

Glycosylated extracellular matrix drives immune suppression by modulating macrophage-T cell crosstalk in triple-negative breast cancer

Tarantola, L^{1,a}, Tyler, EJ^{1,a}, Liu, Y^{1,a}, Maniati, E¹, Thornton, KA¹, Martín-Otal, C¹, Hanna, D¹, Kumar, R¹, Gauthier, V¹, Hirani, P¹, Burger Ramos, M¹, Roth, NJ¹, Bragg, J¹, Puttock, EH¹, McDermott, J¹, Rajeeve, V¹, Cutillas, P¹, Maiques, O¹, Soulier, A², Correa de Sampaio, P², Jones, JL¹, Davies, DM³, Maher, J³, Haslam, SM⁴, Läubli, H⁵ & Pearce, OMT^{*1}.

¹Queen Mary University of London, Barts Cancer Institute, John Vane Science Centre, London EC1M 6BQ, UK.

²Neobe Therapeutics, Salisbury House, Station Road, Cambridge CB1 2LA, UK

³Leucid Bio, Guy's Hospital, Great Maze Pond, London SE1 9RT, UK.

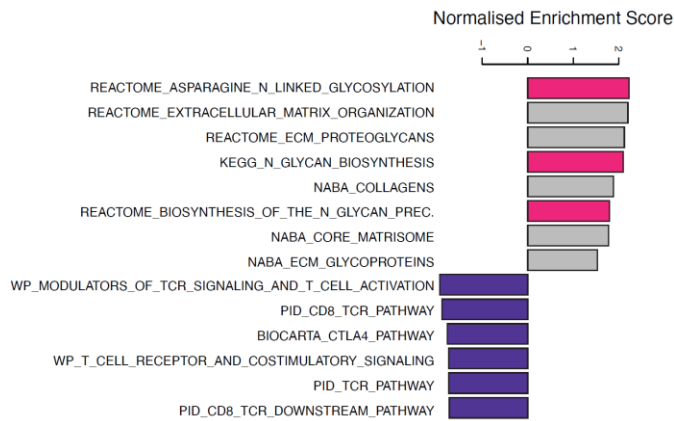
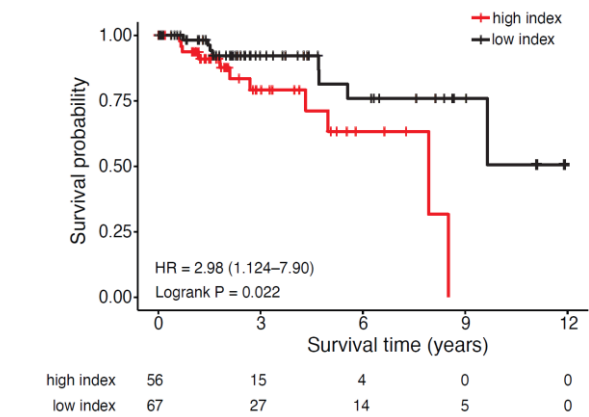
⁴Department of Life Sciences, Imperial College London, London, UK

⁵Department of Biomedicine and Division of Medical Oncology, University Hospital Basel, Hebelstrasse 20, 4031, Basel, Switzerland

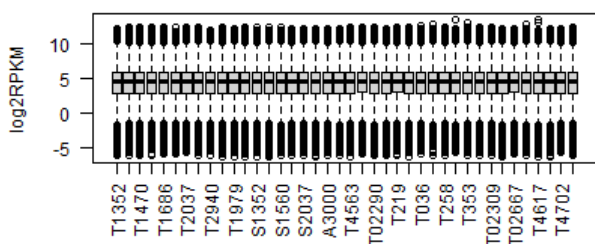
* Corresponding author: Pearce, O.M.T. (o.pearce@qmul.ac.uk)

^a These authors contributed equally: Tarantola, L.; Tyler, E.J.; Liu, Y.

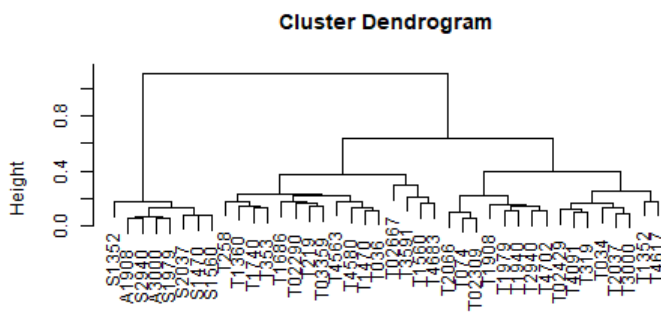
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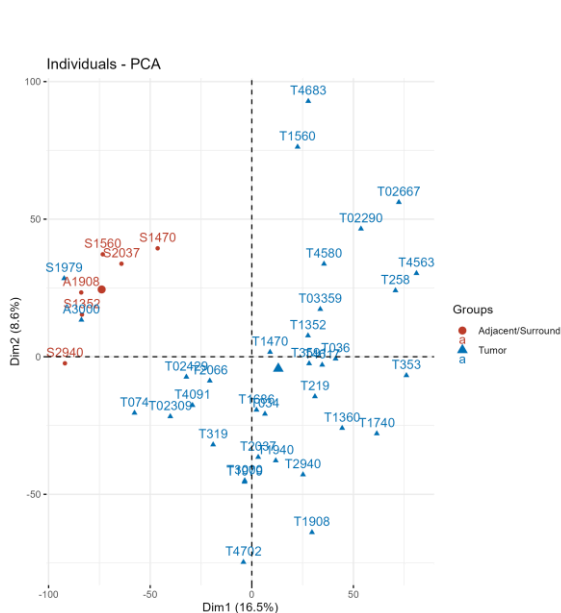
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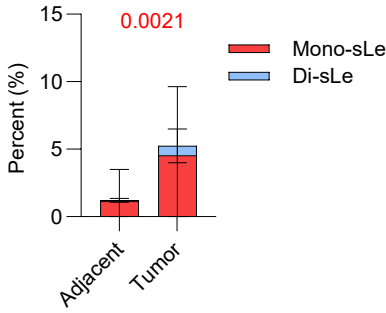
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Supplementary Fig. 1: Characterisation and transcriptomic analysis of TNBC patient tissues. **a**, Kaplan-Meier survival curve for matrix index (MI) high (56 patients) vs MI low (67 patients) from TCGA invasive breast cancer dataset. **b**, Differentially expressed genes between MI high and MI low patient samples are enriched for genes upregulated in ECM remodelling (grey bars) and post-translational glycosylation (pink bars) and genes downregulated in immunity (blue bars). **c**, Exploratory boxplot for sample quality. Boxplots illustrate median (centre of the box) with the upper (Q3: 75th percentile) and lower (Q1: 25th percentile) quartiles (ends of the box); the whiskers correspond to $Q3 + 1.5 \times \text{Interquartile Range (IQR)}$ to $Q1 - 1.5 \times \text{IQR}$; dots beyond the whiskers show potential outliers. **d**, Unsupervised cluster analysis using all genes. **e**, Principal Component Analysis (PCA) using all genes.

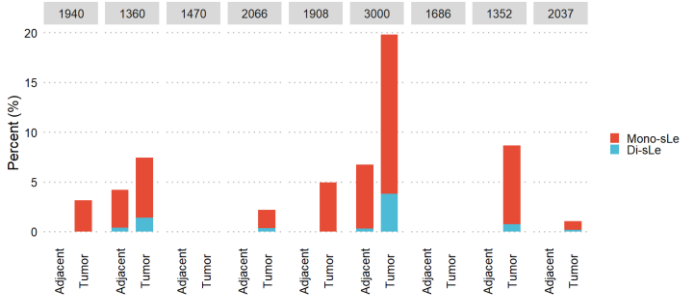
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N-linked Sialyl Lewis in Sialylation (ECM)



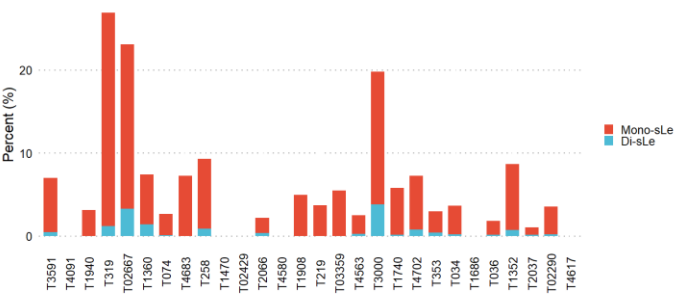
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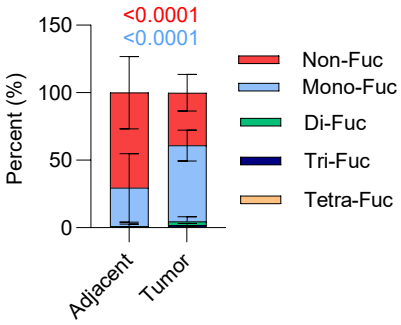
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N-linked Sialyl Lewis in Sialylation (ECM)



d

N-linked Fucosylation (ECM)



e

N-linked Fucosylation (ECM)



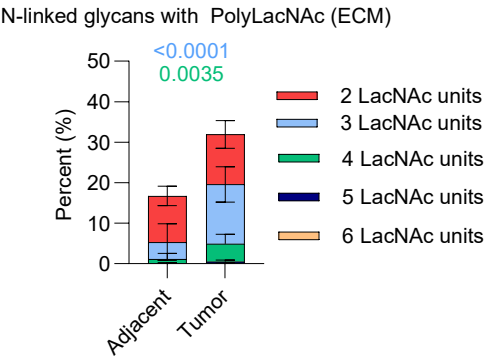
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N-linked Fucosylation (ECM)

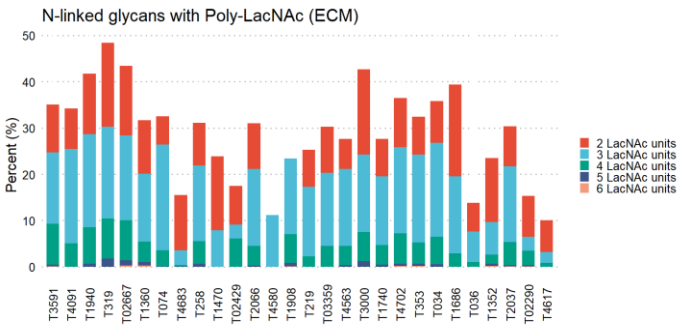


Supplementary Fig. 2: ECM N-linked glycomics analysis. ECM N-linked glycans assessed for **a-c**, Sialyl Lewis groups (Mono-sialyl lewis (sLe), red; Di-sLe, light blue), **d-f**, Fucosylation (Non-fucosylation (Fuc), red; Mono-Fuc, light blue; Di-Fuc, green; Tri-Fuc, dark blue; Tetra-Fuc, peach). **a, d**, average adjacent/surround and tumor samples, Mean with SD. Two-way Repeated Measures ANOVA with Šídák's multiple comparisons test, N=9 each. **b, e**, paired adjacent/surround and tumor samples, N=9 each. **c, f**, all tumor samples ranked by MI. N=28.

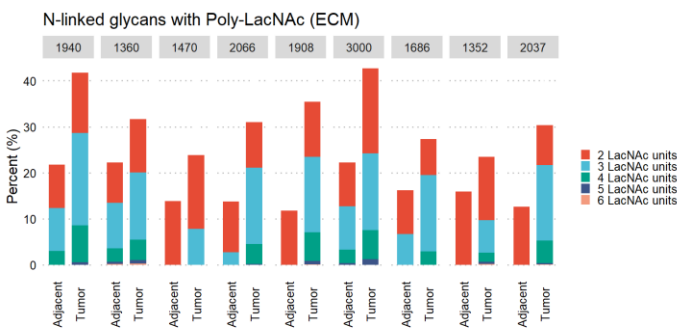
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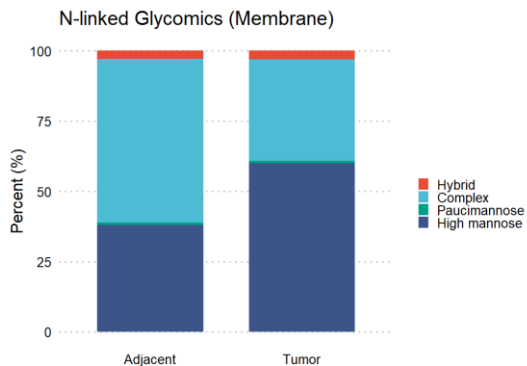


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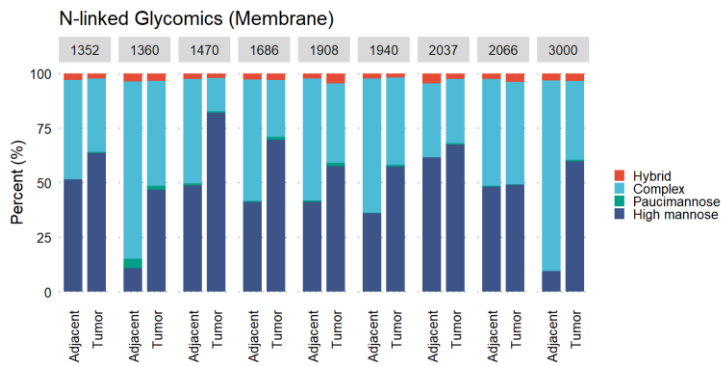


Supplementary Fig. 2: ECM N-linked glycomics analysis. ECM N-linked glycans assessed for **g-i**, Poly-LacNAc (2 LacNAc units, red; 3 LacNAc units, light blue; 4 LacNAc units, green; 5 LacNAc units, dark blue; 6 LacNAc units, peach) structures. **g**, average adjacent/surround and tumor samples, Mean with SD. Two-way Repeated Measures ANOVA with Šídák's multiple comparisons test, N=9 each. N=9 each. **h**, paired adjacent/surround and tumor samples, N=9 each. **i**, all tumor samples ranked by MI. N=28.

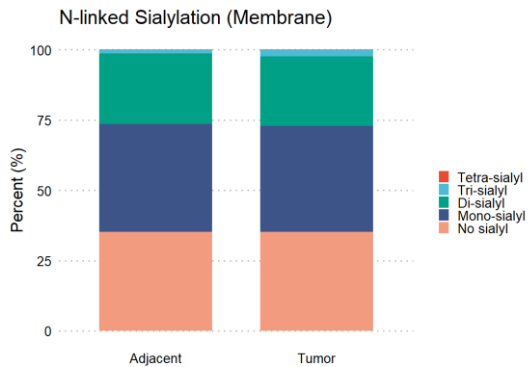
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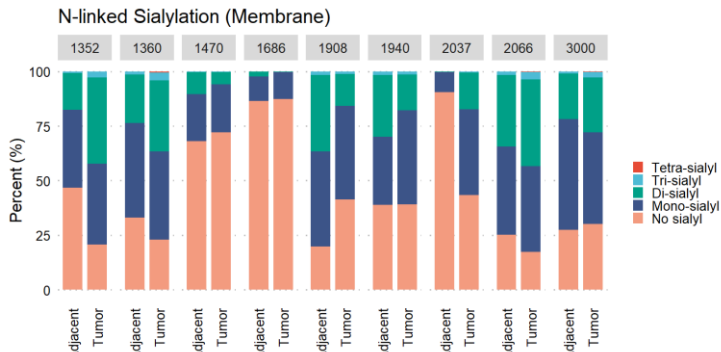
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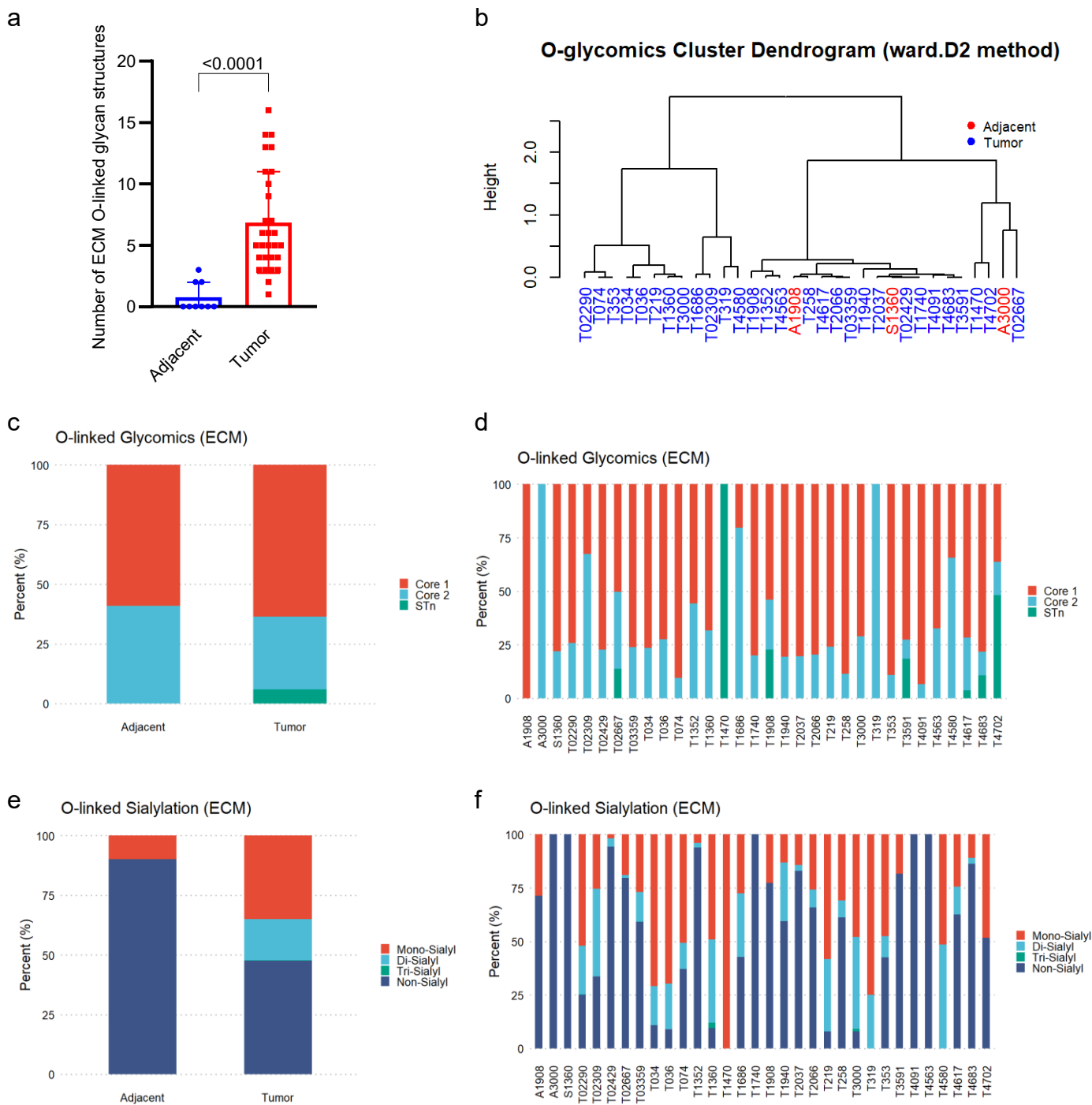
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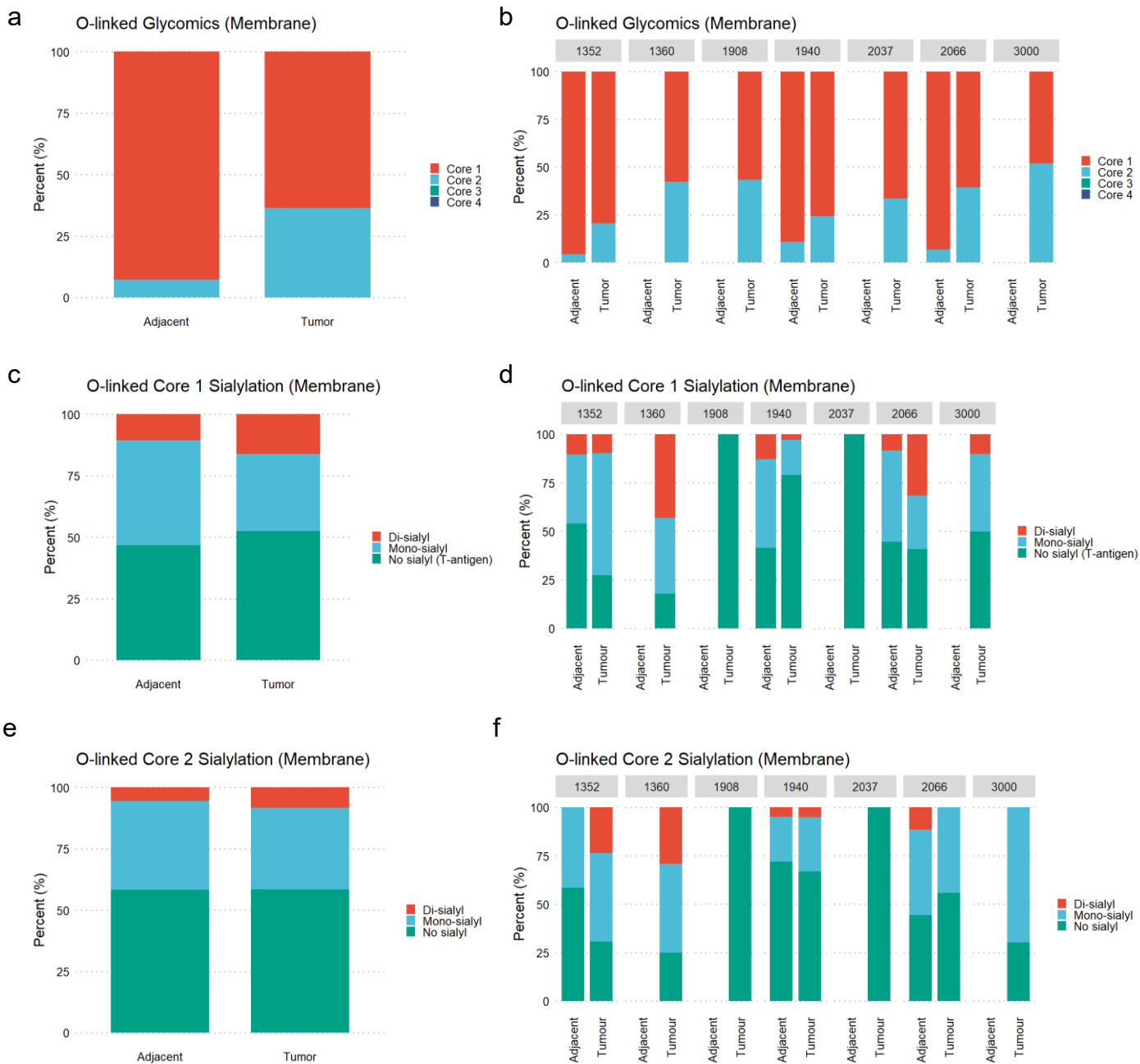
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Supplementary Fig. 3: Membrane N-linked glycomics analysis. **a-b**, Membrane N-linked glycans assigned to four main groups (Complex, dark blue; Hybrid, green; High-mannose, light blue; Paucimannose, red). Stacked barchart displaying the proportion of each family in the N-glycome for **a**, mean adjacent/surround and tumor samples and **b**, paired adjacent/surround samples and tumor samples. N=9 adjacent/surround tissues, N=9 tumor tissues. **c-d**, Membrane N-linked complex glycans broken down into five groups (Non-Sialyl, peach; Mono-Sialyl, dark blue; Di-Sialyl, green; Tri-Sialyl, light blue; Tetra-sialyl, red). Stacked barchart displaying the proportion of each family in the N-glycome **c**, mean adjacent/surround and tumor samples and **d**, paired adjacent/surround samples and tumor samples. N=9 adjacent/surround tissues, N=9 tumor tissues.



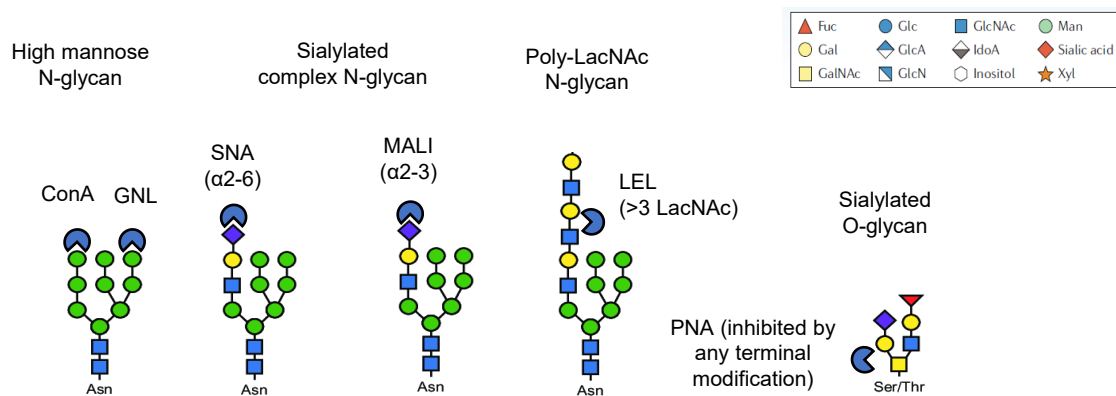
Supplementary Fig. 4: ECM O-linked glycomics analysis. **a**, Number of ECM O-linked glycan structures detected. Mean with SD. Mann Whitney test. N=9 for adjacent/surround ('adjacent'), N=29 for tumor tissues. **b**, Unsupervised cluster dendrogram using ECM O-linked glycan structures from adjacent/surround and tumor tissues. **c-d**, ECM O-linked glycans assigned to three main groups (Core 1, red; Core 2, light blue; STn, green). Stacked barchart displaying the proportion of each family in the O-glycome for **c**, mean adjacent/surround and tumor samples and **d**, all adjacent/surround samples and tumor samples. N=3 adjacent/surround tissues, N=29 tumor tissues. **e-f**, ECM O-linked complex glycans broken down into five groups (Non-Sialyl, dark blue; Mono-Sialyl, red; Di-Sialyl, light blue; Tri-Sialyl, green). Stacked barchart displaying the proportion of each family in the O-glycome **e**, mean adjacent/surround and tumor samples and **f**, all adjacent/surround samples and tumor samples. N=3 adjacent/surround tissues, N=29 tumor tissues.



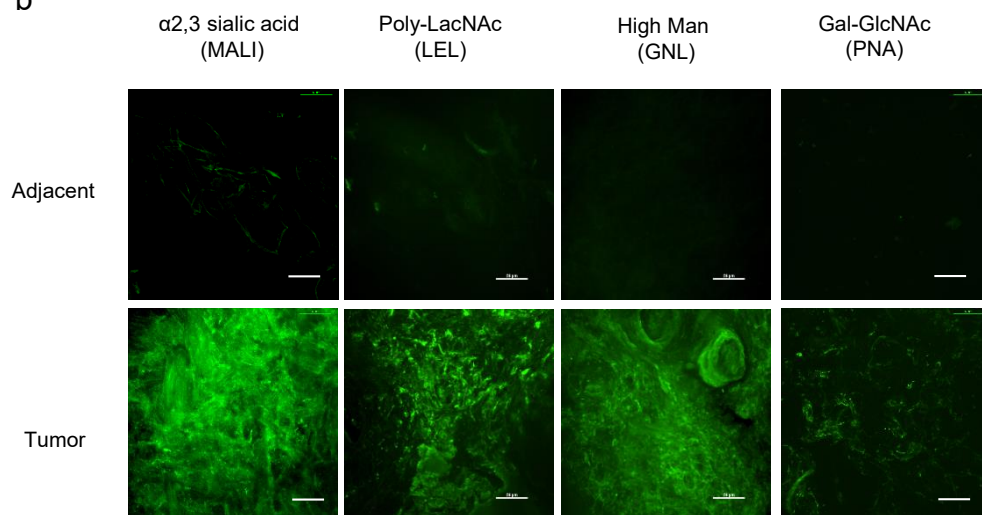
Supplementary Fig. 5: Membrane O-linked glycomics analysis. **a-b**, Membrane O-linked glycans assigned to four main groups (Core 1, red; Core 2, light blue; Core 3, green; Core 4, dark blue). Stacked barchart displaying the proportion of each family in the O-glycome for **a**, mean adjacent/surround and tumor samples and **b**, paired adjacent/surround samples and tumor samples. N=3 adjacent/surround tissues, N=7 tumor tissues. **c-d**, Membrane O-linked Core 1 glycans broken down into three groups (Di-sialyl, red; Mono-sialyl, blue; No Sialyl (T-antigen), green). Stacked barchart displaying the proportion of each family in the O-glycome **c**, mean adjacent/surround and tumor samples and **d**, paired adjacent/surround samples and tumor samples. N=3 adjacent/surround tissues, N=7 tumor tissues. **e-f**, Membrane O-linked Core 2 glycans broken down into three groups (Di-sialyl, red; Mono-sialyl, blue; No sialyl, green). Stacked barchart displaying the proportion of each family in the O-glycome **e**, mean adjacent/surround and tumor samples and **f**, paired adjacent/surround samples and tumor samples. N=3 adjacent/surround tissues, N=7 tumor tissues.

a

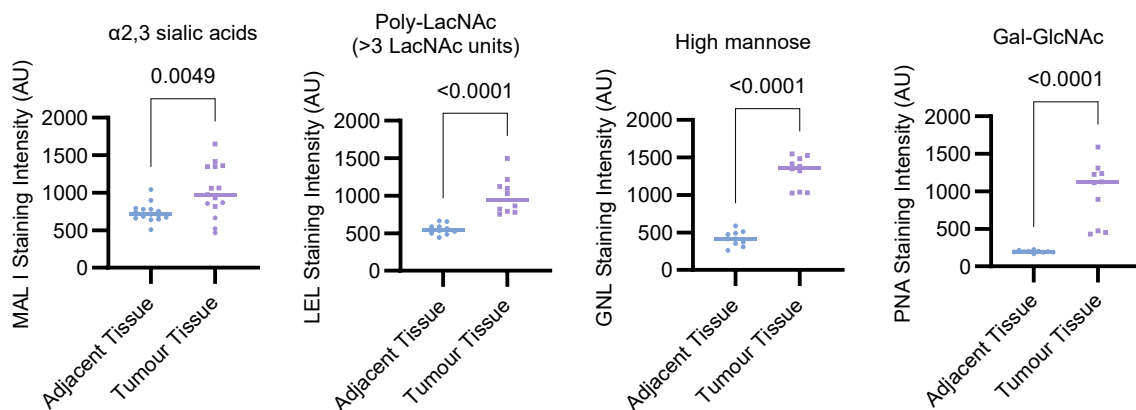
N-glycan, O-glycan & lectin



b

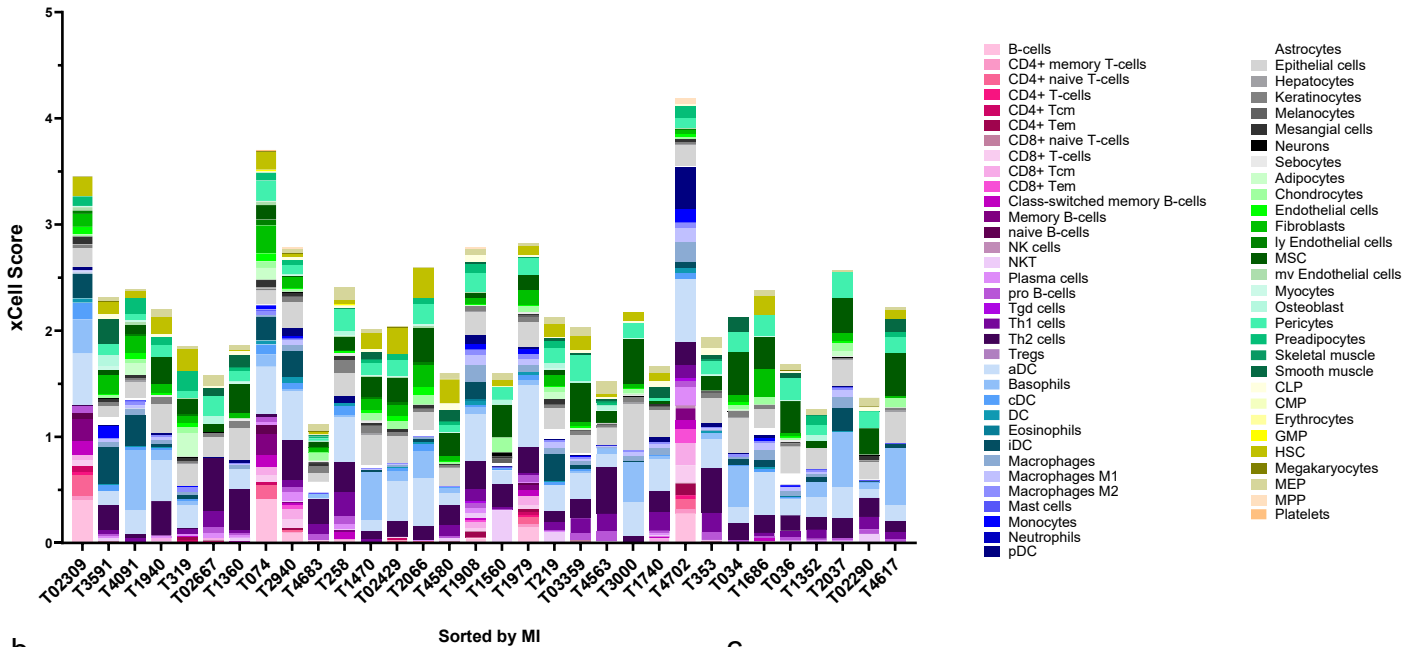


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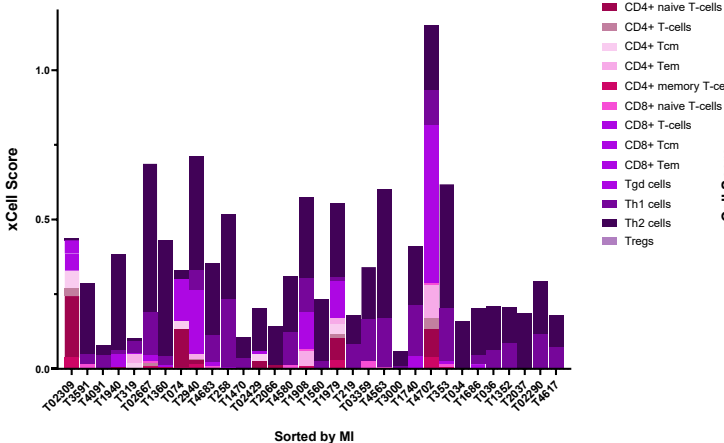


Supplementary Fig. 6: Lectin staining for N-linked and O-linked glycan structures in tumor and adjacent decellularized TNBC patient tissues. **a**, Schematic representing N-linked and O-linked glycan structures and their respective lectin binding partners. **b**, Representative images of matched adjacent and tumor TNBC patient tissue stained with Maackia Amurensis Lectin I (MALI), Fluorescein (FL-1311-2, 10 μ g/mL), Lycopersicon Esculentum (tomato) Lectin (LEL) Dylight 488 (DL-1174-1, 10 μ g/mL), Galanthus Nivalis Lectin (GNL) Fluorescein (FL-1241-2, 10 μ g/mL), and PNA From Arachis hypogaea (peanut), AlexaFluor 488 (L21409, 10 μ g/mL). Scale bar = 50 μ m. **c**, Quantitation of lectin staining intensity using IMARIS image analysis software. Median. Unpaired t test. N=2 matched patient tissues each, 5 FOV per tissue.

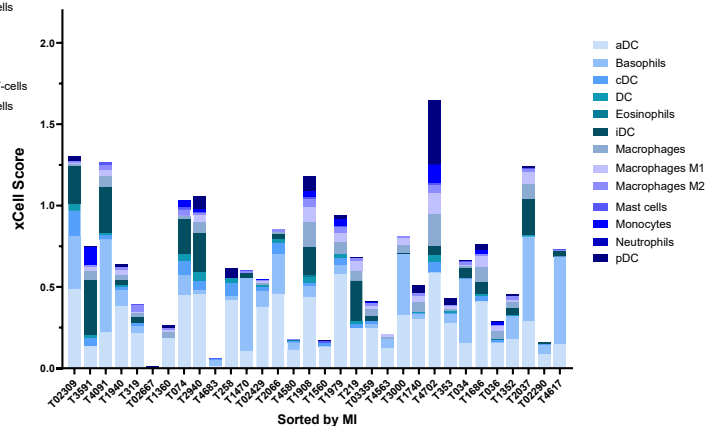
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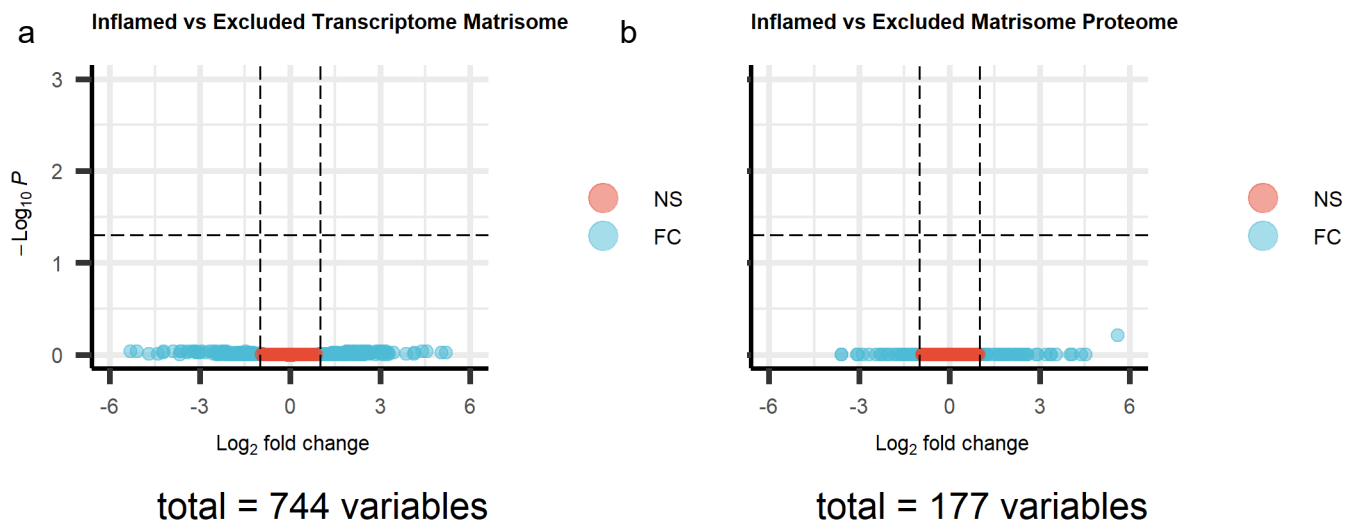
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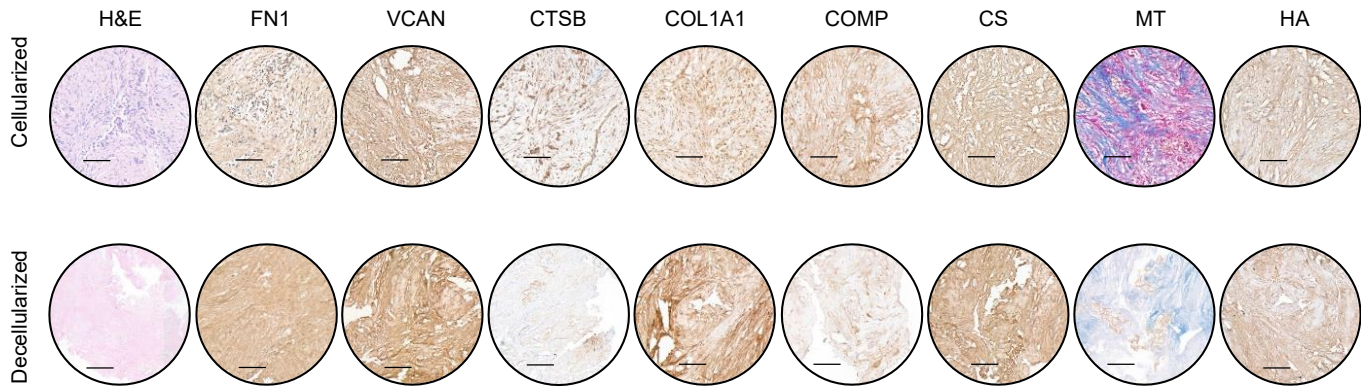


Supplementary Fig. 7: xCell deconvolution of TNBC tumor tissues. **a**, Stacked barchart of 64 xCell signatures for each patient tumor tissue. N=32. **b**, Stacked barchart of xCell lymphoid compartment signatures. N=32. **c**, Stacked barchart of xCell myeloid compartment signatures. N=32.

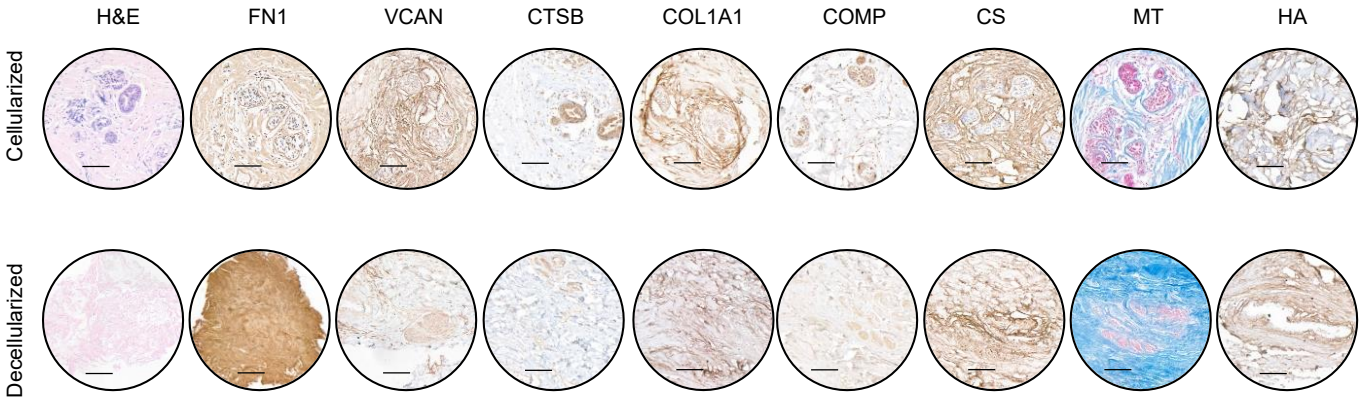


Supplementary Fig. 8: No changes in matrisome transcriptome or proteome between inflamed and excluded samples. **a**, Volcano plot of differentially regulated matrisome genes between inflamed and excluded tumor tissues. N=4 for excluded, N=3 for inflamed. **b**, Volcano plot of differentially regulated matrisome proteins between inflamed and excluded tumor tissues. N=4 for excluded, N=5 for inflamed.

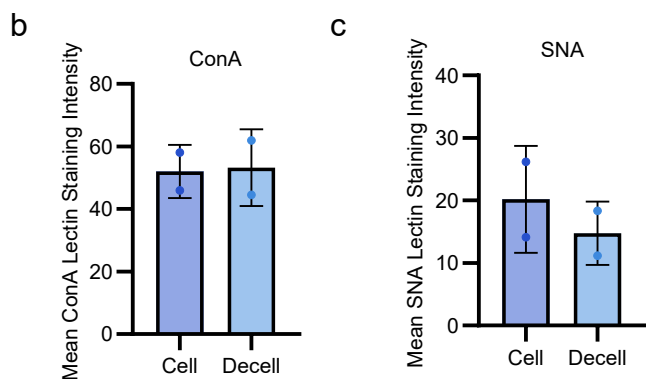
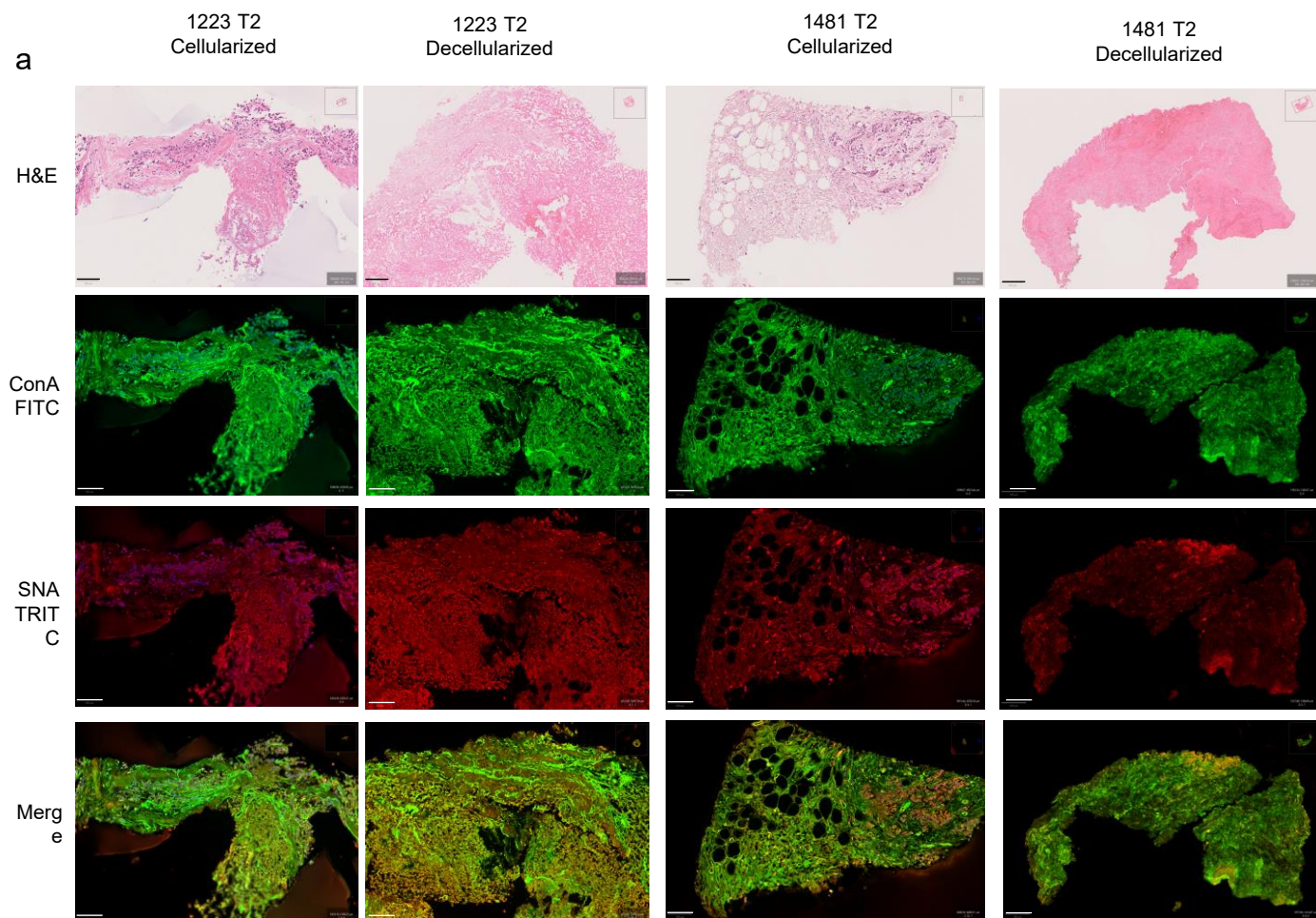
1691 Tumor



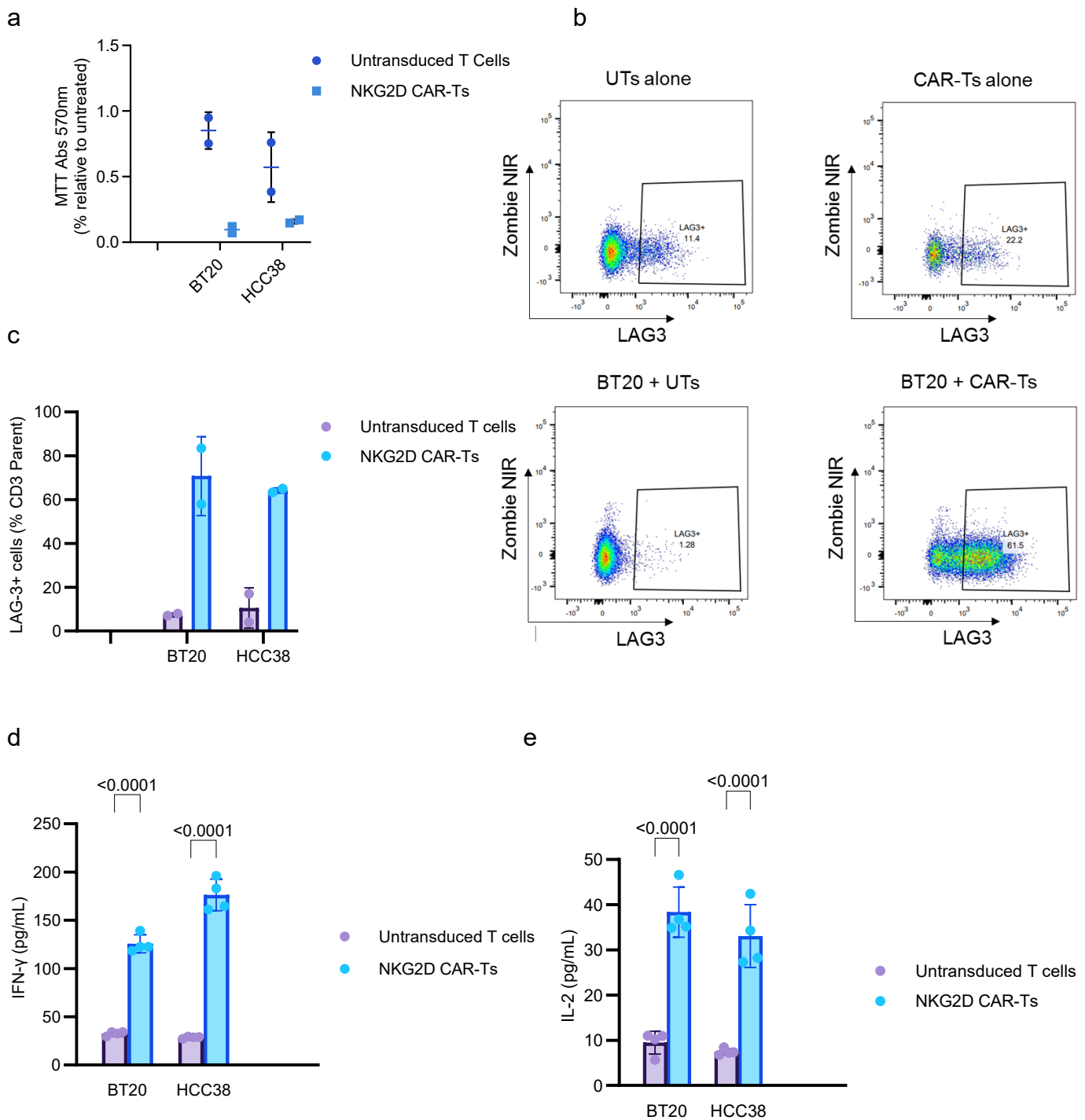
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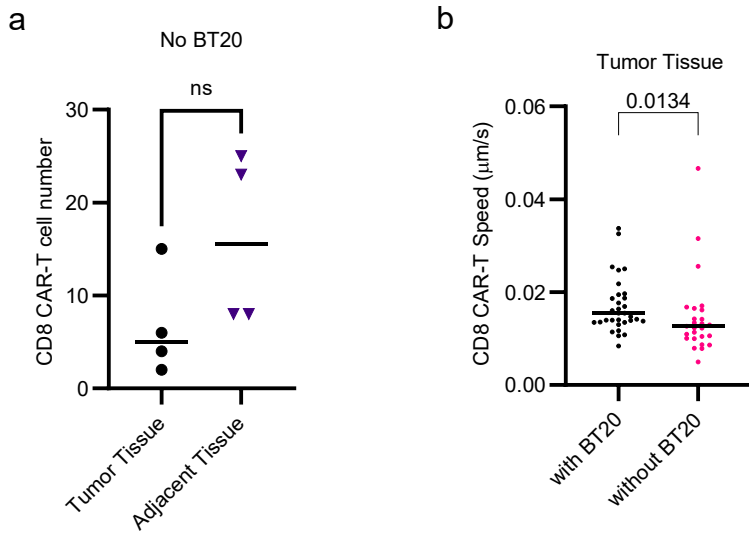
Supplementary Fig. 9: Representative sections of IHC staining for matrix molecules in tumor and surround cellularized and decellularized TNBC patient tissues. Stains included anti-FN1 (ab23750, 1:500), anti-VCAN (HPA004726, 1:200), anti-CTSB (ab58802, 1:400), anti-COL1A1 (HPA011795, 1:300), anti-COMP (ab11056, 1:80), anti-CS (ab11570, 1:600), Masson's Trichrome (MT), and anti-HA (385911, 1:100). Scale bar = 100µm.



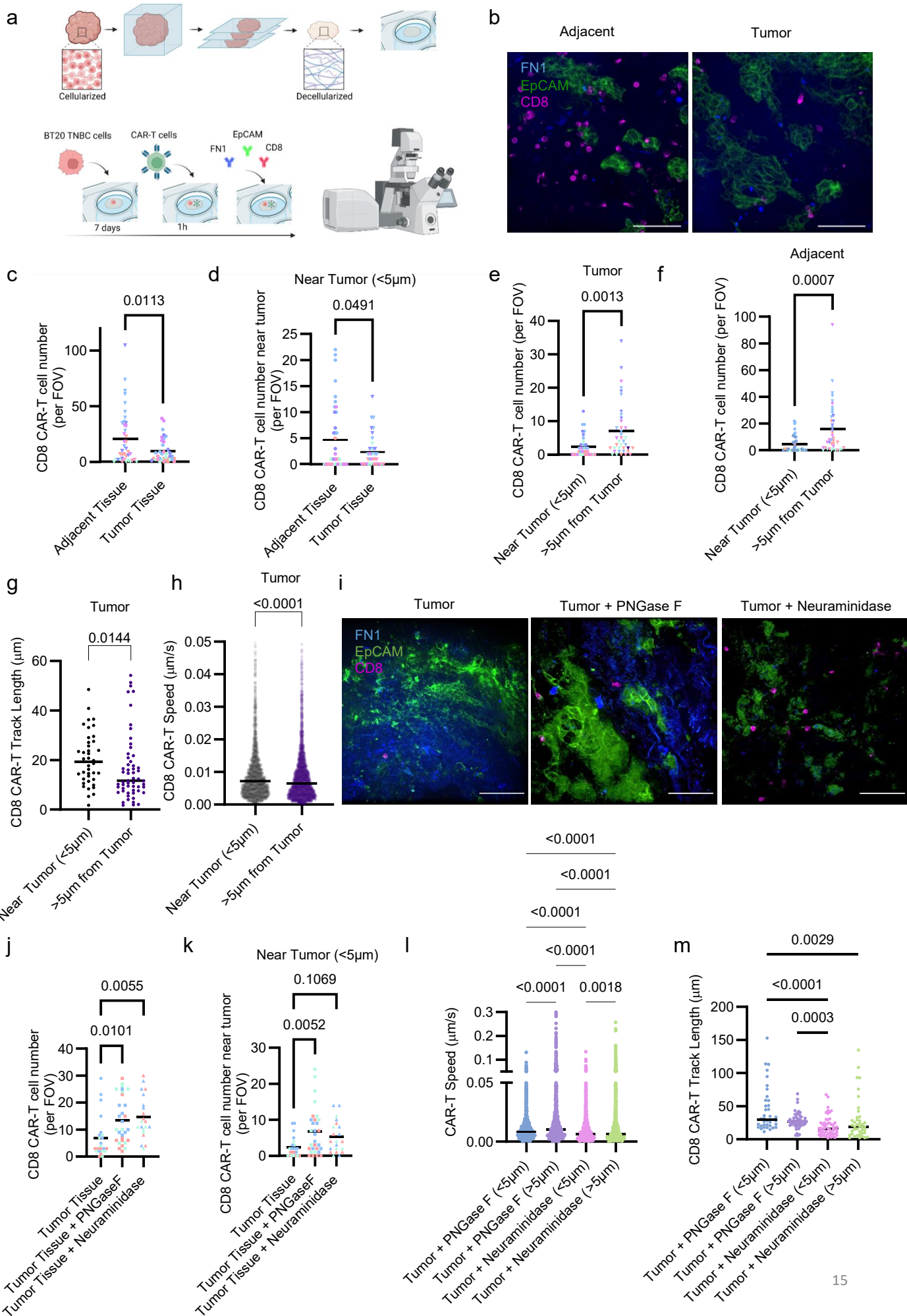
Supplementary Fig. 10: Lectin staining confirms that decellularization does not affect N-linked and sialylated glycan structures in tumor and adjacent TNBC patient tissues. a, Representative images of cellularized or decellularized adjacent or tumor tissue. H&E staining in the top row, staining of a consecutive section with Concavalin A (ConA) lectin conjugated to AF488 for all N-linked glycans in the second row (green) and Sambucus nigra (SNA) lectin conjugated to Cy3 for sialic acids in the third row (red). Scale bar = 100µm. **b-c,** Analysis of **b**, ConA-AF488 and **c**, SNA-Cy3 lectin staining intensity using QuPath. N=2 matched tissue donors for each group, paired T test.



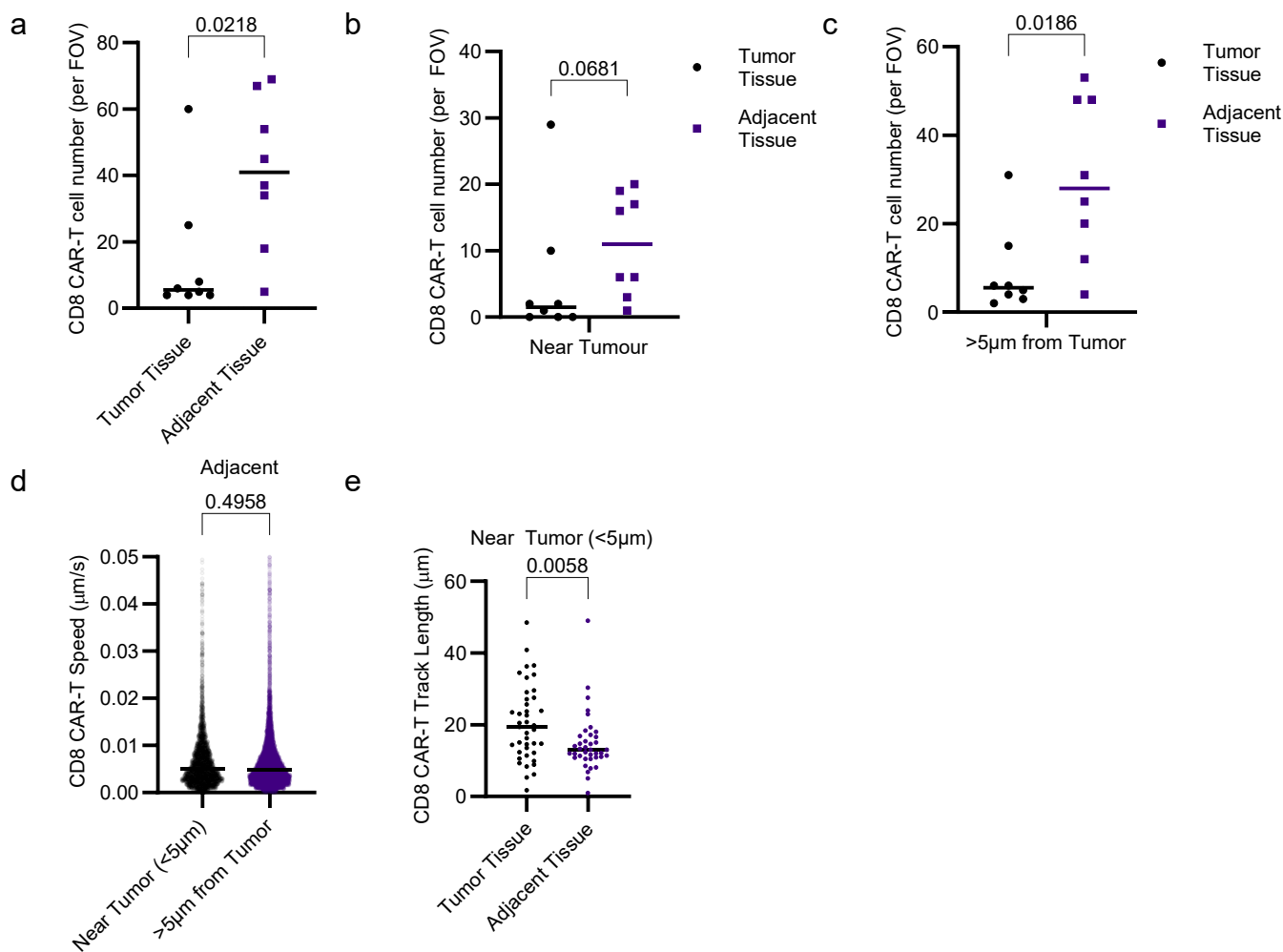
Supplementary Fig. 11: CAR-T targeting of TNBC cell lines in 2D. **a**, MTT assay with three TNBC cell lines, MDA-MB-468, BT20 and HCC38 co-cultured with untransduced T cells or CAR-Ts targeting NKG2D. Mean with SD. Two-way ANOVA with Šídák's multiple comparisons test. N=2. **b**, Representative flow cytometry plots. **c**, Bar plots of flow cytometry expression patterns of LAG3 expression, shown as percentage of CD3⁺ parent cells. Mean with SD. Two-way ANOVA with Šídák's multiple comparisons test. N=2. **d-e**, ELISA was performed to detect secretion of **d**, IFN- γ and **e**, IL-2 by NKG2D-targeting CAR-Ts. Mean with SD. Two-way ANOVA with Šídák's multiple comparisons test. N=4.



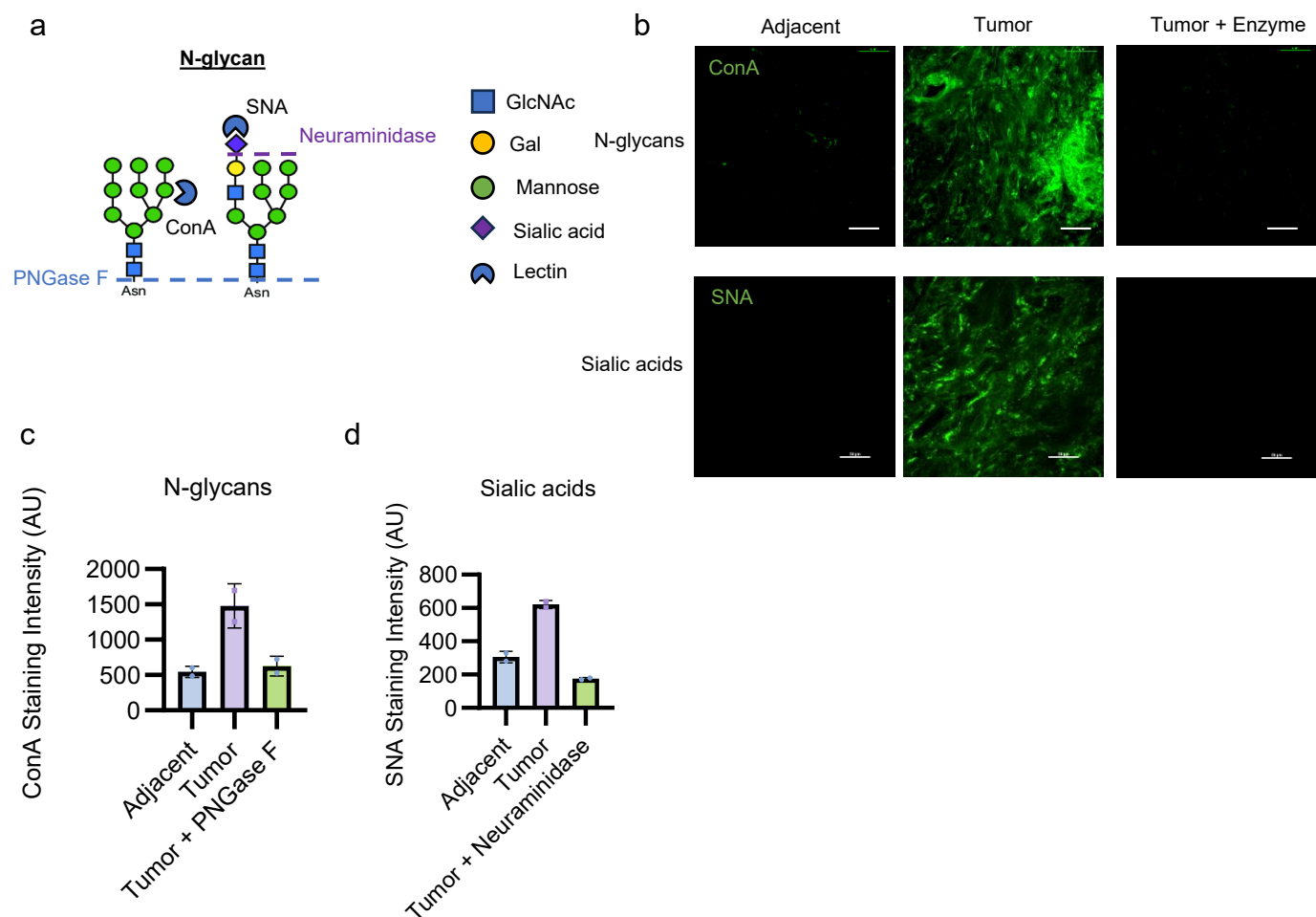
Supplementary Fig. 12: Decellularized tissue timelapse video analysis. a-b, Timelapse confocal microscopy of NKG2D-targeting CAR-Ts in decellularized tissues which were not seeded with BT20 TNBC cells. **a,** Mean CD8 NKG2D-targeting CAR-T cell number per field of view (FOV). Line at median. Unpaired t test. N=4. **b,** Mean speed of CD8+ CAR-Ts. Line at median. Mann Whitney test. N=4.



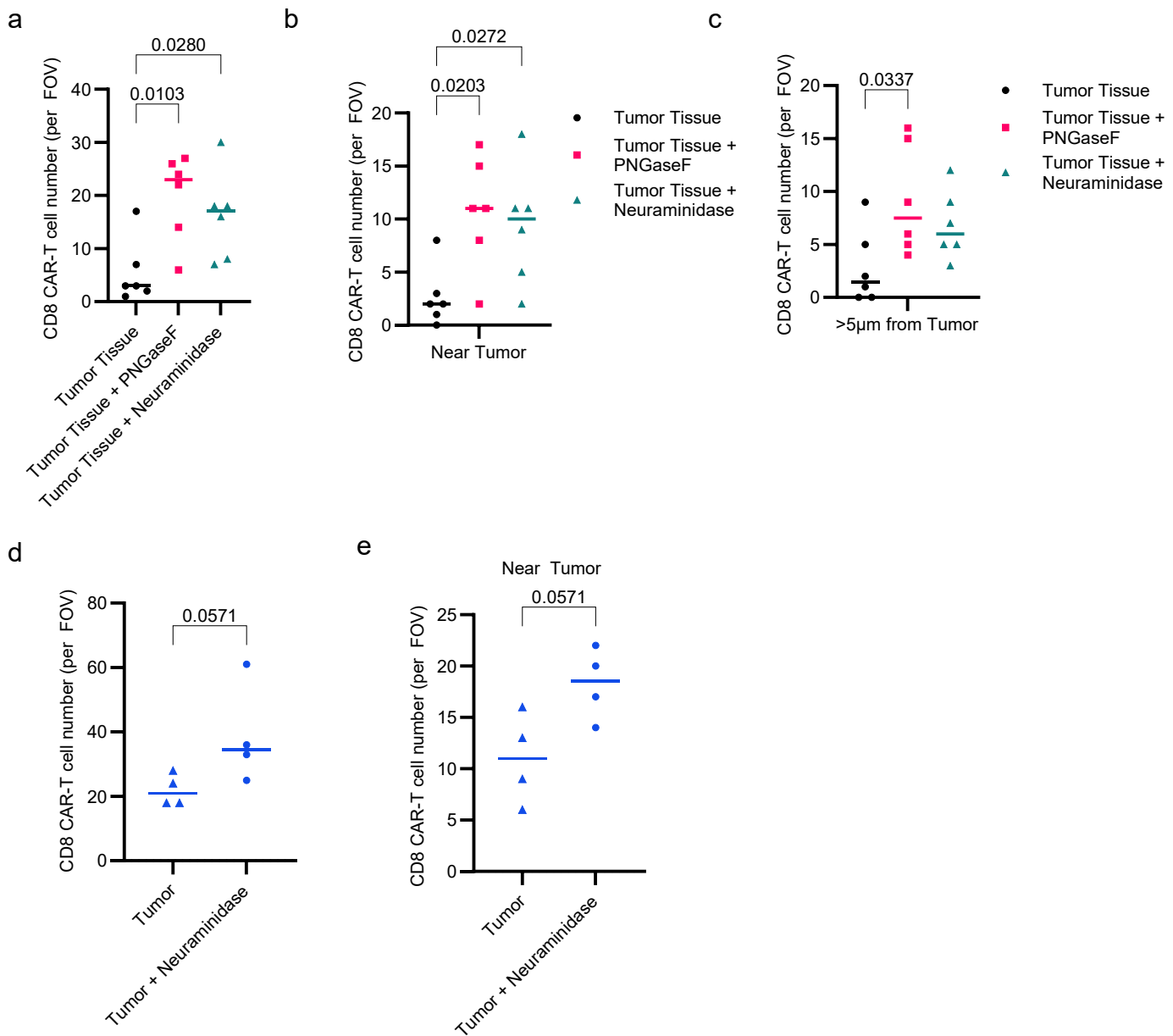
Supplementary Fig. 13: Decellularized TNBC tissue maintains an immune excluded TME in vitro and modifications to ECM-glycans can convert the TME to an immune inflamed phenotype. **a**, Schematic of fluorescence-based live confocal imaging assay using decellularized tissue seeded with BT20 TNBC cells and NKG2D-targeting CAR-T co-culture. Tissues stained with anti-human CD326 (EpCAM) AlexaFluor (AF) 488 (53-8326-42, 1:10) anti-human CD8-AF647 (344725, 1:10) and anti-human fibronectin conjugated to Dylight 405 (F3648, 1:10). Created in BioRender. Tyler, E. (2026) <https://BioRender.com/f72b95i>. **b**, Representative images of adjacent and tumor decellularized tissue recellularized with BT20 TNBC cells (anti-EpCAM-AF488, green) and CAR-T cells (anti-CD8-AF647, purple) with the extracellular matrix stained with anti-FN1-AF405, blue. Scale bar = 100µm. **c-d**, Count of **c**, CD8 CAR-T cell number in each field of view (FOV) and **d**, CD8 CAR-T cell number within 5µm of a tumor cell ('near tumor') and **e-f**, CD8 CAR-T cell number near (>5µm) and far (>5µm) from a tumor cell in **e**, tumor and **f**, adjacent decellularized tissue recellularized with BT20 cells. N=6 tissue donors for each group, 5-10 FOV per tissue. Unpaired T-test. **g**, CD8+ CAR-T cell speed (µm/s) near (<5µm) and far (>5µm) from tumor cells in tumor tissue. **h**, CD8+ CAR-T cell track length (µm) near (<5µm) and far (>5µm) from tumor cells in tumor tissue. **e-h**, Line at median. Mann-Whitney test. N=4, with 2 technical replicates each. **i**, Representative images of tumor control tissue and tumor tissue treated with either PNGase F or neuraminidase enzyme treatment, and recellularized with BT20 TNBC cells (anti-EpCAM-AF488, green), and CAR-T cells (anti-CD8-AF647, purple). Extracellular matrix stained with anti-FN1-AF405 (blue). Scale bar = 100µm. **j-m**, Count of **j**, CD8 CAR-T cell number in each FOV and **k**, CD8 CAR-T cell number within 5µm of a tumor cell ('near tumor'). N=3 tissue donors, 6-10 FOV per tissue. One-way ANOVA with Dunnett's multiple comparison's test. **l**, CD8+ CAR-T cell speed (µm/s) near tumor cells. **m**, CD8+ CAR-T cell track length (µm) near tumor cells. **j-m**, Line at median. Kruskal-Wallis test with Dunn's multiple comparisons test. N=3, with 2 technical replicates each.



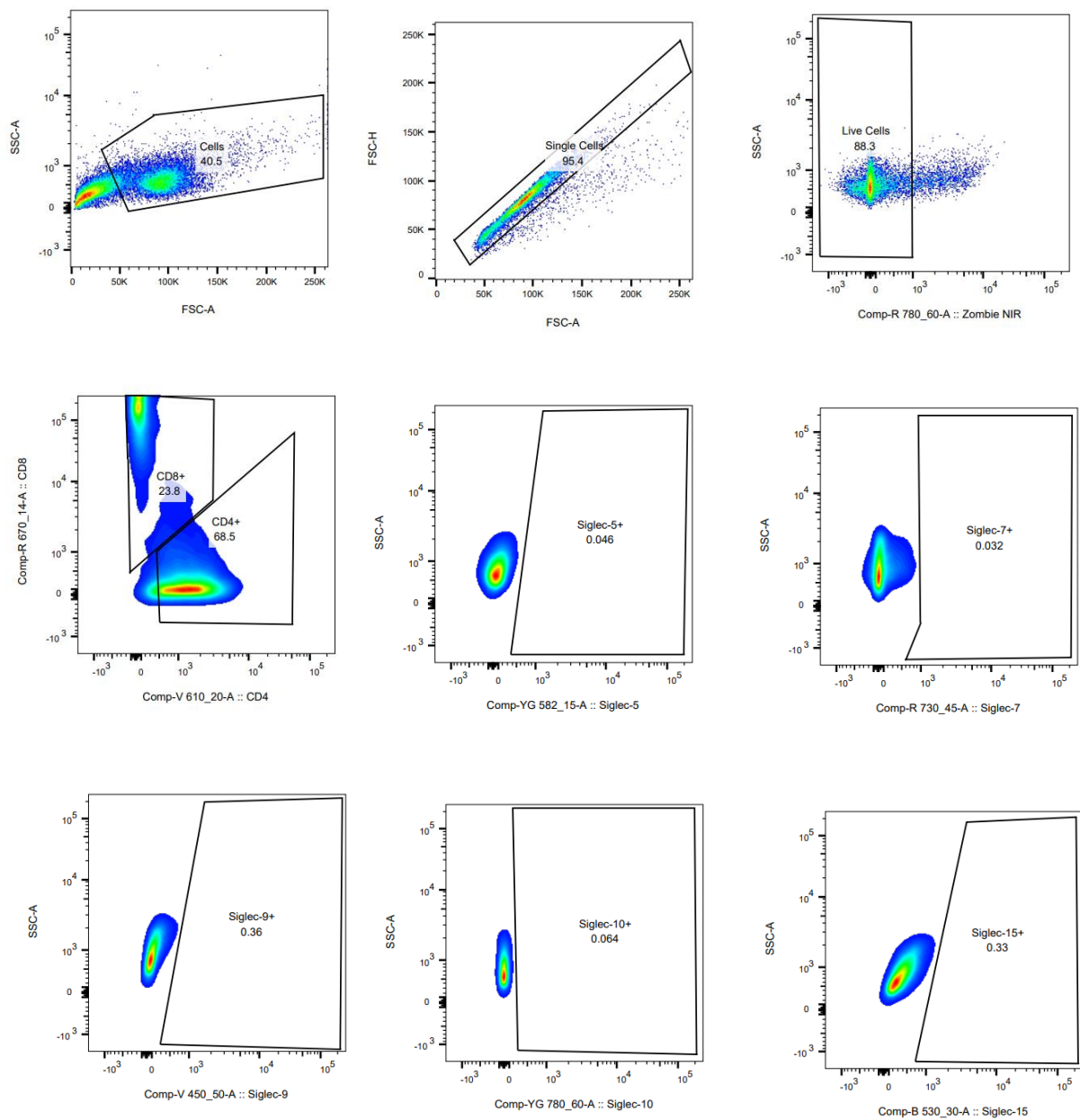
Supplementary Fig. 14: Decellularized tissue timelapse video analysis. Fluorescence-based live confocal timelapse imaging assay using decellularized tissue seeded with BT20 TNBC cells and NKG2D-targeting CAR-T co-culture. Tissues stained with anti-human CD326 (EpCAM) AlexaFluor (AF) 488 (53-8326-42, 1:10) anti-human CD8-AF647 (344725, 1:10) and anti-human fibronectin conjugated to Dylight 405 (F3648, 1:10). Video analysis performed using IMARIS analysis software. **a**, CD8+ CAR-T cell number per field of view (FOV) in tumor and adjacent tissue. **b**, CD8+ CAR-T cell number per field of view (FOV) near EpCAM+ tumor cells (<5μm) in tumor and adjacent tissue. **c**, CD8+ CAR-T cell number per field of view (FOV) >5μm from tumor cells in tumor and adjacent tissue. **d**, CD8+ CAR-T cell speed (μm/s) >5μm from tumor cells and **e**, CD8+ CAR-T cell track length (μm) >5μm from tumor cells in adjacent tissue. **a-e**, Line at median. Mann-Whitney test. N=4, with 2 technical replicates each.



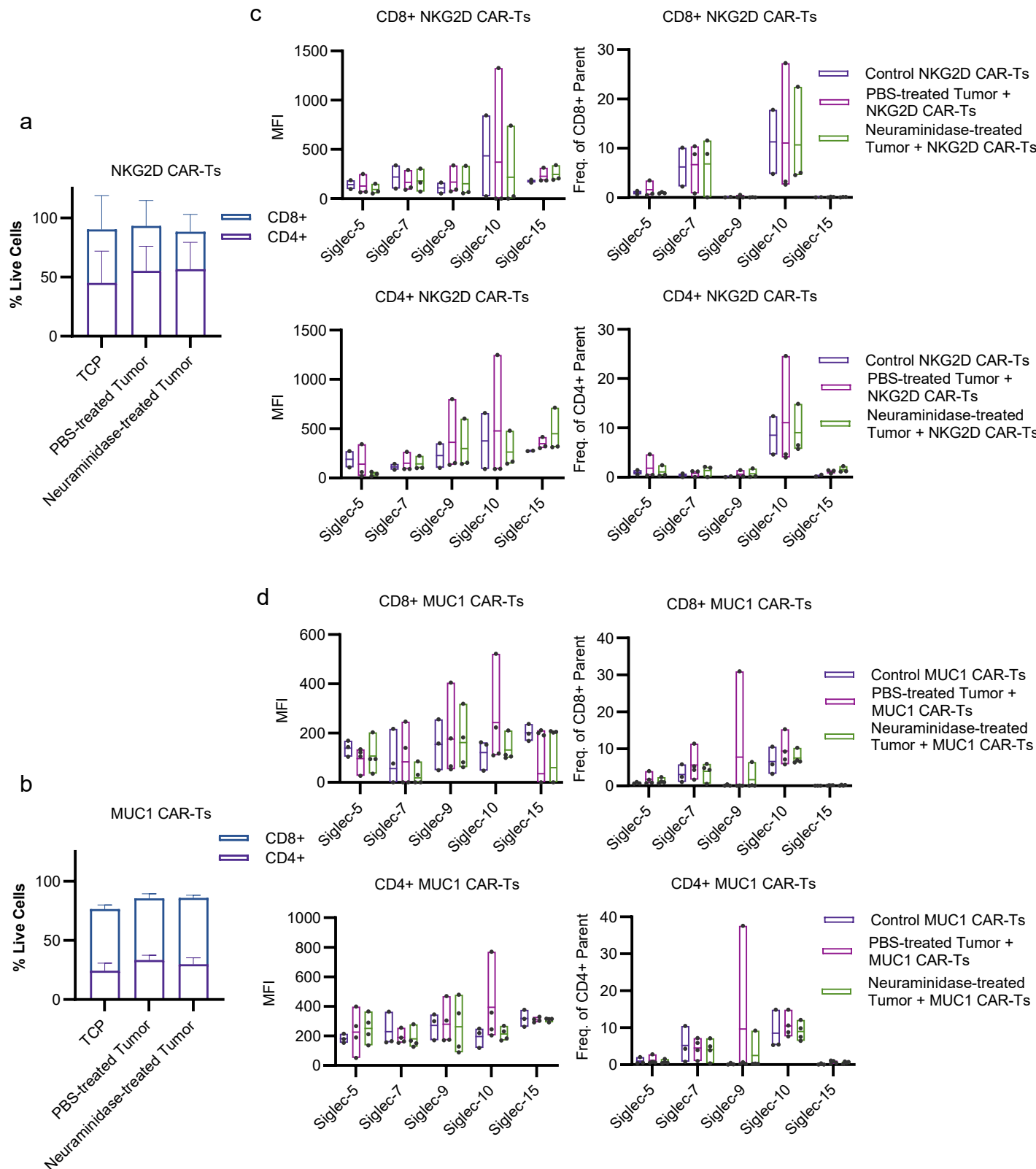
Supplementary Fig. 15: Lectin staining for N-linked and sialylated glycan structures in tumor and adjacent TNBC patient tissues and their cleavage using PNGase F and neuraminidase. a, Schematic of N-linked glycan enzymatic modifications and their respective lectin binding partners for staining. **b**, Representative images of adjacent, tumor, and tumor treated with PNGase F or neuraminidase. Staining with Concavalin A (ConA) lectin conjugated to AF488 for all N-linked glycans in the top row (green) and Sambucus nigra (SNA) lectin conjugated to AF488 for sialic acids in the bottom row (green). Scale bar = 100µm. **c-d**, Analysis of **c**, ConA-AF488 and **d**, SNA-AF488 lectin staining intensity using IMARIS image analysis software. N=2 matched tissue donors for each group, 5 FOV per tissue, One-way ANOVA with Tukey's post-hoc test.



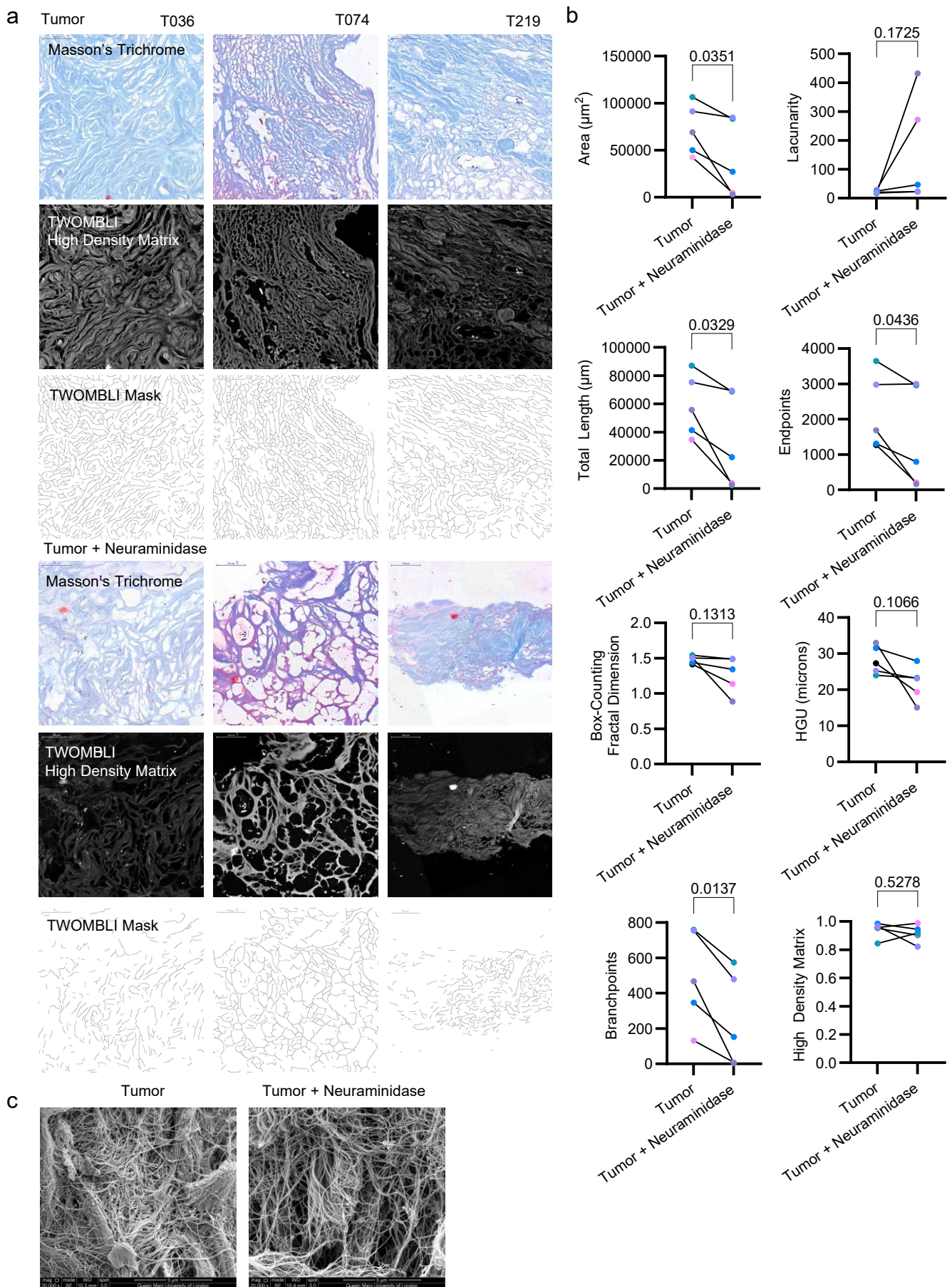
Supplementary Fig. 16: Decellularized tissue timelapse video analysis. Fluorescence-based live confocal timelapse imaging assay using decellularized tumor tissue treated with either PNGase F or neuraminidase enzyme treatment, seeded with BT20 TNBC cells and NKG2D-targeting CAR-T co-culture. Tissues stained with anti-human CD326 (EpCAM) AlexaFluor (AF) 488 (53-8326-42, 1:10) anti-human CD8-AF647 (344725, 1:10) and anti-human fibronectin conjugated to Dylight 405 (F3648, 1:10). Video analysis performed using IMARIS analysis software. **a**, CD8+ CAR-T cell number per field of view (FOV). **b**, CD8+ CAR-T cell number per field of view (FOV) near EpCAM+ tumor cells (<5 μ m). **c**, CD8+ CAR-T cell number per field of view (FOV) >5 μ m from tumor cells. **a-c**, Line at median. One-way ANOVA with Holm-Šidák's multiple comparisons test. N=3, with 2 technical replicates each. **d-e**, MUC1-targeting CAR-T added to BT20-seeded decellularized tumor tissue. **d**, Mean CD8 MUC1-targeting CAR-T cell number per FOV. Line at median. Mann Whitney test. N=4. **e**, Mean CD8 MUC1-targeting CAR-T cell number near tumor (<5 μ m) per FOV. Line at median. Mann Whitney test. N=4.

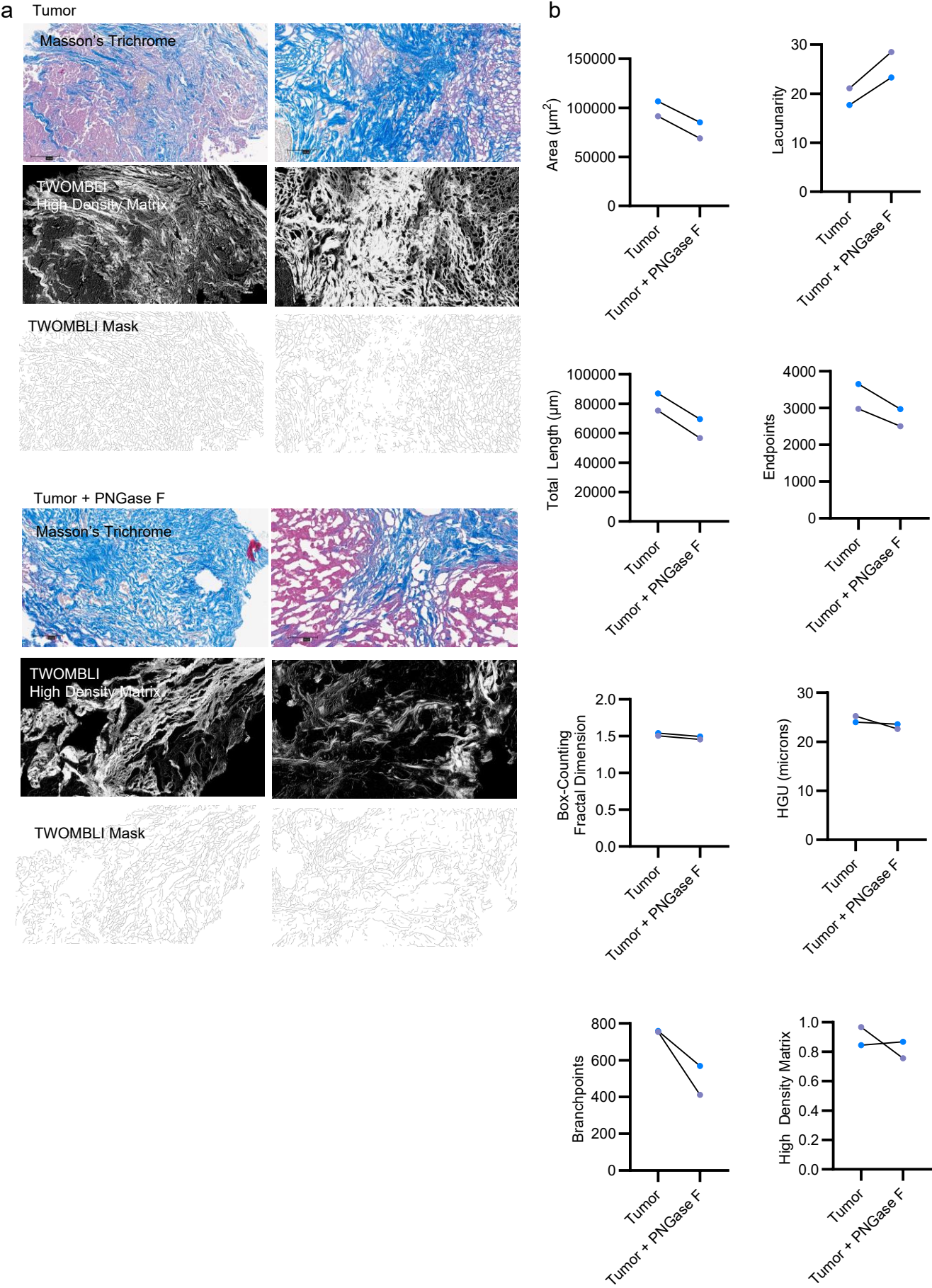


Supplementary Fig. 17: Flow gating strategy and fluorescence minus one (FMO) controls for CAR-T cell Siglec flow cytometry panel. CD4+ and CD8+ CAR-T cells assessed for Siglec expression using flow cytometry after five days. Cells were either cultured alone or co-cultured for five hours with BT20-seeded decellularized tumor tissue or tumor tissue treated with neuraminidase then transferred to a tissue culture plate for five days. N=3.

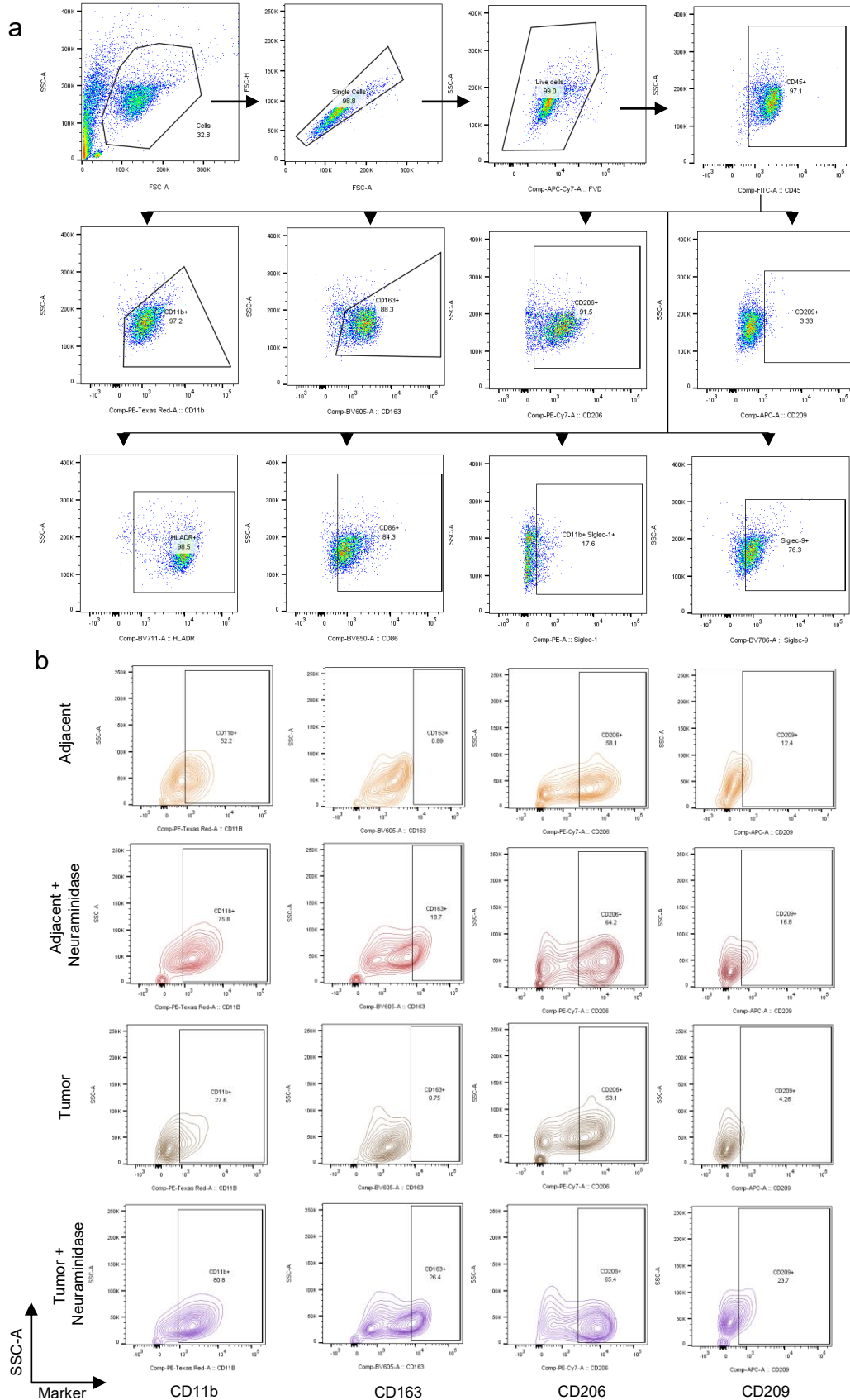


Supplementary Fig. 18: Neuraminidase treatment does not affect Siglec expression on CAR-Ts. **a-b**, Bar plots of flow cytometry expression patterns of CD8 and CD4 expression, shown as percentage of live cell population. **a**, NKG2D-targeting or **b**, MUC-1-targeting CAR-Ts cultured on tissue culture plastic (TCP), PBS-treated TNBC decellularized tumor tissue or neuraminidase-treated tumor tissue after five days. Mean with SD. Two-way ANOVA with Šídák's multiple comparisons test. N=3. **c-d**, Box plots of flow cytometry expression for a panel of Siglec receptors (Siglec-5, Siglec-7, Siglec-9, and Siglec-10) for **c**, NKG2D-targeting or **d**, MUC-1 targeting CAR-Ts. Line at median. Two-way ANOVA with Tukey's multiple comparisons test. N=2-4.

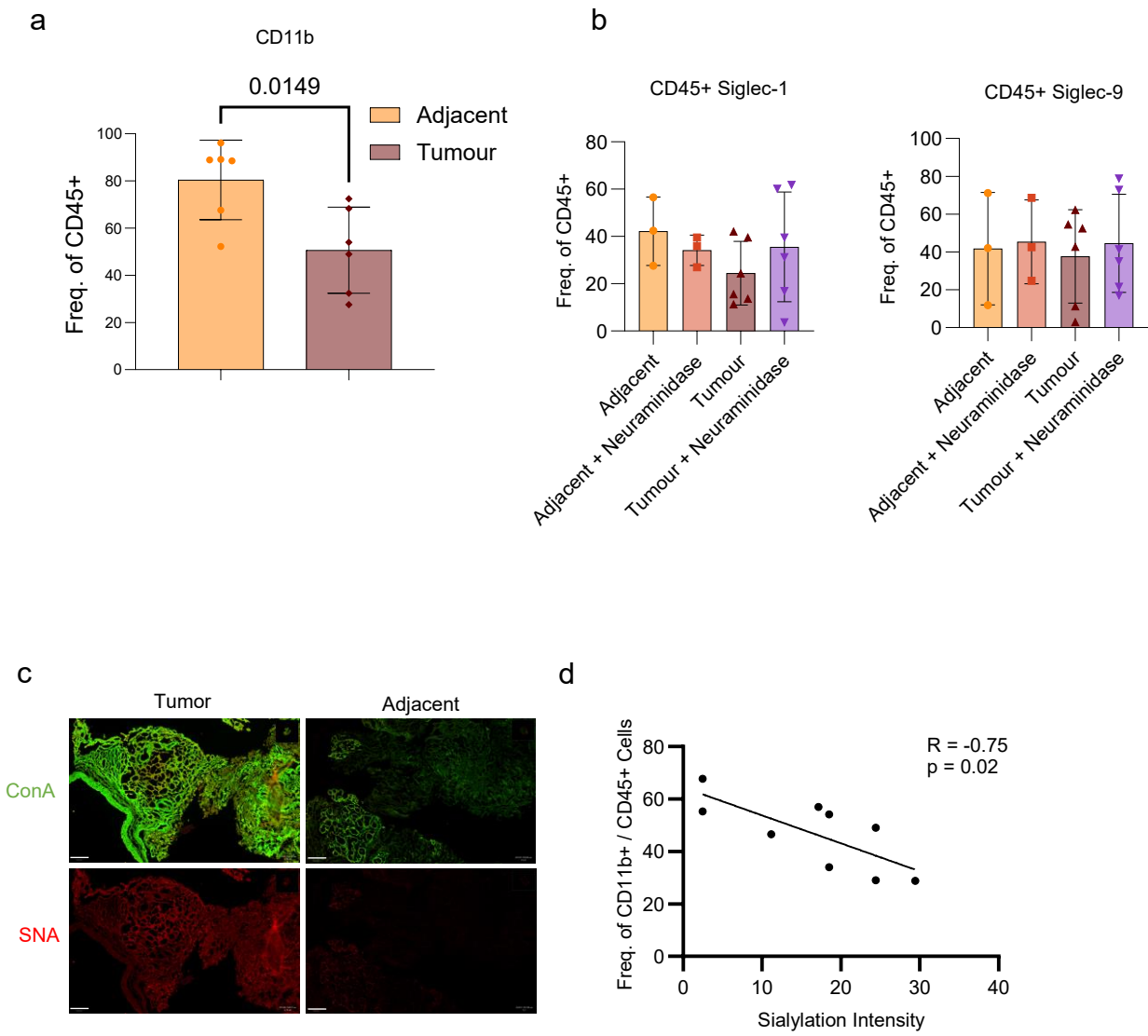




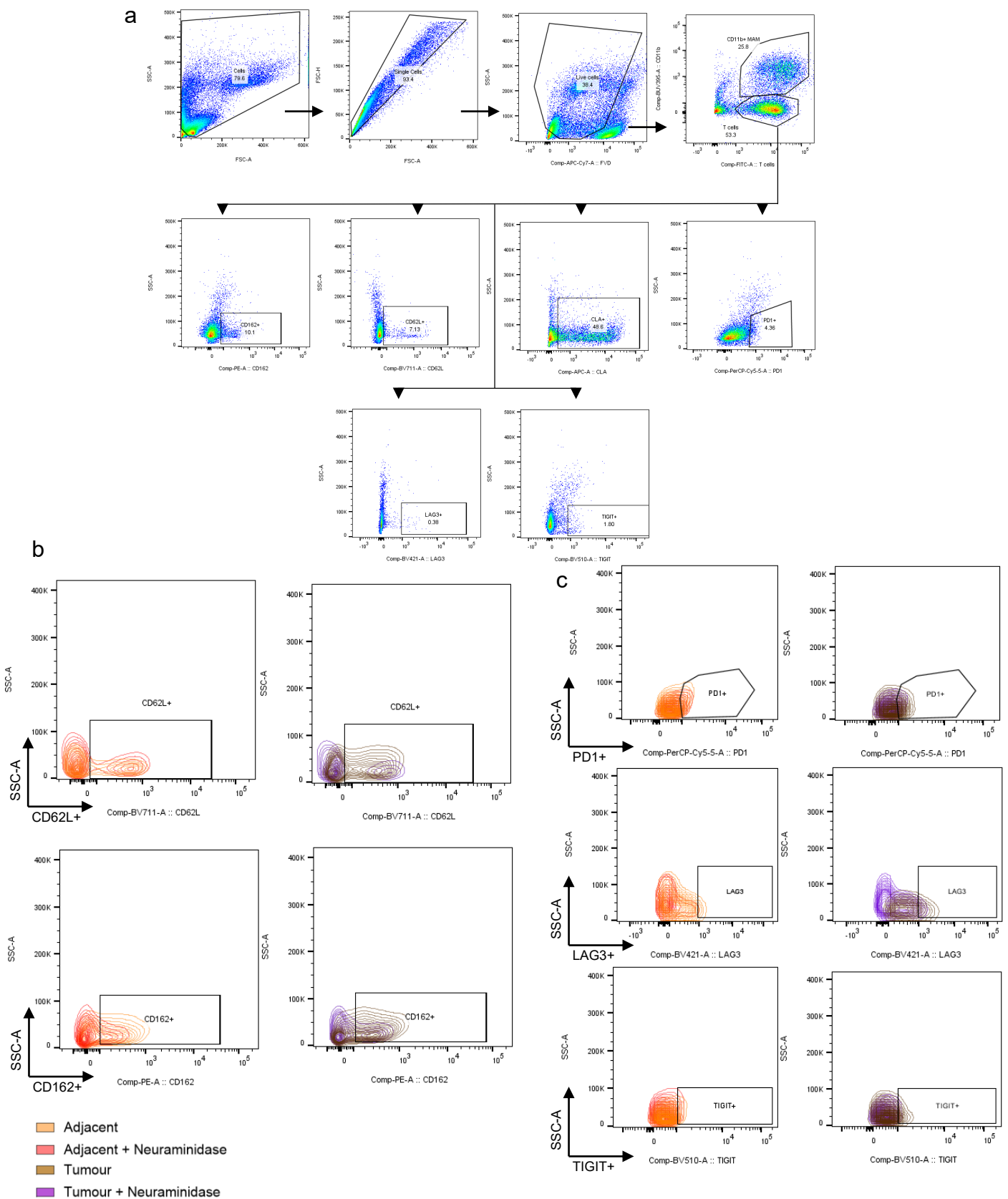
Supplementary Fig. 20: Glycan cleavage enzymes alter tissue architecture. **a**, Representative images of Masson's Trichrome staining & TWOMBLI analysis performed on matched tumor tissue treated with PBS or PNGase F. Scale bar = 100 μm . **b**, TWOMBLI analysis. Two-tailed paired t test. N=2 matched tumor & PNGase F-treated tumor tissues.



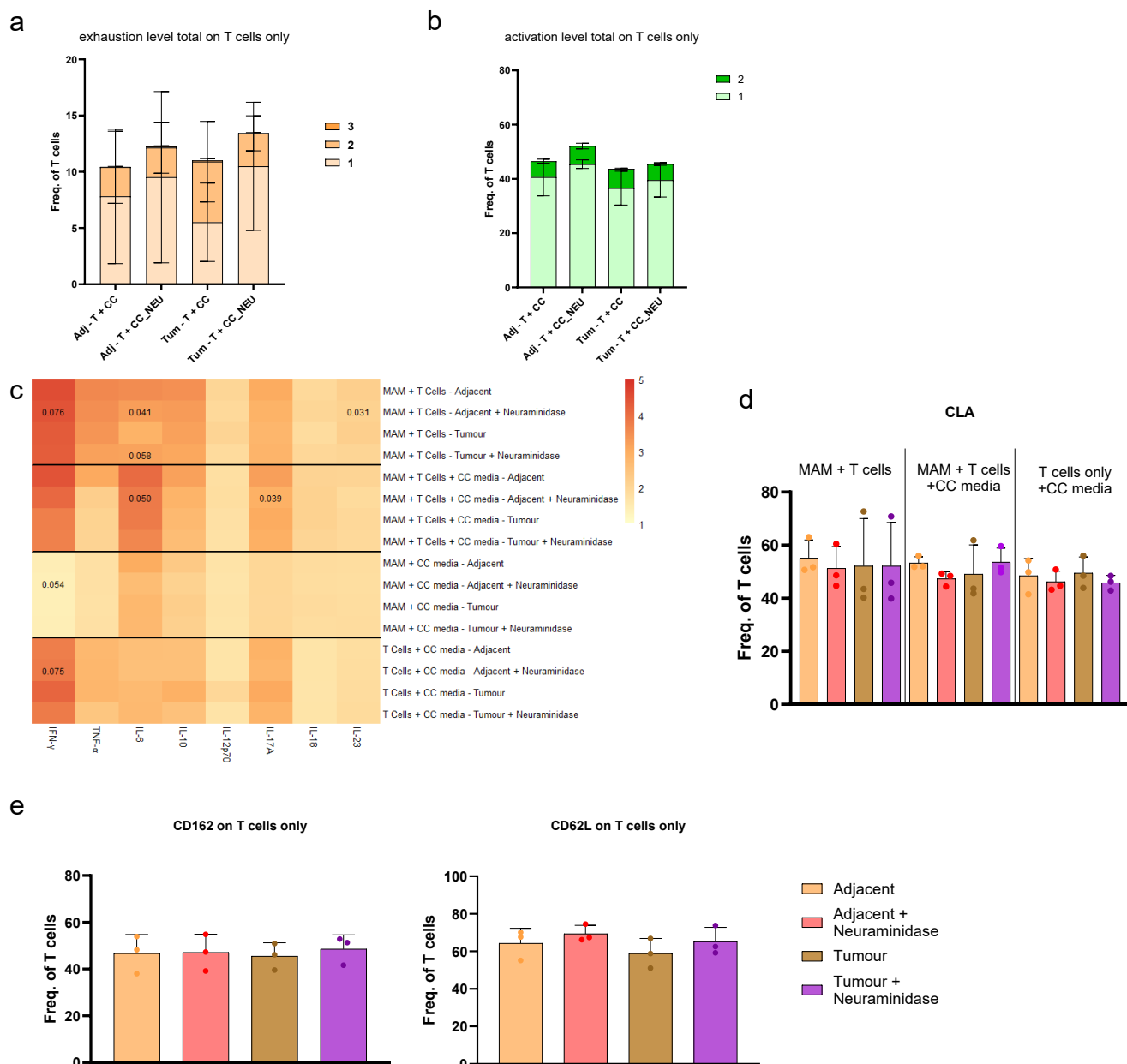
Supplementary Fig. 21. a, Flow gating strategy for macrophage flow cytometry assay. CD45+ macrophages and downstream phenotypic markers as assessed using flow cytometry after 14 days culture on adjacent or tumor tissue with or without neuraminidase treatment (Figure 6). N=3. **b**, Representative contour plots of CD11b+, CD163+, CD206+ and CD209+ macrophages cultured on adjacent or tumor tissues, with or without neuraminidase treatment. N=6 (N=6 adjacent, N=6 tumor, N=6 blood cones).



Supplementary Fig. 22. Macrophages educated on patient tissue express Siglec-1 and Siglec-9 and tissue sialylation correlated with CD11b+ macrophage population educated on matched tissues. **a**, Barchart of flow cytometry expression patterns of CD11b shown as percentage of positive cells from the CD45+ population. Line at mean with standard deviation error bars. Mixed-effects analysis with Tukey's multiple comparisons test. N=6 (N=6 adjacent, N=6 tumor, N=6 blood cones). **b**, Barchart of flow cytometry expression patterns of Siglec-1 and Siglec-9 shown as percentage positive cells from the CD45+ population. Line at mean with standard deviation error bars. Mixed-effects analysis with Tukey's multiple comparisons test. N=3 (N=2 adjacent, N=6 tumor, N=3 blood cones). **c**, Representative images of tumor and adjacent decellularized tissue stained with Concavalin A (ConA) lectin (green) for N-linked glycans, and Sambucus Nigra (SNA) lectin (red) for sialic acids. Scale bar = 100µm. **d**, Pearson's r values for correlation between SNA sialylation intensity of tissue and percentage positive cells from the CD45+ population educated on the tissue. N=9 tissues.



Supplementary Fig. 23. Flow gating strategy for T cell flow cytometry assay. a, T cells and downstream phenotypic markers as assessed using flow cytometry after 2 days in co-culture with MAMs (Figure 7). N=3. **b-c**, Representative contour plots of CD62L+, CD162+, PD1+, LAG3+, TIGIT+ T cells cultured on adjacent or tumor tissues with MAMs and cancer cell media, with or without neuraminidase treatment. N=3 (N=3 adjacent, N=3 tumor, N=3 blood cones).



Supplementary Fig. 24. MAMs influence T cell phenotype in a glycan-dependent manner. Stacked bar charts of combinatorial gating for **a**, exhaustion markers (PD1, LAG3, TIM3) or **b**, activation markers (ICOS & CD137) shown as percentage positive cells expressing one marker, two markers or three markers from the T cell population. Line at mean with standard deviation error bars. Mixed-effects analysis with Tukey's multiple comparisons test. N=3 (N=3 adjacent, N=3 tumor, N=3 blood cones). **c**, Heatmap showing log₁₀-transformed concentrations (pg/mL) of 8 cytokines (IFN- γ , TNF- α , IL-6, IL-10, IL-12p70, IL-17A, IL-18 and IL-23) measured in culture supernatants from macrophage (MAM) and T cell co-cultures (or monocultures) grown on decellularized adjacent or tumor ECM scaffolds, in the absence or presence of cancer cell-conditioned medium (CC). ECM scaffolds were either untreated or treated with neuraminidase (Neu) prior to cell seeding. N=3 (N=3 adjacent, N=3 tumor, N=3 blood cones). Overlaid numbers indicate paired t-test p-values for comparisons between untreated and neuraminidase-treated ECM within the same tissue and CC condition. **d-e**, Bar chart of flow cytometry expression patterns of CLA, CD62L and CD162 on T cells shown as percentage of positive cells from T cell population. Line at mean with standard deviation error bars. Mixed-effects analysis with Tukey's multiple comparisons test. N=3 (N=3 adjacent, N=3 tumor, N=3 blood cones).

Marker	Secondary	Concentration	Catalog
panCK	Rabbit	1:1000	Z0622
CD8	Mouse	1:500	M7103
ConA-AF488	-	1:5	C11252
SNA-Cy3	-	1:100	CL-1303-1

Supplementary Table 1: Optimized antibodies for IHC.