

SUPPLEMENTAL FIGURE LEGENDS:

Figure S1: A single-cell transcriptomic atlas of exhausted and non-exhausted breast tumor microenvironments

- A.** Number of patient samples subjected to sequencing by tumor subtype (left) and tumor grade (right).
- B.** Number of sequenced cells per patient sample before and after cell filtering.
- C.** UMAP plot of scRNA-seq data colored by cluster from initial high-resolution Seurat clustering.
- D.** DotPlot of scRNA-seq data showing expression of indicated markers in the high-resolution clusters.

Figure S2: IMC setup and cell type annotation based on IMC Protein Panel data

- A.** List of antigens and transcripts detected using both panels (left), only the Protein Panel (middle), or only the RNA Panel (right).
- B.** Exemplary IMC images of regions classified as non-TLS, immature TLS (pre-TLS), or mature TLS.
- C.** Line plot comparing total cell numbers in matched images from the Protein Panel and the RNA Panel datasets.
- D.** UMAP plot of IMC Protein Panel data of a random subset of 10,000 cells colored by cell class.
- E.** UMAP plots of IMC Protein Panel data colored by marker expression.
- F.** Heatmap of IMC Protein Panel data showing normalized average expression of selected marker genes for all stromal/immune subclusters.
- G.** Stacked barplot of IMC protein panel data displaying cell type compositions for all individual images (annotated with information about patient sample, immune environment (IE) classification and TLS status).
- H.** Neutrophil frequency in IE1 and IE2 tumors (IMC Protein Panel data). Wilcoxon rank sum test was used for statistical analysis. Boxplot body shows IQR, and whiskers extend to the largest and the smallest value lying within 1.5 times the IQR above the 75th percentile and below the 25th percentile, respectively. TLS and pre-TLS images were excluded to avoid bias.

Figure S3: Cell type annotation and cytokine expression definition based on IMC RNA Panel data

- A.** UMAP plot of IMC RNA Panel data of a random subset of 10,000 cells colored by cell class.
- B.** Heatmap of IMC RNA panel data showing normalized average expression of selected marker genes for all stromal/immune subclusters.
- C.** UMAP plots IMC RNA Panel data of all T and NK cells colored by subtype (top) and marker expression (bottom).

Figure S4: Detailed transcriptomics-based T and NK cell subcluster analysis

- A. Tree plot of scRNA-seq data for T and NK cell clusters using different clustering resolution parameters.
- B. UMAP plot of scRNA-seq data of 36,000 T and NK cell cells colored by IE (left) and patient sample (right).
- C. Stacked barplots of the absolute cell number originating from each patient sample in each T and NK cell cluster (scRNA-seq data).
- D. Heatmap of normalized average expression of the top 10 differentially expressed genes of each T and NK cell cluster (see also Supplementary Table 2A).
- E. Ratio of mean *CD8* and mean *CD4* expression for each T cell cluster. To obtain mean *CD8* expression, mean *CD8A* and mean *CD8B* expression were averaged. Dashed line indicates where cutoff for *CD8*⁺ T cell clusters was made.
- F. Bar chart indicating proportion of cells that express either *KLRC1* or *CD3E* or both for each T and NK cell cluster.
- G. Frequency of *PDCD1*-expressing T and NK cells in scRNA-seq data versus frequency of T cells belonging to PD-1^{high} clusters in CyTOF data for all patient samples.
- H. Bar plot showing the log2-fold expression change of *MHC-I* transcripts and *CD274* in the epithelial subset and *CD274* in the myeloid subset from IE1 relative to IE2 (patient-averaged pseudobulk data).
- I. Boxplot of IMC protein panel data comparing the mean PD-L1 protein expression for the epithelial subset of IE1 versus IE2 samples. Wilcoxon rank sum test was used for statistical analysis. Boxplot body shows IQR, and whiskers extend to the largest and the smallest value lying within 1.5 times the IQR above the 75th percentile and below the 25th percentile, respectively.
- J. Stacked barplots indicating the slide-wide *CD8*⁺ T cell infiltration status for a cohort of 14 IE1 samples and 11 IE2 samples (including the samples analyzed by IMC).
- K. Scatterplot of Tfh frequency versus the frequency of *CD8*⁺ exhausted T cells (left), and B cells (middleright) and plasma cells (right) as defined by scRNA-seq.
- L. Scatterplot of mean *CXCL13* transcript abundance versus mean *PDCD1* transcript abundance for each T and NK cell cluster (top) and each patient sample (bottom).

For scatterplots, Spearman correlation coefficient and p value are indicated.

Figure S5: IMC-based T cell subclustering and comparison to scRNA-seq

- A. UMAP plot of all T and NK cells colored by subcluster based on IMC data (Protein Panel). Cluster 9 was excluded from further T cell subtype analysis as it was high for all markers and present in only one sample.
- B. Heatmap of normalized average expression of the most relevant markers for each T and NK cell subcluster in IMC data (Protein Panel). Subtype annotation is displayed above the heatmap.

- C. Boxplot comparing PD-1^{high} cell proportions out of all CD8⁺ T cells (left) and CD4⁺ T cells (right) between IE1 and IE2 samples based on IMC data (Protein Panel). Wilcoxon rank sum test was used for statistical analysis.
- D. Boxplot comparing the mean TCF7 counts between CD8⁺ T cells from IE1 and IE2 samples based on IMC data (Protein Panel).
- E. Barplots showing the proportion of Ki-67⁺ cells for PD-1^{high} and PD-1^{low} T cell subsets based on IMC data (Protein Panel).
- F. Scatterplot of the CXCL13⁺ T cell proportion versus the PD-1^{high} T cell proportion for matched ROIs from the RNA Panel and the Protein Panel, respectively.
- G. Scatterplot of CXCL13^{high} cell proportions as defined by IMC versus scRNA-seq for CD8⁺ T cells (left) and CD4⁺ T cells (right). A significant correlation persists even after removal of the apparent outlier.

For scatterplots, Spearman correlation coefficient and p value are indicated. For boxplots, Wilcoxon rank sum test was used for statistical analysis. Boxplot bodies show IQR, and whiskers extend to the largest and the smallest value lying within 1.5 times the IQR above the 75th percentile and below the 25th percentile, respectively. For panels C and G, only non-TLS images were included in the analysis in order to avoid region-selection bias.

Figure S6: Marker genes associated with exhaustion over pseudotime and IMC validation of exhaustion-related cytotoxic signatures

- A. Boxplot of IMC Protein Panel data comparing the mean Granzyme B protein expression between CD8⁺ T cells from IE1 and IE2 samples.
- B. Single-cell expression along pseudotime for transcript levels used as input of the Ouija pseudotime computation (*GZMB*, *FASL*, *TNF*, *IFNG*, and *GZMK* were also used as input and are included in the main figure). Red line corresponds to locally estimated scatterplot smoothing (LOESS) curve.
- C. Boxplot of IMC RNA Panel Data comparing the NKT cell proportions in IE1 and IE2 tumors.

For scatterplots, Spearman correlation coefficient and p value are indicated. For boxplots, Wilcoxon rank sum test was used for statistical analysis. Boxplot bodies show IQR, and whiskers extend to the largest and the smallest value lying within 1.5 times the IQR above the 75th percentile and below the 25th percentile, respectively.

Figure S7: Detailed transcriptomics-based myeloid cell subcluster analysis

- A. Bar plots showing differential expression of the indicated genes between myeloid cells from IE1 and IE2 tumors in pseudobulk patient-averaged scRNA-seq data. The four graphs show levels of transcripts encoding (left to right) selected cytokines, complement system activation components, matrix metalloproteinases, and metallothionein proteins. For each gene group, with the exception of the cytokine group, all genes with an expression level >1.5 cpm in the higher

expressing IE were included in the plot. For the cytokine group, the eight genes with the lowest FDR were included.

- B. Boxplots comparing the T and NK patient-averaged pseudobulk expression of *CD46* and *CD55* in IE1 and IE2 samples.
- C. UMAP plot of 26,000 myeloid cells colored by patient (left) and IE (right).
- D. Stacked barplots of relative myeloid cluster frequencies for each patient.
- E. Stacked barplots showing myeloid cluster composition by patient.
- F. Heatmap showing normalized average expression of myeloid markers commonly used for subtype classification and/or FACS and CyTOF experiments.
- G. Boxplots comparing distances to the next T cell from myeloid cells that express at least one of the measured T cell-attracting cytokines versus other myeloid cells, based on IMC RNA Panel data. Only non-TLS images were included.
- H. Boxplots comparing the proportion of PD-1^{high} T cells in the 10 T cells spatially closest to myeloid cells with and without a suppressive phenotype based on IMC Protein panel data. T_{regs} were excluded for this analysis.
- I. Single-cell scatterplot of the T cell-attraction score versus the T cell-suppression score.
- J. Scatterplot of the mean T cell-suppression score versus the mean T cell-attraction score for all patients colored by IE.
- K. Single-cell scatterplot of the M1 score versus the M2 score.
- L. Boxplots comparing the mean T cell-attraction, T cell-suppression, M1, and M2 scores for myeloid cells from IE1 and IE2 tumors.

For boxplots, Wilcoxon rank sum test was used for statistical analysis. Boxplot bodies show IQR, and whiskers extend to the largest and the smallest value lying within 1.5 times the IQR above the 75th percentile and below the 25th percentile, respectively. ****
p < 0.0001.

Figure S8: Expression profile and pseudotime developmental trajectory of migDCs

- A. DotPlot of scRNA-seq data showing expression of *CD274*, *LAMP3*, and *CCR7* across all original high-resolution clusters of the full dataset.
- B. Heatmap of IMC Protein Panel data showing normalized average expression of LAMP3, PD-L1, IDO, and CD40 proteins for all cell subtypes.
- C. Bar plots of IMC RNA Panel data displaying percentage of each cell type that expresses *CCL17* (left) or *CCL22* (right) mRNA.
- D. Monocle2 trajectory inference of cDC subsets and migDCs based on scRNA-seq data.

Figure S9: A map of predicted ligand-receptor interactions across exhausted and non-exhausted breast tumor microenvironments

- A.** Heatmap depicting the number of interactions with an LR score > 0.4 between all main cell type pairs, as given by SingleCellSignalR.
- B.** Heatmap depicting the number of significant interactions between all main cell type pairs as given by CellPhoneDB. The heatmap is symmetric because LR pairs are not treated as directional in CellPhoneDB. Significance of interactions was calculated directly by the CellPhoneDB algorithm.
- C.** Heatmap showing the LR score for a selection of ligand-receptor interactions over the five most frequent cell type pairs. From the 100 top-scoring ligand-receptor pairs for each cell type pair, the five ligand-receptor pairs with the highest coefficient of variation across cell type pairs were extracted. Ligand-receptor pairs were then selected from this subset based on the strength of literature evidence and biological interpretability. Full SingleCellSignalR results are available in Supplementary Table 6.
- D.** Boxplots comparing LR scores (from SingleCellSignalR, top) and LR means (from CellPhoneDB, bottom) for a selection of ligand-receptor pairs in IE1 and IE2 tumors. Wilcoxon rank sum test was used for statistical analysis. Variations in p values can be explained by the inherently different interaction scoring approach employed by the two methods. Boxplot bodies show IQR, and whiskers extend to the largest and the smallest value lying within 1.5 times the IQR above the 75th percentile and below the 25th percentile, respectively.
- E.** Heatmap showing the LR scores for a selection of LR interactions over the main T and NK-myeloid metacluster pairs. From the 100 top-scoring LR pairs for each metacluster pair, the five LR pairs with the highest coefficients of variation across metacluster pairs were extracted. LR pairs were then selected from this subset based on the strength of literature evidence and biological interpretability (full SingleCellSignalR results are available in Supplementary Table 6). Less relevant cell types (LC, pDC, T-mixed) as well as myeloid-myeloid and T and NK autopairs were excluded for clarity of the figure. M indicates myeloid metacluster, T indicates T and NK cell metacluster.

Figure S10: IMC analysis confirms elevated cytokine expression in IE1 and reveals TLS-specific cell type distribution

- A.** Boxplot comparing the proportion of cytokine-expressing cells for IE1 and IE2 samples. Each dot represents a sample. Only non-TLS images were included and Wilcoxon rank sum test was used for statistical analysis.
- B.** Boxplot comparing the proportion of cytokine-expressing cells for IE1 and IE2 samples separated by cell type.
- C.** Boxplots comparing the proportions of cytokine-expressing cells that are part of a cytokine motif for IE1 and IE2 samples. Only non-TLS images were included and Wilcoxon rank sum test was used for statistical analysis.
- D.** Boxplots comparing the frequency of all cell subtypes for non-TLS, immature TLS, and mature TLS images. The analysis is based on the Protein Panel data and Wilcoxon rank sum test was used for statistical analysis.

- E. Boxplots comparing the frequency of CXCL13⁺/CD8⁺ and CXCL13⁺/CD4⁺ T cells for non-TLS, immature TLS, and mature TLS images. Wilcoxon rank sum test was used for statistical analysis.
- F. Heat map indicating significant pairwise cell type interaction or avoidance summarized across the two-sided permutation tests on all TLS images (mature and immature) of the Protein Panel dataset ($n = 19$ images, 1,000 permutations each). Square color indicates the percentage of images with a significant cell-cell interaction or avoidance ($p < 0.01$).

Boxplot bodies show IQR, and whiskers extend to the largest and the smallest value lying within 1.5 times the IQR above the 75th percentile and below the 25th percentile, respectively.

Fig. S11: Tumor grade does not account for the observed differences between exhausted and non-exhausted immune environments

- A. Volcano plot showing differential gene expression between T and NK cells of IE1 and IE2 samples in pseudobulk patient-averaged scRNA-seq data when tumor grade is used as a blocking factor in the model (compare Fig. 2A). Dashed lines indicate a false discovery rate (FDR) of 0.1 and a log2 fold change (logFC) of 0.5.
- B. Enrichment of T/NK cluster frequencies, annotated by cell type, in lower grade (G1/G2) or higher grade (G3) samples. Wilcoxon rank sum test was used for statistical analysis and dashed lines indicate a p value of 0.05. Enrichment of the respective cluster in IE1 or IE2 is indicated by a colored box (compare Fig. 2E).
- C. Boxplot comparing the mean single-cell HLA-ABC expression in IMC data for the epithelial subsets of lower grade (G1/G2) versus higher grade samples (G3) (compare Fig. 2I).
- D. Boxplot comparing CXCL13^{high} cell proportions out of all CD8⁺ T cells (left) and CD4⁺ T cells (right) between IMC samples of lower grade (G1/G2) versus higher grade (G3) (compare Fig. 2K). Only non-TLS images were included and Wilcoxon rank sum test was used for statistical analysis.
- E. Volcano plot showing differential gene expression between myeloid cells of IE1 and IE2 samples in pseudobulk patient-averaged scRNA-seq data when tumor grade is used as a blocking factor in the model (compare Fig. 4A). Dashed lines indicate an FDR of 0.1 and a logFC of 0.5. Genes are colored by functional group.
- F. Enrichment of myeloid cluster frequencies in lower grade (G1/G2) or higher grade samples (G3). Wilcoxon rank sum test was used for statistical analysis and dashed lines indicate a p value of 0.05. Enrichment of the respective cluster in IE1 or IE2 is indicated by a colored box (compare Fig. 4C).

Fig. S12: Staining patterns of all antibodies included in the Protein Panel on exemplary IMC images of breast cancer samples from this cohort.

Fig. S13: Staining patterns of all antibodies included in the RNA Panel on exemplary IMC images of breast cancer samples from this cohort.

Fig. S1

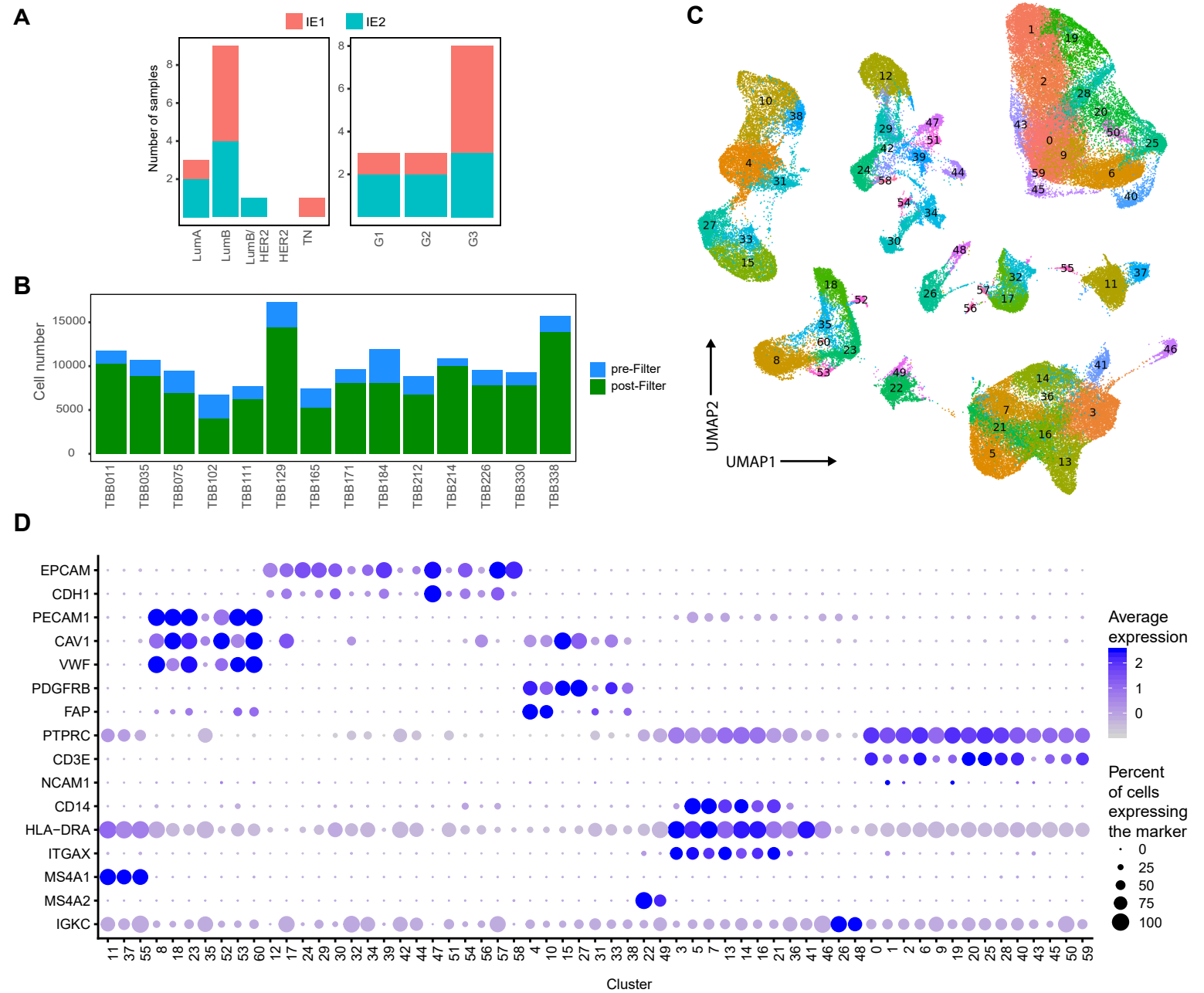


Fig. S2

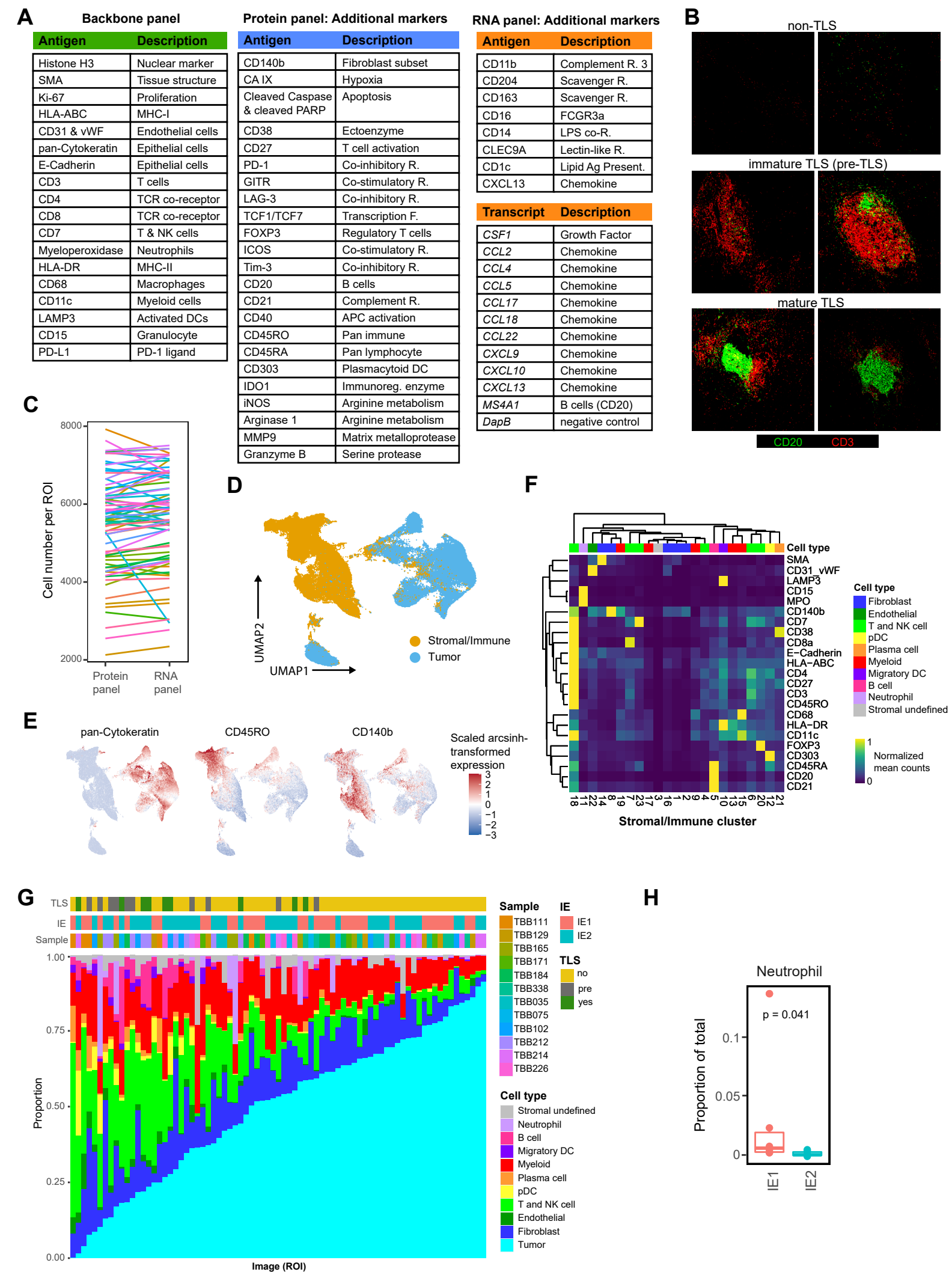
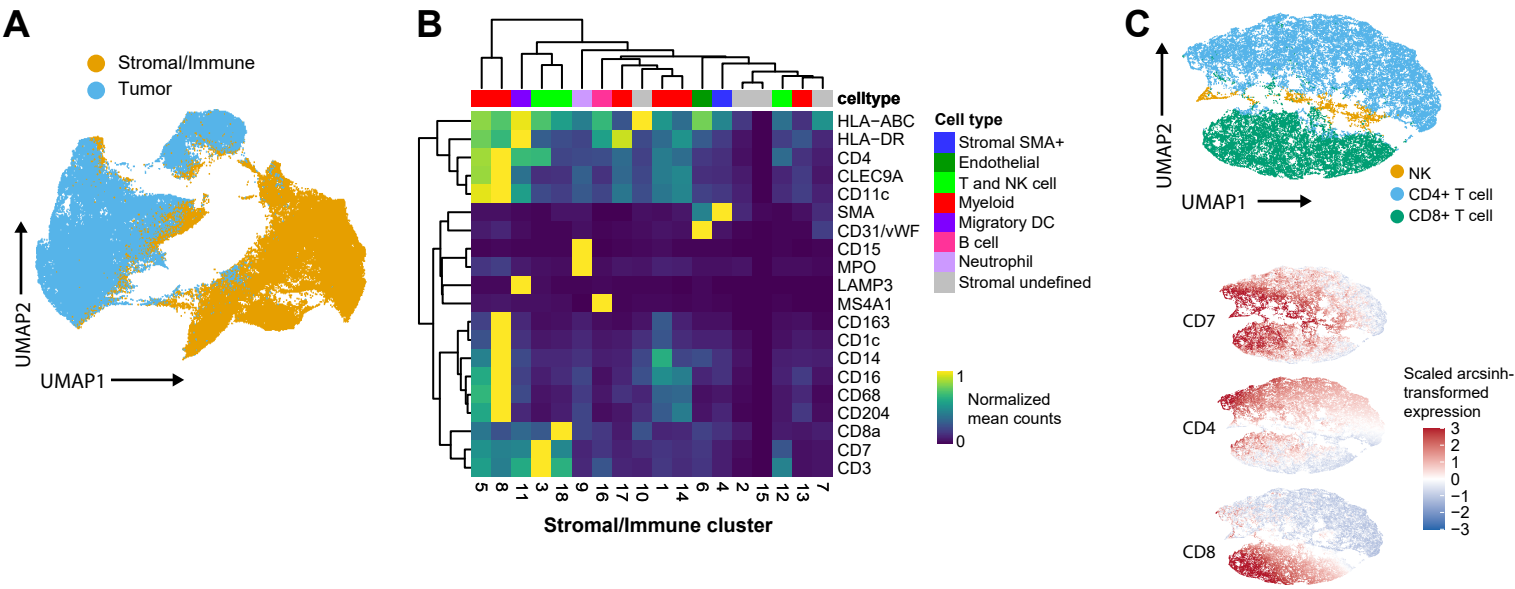


Fig. S3



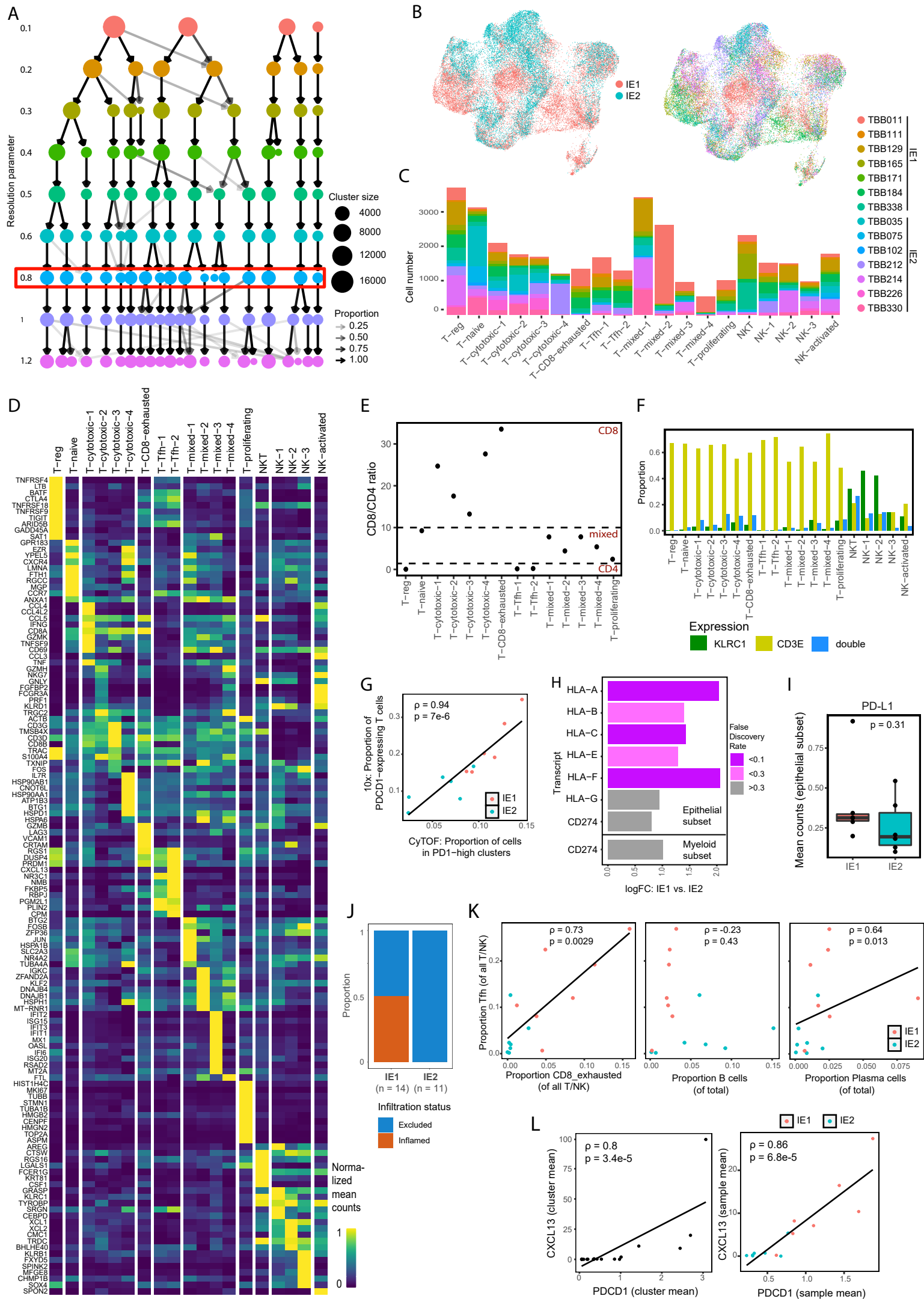


Fig. S5

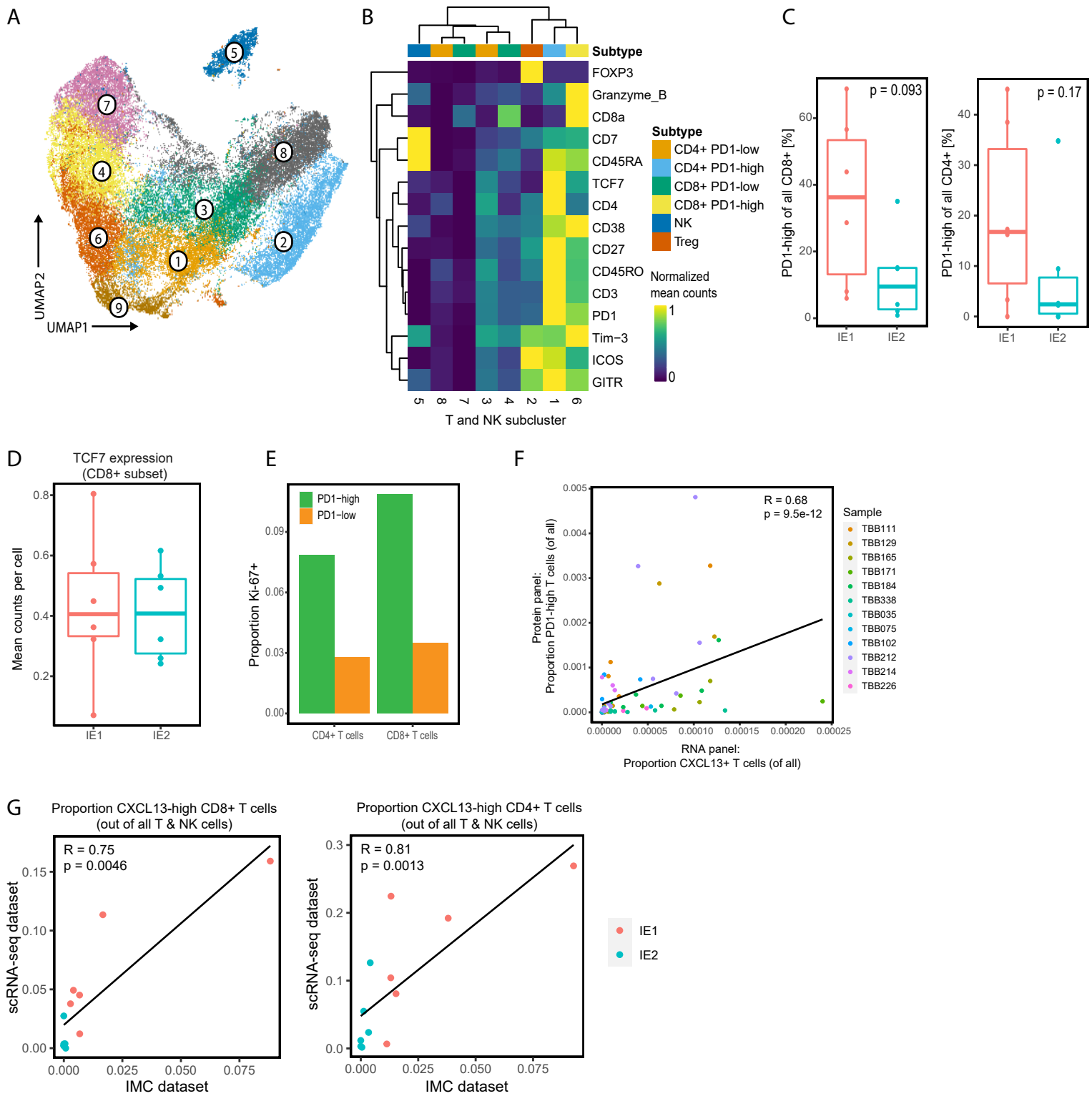
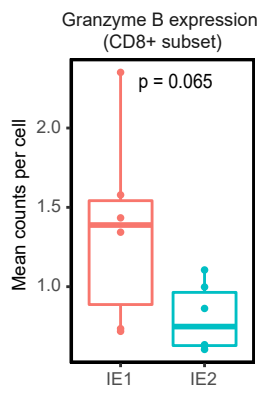
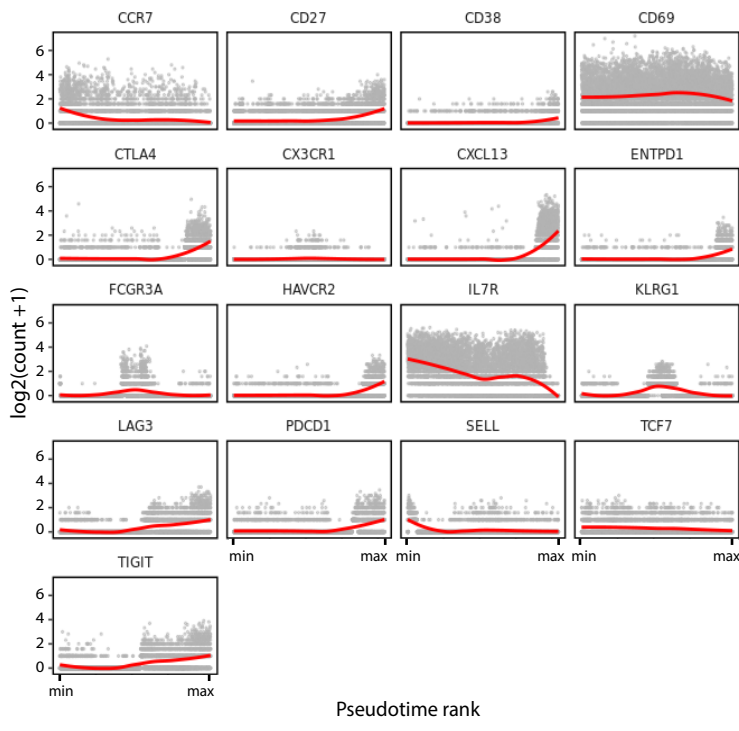


Fig. S6

A



B



C

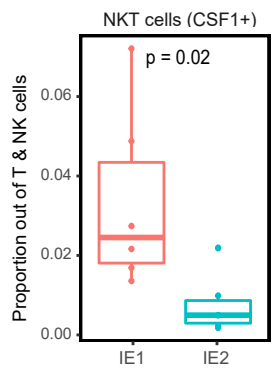


Fig. S7

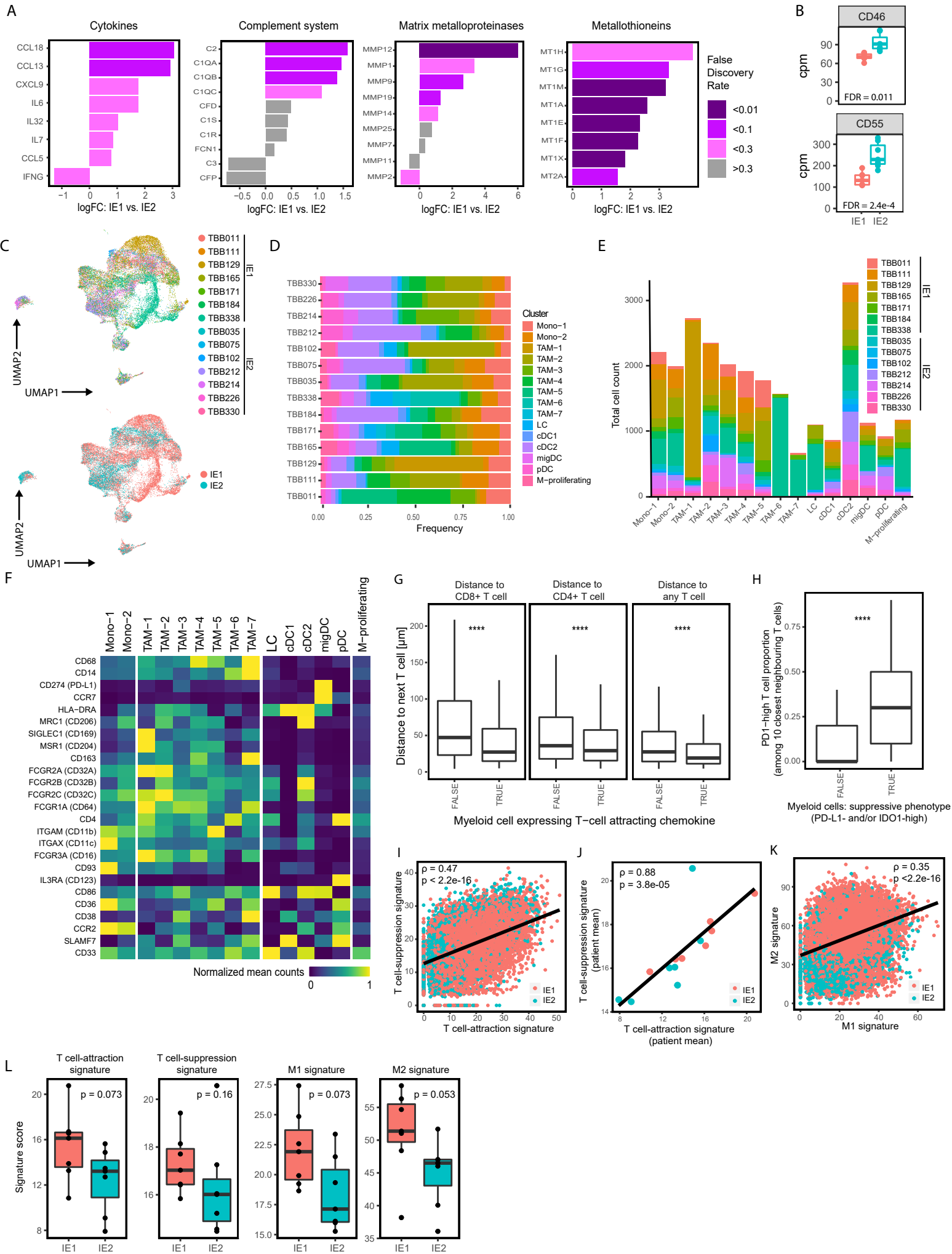
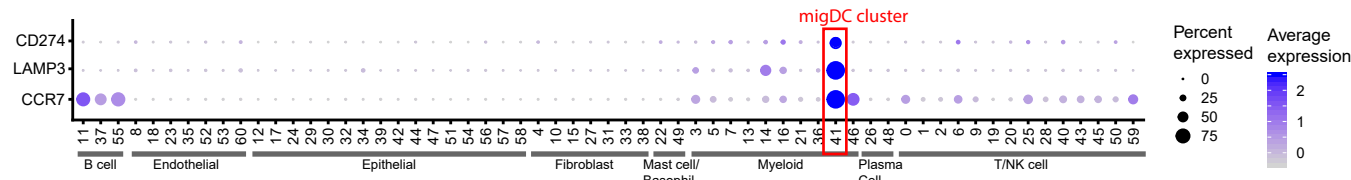
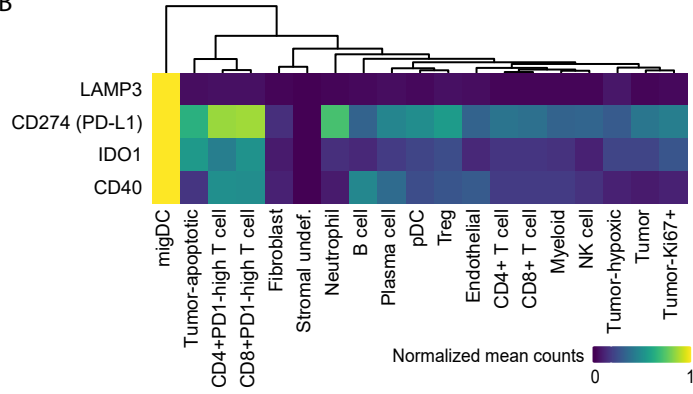


Fig. S8

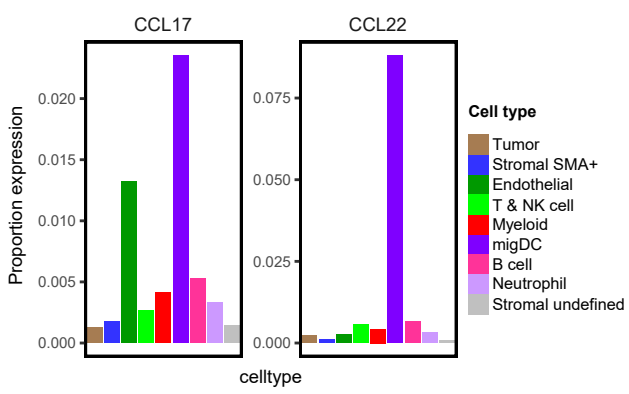
A



B



C



D

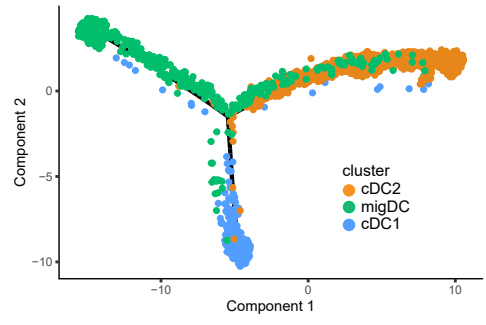


Fig. S9

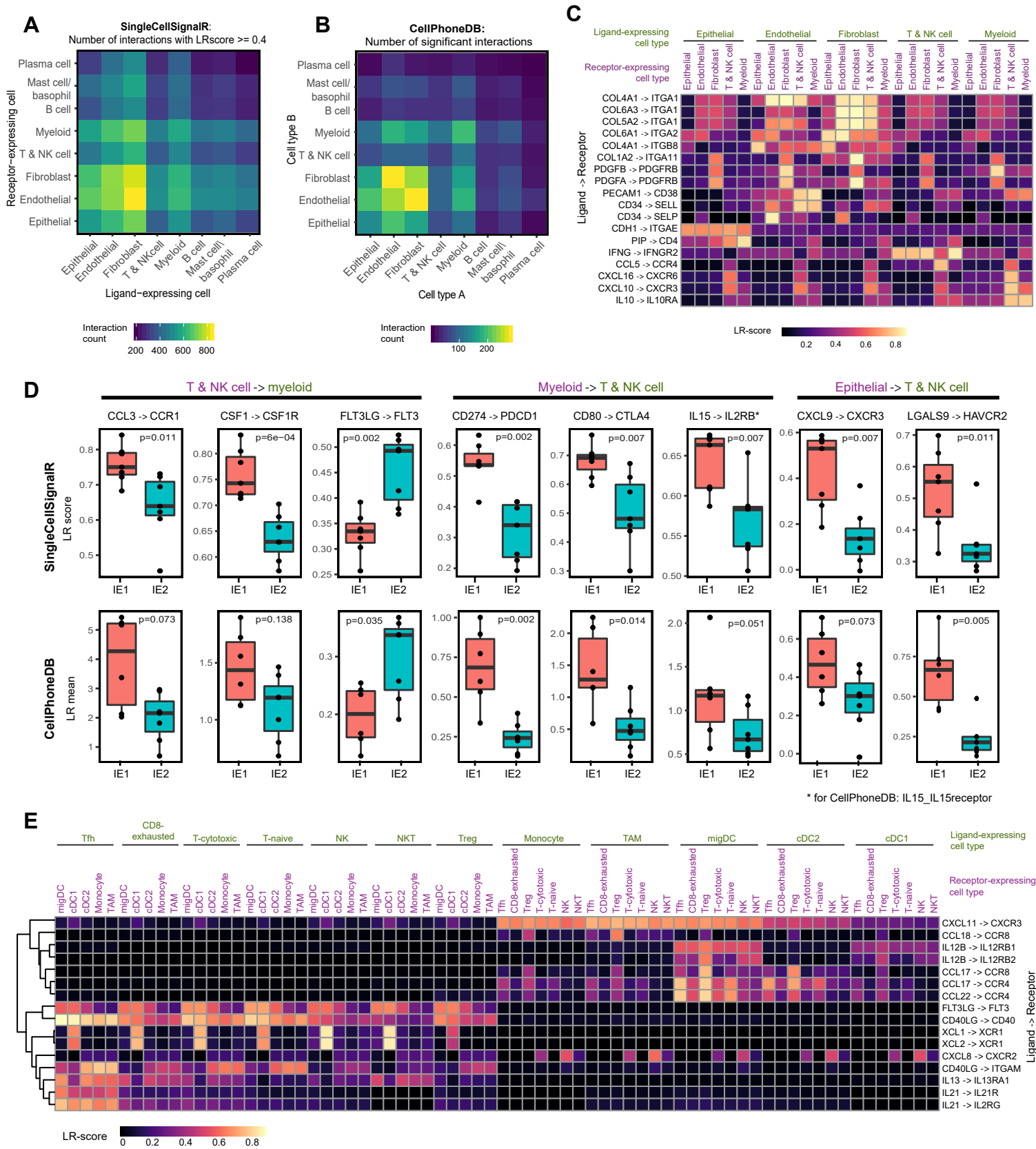


Fig. S10

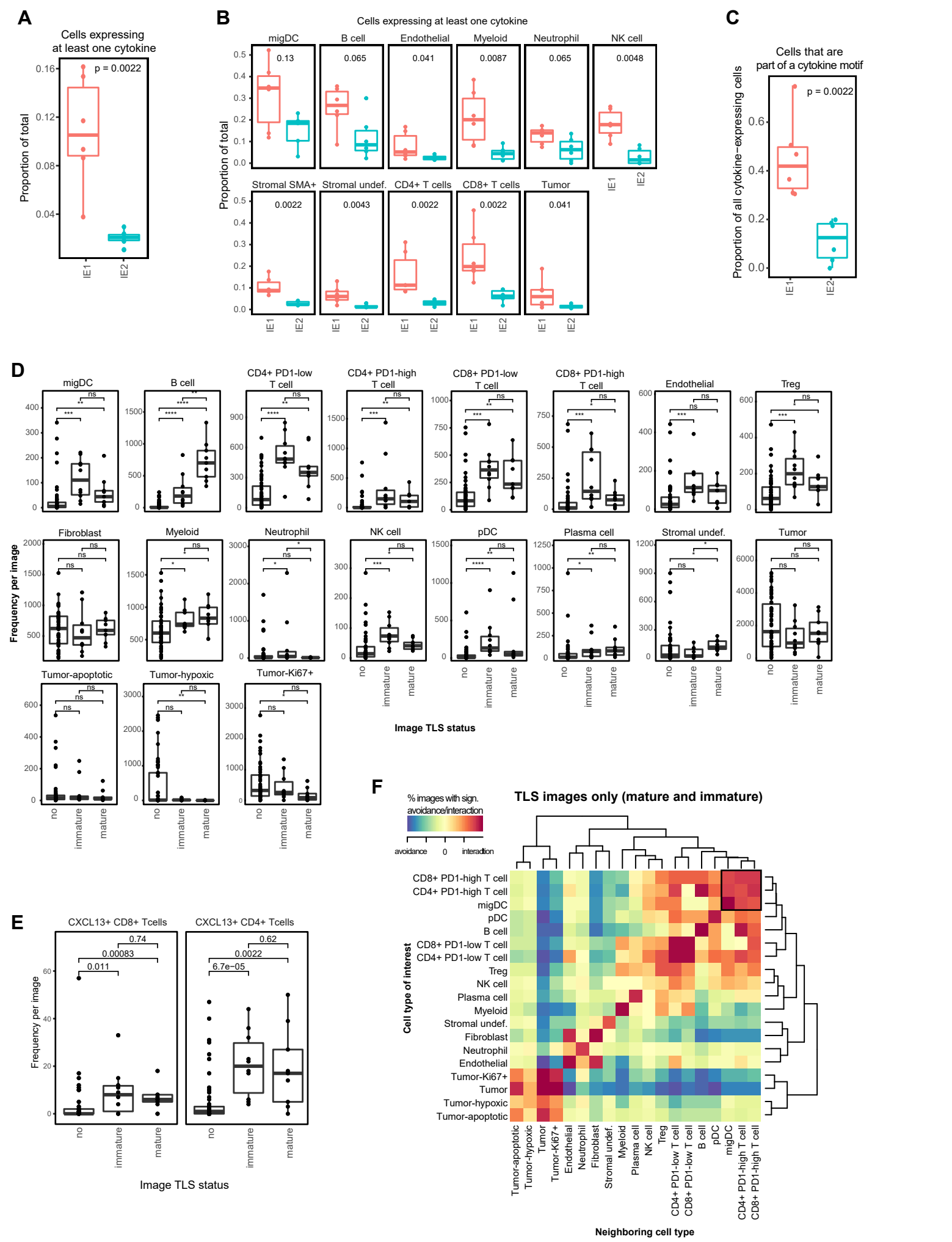


Fig. S11

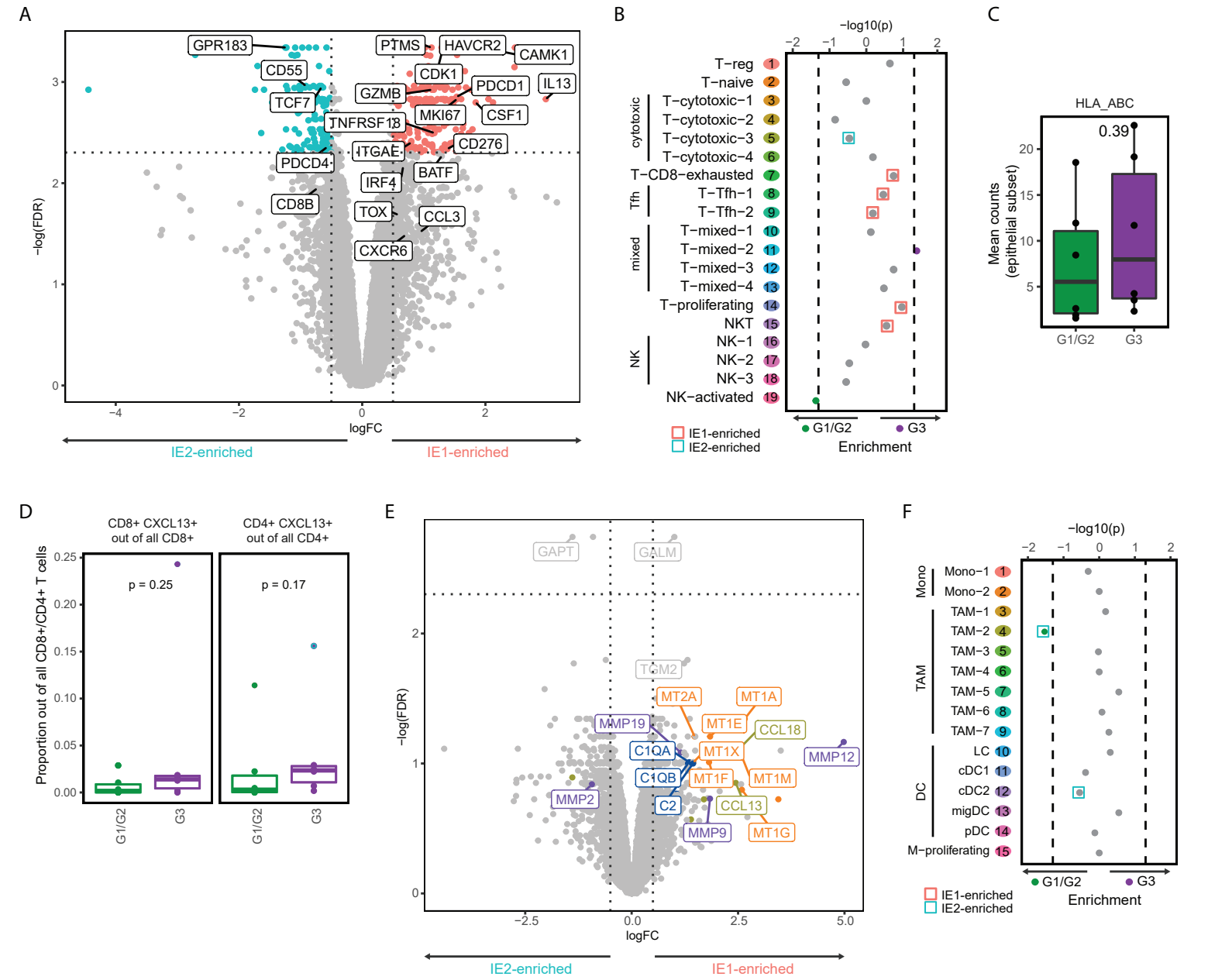
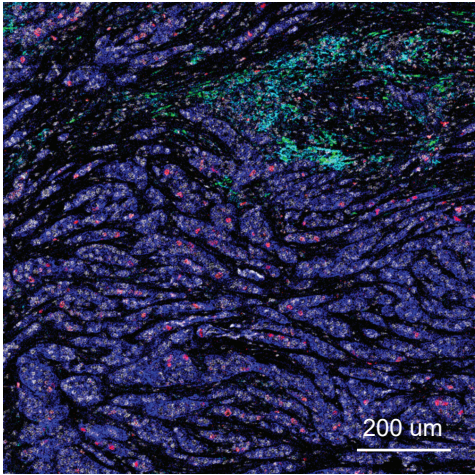
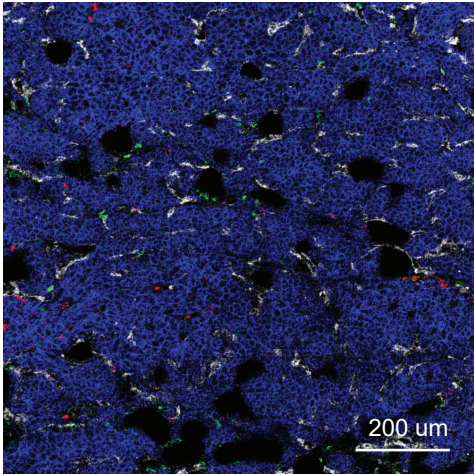


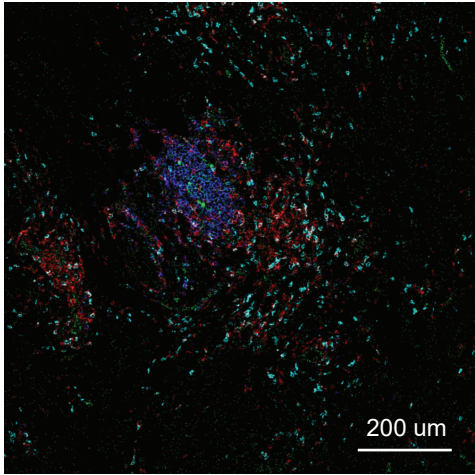
Fig. S12
Protein Panel: Example images for each marker



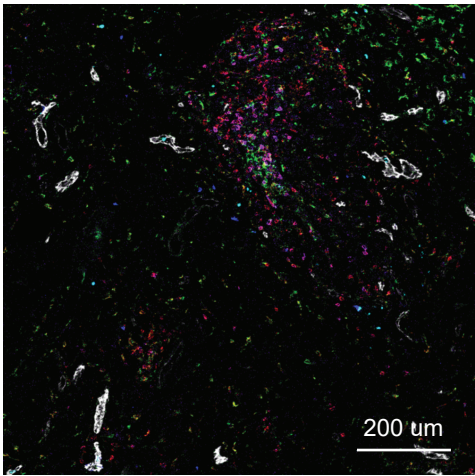
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E/P-Cadherin Ki-67



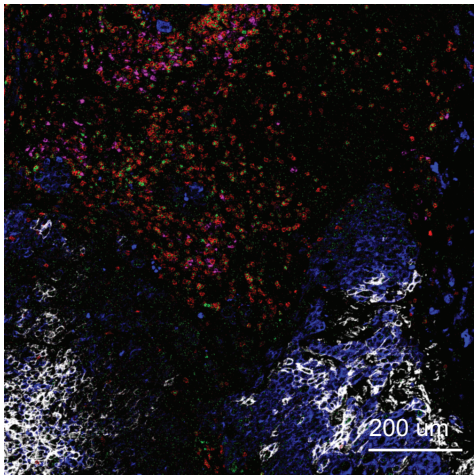
CD140b Cleaved Caspase/CD7
E/P-Cadherin Cleaved PARP



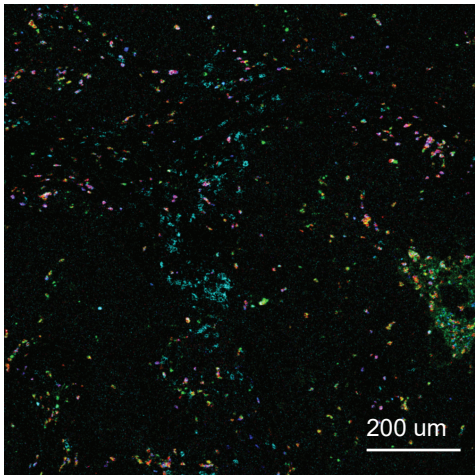
CD45RO CD21
CD45RA CD8a



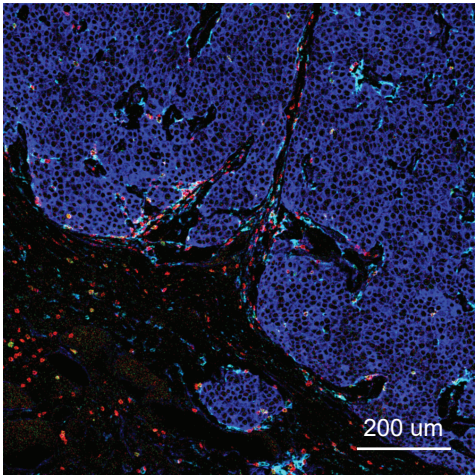
CD31/vWF Tim-3 CD15
PD-L1 CD4 ICOS



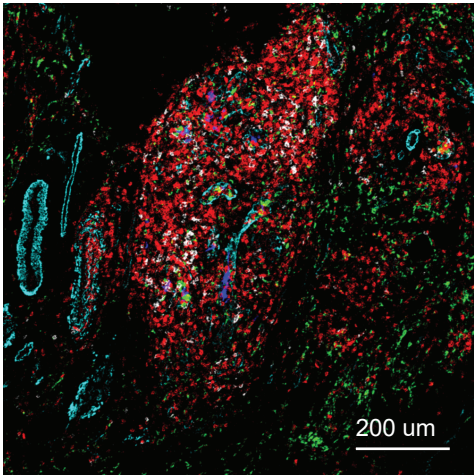
CAIX TCF1 LAMP3
panCK CD3



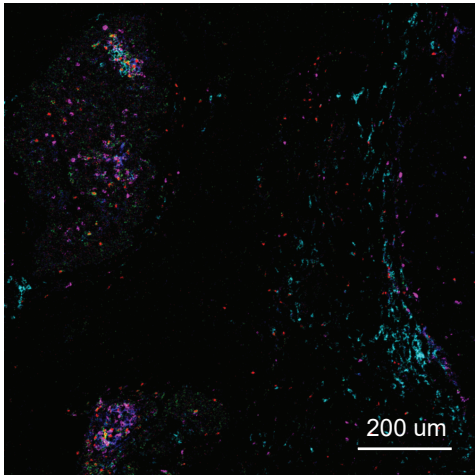
CD303 MPO
MMP9 Arginase-1



HLA-DR PD-1
HLA-ABC CD3



CD20 CD68 SMA
IDO1 CD27



Granzyme B GITR CD38
CD40 FOXP3

Fig. S13
RNA Panel: Example images for each marker

