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Supplemental information

Bidirectional signals generated by Siglec-7 and its crucial ligand tri-sialylated T to escape of cancer cells from immune surveillance

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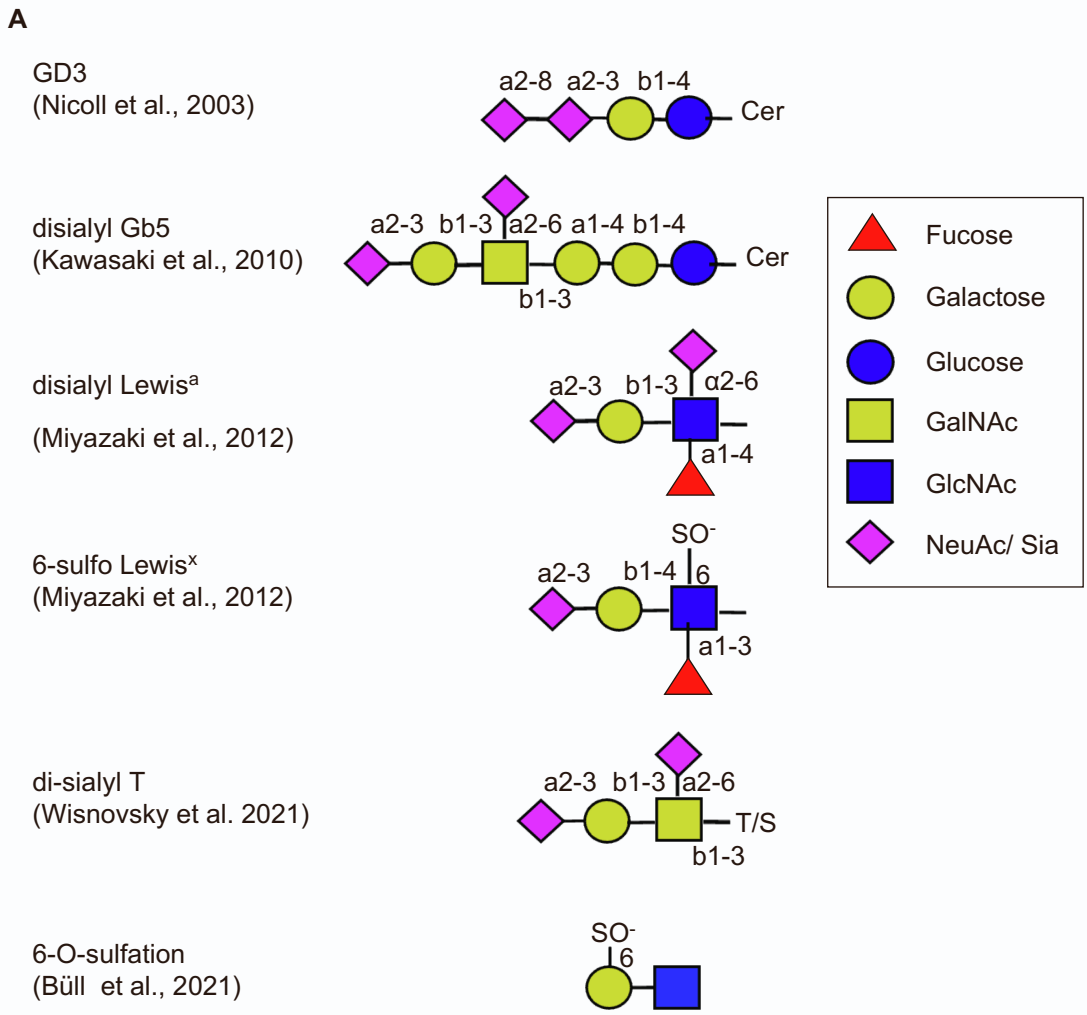

Figure S1. All reported Siglec-7-recognizing glycans are mono- or di-sialylated structures, related to Figure 1

(A) Among glycosphingolipids, disialylganglioside GD3 is the best ligand for Siglec-7,[6] and a branched-type disialyl GSLs, disialyl Gb5, has also been reported to be the ligand.[7] Carbohydrates with branched disialyl structures on core 1 and/or core 2 are also specific ligands.[8] They might be carried on both GSLs and O-glycans. Recently, di-sialyl T was identified as Siglec-7 ligand.[7,15,17,18] Instead of sialic acid being modified to galactose 6-o-sulfation, which is sulfated, was also reported as a ligand glycan.[19] chain.Essential glycan structures commonly recognized by Siglec-7 are not clear from these carbohydrate structures

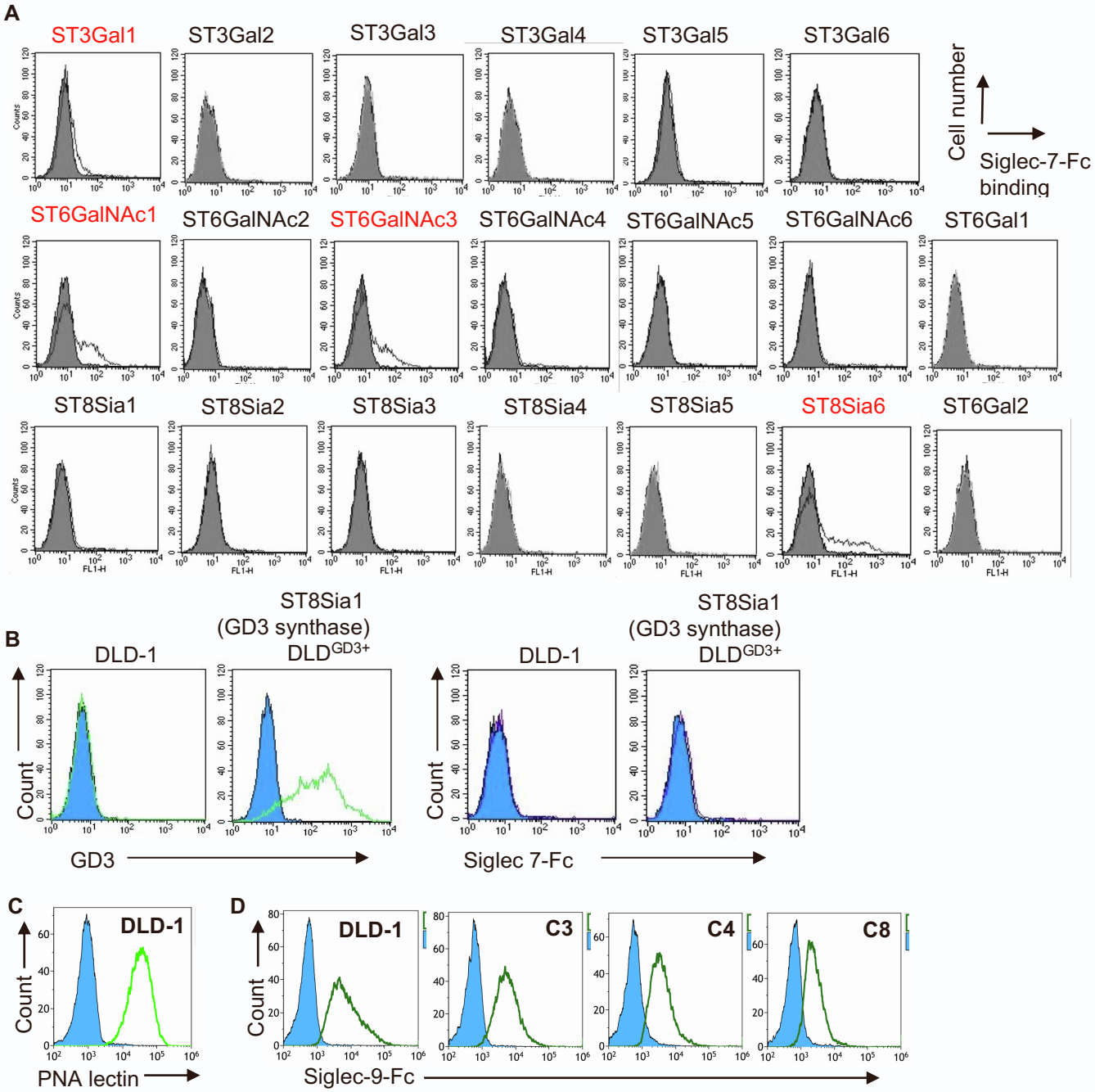

Figure S2. Identification of sialyltransferase(s) that bring about definite Siglec-7-Fc binding on the cell surface when separately transfected, related to Figure 1

(A) cDNAs of 20 sialyltransferases genes were transfected into DLD-1 using Lipofectamine 2000. Two days later, the cells were stripped with trypsin and reaction with Siglec-7-Fc was analyzed using flow cytometry. Consequently, siglec-7-Fc bound to DLD-1 cells transfected with the cDNA of ST3Gal1, ST6GalNAc1, ST6GalNAc3 or ST8Sia6 (red letters).

(B) Sialyltransferase ST8Sia1 (GD3 synthase)-transfectant DLD-1 cells (DLD^{GD3}) showed binding of anti-GD3 antibody, but no binding of Siglec-7-Fc could be seen.

(C) DLD-1 were stained with PNA-biotin and avidin-FITC and analyzed using FACS. PNA lectin recognize non-sialyleted Gal-b1-3GalNAc O-glycan.

(D) Binding of Siglec-9-Fc.

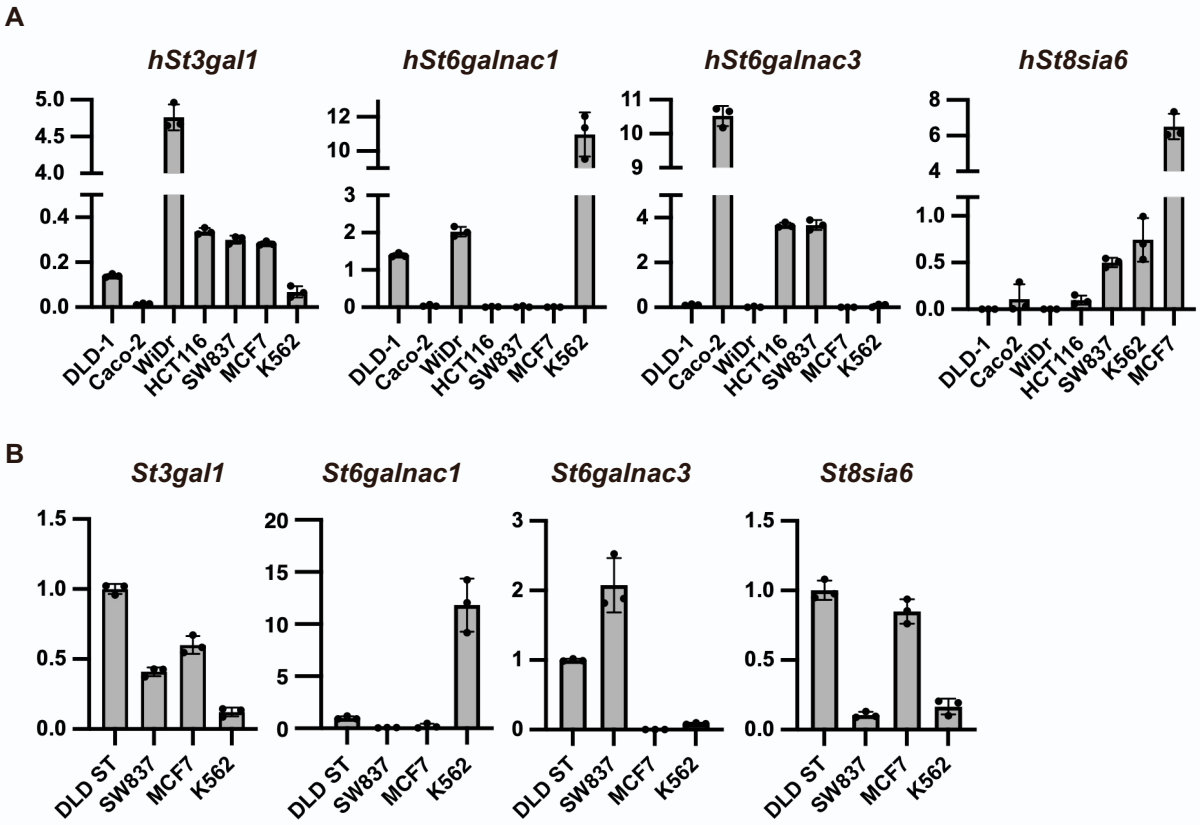

Figure S3. Expression of four sialyltransferases in various cancer cell lines , related to Figure 1

(A) The expression of their sialiltransferase in human colorectal adenocarcinoma cell lines DLD-1, Caco2, WiDr, HCT116 and SW837, human leukemia cell line K562 and human breast cancer cell line MCF7 were analyzed using qPCR. Relative expression levels against human gapdh are shown graphically. Data are mean +/- SD, n = 3 biological replicates.

(B) The expression levels of transfected mouse genes in DLD-1 ST transfectants were compared using the delta-delta Ct method to determine the extent to which they are expressed in transfectants compared to cell lines. Primers for mice were used for the transfectants and primers for humans were used for the cell lines to obtain their Ct values. Data are mean +/- SD, n = 3 biological replicates.

A

Podocalyxin (PODXL)

Protein sequence coverage: 32%

Matched peptides shown in ***bold red***.

```
  1 MRCALALSAL  LLLLSTPPLL  PSSPSPSPSP  SQNATQTTTD  SSNKTAPTPA
 51 SSVTIMATDT  AQQSTVPTSK  ANEILASVKA  TTLGVSSDSP  GTTTLAQQVS
101 GPVNTTVARG  GGSGNPTTTI  ESPKSTKSAD  TTTVATSTAT  AKPNTTSSQN
151 GAEDTTNSGG  KSSHSVTTDL  TSTKAEHLTT  PHPTSPLSPR  QPTSTHPVAT
201 PTSSGHDHLM  KISSSSSTVA  IPGYTFTSPG  MTTTLLETVF  HHVSQAGLEL
251 LTSGDLPTLA  SQSAGITASS  VISQRTQQTS  SQMPASSTAP  SSQETVQPTS
301 PATALRTPTL  PETMSSSPTA  ASTTHRYPKT  PSPTVAHESN  WAKCEDLETQ
351 TQSEKQLVLN  LTGNTLCAGG  ASDEKLISLI  CRAVKATFNP  AQDKCGIRLA
401 SVPGSQTVVV  KEITIHTKLP  AKDVYERLKD  KWDELKEAGV  SDMKLGDQGP
451 PEEAEDRFSM  PLIITIVCMA  SFLLLVAALY  GCCHQRLSQR  KDQQRLTEEL
501 QTVENGYHDN  PTLEVMETSS  EMQEKKVVSL  NGELGDSWIV  PLDNLTKDDL
551 DEEEDTHL
```

Mucin-13 (MUC13)

Protein sequence coverage: 38%

Matched peptides shown in ***bold red***.

```
  1 MKAIIHLTLL  ALLSVNTATN  QGNSADAVTT  TETATSGPTV  AAADTTETNF
 51 PETASTTANT  PSFPTATSPA  PPIISTHSSS  TIPTPAPPII  STHSSSTIPI
101 PTAADSESTT  NVNSLATSDI  ITASSPNDGL  ITMVPSETQS  NNEMSPTTED
151 NQSSGPPTGT  ALLETSTLNS  TGPSNPCQDD  PCADNSLCVK  LHNTSFCLCL
201 EGYYYNSSTC  KKGKVFPGKI  SVTVSETFDP  EEKHSMAYQD  LHSEITSLFK
251 DVFGTSVYGQ  TVILTVSTSL  SPRSEMRADD  KFVNVTIVTI  LAETTSDNEK
301 TVTEKINKAI  RSSSSNFLNY  DLTLRCDYYG  CNQTADDCLN  GLACDCKSDL
351 QRPNPQSPFC  VASSLKCPDA  CNAQHKQCLI  KKSGGAPECA  CVPGYQEDAN
401 GNCQKCAFGY  SGLDCKDKFQ  LILTIVGTIA  GIVILSMIIA  LIVTARSNNK
451 TKHIEEENLI  DEDFQNLKLR  STGFTNLGAE  GSVFPKVRIT  ASRDSQMQNP
501 YSRHSSMPRP  DY
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B

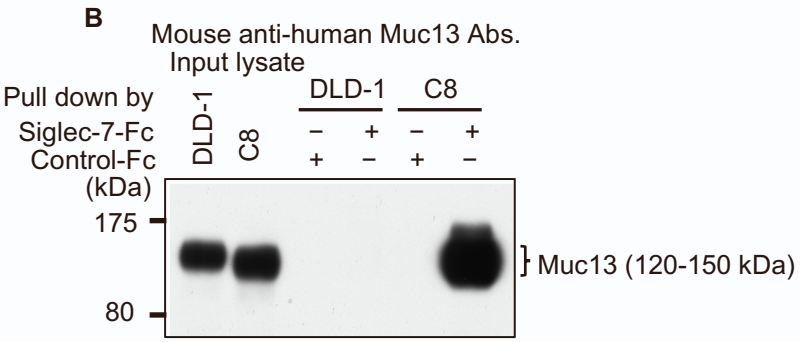


Figure S4. MS results of pull-down products with Siglec-7-Fc, related to Figure 1.

(A) Siglec-7-Fc pull-down products from ST8Sia6-transfected DLD-1 and parent cells were analyzed using LC/MS. In the pull-down samples derived from clone 8, two proteins, Mucin 13 and Podocalyxin, were identified using the MASCOT search. The identified peptides corresponded to 32, and 38% of the respective proteins, as indicated using red letters. The sequences of the extracellular regions did not match because the information on post-translational modification related to glycans is not included in the database search.

(B) Immunoblotting of MUC13 in the Siglec-7-Fc pull down samples from DLD-1 and Clone 8. Their samples were applied for immuno-blotting using anti-MUC13 antibody. Muc13 shows non-covalent re-association, i.e., an alpha subunit, which is modified via O-glycosylation, and a beta subunit, which has no glycosylation site.[13,14]

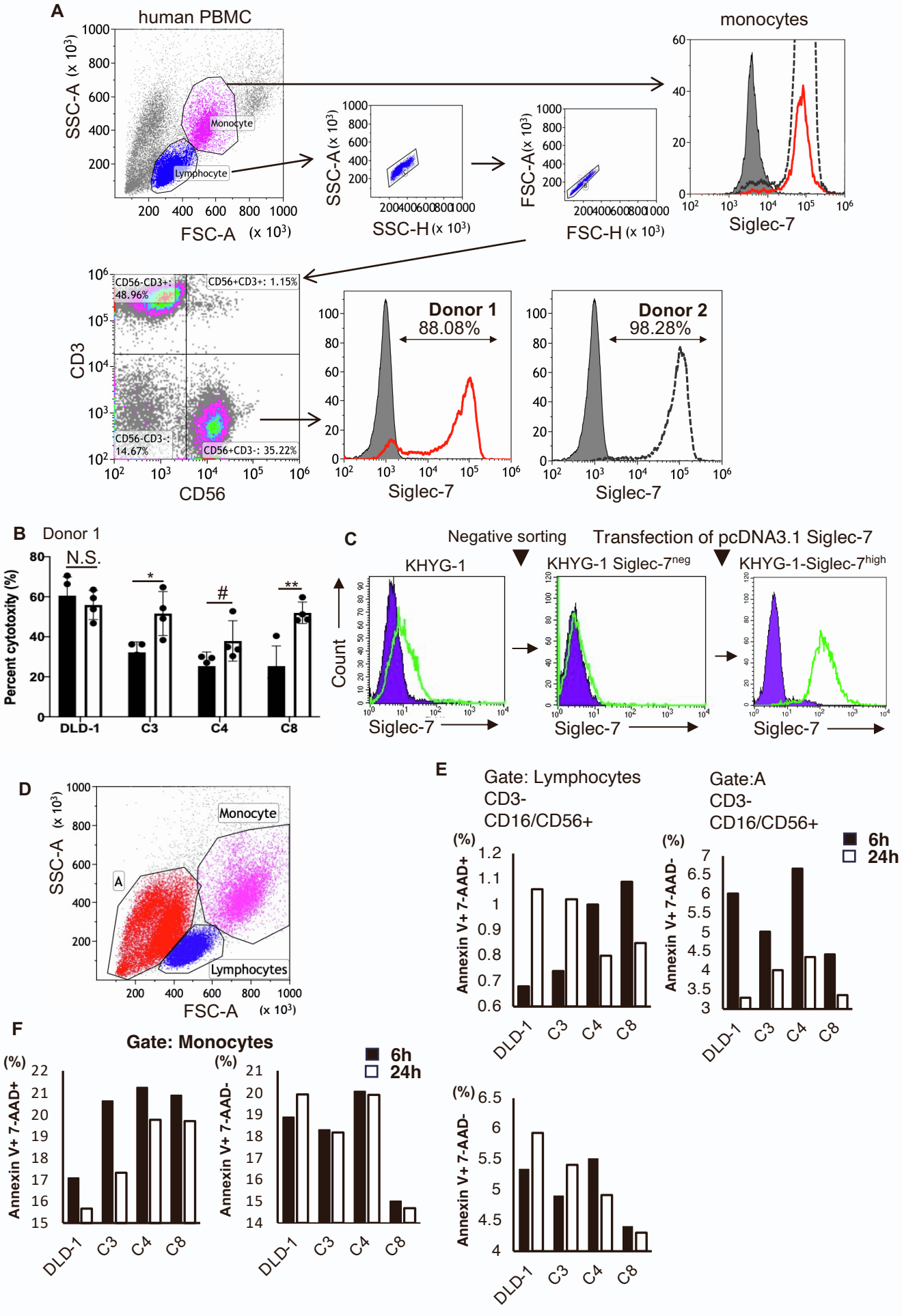

A
human PBMC
SSC-A (x 10³)
FSC-A (x 10³)
Monocyte
Lymphocyte
SSC-H (x 10³)
FSC-H (x 10³)
monocytes
Siglec-7
CD3
CD56
CD56-CD3+: 48.96%
CD56+CD3+: 1.15%
CD56-CD3-: 14.67%
CD56+CD3-: 35.22%
Donor 1
88.08%
Donor 2
98.28%
B
Donor 1
N.S.
Percent cytotoxity (%)
DLD-1
C3
C4
C8
C
Negative sorting
Transfection of pcDNA3.1 Siglec-7
KHYG-1
KHYG-1 Siglec-7neg
KHYG-1-Siglec-7high
Count
Siglec-7
D
Monocyte
Lymphocytes
A
E
Gate: Lymphocytes CD3- CD16/CD56+
Gate:A CD3- CD16/CD56+
6h
24h
Annexin V+ 7-AAD+
Annexin V+ 7-AAD-
F
Gate: Monocytes

Figure S5. Expression of Siglec-7 on some populations of human PBMCs and establishment of NK cell line KHYG-1 strongly expressing Siglec-7, related to Figure 2

(A) Human peripheral blood mononuclear cells (PBMCs) were collected via Ficoll-Hypaque centrifugation. These cells were stained with antibodies against Siglec-7 (V450), T cell marker (anti-CD3), B cell marker (CD19), and NK cell marker (CD16+CD56) and analyzed using flow cytometry. First, cells were divided into monocytes and other mononuclear cells by gating on FSC and SSC plots. Siglec-7 was detected in almost 100% of monocytes. Lymphocytes were divided into B cells, T cells, and NK cells. Among them, approximately 78% in NK cells were Siglec-7-positive.

(B) Rescue effect of blocking antibodies on cytotoxic activity using Donor 2-derived PBMCs.Apoptosis of primary NK cells. DLD-1 Control IgG vs DLD-1 Anti-Sig-7, $p = 0.078$, C3 Control IgG vs C3 Anti-Sig-7, $p = 0.0083$, C4 Control IgG vs C4 Anti-Sig-7, $p = 0.067$, C8 Control IgG vs C8 Anti-Sig-7, $p = 0.0005$, Data are mean +/- SD, n = 4 biological replicates.

(C) The NK cell line KHYG-1 expressed a low level of endogenous Siglec-7. Siglec-7 negative population was sorted using anti-Siglec-7 antibody on BD Aria2 to prepare KHYG-1 Siglec-7^{neg}. Then, KHYG-1 Siglec-7^{neg} cells were transfected with the pcDNA3.1-Siglec-7 or pcDNA3.1 empty vector using the Amaxa Nucleofector^{TM} system (LONZA). The transfectant cells were selected by G418, and a Siglec-7-highly expressing population was sorted, followed by cloning with limiting dilution, leading to the establishment of KHYG-1-Siglec-7^{high} .

(D) Gating Information of PBMCs using flow cytometry.

(E) Ratio of early to late apoptosis in each gate of NK cells during co-culture with DLD-1 and the transfectants.

(F) Ratio of early to late apoptosis in monocytes.

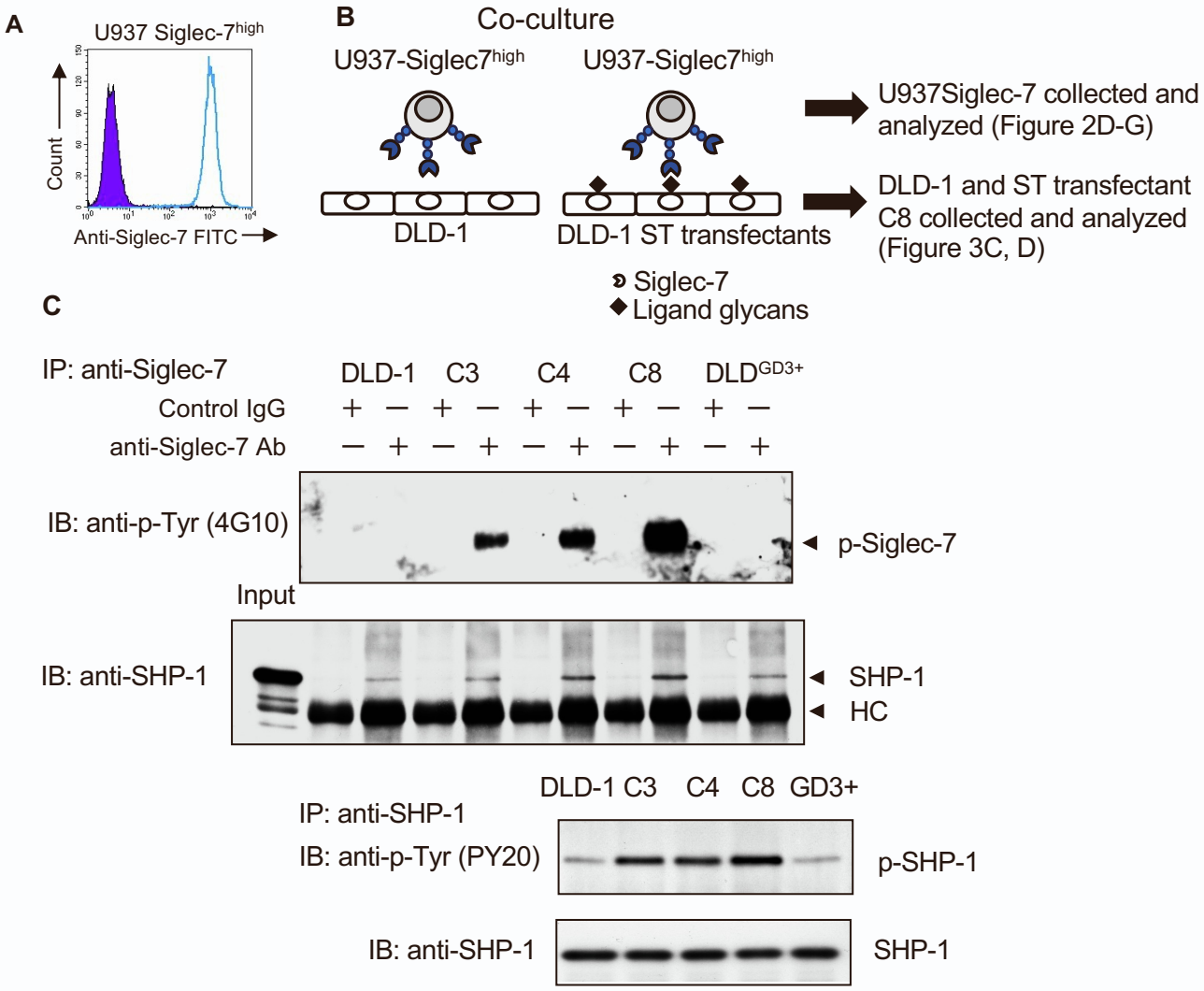


Figure S6. Interaction between Siglec-7 on U937 and carbohydrate ligands on DLD-1 cells caused phosphorylation of Siglec-7 and SHP-1 in U937, related to Figure 2

(A) Expression of Siglec-7 on U937-Siglec7^{high}.

(B) Experimental procedures performed in Figures 2D-G and S6B for analysis of U937-Siglec7^{high} and in Figures 3C–D for DLD-1 and C8. U937-Siglec-7^{high} was co-cultured with DLD-1, its transfectants with STs (C3, C4, C8) or DLD^{GD3+} cells, and incubated in the presence of pervanadate for 10 min. Then, U937 was collected and lysed.

(C) The lysates were immunoprecipitated with anti-Siglec-7 or anti-SHP-1 antibodies. Subsequently, the precipitates were served for immuno-blotting with anti-phosphotyrosine antibody (4G10) or anti-phosphotyrosine antibody (PY20) and anti-SHP-1, respectively. These results are identical with those of Figure 2 except for DLD^{GD3+}. DLD^{GD3+}, which are negative for Siglec-7 binding, did not show enhancement of phosphorylation of Siglec-7 and SHP-1.

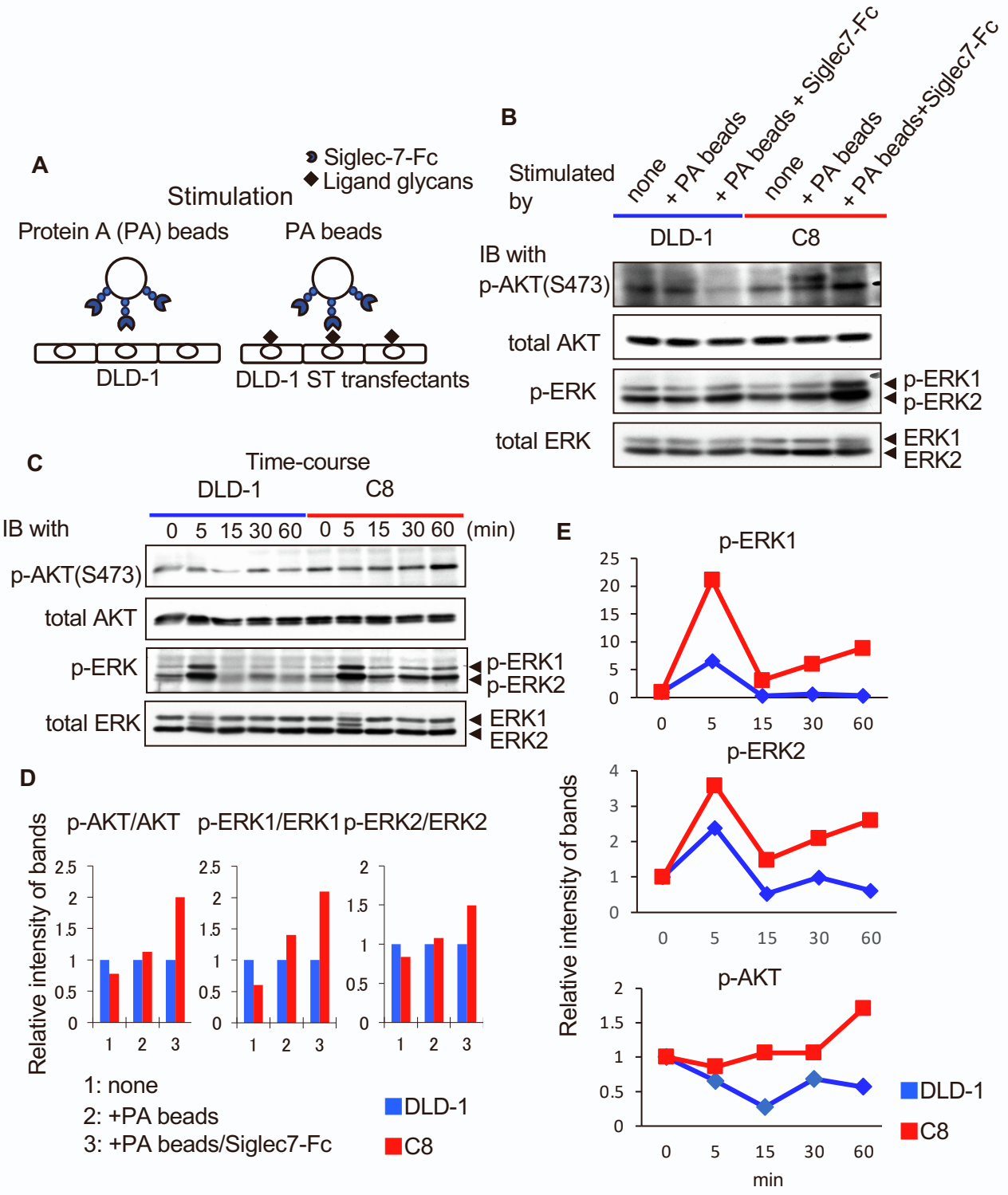


Figure S7. Phosphorylation of downstream molecules of PODXL upon Siglec7-Fc stimulation, related to Figure 3

(A) The fusion protein Siglec-7-Fc was fixed on the protein A-beads in advance. These beads were added to DLD-1 and C8 cells in the culture dishes, and then they were incubated for 15 min.

(B) After washing out the beads, cells were lysed, and the lysates were analyzed using immunoblotting using antibodies reactive with p-AKT (s473), p-ERK, total AKT, or total ERK.

(C) These cells were stimulated with Siglec-7-Fc fixed on the protein A-beads for individual time points. Those samples were analyzed for phosphorylation of AKT and ERK via IB of the individual antibodies.

(D) The immunoblotting bands (B) were quantified using ImageJ software, and band intensities are presented after correction with the bands of individual total proteins.

(E) The immunoblotting bands (C) were quantified using ImageJ software, and band intensities are presented after correction with the bands of individual total proteins.

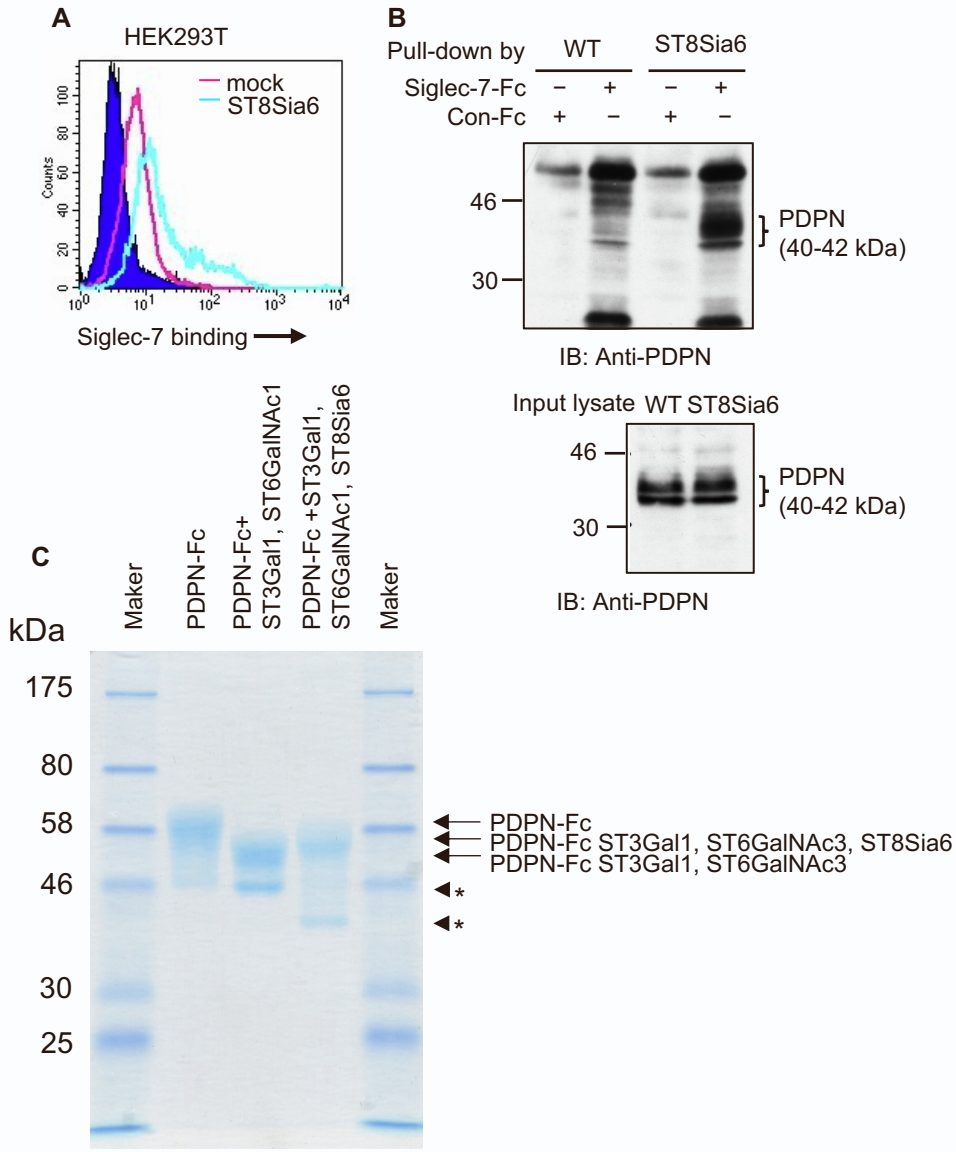


Figure S8. Flow cytometry and immuno-blotting of ST8Sia6 forcibly expressed HEK293T and CBB staining of three types of purified PDPN-Fc generated in HEK293T cell, related to Figure 5

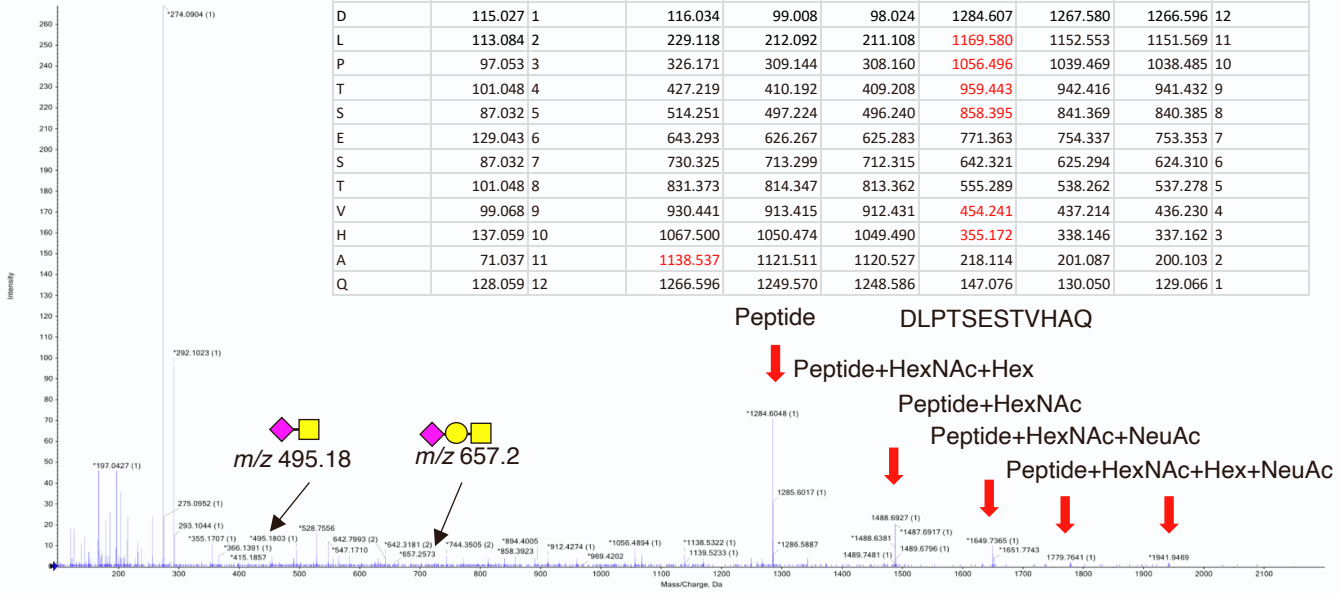
(A) Siglec-7-Fc is primarily weakly positive, as shown in flow cytometry of vector control HEK293T cells. Pink and cyan lines indicate vector control (mock) and ST8Sia6 expression vector-transfected cells, respectively. Transient expression of ST8Sia6 enhanced Siglec-7-binding to HEK293T.

(B) PDPN is a Siglec-7 ligand carrier protein especially in ST8Sia6-transfected HEK293T cells, as shown in immunoblotting (right).

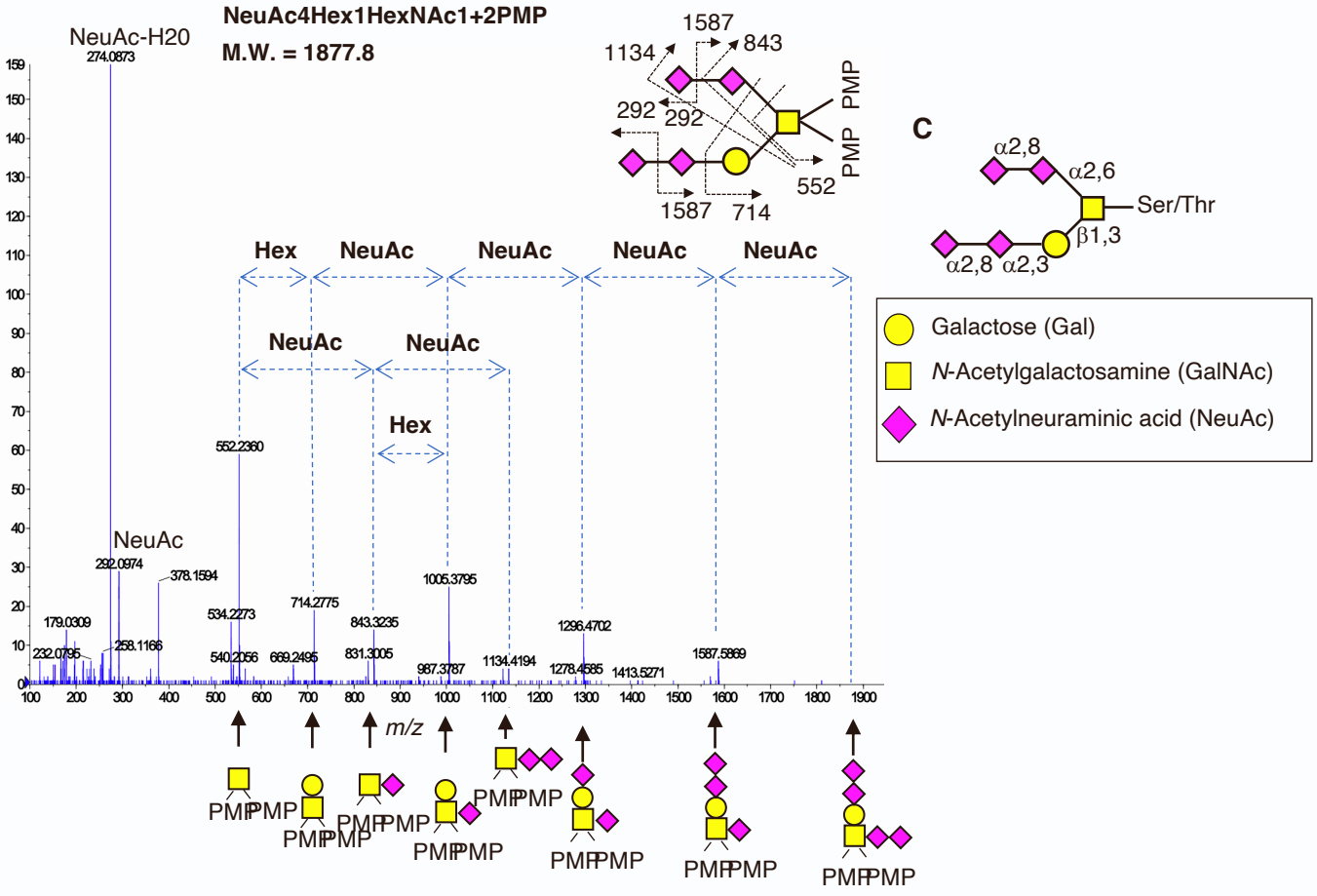
(C) A PDPN-Fc expression vector for recombinant fusion proteins was used for the transfection into HEK293T under three conditions. Lane 1: PDPN-Fc cDNA only. Lane 2: Co-transfected with ST3Gal1 and ST6GalNAc3 cDNAs. Lane 3: Co-transfected with ST3Gal1, ST6GalNAc3, and ST8Sia6 cDNAs. The PDPN-Fc proteins were purified using a protein A beads column. Five μg PDPN-Fc(s) were subjected to SDS-PAGE and stained using Coomassie brilliant blue (CBB). * indicates extra bands.

A Tandem mass spectrum of 938.710, 3+

Symbol	Res. Mass	# (N)	b	b - 17	b - 18	y	y - 17	y - 18	# (C)
D	115.027	1	116.034	99.008	98.024	1284.607	1267.580	1266.596	12
L	113.084	2	229.118	212.092	211.108	1169.580	1152.553	1151.569	11
P	97.053	3	326.171	309.144	308.160	1056.496	1039.469	1038.485	10
T	101.048	4	427.219	410.192	409.208	959.443	942.416	941.432	9
S	87.032	5	514.251	497.224	496.240	858.395	841.369	840.385	8
E	129.043	6	643.293	626.267	625.283	771.363	754.337	753.353	7
S	87.032	7	730.325	713.299	712.315	642.321	625.294	624.310	6
T	101.048	8	831.373	814.347	813.362	555.289	538.262	537.278	5
V	99.068	9	930.441	913.415	912.431	454.241	437.214	436.230	4
H	137.059	10	1067.500	1050.474	1049.490	355.172	338.146	337.162	3
A	71.037	11	1138.537	1121.511	1120.527	218.114	201.087	200.103	2
Q	128.059	12	1266.596	1249.570	1248.586	147.076	130.050	129.066	1



B Tandem mass spectrum of 939.9, 2+



C

Figure S9. Quadro-sialyl T was identified, related to Figure S6

(A) Precursor ion at m/z 938.710 (3+) corresponded to ^{82}DLPTSESTVHAQ^{93} of PDPN and $HexNAc_1Hex_1Neu5Ac_4$.

(B) MS/MS spectrum of released and labeled quadro-sialylated O-glycan. O-glycans were released by β-elimination and labeled with 1-phenyl-3-methyl-5-pyrazolone (PMP). Tandem mass spectrum of *m/z* 939.9 (2+) were analyzed using TOF MS/MS. However, this was present on a peptide other than the PLAG 3 domain of PDPN, since the Siglec-7 ligand glycan is present on the PLAG3 domain. The quadro-sialyl T has two tandem di-sialyl structures similar to di-sialyl ganglioside GD3.

(C) Predicted Glycan structure.