

Supplementary information

Tractable mouse TNBC models capture the heterogeneous tumor immune microenvironment and adaptation to PD-L1 blockade

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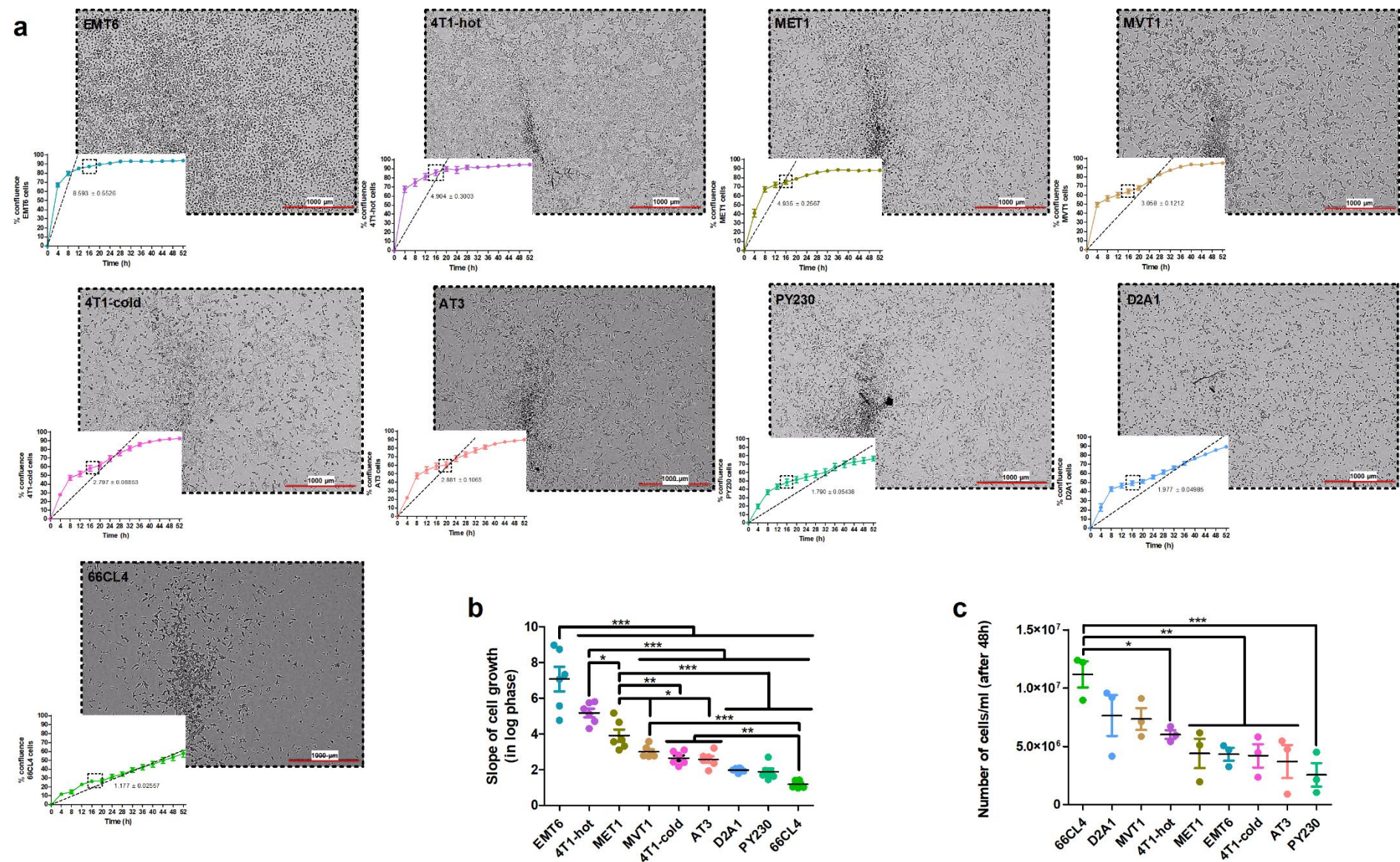
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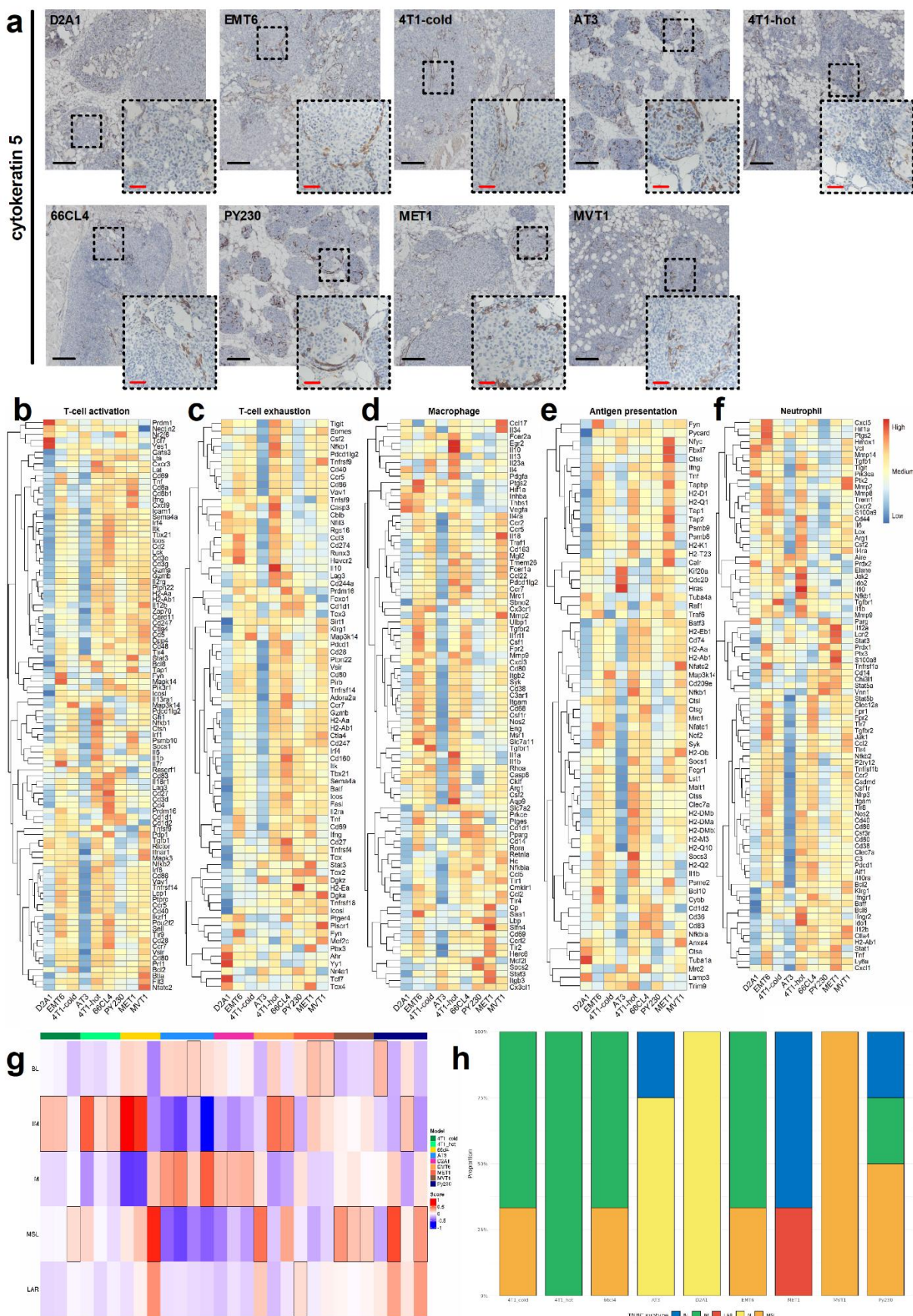
Supplementary Fig. 1



Supplementary Figure 1. In vitro growth kinetics of 9 complementary mouse TNBC cell lines.

(a) Cell growth slopes were calculated during the log phase of cell growth as indicated on the graphs for the different mouse TNBC cell lines. Representative images of the mouse TNBC cell lines after 16 h in culture highlight their differential morphology and tendency to cluster. (b) Slope of cell growth during log phase for the 9 mouse TNBC cell lines (n = 6 for each cell line). (c) Number of cells per ml after 48 h growth in a T75 flask (n = 3 for each cell line). Data in panels b and c are presented as the means +/- standard error of the mean (SEM). *p < 0.05, **p < 0.01, ***p < 0.001.

Supplementary Fig. 2



Supplementary Figure 2. Mouse TNBC cell line-derived primary tumors at an early timepoint capture the heterogeneous TIME.

(a) Staining for the myoepithelial cell marker cytokeratin 5 on sections of primary tumors at 2 w p.i. Dashed inserts highlight stained tissue at a larger magnification. Black scale bars = 200 μm , red scale bars = 50 μm . (b-f) Heatmaps showing row-normalized expression across primary tumors compared to healthy mammary glands for genes associated with T-cell activation (b), T-cell exhaustion (c), macrophages/TAMs (d), antigen presentation (e) and neutrophils/TANs (f). (g,h) Heterogeneity between the mouse TNBC cell line-derived primary tumors based on gene signatures from recently identified TIME landscapes (22).

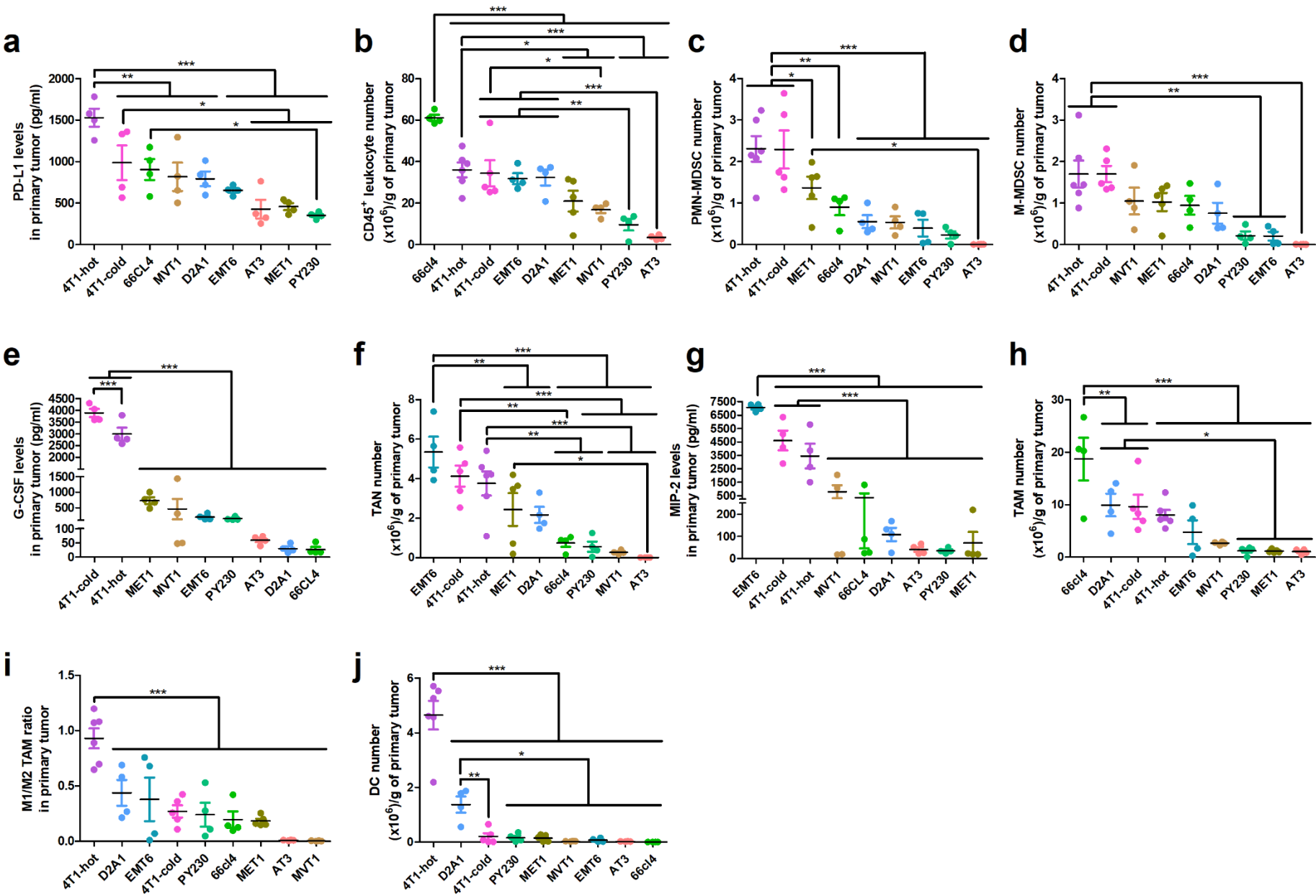
Supplementary Fig. 3



Supplementary Figure 3. Extended hallmarks of cancer associated with 9 mouse TNBC cell line-derived primary tumors at an early timepoint.

GSEA bar graph showing the NES of selected cancer hallmarks for each primary tumor compared to healthy mammary glands.

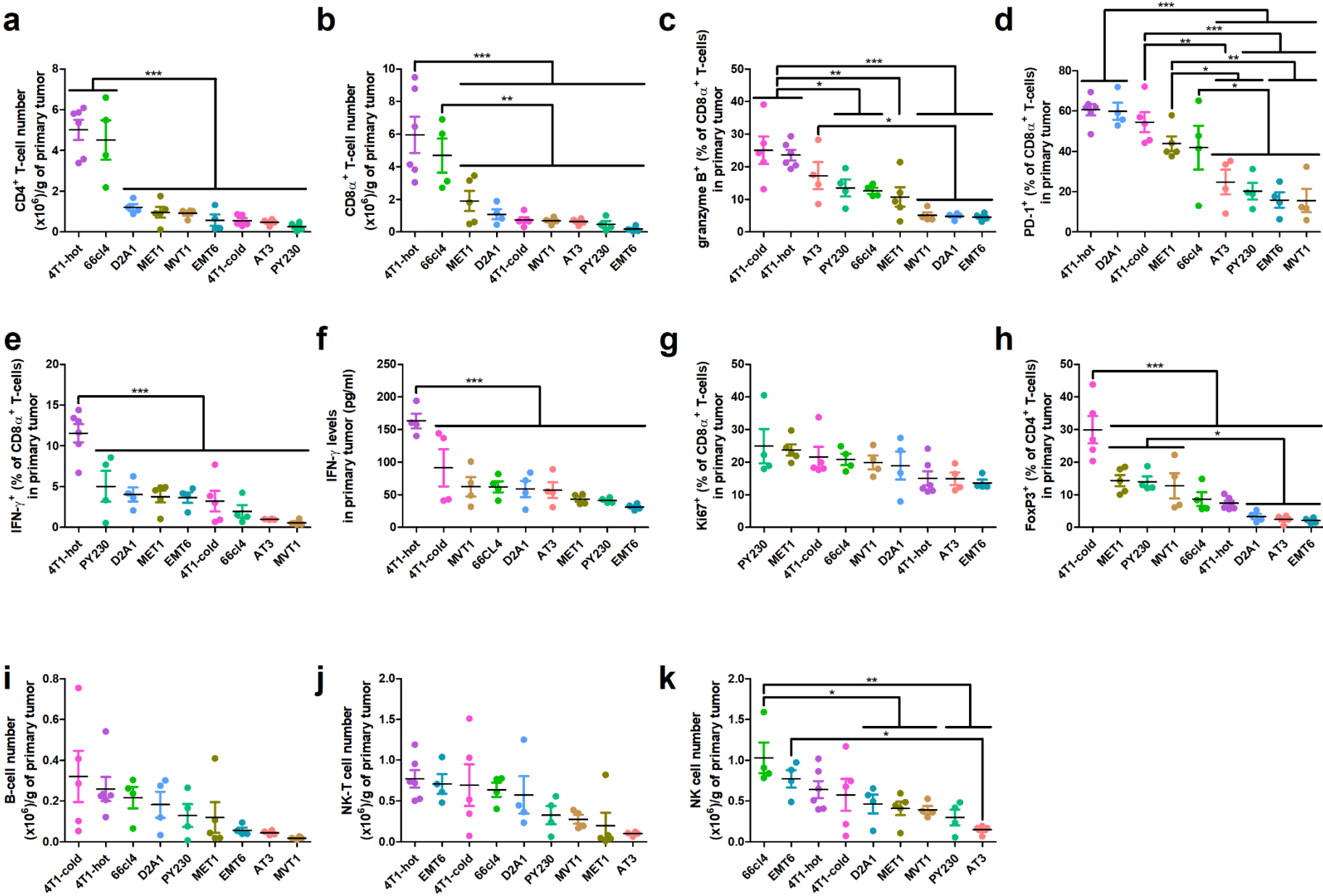
Supplementary Fig. 4



Supplementary Figure 4. Flow cytometric immunophenotyping of myeloid cell subtypes and related cytokine levels in 9 mouse TNBC cell line-derived primary tumors at a late timepoint.

(a) Levels of PD-L1 in lysates of the 9 mouse TNBC cell line-derived primary tumors. (b-d) Number of CD45⁺ leukocytes (b), PMN-MDSCs (c) and M-MDSCs (d) per gram of primary tumor at 1 cm³ size. (e) Levels of G-CSF in primary tumors at 1 cm³ size. (f) Number of TANs per gram of primary tumor at 1 cm³ size. (g) Levels of MIP-2 in primary tumors at 1 cm³ size. (h,i) Number of TAMs per gram of primary tumor at 1 cm³ size (h), with also calculated M1/M2 TAM ratio (i). (j) Number of DCs per gram of primary tumor at 1 cm³ size. Data are presented as the means +/- SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

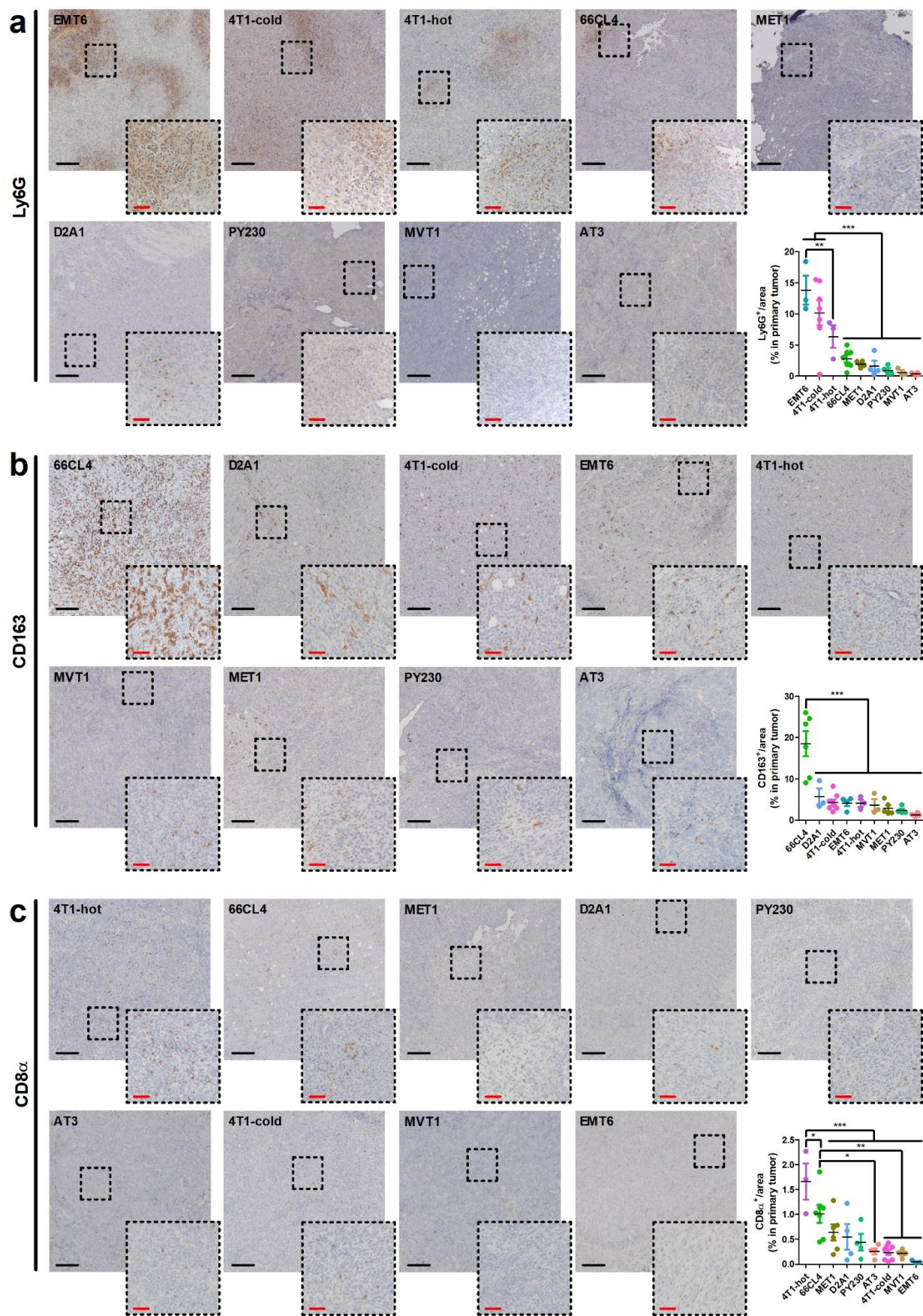
Supplementary Fig. 5



Supplementary Figure 5. Flow cytometric immunophenotyping of lymphocytic cell subtypes and related cytokines in 9 mouse TNBC cell line-derived primary tumors at a late timepoint.

(a,b) Number of CD4⁺ **(a)** and CD8 α ⁺ T-cells **(b)** per gram of primary tumor at 1 cm³ size. **(c-e)** Percentage of granzyme B⁺ **(c)**, PD-1⁺ **(d)** and IFN- γ ⁺ cells **(e)** within the CD8 α ⁺ T-cell population of primary tumors. **(f)** Levels of IFN- γ in primary tumors at 1 cm³ size. **(g)** Percentage of Ki67⁺ cells within the CD8 α ⁺ T-cell population of primary tumors. **(h)** Percentage of FoxP3⁺ cells within the CD4⁺ T-cell population of primary tumors. **(i-k)** Number of B-cells **(i)**, NK-T **(j)** and NK cells **(k)** per gram of primary tumor at 1 cm³ size. Data are presented as the means +/- SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

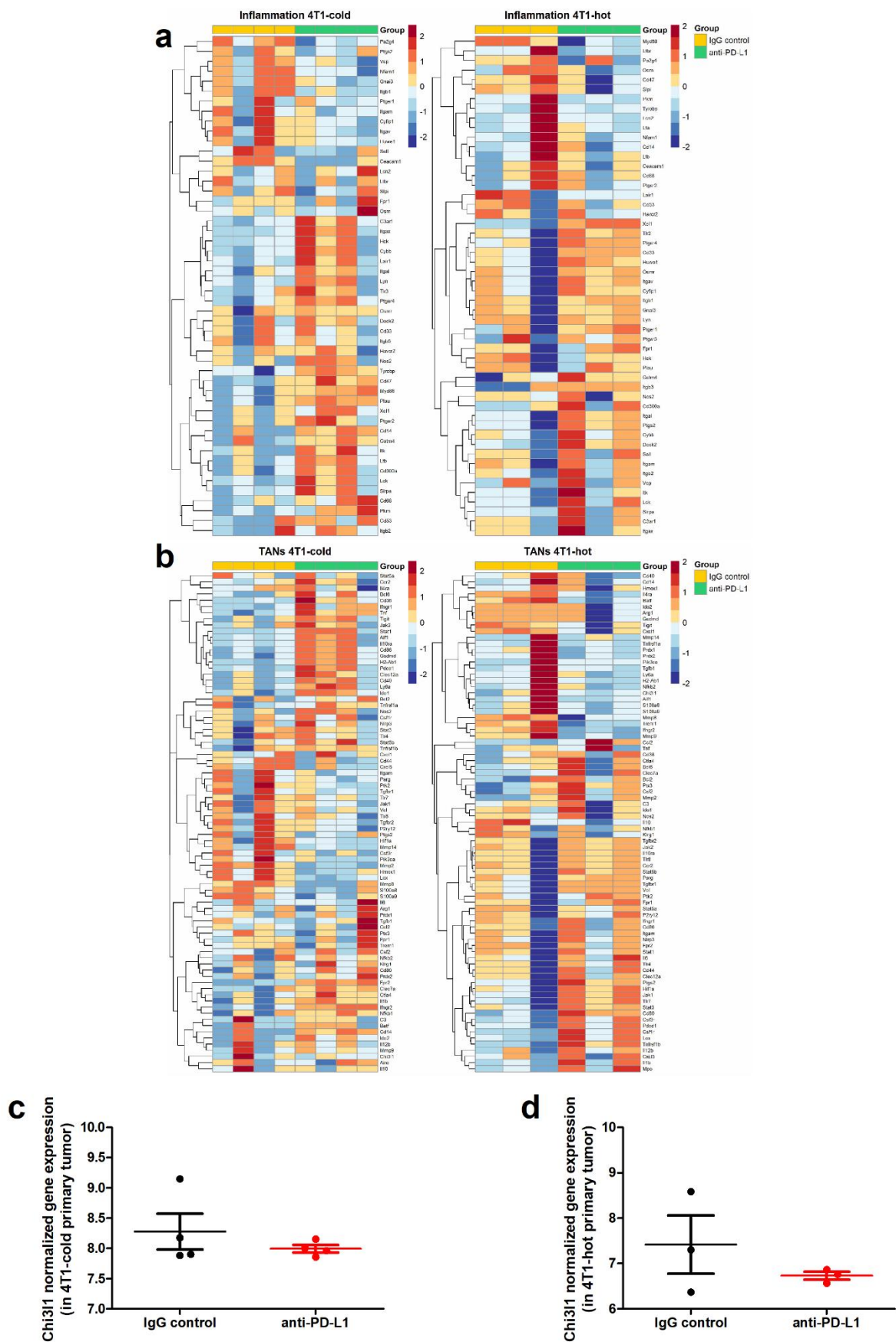
Supplementary Fig. 6



Supplementary Figure 6. Immunohistochemical analysis of immune cell markers in 9 mouse TNBC cell line-derived primary tumors at a late timepoint.

Primary tumors were isolated when reaching 1 cm³ size (i.e. at 4-6 w p.i.), fixed in formaldehyde, embedded in paraffin and processed into tissue sections for immunohistochemistry. **(a-c)** Staining for the TAN marker Ly6G **(a)**, the M2 TAM marker CD163 **(b)** and the CD8 α ⁺ T-cell marker CD8 α **(c)** on primary tumor sections (Ly6G stainings: n = 7 for 4T1-cold and 66CL4, n = 4 for D2A1, AT3, PY230 and MET1, n = 3 for EMT6, 4T1-hot and MVT1; CD163 stainings: n = 8 for 4T1-cold, n = 5 for MET1, n = 6 for 66CL4, n = 4 for EMT6, AT3, PY230, n = 3 for 4T1-hot, D2A1 and MVT1; CD8 α stainings: n = 8 for 4T1-cold, n = 7 for 66CL4, n = 6 for MET1, n = 4 for D2A1, AT3, PY230 and MVT1, n = 3 for 4T1-hot and EMT6). Dashed inserts highlight stained tissue at a larger magnification. Black scale bars = 200 μ m, red scale bars = 50 μ m. Data are presented as the means $\pm$ SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

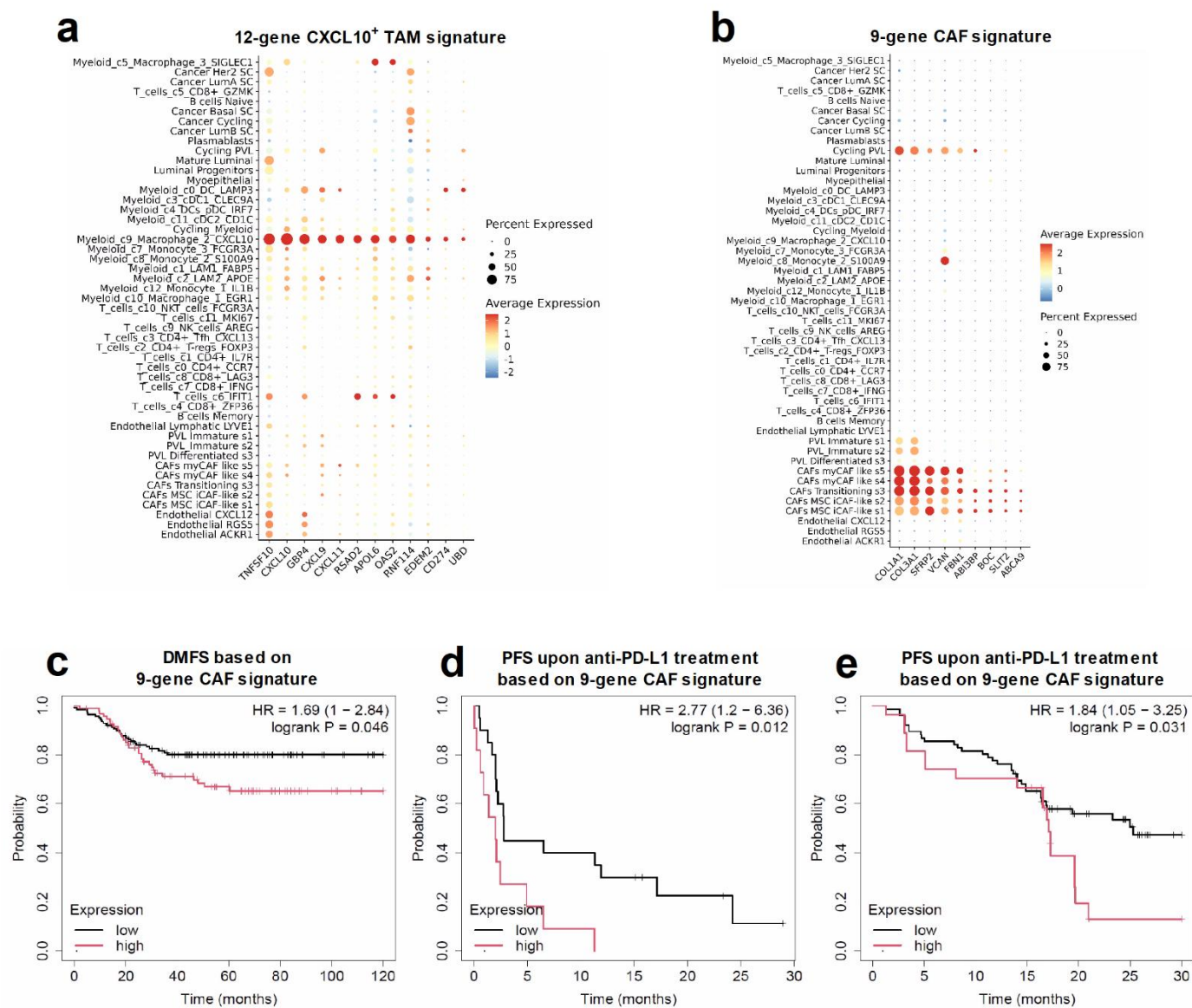
Supplementary Fig. 7



Supplementary Figure 7. General inflammation- and neutrophil-associated gene signatures in 4T1-cold and 4T1-hot primary tumors upon PD-L1 treatment.

(a,b) Heatmaps showing row normalized expression levels of genes associated with general inflammation **(a)** and TANs **(b)** in anti-PD-L1- and IgG-treated 4T1-cold (left) and 4T1-hot primary tumors (right). **(c,d)** Normalized gene expression levels of the TAN-associated gene *Chi3l1* in anti-PD-L1- and IgG control-treated 4T1-cold **(c)** and 4T1-hot primary tumors **(d)**. Data in panel c and d are presented as the means $\pm$ SEM.

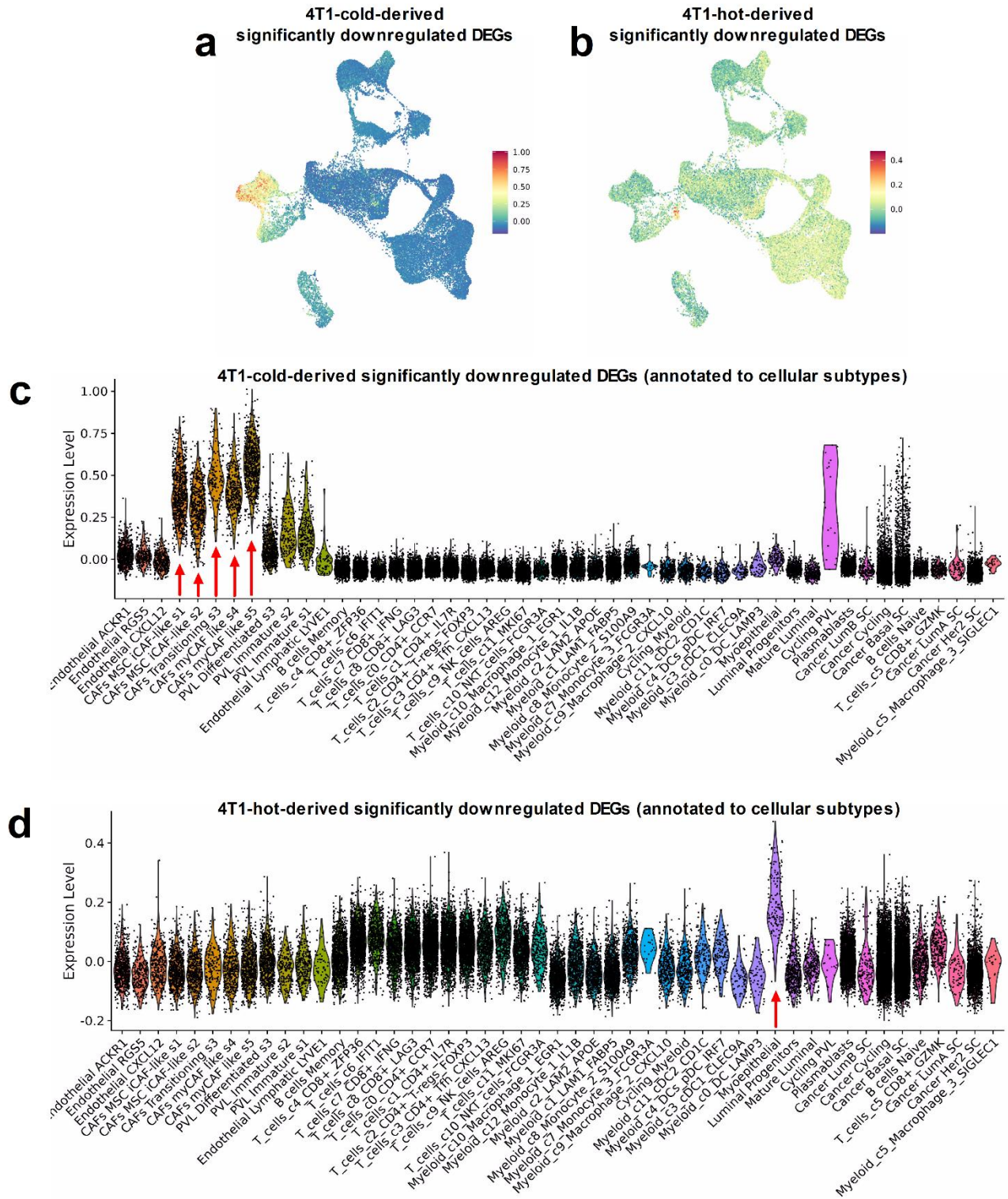
Supplementary Fig. 8



Supplementary Figure 8. Defining a 12-gene CXCL10⁺ TAM and 9-gene CAF signature upon anti-PD-L1-treatment in respectively 4T1-cold and 4T1-hot primary tumors.

(a,b) Annotation of a 12-gene signature to CXCL10⁺ TAMs **(a)** and a 9-gene signature to CAFs **(b)** using an existing scRNA-seq atlas of human TNBC (25). **(c)** Kaplan-Meier plots showing distant metastasis-free survival (DMFS) over 10 years time in TNBC patients based on mean expression of a 9-gene CAF signature. Number of included TNBC patients for analysis: 235, with PAM50 and StGallen basal classification. Patients were split in high vs low expressors based on auto select of the best cutoff (percentile) by the KM-plotter tool for breast cancer mRNA gene chip data. **(d,e)** Kaplan-Meier plot showing progression-free survival (PFS) over 30 months time upon anti-PD-L1 treatment in bladder cancer **(d)** and esophageal adenocarcinoma **(e)** based on mean expression of a 9-gene CAF signature. Number of included patients for analysis: 31 for bladder cancer and 103 for esophageal adenocarcinoma, treated with either Atezolizumab or Durvalumab. Patients were split in high vs low expressors based on auto select of the best cutoff (percentile) by the KM-plotter tool for immunotherapy.

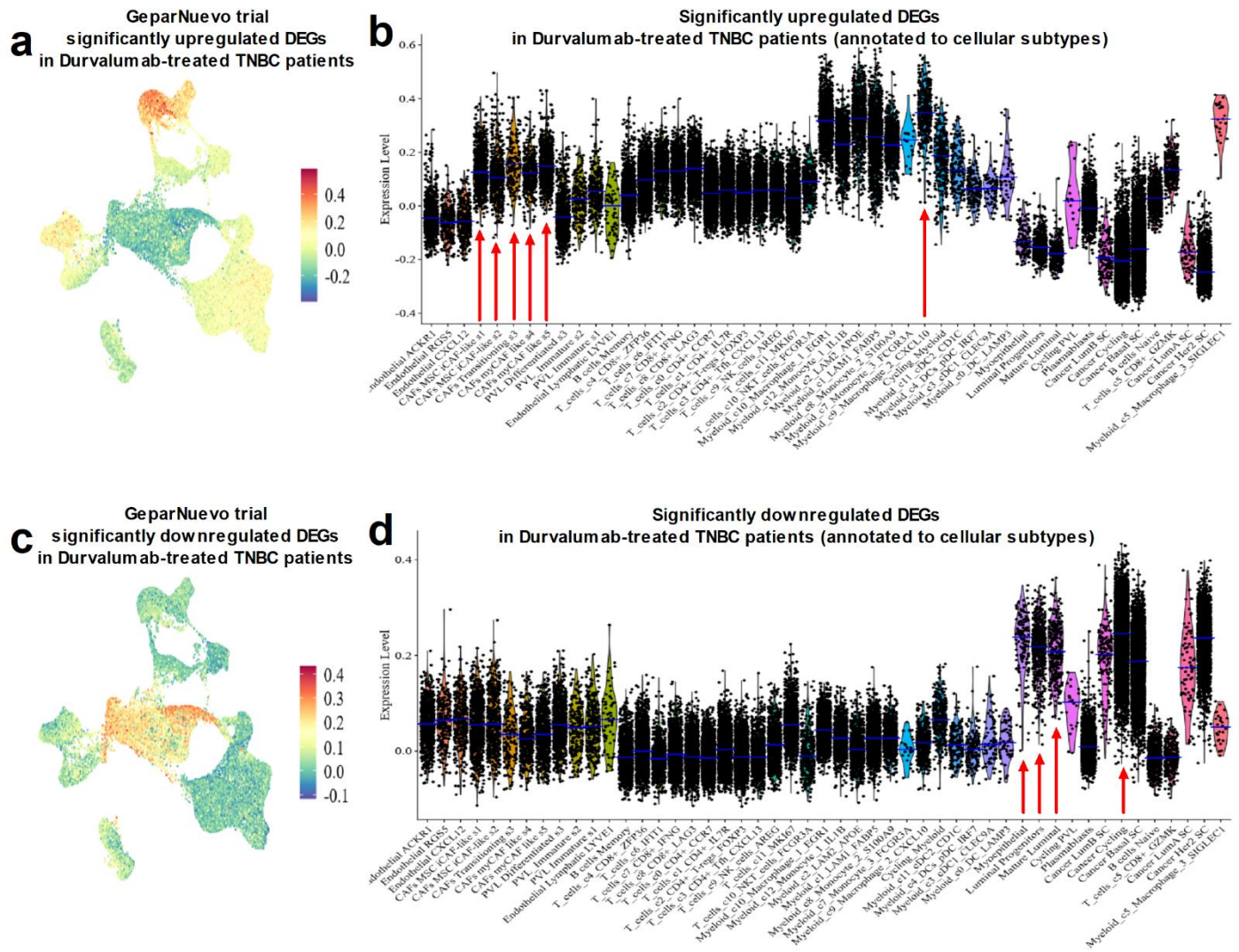
Supplementary Fig. 9



Supplementary Figure 9. Mapping downregulated gene sets in anti-PD-L1-treated 4T1-cold and 4T1-hot primary tumors to respectively CAFs and myoepithelial cells.

(a,b) UMAP of calculated gene set expression of significantly downregulated DEGs in 4T1-cold **(a)** and 4T1-hot primary tumors **(b)** upon anti-PD-L1 treatment across all cell type clusters. **(c,d)** Violin plots showing distribution of significantly downregulated DEGs in 4T1-cold **(c)** and 4T1-hot primary tumors **(d)** upon anti-PD-L1 treatment across specific cellular subtype clusters.

Supplementary Fig. 10



Supplementary Figure 10. Mapping up- and downregulated gene sets in primary breast carcinoma from Durvalumab-treated TNBC patients with absence of disease reduction.

(a) UMAP of calculated gene set expression of significantly upregulated DEGs in primary breast carcinoma from Durvalumab-treated TNBC patients across all cell type clusters. (b) Violin plots showing distribution of significantly upregulated DEGs in primary breast carcinoma from Durvalumab-treated TNBC patients across specific cellular subtype clusters. (c) UMAP of calculated gene set expression of significantly downregulated DEGs in primary breast carcinoma from Durvalumab-treated TNBC patients across all cell type clusters. (d) Violin plots showing distribution of significantly downregulated DEGs in primary breast carcinoma from Durvalumab-treated TNBC patients across specific cellular subtype clusters.