPBS twice to remove platelets. To prepare CD3/CD28 activated human T cells, the PBMC cells were rested in a plate for 3 hours to remove monocytes and DC cells. Then, the unattached cells were collected and activated with Dynabeads^{TM} human T-activator CD3/CD28 (GIBCO, 11131D) for 3 days following supplier instructions. After removing magnetic beads, the cells were cultured in 300 U/mL rhIL-2 (TECIN^{TM}, Roche) supported T cell medium for another 10 days before use.

Preparation of peripheral NK Cells: The peripheral NK cells were directly isolated from the blood of healthy donors using a negative isolation kit (Stem Cell, EasySep^{TM} Direct human NK cell isolation kit, 19665), following the protocol provided by commercial suppliers. All individuals provided informed consent for blood donation according to a protocol approved by the Internal Review Board and Ethics Committee. Briefly, a 15 mL (˜7 mL x 2 samples per donor) blood sample per donor was used to prepare NK cells. After the magnetic beads-based negative isolation, the resultant NK cells were collected, washed with fresh medium, and seeded in culture flasks at a concentration of 2 x 10^6 cells/mL in RPMI-1640 medium (Gibco) containing 10% (vol/vol) FBS (Sigma), MEM NEAA (Gibco), and 10 mM HEPES (Gibco), 100 U/mL penicillin, and 100 U/mL streptomycin (Gibco). The rhIL-2 (100 U/mL) and rhIL-15 (50 ng/mL, NCI, NSC# 745101) were added on day 1. Primary NK cells were kept for 10 days before used for glycocalyx modification and killing assays. Fresh medium, rhIL-2, and rhIL-15 were added every 2 days during culture, and the cell concentration was maintained at 2 x 10^6 cells/mL. The Siglec-7 high NK-92MI and peripheral NK cells were sorted by flow cytometry.

Glycan metabolic labeling: The Ac_4ManNAl was dissolved in ethanol to prepare a 10 mM stock solution. For metabolic labeling of NK-92MI, Raji, or HEK293T cells, Ac_4ManNAl was preadded to the culture dishes at a final concentration of 50 μM, and cells were seeded at a density of 2 x 10^5 cells/mL after the complete volatilization of ethanol. Cells were then kept in normal culture for 48 h. The resulting SiaNAl-labeled glycoconjugates on cells were further labeled by biotin in PBS buffer (pH 7.4) containing 2% FBS, 50 μM azide-PEG4-biotin, 300 μM BTTPS and 50 μM Cu^{2+} premixture, and 2.5 mM freshly prepared sodium ascorbate for 5 mins. Finally, cell-surface biotin was further probed by 2 μg/mL streptavidin-Alexa fluor 647 conjugate and quantified via flow cytometry. For co-culture treatment, the target cells and effector NK cells were mixed and maintained at 37 ^oC for 1h before the biotin conjugation.

General Procedure for the incorporation of Sia-CMP onto live cells via STs-assisted cell-surface glycan editing: Truncated human sialyltransferases were prepared as previously described[9], including hST6Gal1, hST3Gal1, and hST3Gal4. For STs-assisted cell-surface glycan editing, the live cells were washed three times with PBS buffer (pH 7.4) and resuspended in HBSS buffer (pH 7.4) containing 3 mM HEPES, 20 mM $MgSO_4$, 40 μg/mL hSTs, and indicated concentrations of CMP-Sia. The Siglec staining buffer was prepared by premixing the Siglec-7-Fc[10] or Siglec-9 Fc[10] developed in the Macauley lab (5 μg/mL) with anti-human Fc APC conjugate at a 1:1.2 molar ratio on ice for 1 hr (7 mutations were introduced in the Fc domain, L234A, L235A, G237A, H268A, P238S, A330S, P331S, to eliminate FcγR binding. hSTs-edited cells were washed with PBS containing 1% (vol/vol) FBS three times, resuspended in Siglec staining buffer, and incubated for 30 min on ice in the dark. Then, the cells were washed and resuspended in FACS buffer for flow cytometry analysis. For fluorescence microscopy imaging, hSTs-edited NK-92MI cells were washed with PBS containing 1% (vol/vol) FBS three times and incubated with Raji target cells at an E/T ratio of 5:1 on glass-bottom dishes (35 mm) for 1 hr at 30 ^oC. The resulting cell-cell clusters were stained in 1:1 immunoblot buffer (PBS, pH 7.4, 2 % (vol/vol) FBS, 2 μg/mL antiSiglec-7 PE conjugate) Siglec staining buffer, and incubated for 30 min on ice in dark. Finally, the cells were carefully washed with PBS twice and imaged via fluorescence microscopy.

LDH assay: NK-92MI cell cytotoxic function was evaluated by the LDH assay kit (CytoTox® 96 Non-Radioactive Cytotoxicity Assay, Promega, G1782) using Raji and Daudi B lymphoma cells as targets for cytotoxicity. The number of effector cells available was determined via hemocytometer, and the viability of cells was assessed by trypan blue exclusion. Effector cells and target cells were used as controls for assays. The counts of peripheral NK cells were determined via flow cytometry by gating anti-CD3-FITC (negative) and anti-CD56-PE

(positive). The LDH assays were performed by following the instructions from the commercial supplier. In brief, E/T coculture of the indicated cells in about 100 μL media was maintained at 37 °C for 4~5 hr, and 40 μL supernatant was collected and subjected to LDH assay. The pretreatment of NK cells with Siglec-7 blocking antibody (clone s7.7, 2.5 μg/mL, Biolegend) was performed by incubating cells at 37 °C for 30 min, and α2-3,6,8,9-neuraminidase A (NEB) assisted desialylation of NK cells or target cells was performed by incubating cells at 37 °C for 60 min, before coincubation and target cell killing assays. In brief, live cells (per million) were resuspended in 10 μL DMEM media (serum-free) containing one unit α2-3,6,8,9-neuraminidase A and incubated at 37 °C for 60 min before the cells were washed with PBS for further analysis.

ELISA quantification of IFN-γ and granzyme B: The NK-92MI cells and Raji cells were co-cultured at an E/T ratio of 5:1 for 1 hour. Samples were triplicated and the supernatant was collected for ELISA analysis. The Max™ deluxe set human IFN-γ ELISA kit (430104) and Legend Max™ human granzyme B precoated ELISA kit (439207) were got from Biolegend and used following assay procedure provided by suppliers. 50 μL medium for each sample was diluted to 100 μL with DPBS for IFN-γ quantification, and 20 μL medium for each sample was diluted to 100 μL with DPBS for granzyme B measurement. All the samples were run in duplicate. The acute concentration was calculated from the standard curve.

Flow cytometry assays: The Siglec-7 on NK-92MI cells were stained with mouse anti-Siglec-APC antibody on ice for 30 min in dark (clone 6-434, 1:200, Biolegend). The Siglec-7 ligands were detected with the premixture (1h on ice in dark) of Siglec-7-Fc and anti-human Fc-APC antibody on ice for 30 min in dark (1:200, Biolegend). Lectin stainings were performed with SNA-biotin (10 μg/mL, Vector) and MAA-II-biotin (10 μg/mL, Vector) on ice for 30 min in dark, following streptavidin-Alexa Fluor 647 (2 μg/mL, Life) after three times washing with DPBS (pH 7.4). In the co-culture system, the NK-92MI cells were detected via anti-CD56-PE (1:200, Biolegend), peripheral NK cells were probed by anti-CD3-FITC (negative), and anti-CD56-PE (positive), the Raji cells were detected with anti-CD19-APC (1:200, Biolegend). After staining, the cells were spun down, washed with DPBS three times, and resuspended in FACS buffer for flow cytometry assays.

Fluorescence microscopy imaging: For imaging of live cells, the NK-92MI cells modified with or without Siglec-7 specific high-affinity ligands were further incubated with Raji target cells at a 5:1 E/T ratio for 1h at 37 oC, or not. Then, the Siglec-7 on NK-92MI cells were stained directly by mouse anti-Siglec-7-PE antibody (clone 6-434, 1:200, Biolegend). The signal of PE and fluorescein was directly imaged after washing off the free dyes. The neuraminidase-assisted desialylation of NK cells was performed by incubating cells at 37 °C for 60 min, before coincubation with or without target Raji cells. For immunostaining of the tissue specimens, the frozen lung tumor tissue and healthy lung tissue specimens were got from US Biomax, including FLU401c, G035, G036, and G037. The frozen tissue array slides were put on ice for 10 min before blocking with DPBS buffer supported by 5% FBS on ice for 1 hour. After washed with DPBS for 3times, the slides were ready for antibody and Siglec-7-Fc profiling. The paraffin-embedded human tumor and adjacent tissue specimens from a number of malignancies were also purchased from commercial suppler (US Biomax), including MC245a (c040 and c041), lung tissue array BC041114 (E094 and E095), and (LC1201a). The Histo-clear II (Electron Microscopy Sciences) was used to remove the paraffin following the supplier instructions. In brief, the slides were washed in Histo-clear II 2 times (5 min), 10% ethanol (vol:vol) in Histo clear II for 1 time (5 min), ethanol for 2 times (5 min), 95% ethanol (vol:vol) in H_2O for 1 time (5 min), 80% ethanol (vol:vol) in H_2O for 1 time (5 min), and 70% ethanol (vol:vol) in H_2O for 1 time (5 min). The tissue samples were then blocked by incubation with DPBS buffer (pH7.4) containing 5% (w/vol) bovine serum albumin (BSA) at rt for 1h. After washed with DPBS three times, the slides were further stained with antibodies by incubation at 4°C overnight. To probe the sialyl-ligands of Siglec-7 on tissues, the Siglec-7-Fc (2 μg/mL) were premixed with anti-human IgG Fc-APC conjugated monoclonal antibody (5 μg/mL, Biolegend) at 4 °C for 30 min, before added to tissue samples. The NK cells in tissue samples were probed by the human CD56 antibody (2 μg/mL, Biolegend). GD3 ganglioside was profiled with GD3 specific antibody (clone R24, 2 μg/mL, Abcam). The $CD8^+$ T cells were probed by the CD8α antibody (5 μg/mL, Biolegend). Then, the slides were washed with DPBS 3 times, mounted with the prolonged gold antifade media with DAPI (Cell Signaling), and sealed for fluorescent imaging.

Confocal imaging: After treating with ST6Gal1 and CMP-^{FTMC}Neu5Ac (100 nM), NK-92MI cells (1 x 10^6 cells) were washed with pre-warmed (37 °C) PBS for three times. The resultant NK-92MI cells loading high-affinity ligands against Siglec-7 were then co-incubated with Raji cells pre-stained with Hochest 33342 (2 μg/mL, 10 min, rt) at an E/T ratio of 5:1 in 30 μL RPMI1640 complete media for 1 hr. Next, the cell mixture was gently moved into a bottom-glass dish and subjected to confocal imaging. The dishes were freshly coated with ConA to immobilize the cells. The images were collected on a Zeiss LSM880 with a 63x oil lens (NA 1.4).

Western blot and immunoprecipitation (IP): The NK-92MI-S7^{high} cells (˜10 x 10^6) modified with or without ^{FTMC}Neu5Ac were mixed with target cells in RPMI 1640 medium (100 uL) at the indicated effector to target ratio for 30 min or 1h and pelleted down. The cells were washed with DPBS and lysed with RIPA buffer (lipid, Alfa Aesar, J63306) containing 5 μg/mL DNase 1 and protease inhibitor cocktail (Pierce™ protease inhibitor tablets, EDTA-free, Thermo, A32955). For Siglec-7 IP, the resultant lysates were first incubated with 10 μg Rabbit anti-Siglec-7 antibody (clone H-48, Santa Cruz Biotech) for 3h, followed by protein A/G agarose incubation overnight at 4 °C. The beads were washed three times with the lysis buffer and eluted by boiling in 4 x SDS loading buffer containing β-mercaptoethanol. For Western blotting, proteins were resolved by SDS-PAGE on Bis-Tris Criterion Gels (4-12%; Bio-Rad) and transferred to a nitrocellulose membrane by wet transfer (Tris-glycine, 20% MeOH) at 100 V for 1h. the membranes were blocked with PBST buffer (DPBS with 0.05% Tween-20) containing 5% BSA, and the primary antibody incubation conditions were conducted in PBST buffer containing 1% BSA overnight. The antibodies used in this study were mouse anti-Siglec-7 antibody (clone 194212, 1:500, R&D)/HRP goat anti-mouse IgG (1:10,000, Pierce®), biotin anti-phosphotyrosine (clone PY20, 1:200, Biolegend)/HRP anti-biotin antibody (1:10,000, Jackson), rat anti-SHP-1 antibody (clone W17240D, 1:500, Biolegend)/HRP goat anti-rat IgG antibody (1:5000, Biolegend).

Data and software availability: The fluorescent images were processed with ImageJ and the flow cytometry data were processed using Flowjo. The tissue area was defined with bright-field images in ImageJ. To profile NK and $CD8^+$ T cell infiltration into the tissue, the raw integrated density (RawIntDen) of CD56 and CD8α signal was used for correlation analysis. To minimize the CD56 staining background on other cells of tissue specimens, and intensity cut-off of 50 (a.u.) was used for ImageJ quantification. The raw data that supported the findings of this study are available from the authors upon reasonable request.

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