Glycosylated extracellular matrix drives immune suppression by controlling T cell movement, macrophage phenotype, and macrophage-T cell crosstalk in triple negative breast cancer.

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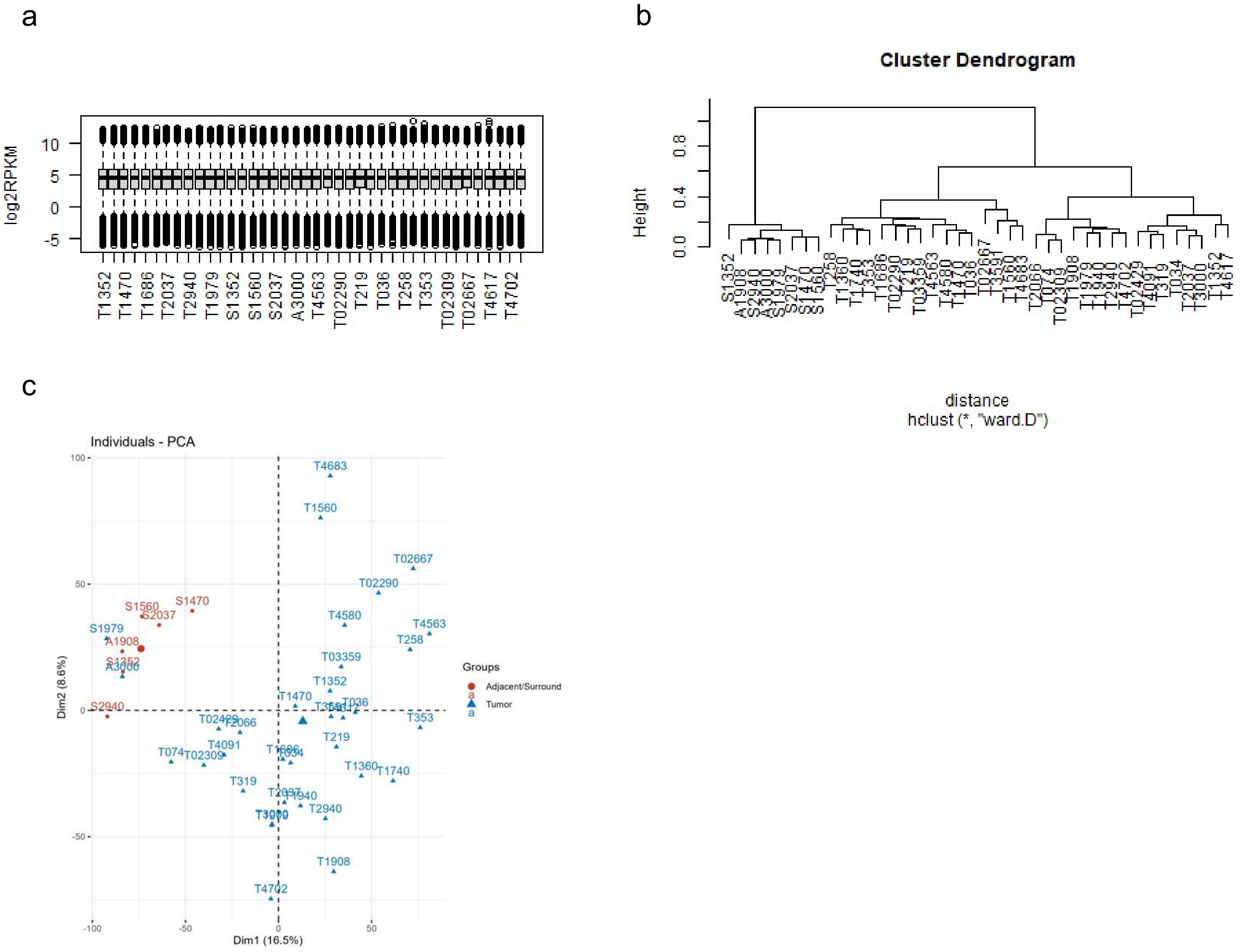
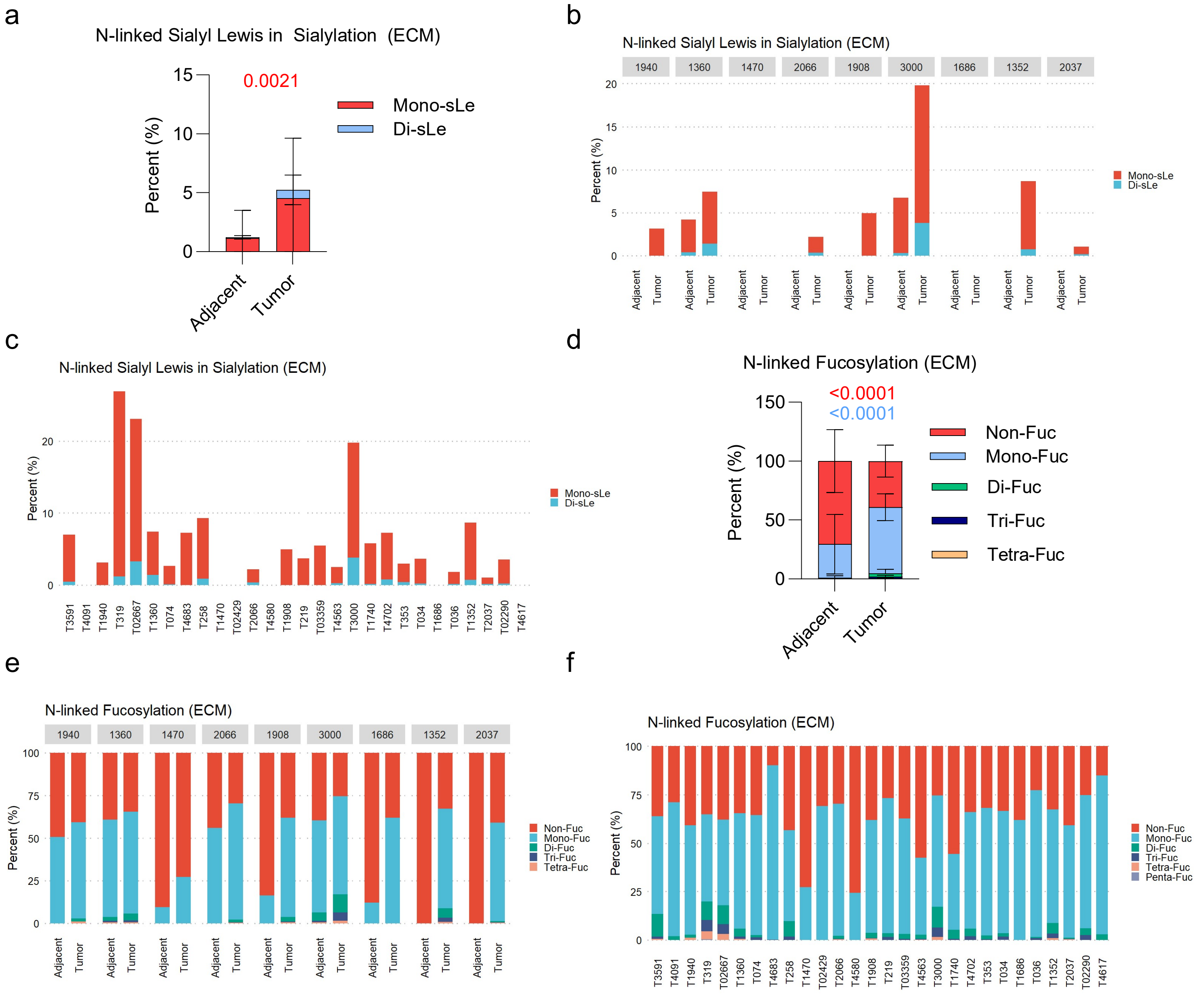
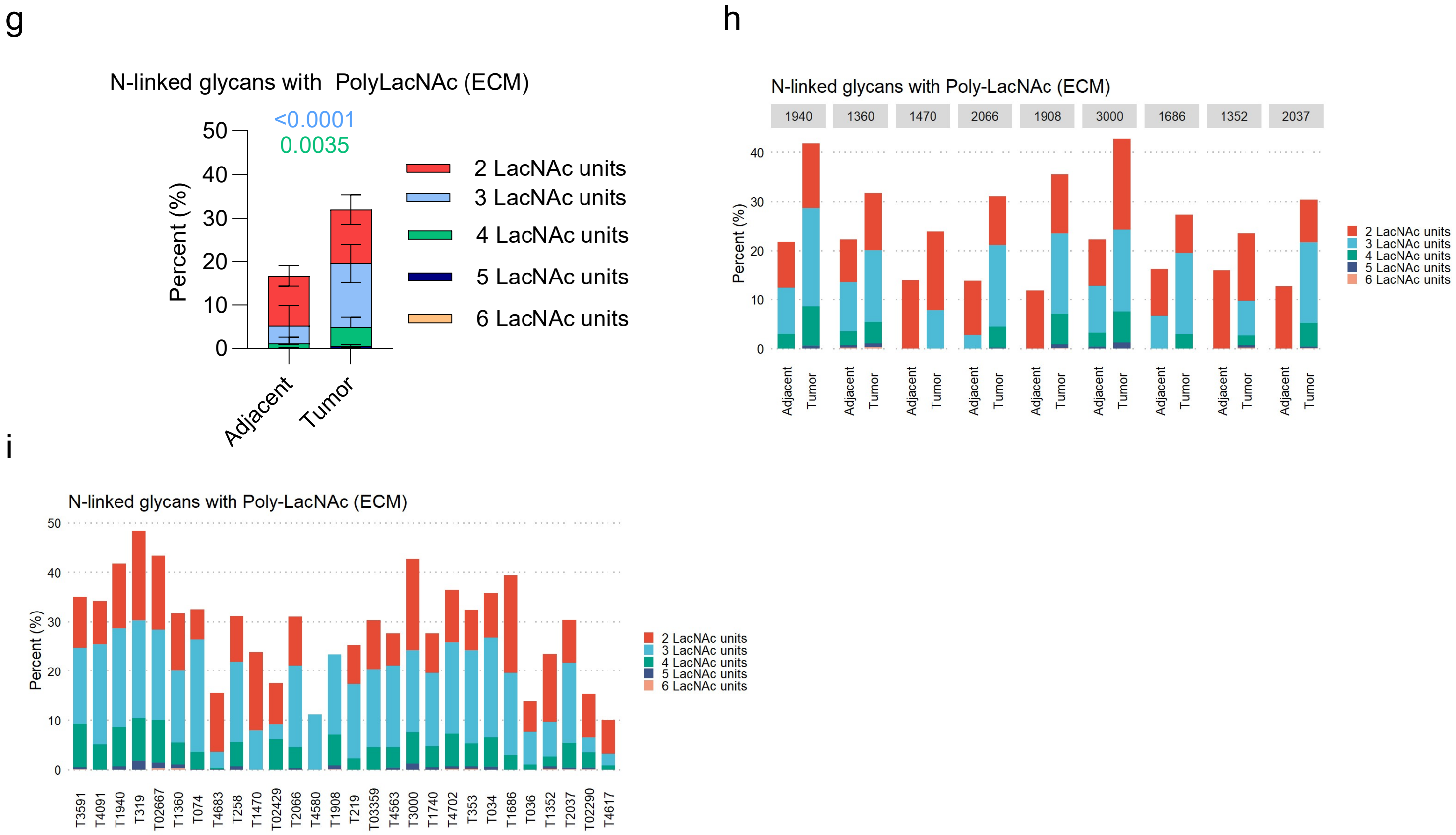


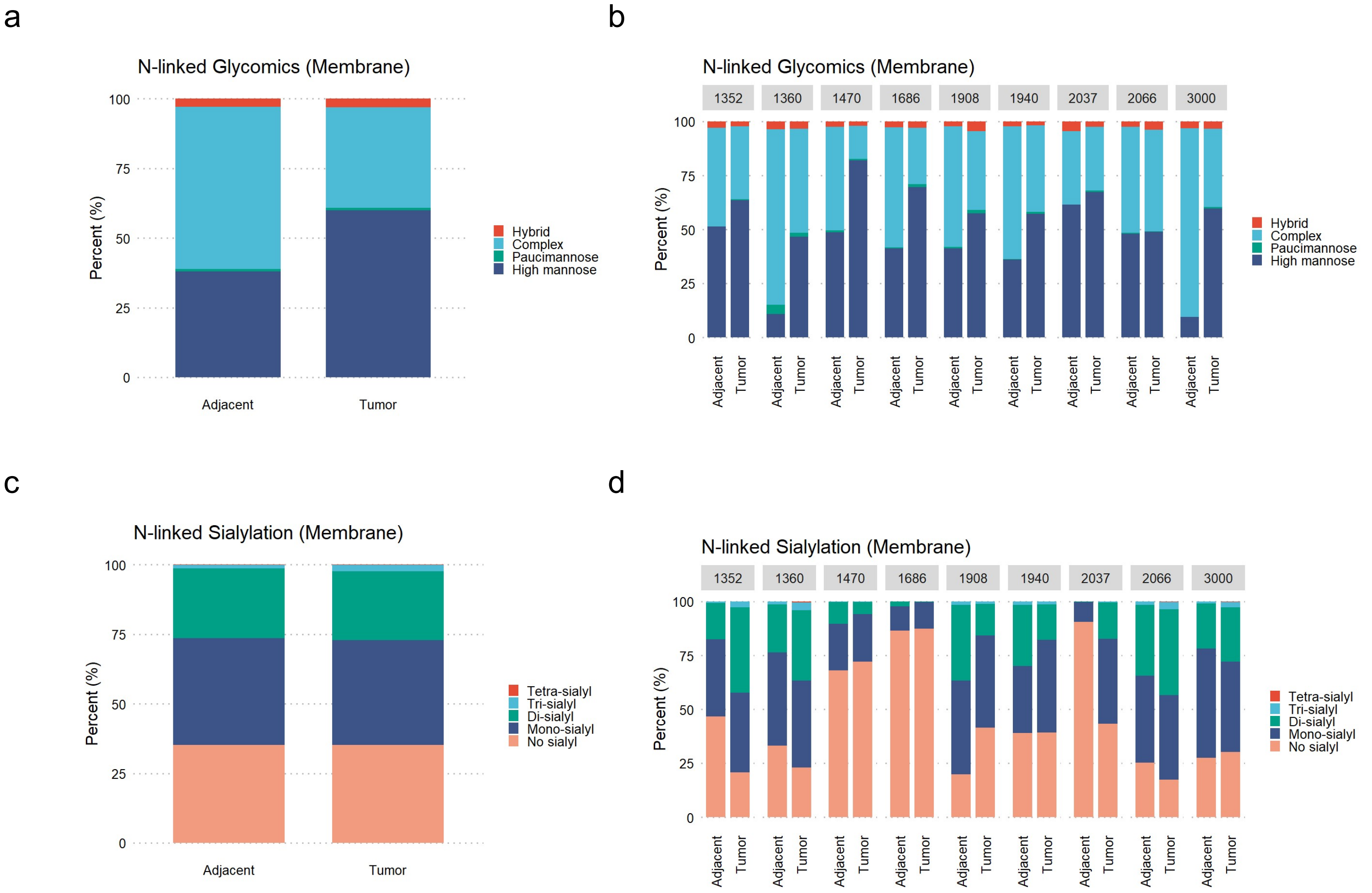
Supplementary Fig. 1: Transcriptomic analysis of TNBC patient tissues. a, Exploratory boxplot for sample quality. Boxplots illustrate median (centre of the box) with the upper (Q3: 75th percentile) and lower (Q1: 25th percentile) quartiles (ends of the box); the whiskers correspond to Q3 + 1.5 x Interquartile Range (IQR) to Q1 – 1.5 x IQR; dots beyond the whiskers show potential outliers. N=. **b,** Unsupervised cluster analysis using all genes. **c,** Principal Component Analysis (PCA) using all genes.



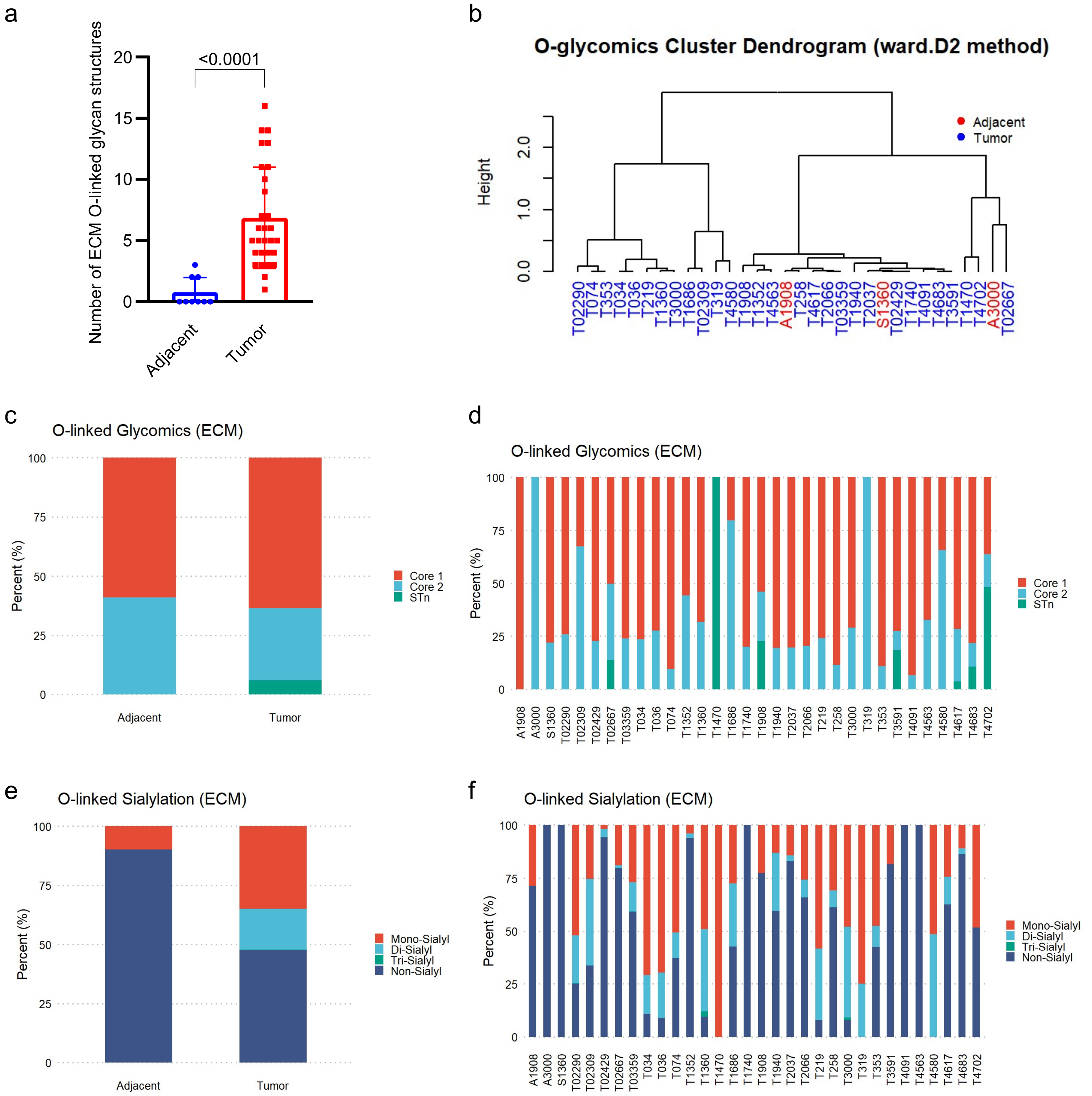
Supplementary Fig. 2: ECM N-linked glycomics analysis. ECM N-linked glycans assessed for **a-c,** Sialyl Lewis groups (Mono-sialyl lewis (sLe), red; Di-sLe, light blue) , **d-f,** Fucosylation (Non-fucosylation (Fuc), red; Mono-Fuc, light blue; Di-Fuc, green; Tri-Fuc, dark blue; Tetra-Fuc, peach). **a,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



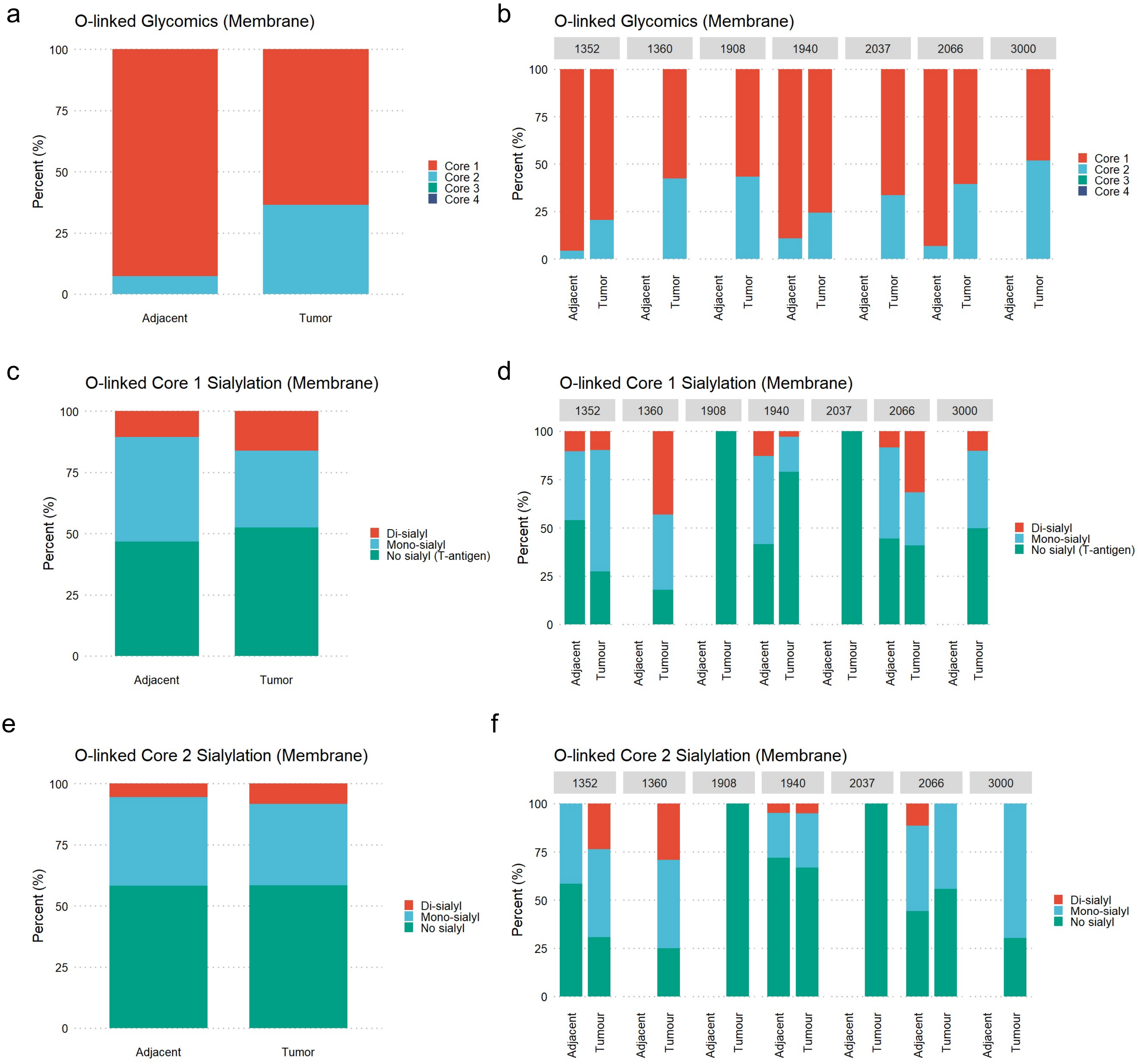
Supplementary Fig. 2: ECM N-linked glycomics analysis. ECM N-linked glycans assessed for **g-i,** Poly-LacNAc (2 LacNAc units, red; 3 LacNAc units, light blue; 4 LacNAc units, green; 5 LacNAc units, dark blue; 6 LacNAc units, peach) structures. **g,** average adjacent/surround and tumor samples, Mean 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.



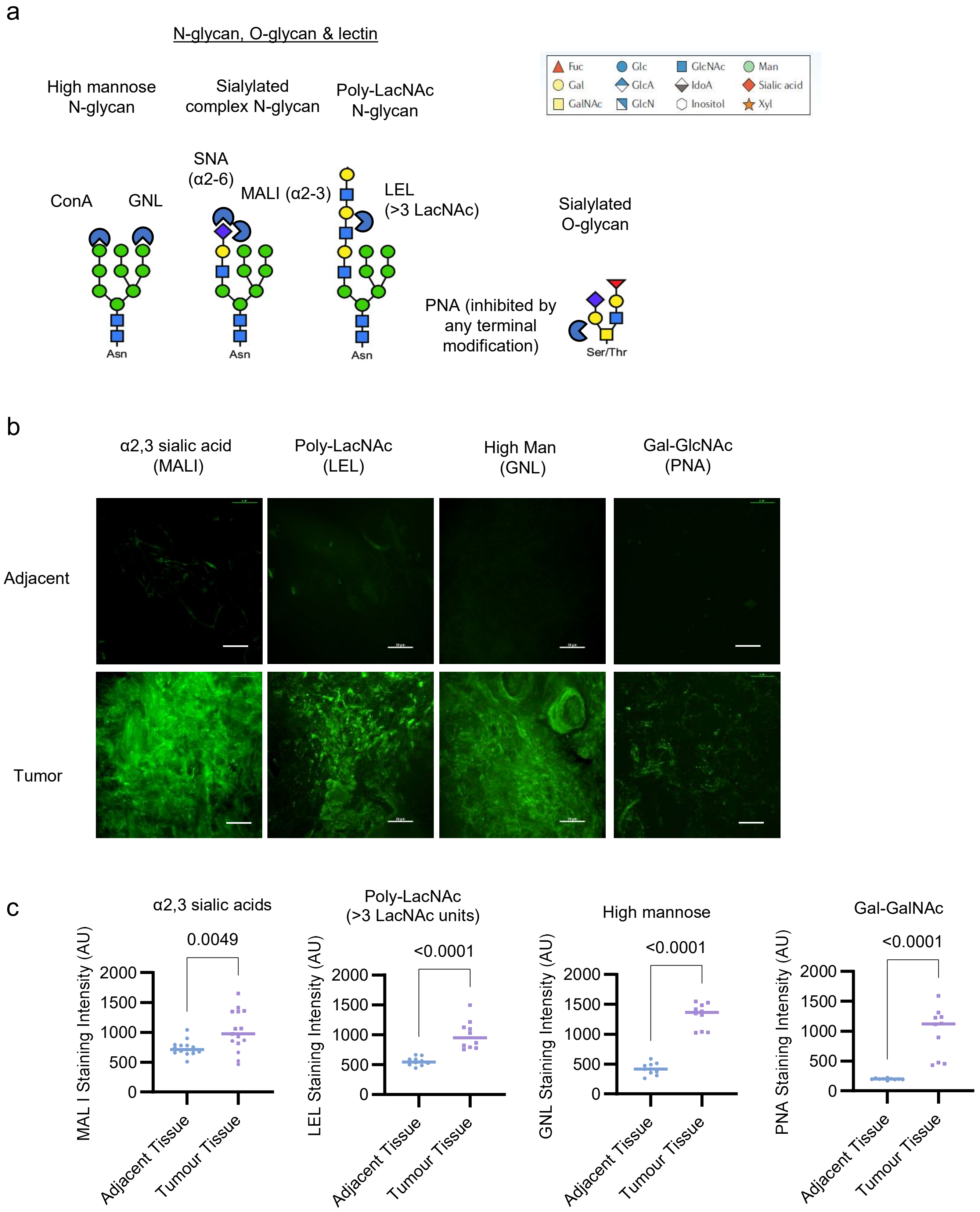
Supplementary Fig. 3: Membrane N-linked glycomics analysis. a-b, Membrane N-linked glycans assigned to four main groups (Complex, dark blue; Hybrid, green; High-mannose, light blue; Paucimannose, red). Stacked barchart displaying the proportion of each family in the N-glycome for **a,** mean adjacent/surround 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.



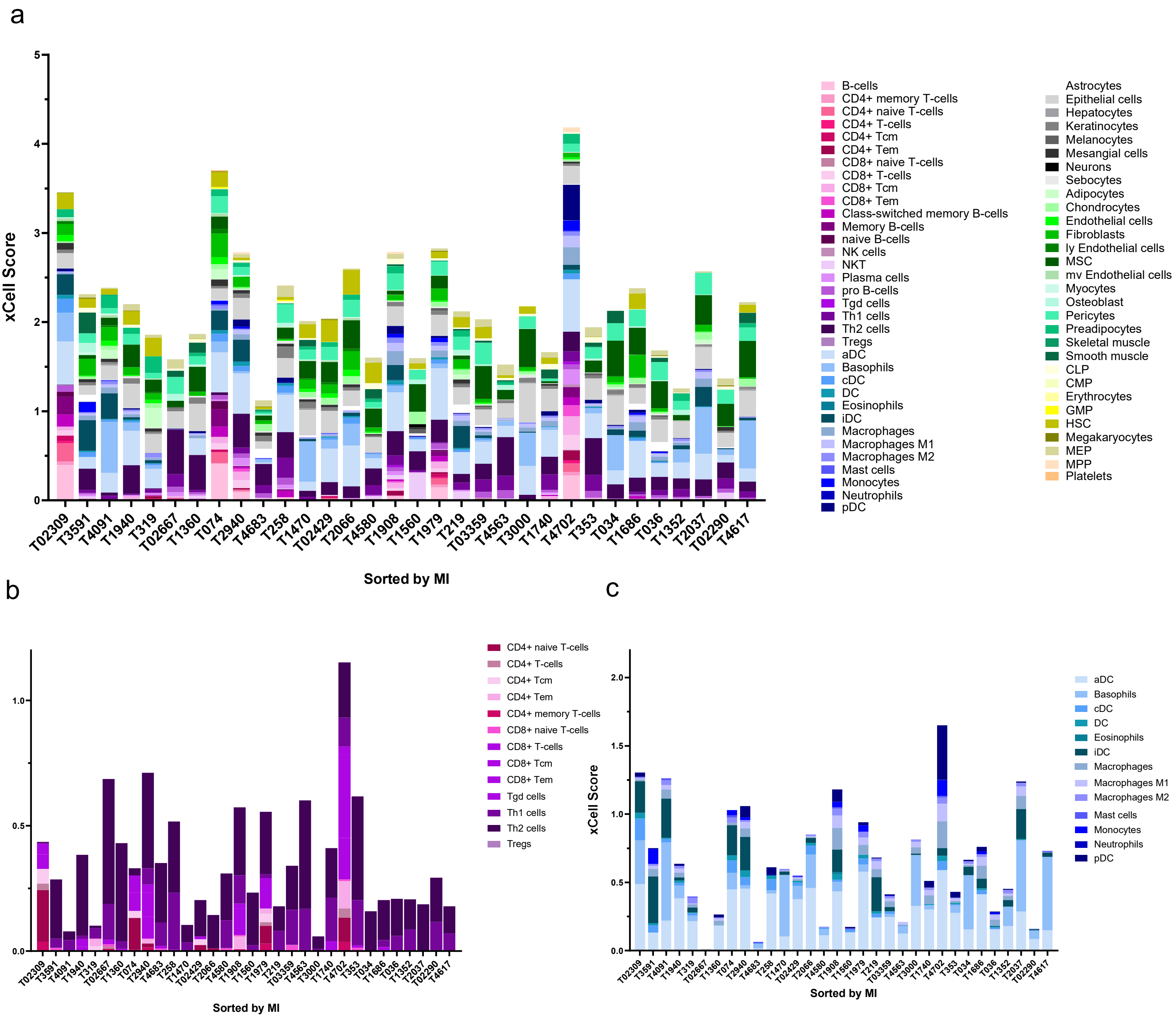
Supplementary Fig. 4: ECM O-linked glycomics analysis. a, Number of ECM O-linked glycan structures detected. Mean with SD. Mann Whitney test. N=9 for adjacent/surround ('adjacent'), N=29 for tumor tissues. **b,** Unsupervised cluster dendrogram using ECM O-linked glycan structures from adjacent/surround 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.



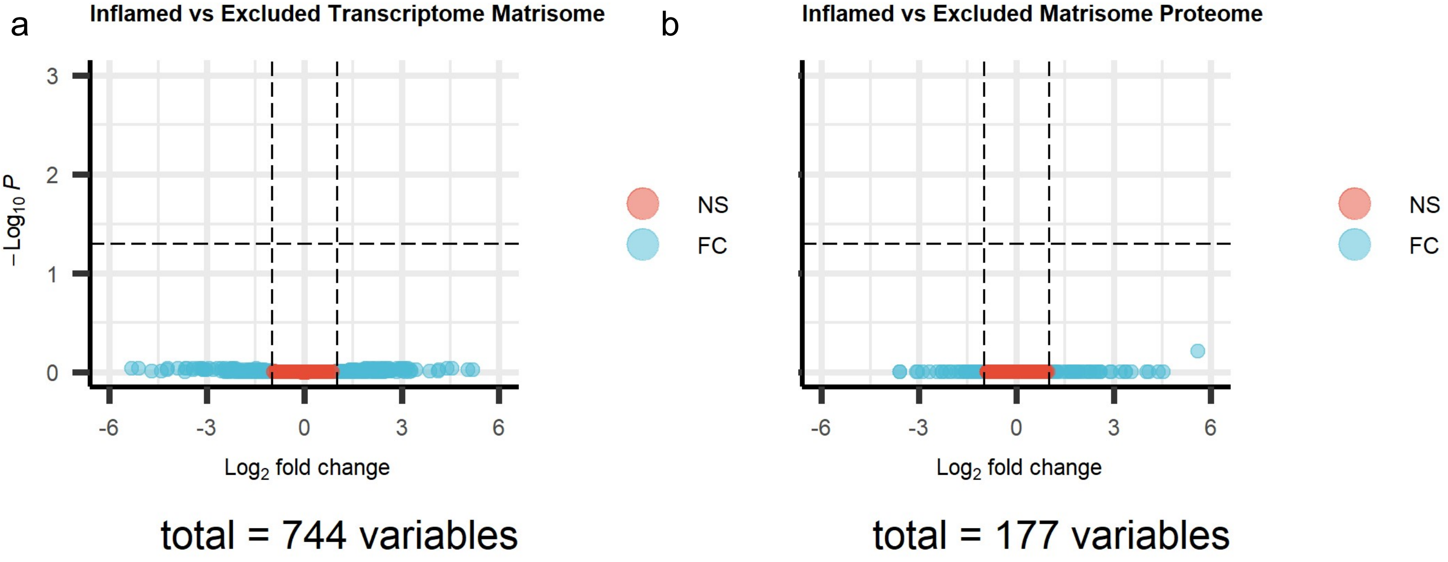
Supplementary Fig. 5: Membrane O-linked glycomics analysis. a-b, Membrane O-linked glycans assigned to four main groups (Core 1, red; Core 2, light blue; Core 3, green; Core 4, dark blue). Stacked barchart displaying the proportion of each family in the O-glycome for **a,** mean adjacent/surround and 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.



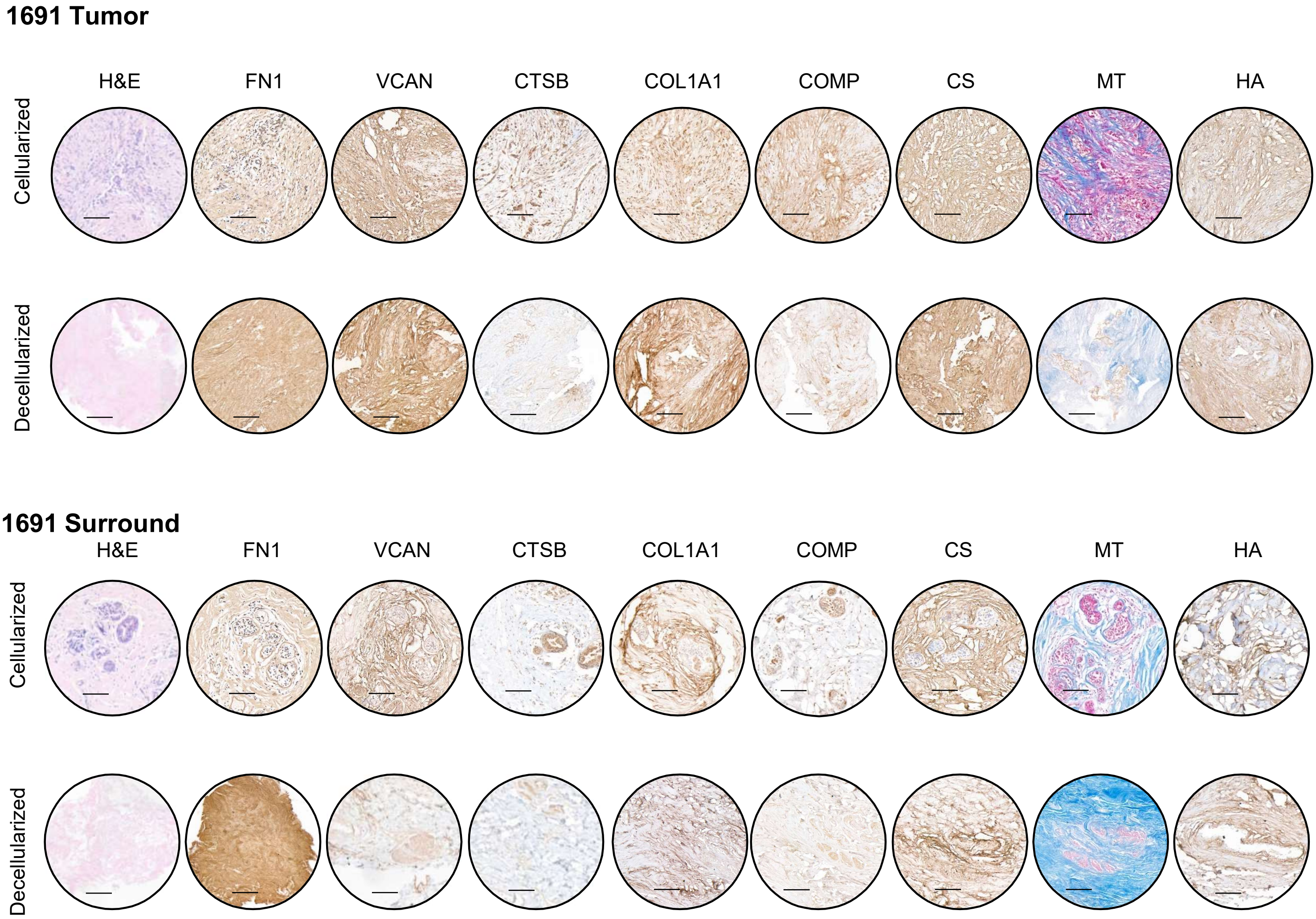
Supplementary Fig. 6: Lectin staining for N-linked and O-linked glycan structures in tumor and adjacent decellularized TNBC patient tissues. a, Schematic representing N-linked and O-linked glycan structures and their respective lectin binding partners. **b,** Representative images of matched adjacent and tumor TNBC patient tissue stained with Maackia Amurensis Lectin I (MALI), Fluorescein (FL-1311-2, 10µg/mL), Lycopersicon Esculentum (tomato) Lectin (LEL) Dylight 488 (DL-1174-1, 10µg/mL), Galanthus Nivalis Lectin (GNL) Fluorescein (FL-1241-2, 10µg/mL), and PNA From Arachis hypogaea (peanut), AlexaFluor 488 (L21409, 10µg/mL). Scale bar = 50µm. **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.



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.

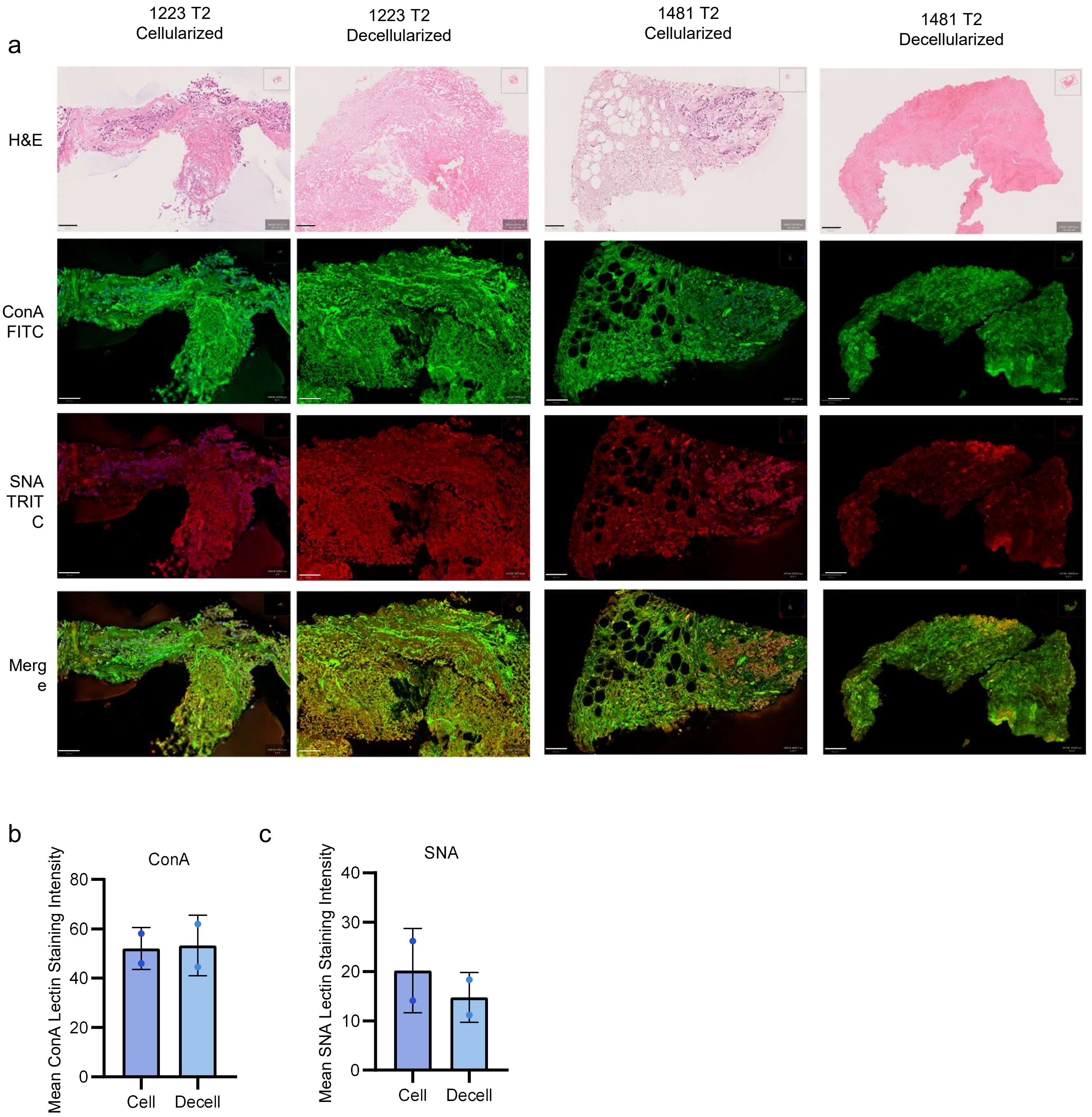


Supplementary Fig. 8: No changes in matrisome transcriptome or proteome between inflamed and excluded samples. a, Volcano plot of differentially regulated matrisome genes between inflamed and excluded tumor tissues. N=4 for excluded, N=3 for inflamed. **b,** Volcano plot of differentially regulated matrisome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

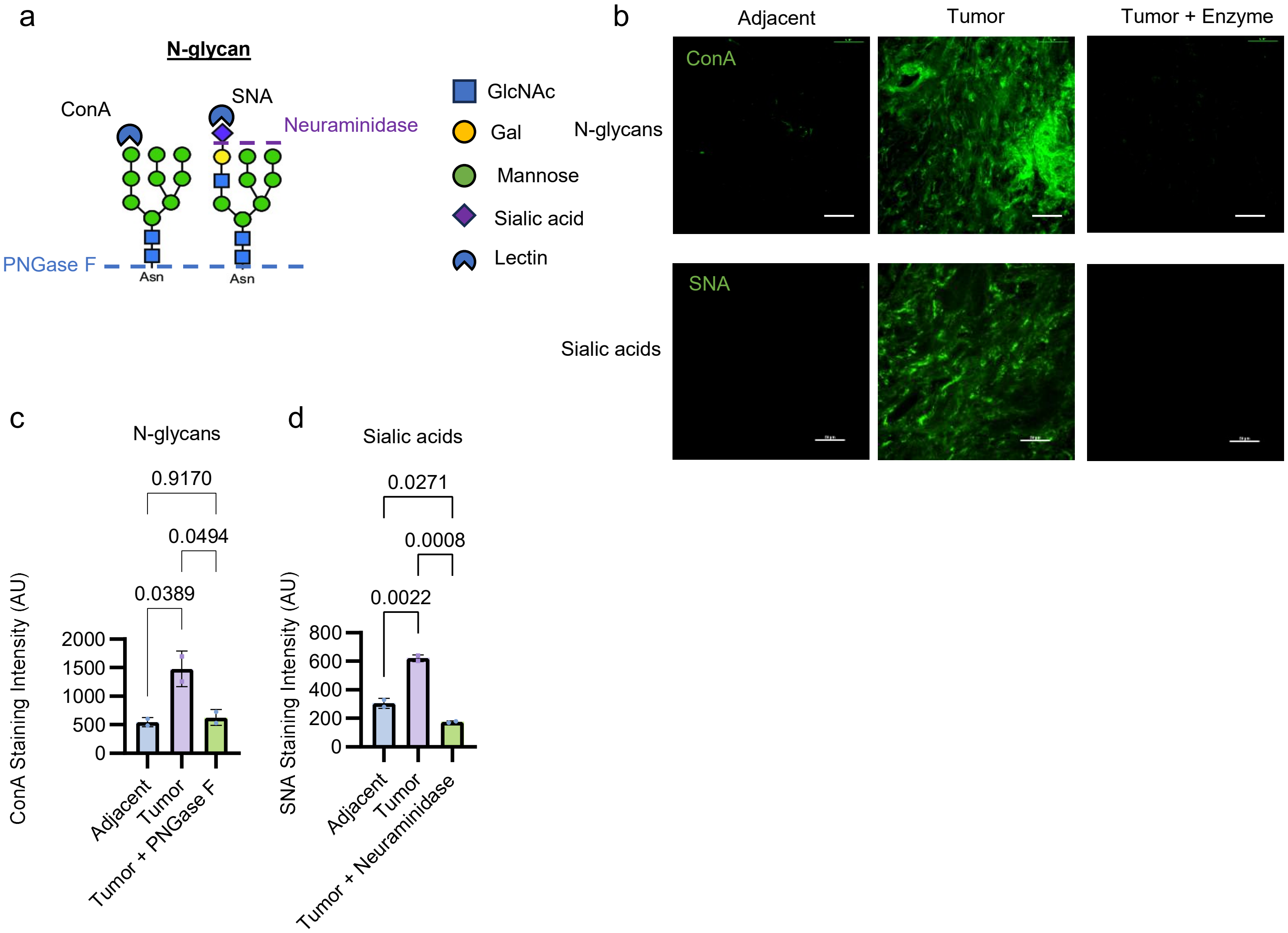

1691 Tumor

1691 Surround

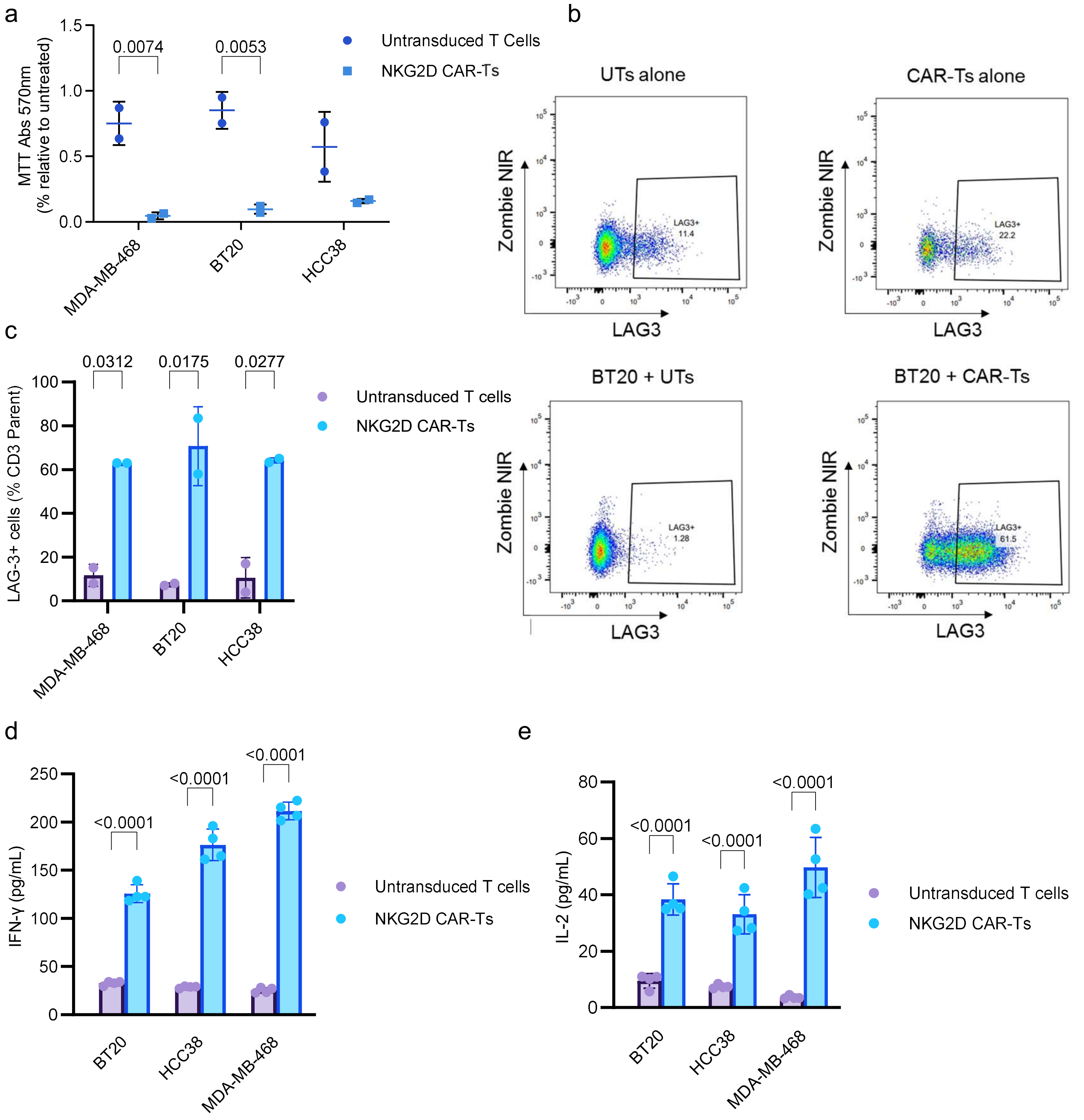
Supplementary Fig. 9:. Representative sections of IHC staining for matrix molecules in tumor and surround cellularized and decellularized TNBC patient tissues. Stains included anti-FN1 (ab23750, 1:500), anti-VCAN (HPA004726, 1:200), anti-CTSB (ab58802, 1:400), anti-COL1A1 (HPA011795, 1:300), anti-COMP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



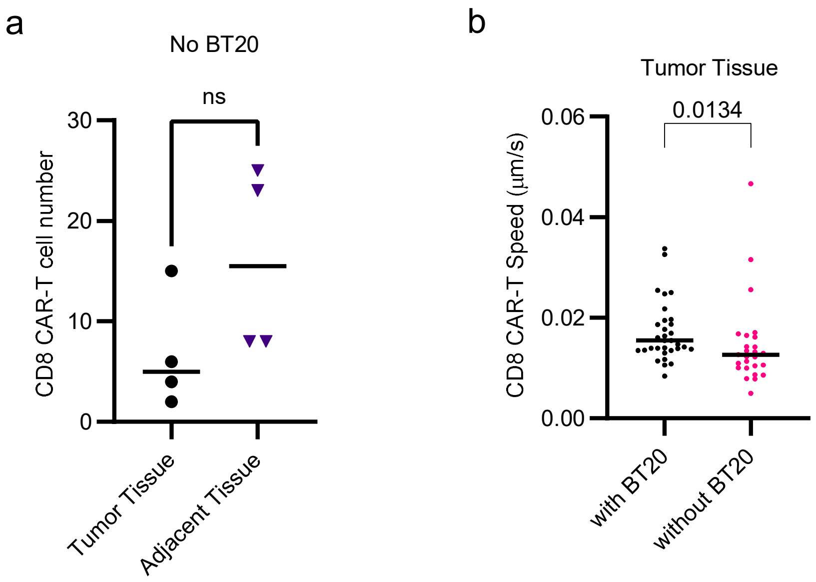
Supplementary Fig. 10: Lectin staining confirms that decellularization does not affect N-linked and sialylated glycan structures in tumor and adjacent TNBC patient tissues. a, Representative images of cellularized or decellularized adjacent or tumor tissue. H&E staining in the top row, staining of a 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.



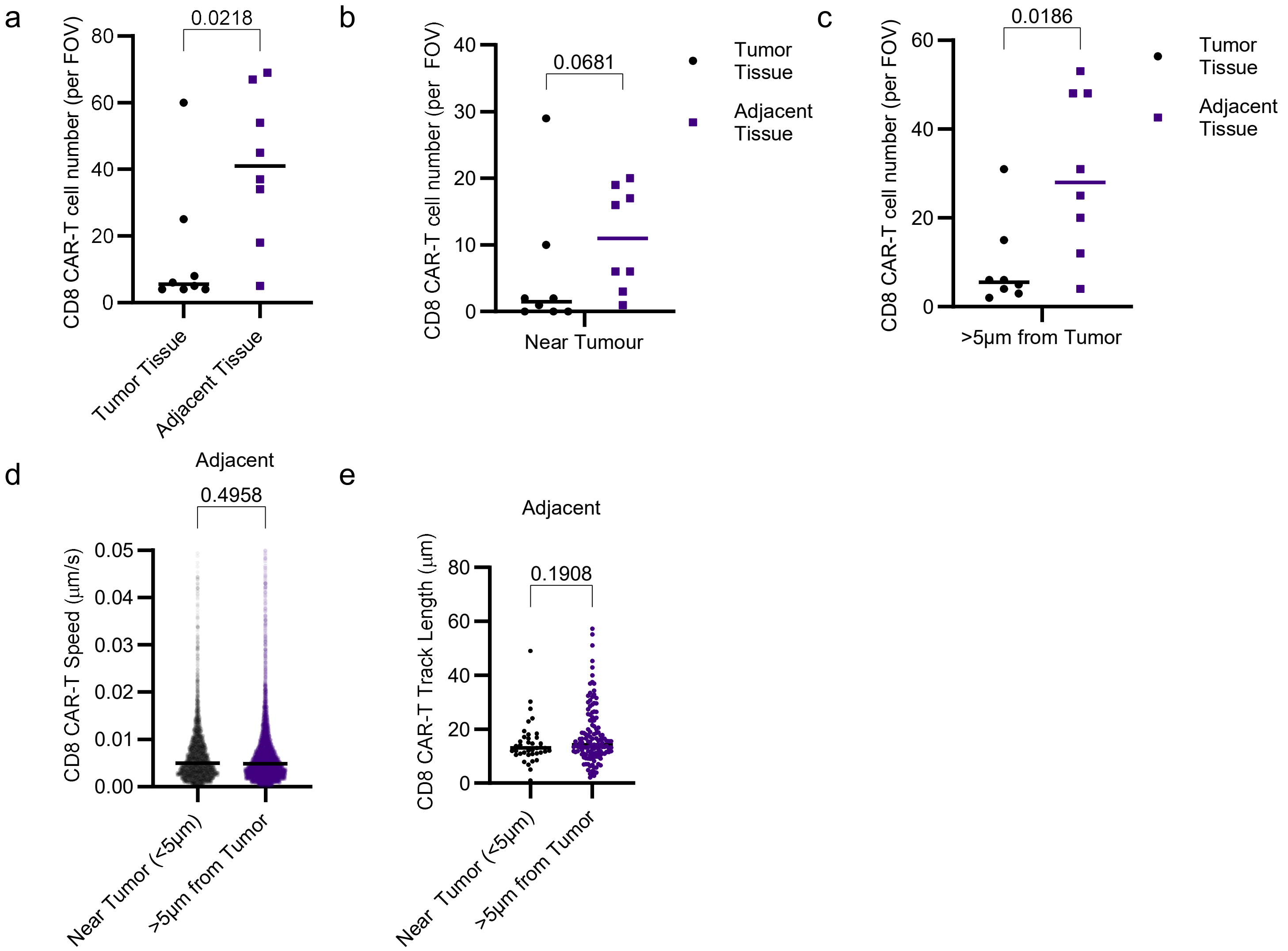
Supplementary Fig. 11: Lectin staining for N-linked and sialylated glycan structures in tumor and adjacent TNBC patient tissues and their cleavage using PNGase F and neuraminidase. a, Schematic of N-linked glycan enzymatic modifications and their respective lectin binding partners for staining. **b,** 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**.**



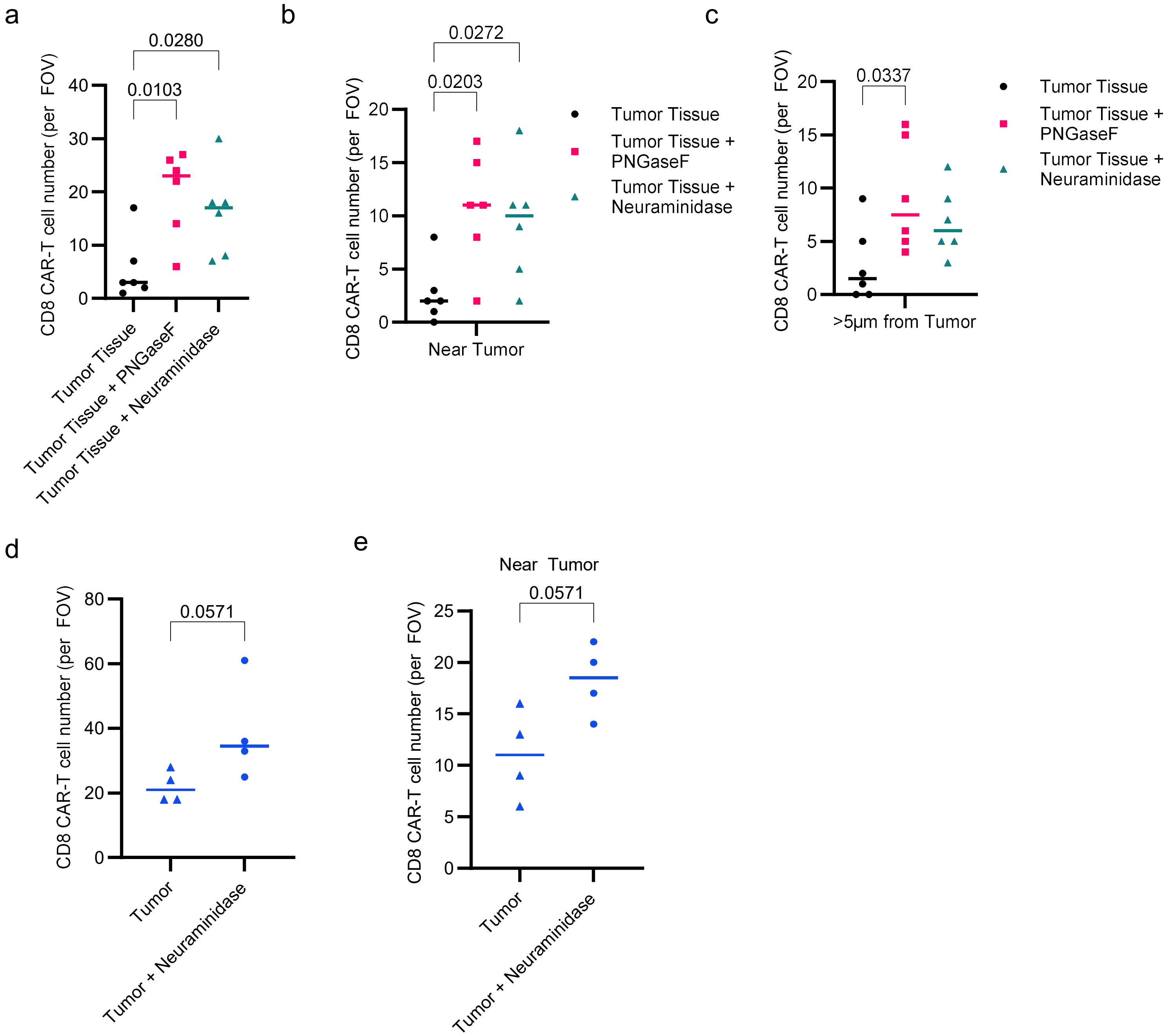
Supplementary Fig. 12: CAR-T targeting of TNBC cell lines in 2D. a, MTT assay with three TNBC cell lines, MDA-MB-468, BT20 and HCC38 co-cultured with untransduced T cells or CAR-Ts targeting NKG2D. Mean with SD. Two-way ANOVA with Šídák's multiple comparisons test. N=2. **b,** Representative flow cytometry 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.



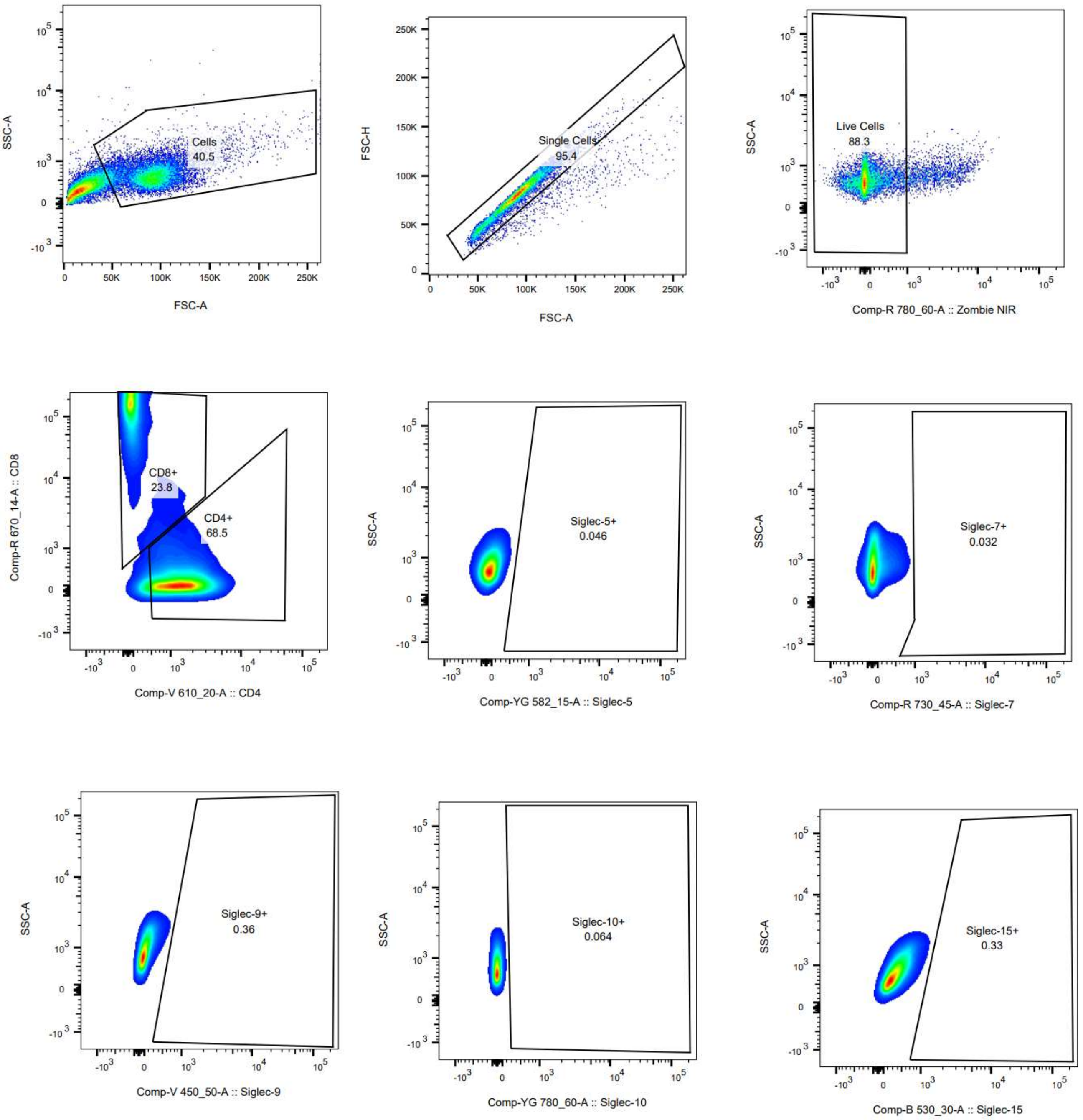
Supplementary Fig. 13: Decellularized tissue timelapse video analysis. a-b, Timelapse confocal microscopy of NKG2D-targeting CAR-Ts in decellularized tissues which were not seeded with BT20 TNBC cells. **a,** Mean CD8 NKG2D-targeting CAR-T cell number per field of view (FOV). Line at median. Unpaired t test. N=4. **b,** Mean speed of CD8+ CAR-Ts. Line at median. Mann Whitney test. N=4.



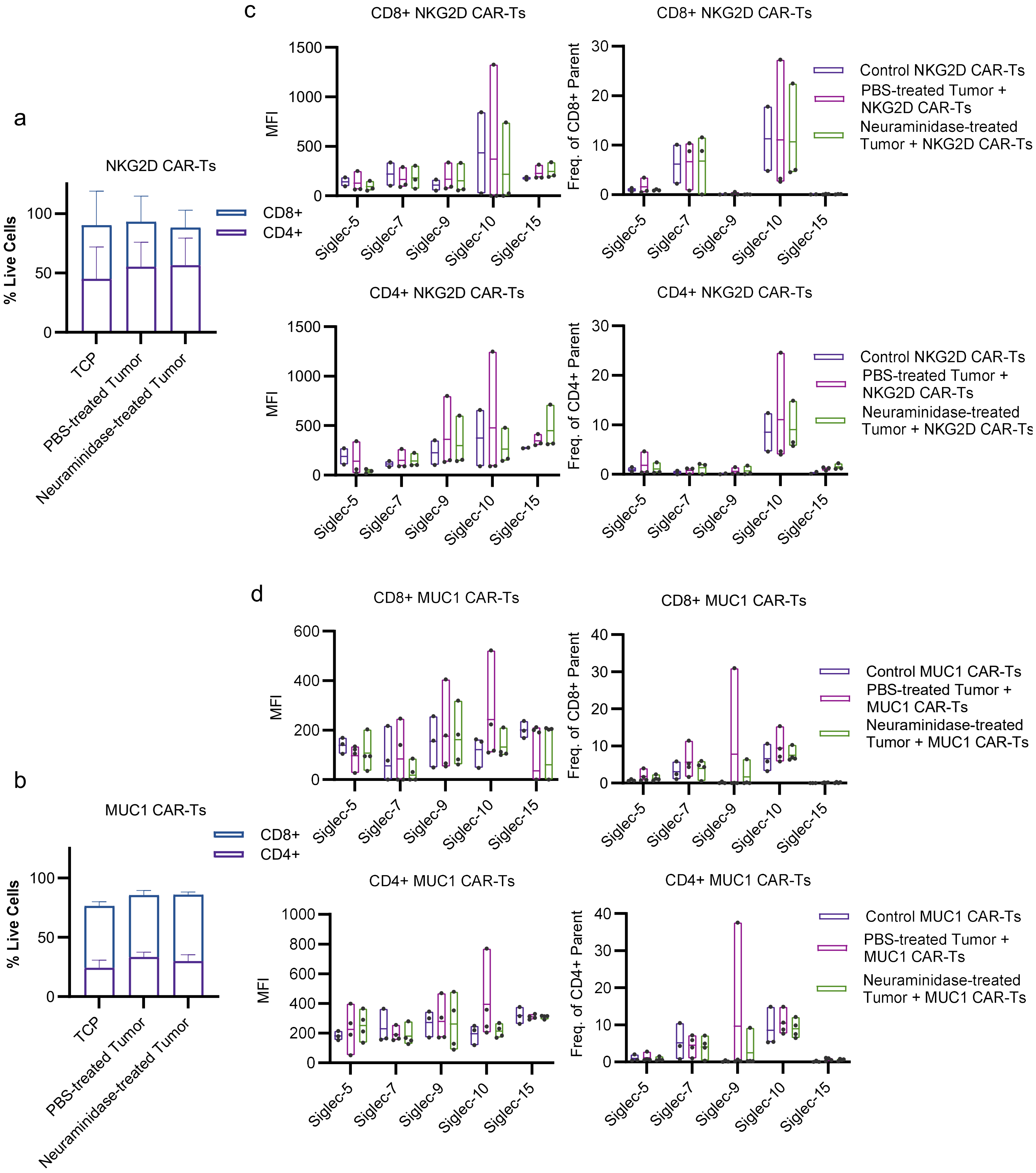
Supplementary Fig. 14:. Decellularized tissue timelapse video analysis. Fluorescence-based live confocal timelapse imaging assay using decellularized tissue seeded with BT20 TNBC cells and NKG2D-targeting CAR-T co-culture. Tissues stained with anti-human CD326 (EpCAM) AlexaFluor (AF) 488 (53-8326-42, 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.



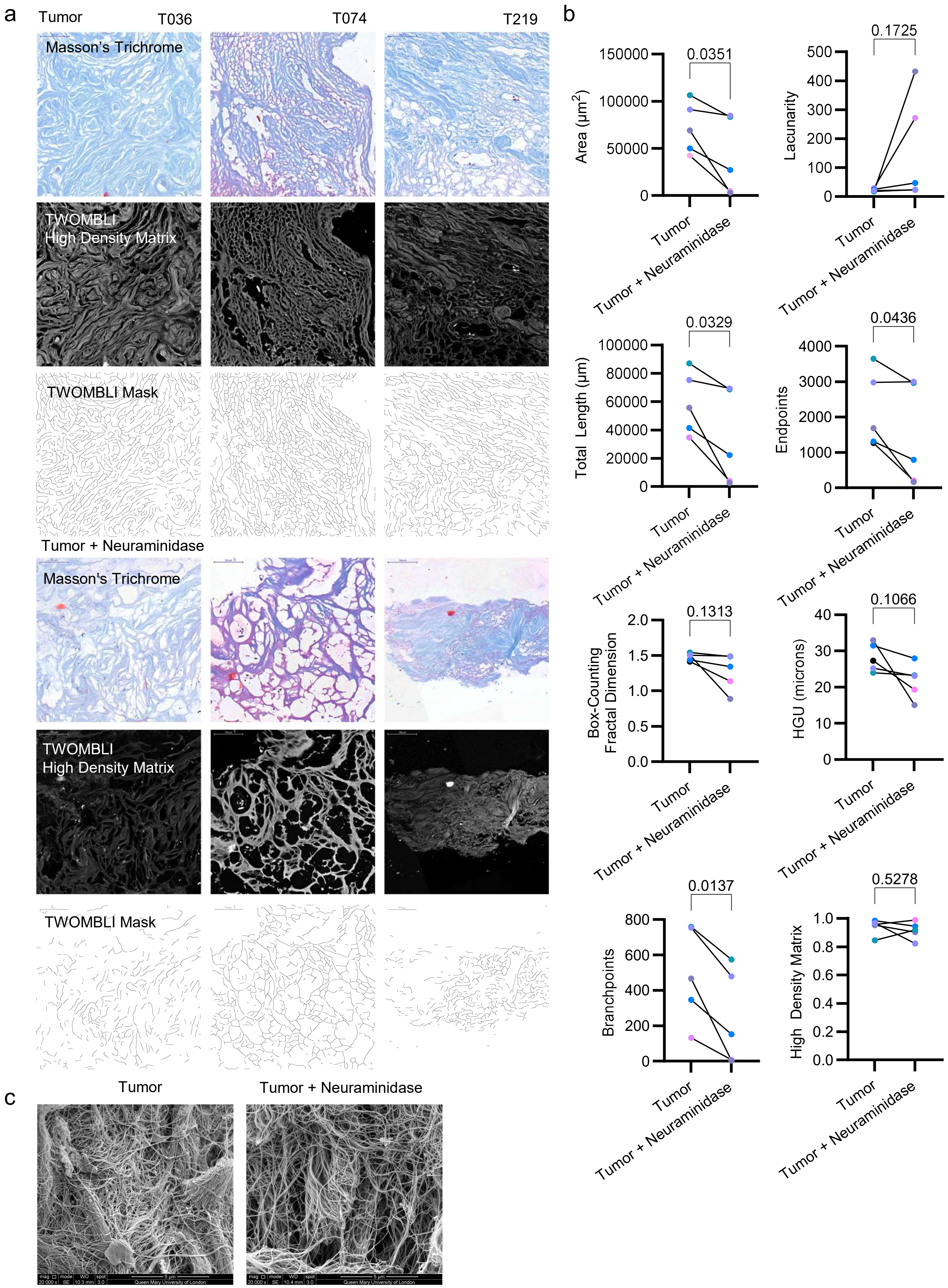
Supplementary Fig. 15: Decellularized tissue timelapse video analysis. Fluorescence-based live confocal timelapse imaging assay using decellularized tumor tissue treated with either PNGase F or neuraminidase enzyme treatment, seeded with BT20 TNBC cells and NKG2D-targeting CAR-T co-culture. Tissues stained 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 (<5µm). **c,** CD8+ CAR-T cell number per field of view (FOV) >5µm from tumor cells. **a-c,** Line at median. One-way ANOVA with Holm-Šídák'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 (<5µm) per FOV. Line at median. Mann Whitney test. N=4.



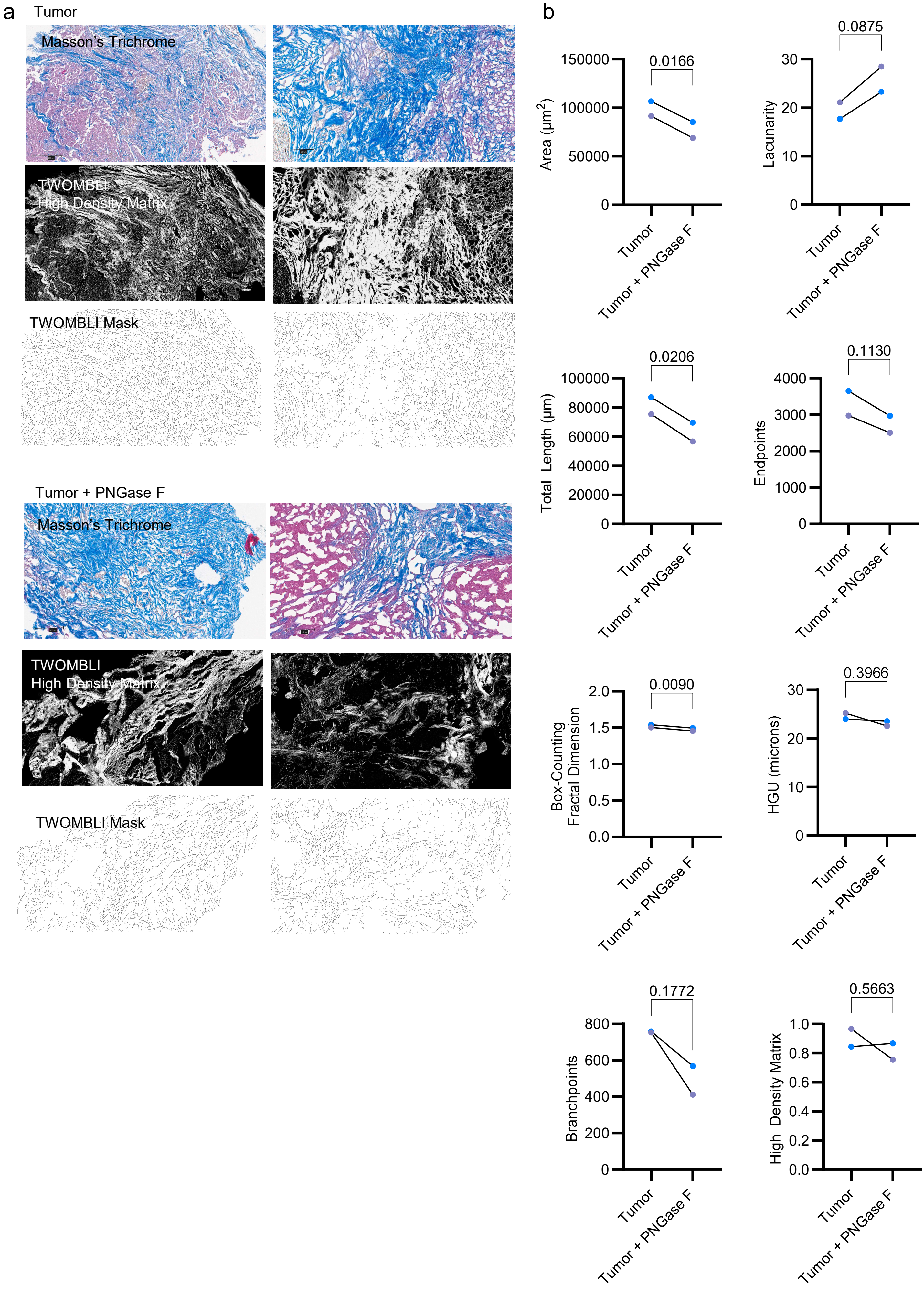
Supplementary Fig. 16: Flow gating strategy and fluorescence minus one (FMO) controls for CAR-T cell Siglec flow cytometry panel. CD4+ and CD8+ CAR-T cells assessed for Siglec expression using flow cytometry after five days. Cells were either cultured alone or co-cultured for five hours with BT20-seeded decellularized tumor tissue or tumor tissue treated with neuraminidase then transferred to a tissue culture plate for five days. N=3.



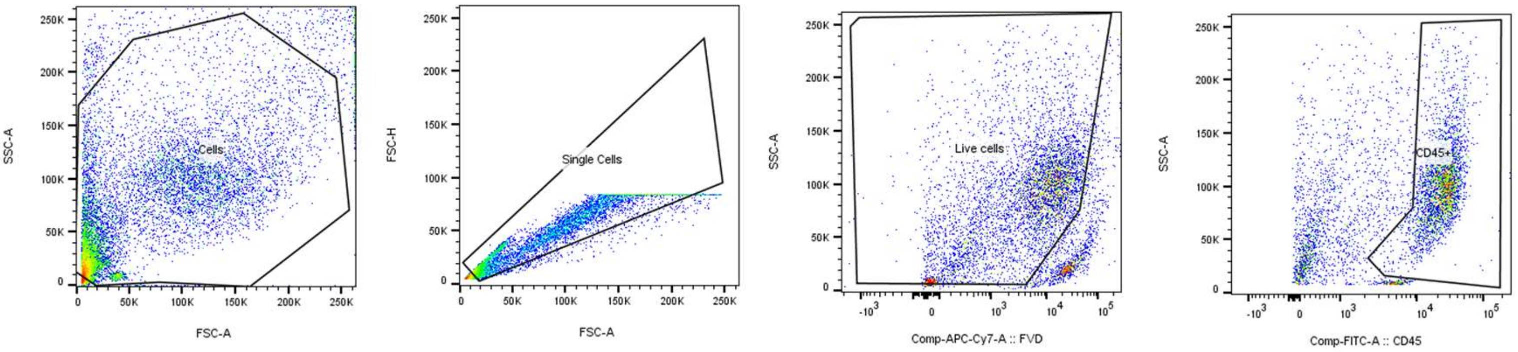
Supplementary Fig. 17: Neuraminidase treatment does not affect Siglec expression on CAR-Ts. a-b, Bar plots of flow cytometry expression patterns of CD8 and CD4 expression, shown as percentage of live cell population. **a,** NKG2D-targeting or **b,** MUC-1-targeting CAR-Ts cultured on tissue culture plastic (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.



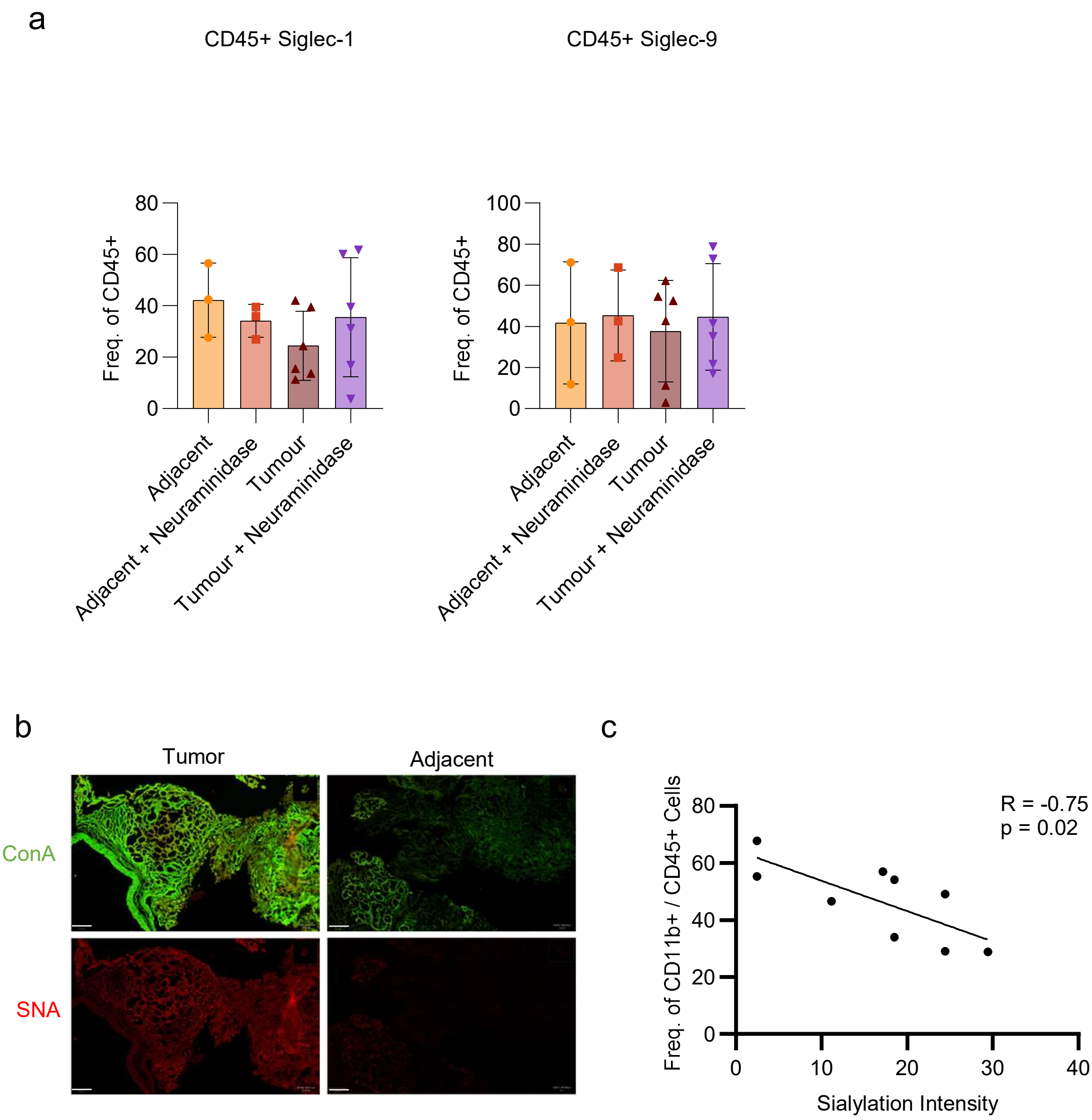
Supplementary Fig. 18: Glycan cleavage enzymes alter tissue architecture. a, Representative images of Masson's Trichrome staining & TWOMBLI analysis performed on matched tumor tissue treated with PBS or neuraminidase. Scale bar = 100μm. **b,** TWOMBLI analysis. Two-tailed paired t test. N=5 matched tumor & neuraminidase-treated tumor tissues. **c,** Representative scanning electron microscopy (SEM) images on matched tumor tissue treated with PBS or neuraminidase. Scale bar = 5μm.



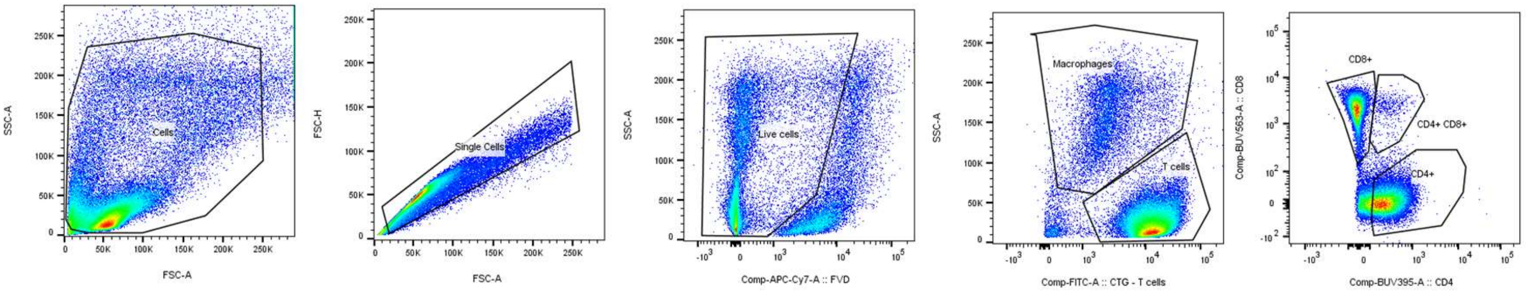
Supplementary Fig. 19: Glycan cleavage enzymes alter tissue architecture. a, Representative images of Masson's Trichrome staining & TWOMBLI analysis performed on matched tumor tissue treated with PBS or PNGase F. Scale bar = 100μm. **b,** TWOMBLI analysis. Two-tailed paired t test. N=2 matched tumor & PNGase F-treated tumor tissues.



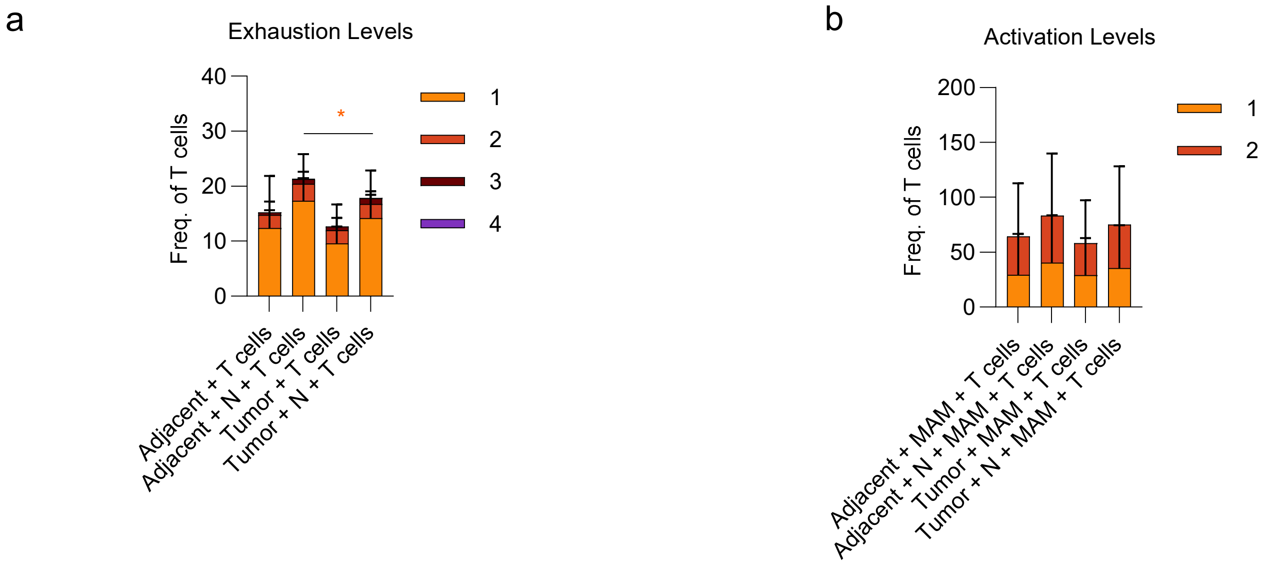
Supplementary Fig. 20. Flow gating strategy for macrophage flow cytometry assay. CD45+ macrophages as assessed using flow cytometry after 14 days culture on adjacent or tumor tissue with or without neuraminidase treatment (Figure 7). N=3.



Supplementary Fig. 21. 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 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). **b,** 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. **c,** 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.



Supplementary Fig. 22. Flow gating strategy for T cell flow cytometry assay. T cells as assessed using flow cytometry after 2 days culture in co-culture with MAMs (Figure 8). N=3.



Supplementary Fig. 23. MAMs influence T cell phenotype in a glycan-dependent manner. Stacked bar charts of combinatorial gating for **d,** exhaustion markers (PD1, LAG3, TIM3) or **f,** activation markers (ICOS & CD137) shown as percentage positive cells expressing one marker, two markers, three, or four 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=2 adjacent, N=2 tumor, N=3 blood cones).