Wright et al., Supplementary Methods

Case series

Cases included formalin-fixed paraffin-embedded (FFPE) excisional biopsies taken before treatment and from an unselected patient population (ages 27-87) with nearly equal numbers of males and females (28; 23). All biopsies were lymph nodes obtained for primary diagnosis. Biopsies from patients with a history of lymphoma were excluded, as were core needle biopsies.

Tissue imaging, image processing, and normalization (Figure 1)

Multiplexed images were acquired on the MIBI-TOF using the following parameters: pixel dwell time: 12 ms, image size: 500 μm^2 at 1024 x 1024 pixels, probe size: ~500 nm, primary ion current: 5 nA as measured via a Faraday cup on the sample holder. After imaging, MIBI pseudo-images were extracted with MIBI/O software (IonPath) and denoised using MIBIAnalysis tools (https://github.com/lkeren/MIBIAnalysis) as described.[1,2]

Image processing employed MIBItracker data visualization software (Ionpath) to assign the mean signal intensity for each biomarker to each cell in each field of view (FOV). We subsequently normalized the marker expression as follows. First, we established which cells contained an ion count of at least ten for the lineage markers (i.e., CD20, PAX5, CD3, CD4, CD8, CD11c, CD11b, CD68, CD163, and CD56). Then we computed each image's overall expression among these cells by summing the per-cell mean dsDNA and Na-K-ATPase+/MHC Class I ion abundance. For cells with a greater than 0.1 mean ion count, we calculated the image's overall expression as the median of this value. Next, cell marker expression was normalized by dividing each image's median overall expression. The image expression-normalized cell marker expressions were then transformed by Arcsinh with a cofactor of one. The cell marker expression values were standardized within each marker to be between zero and one by subtracting the lowest value and dividing by the difference between the highest and lowest. This standardized marker expression was used for downstream expression-based analysis other than cell phenotyping.

Cell segmentation

Cell segmentation was performed using the HighPlex FL module (v3.1.0) on Halo software (v3.0.311.299; Indica Labs, Albuquerque, NM). Pathologist review of segmented images confirmed that accurate nuclear/ cell segmentation was achieved in > 90% of cells and that no critical cell populations (tumor, immune, and CD31-positive cells) were missed.

Cell lineage assignments

In tissue sections, there is significant overlap between cell boundaries that are in contact, very near one another, and above or below the plane of the section. Thus, a major challenge in our assigning cell lineages was to avoid false positive annotations due to overlapping markers on adjacent cells. To address this, we purposefully over-clustered cells during lineage marker assignment with the goal of later merging or eliminating clusters through a visual review of identified cell phenotypes in the raw data, and based upon our prior knowledge of immunology, cell morphologies and hematopathology (*Supplementary Figure 1*).

We followed a multi-step process. First, the standardized per-cell expression values of lineage markers were scaled within each cell using Python Scikit-learn's MinMaxScaler. Second, these values were used to generate a UMAP and fed into FlowSOM software (Bioconductor v3.15) to yield 50 clusters (*Supplementary Figure 1*).[3] Over-clustering (n=50) was important because an "optimized" application of a clustering algorithm may miss small but important cell populations. Third, each cell cluster's relative lineage marker expression was visualized, and then each cluster was annotated by expert hematopathologists' review (KTW, SJR). Fourth, the intentionally over-clustered groups with similar lineage marker expression were merged into eight defined cell lineages ($CD4^+$ T cells, $CD8^+$ T cells, $CD11C^+CD68^{+/-}CD163^-$ dendritic cells, $CD11b^+$ myeloid cells, $CD68^+CD163^-CD11c^-$ "undifferentiated" or "M1-like" macrophages/dendritic cells, $CD163^+CD68^{+/-}CD11c^-$ "M2-like" macrophages, NK cells, tumor cells) and unphenotyped "other" cells without lineage assignment.[4] Of note, we used CD20 and PAX5 to detect and define the DLBCL tumor cells, but a small number of non-neoplastic B cells may have been inadvertently counted. Fifth, the defined cell lineage

assignments were re-mapped onto the cell segmentation mask to generate cell lineage maps of each FOV and compared to the original MIBI-TOF pseudo-images through expert pathologist review (KTW, SJR) to ensure that the lineage maps accurately represented the raw data and specific antigen staining (*Supplementary Figure 1*).

Derivation of composite cell neighborhood types (CNTs, Figure 2)

Cell Neighborhoods were defined by anchoring each cell into groups of 20 (self and 19 nearest neighbors) using previously published methods.[3] The scripts for performing NT identification can be found at: https://github.com/nolanlab/NeighborhoodCoordination.

We used k-means clustering to identify six cellular neighborhood types (CNTs), which were used as a model for labeling and quantifying local cell microenvironments in a spatially resolved manner. The division was supported by an elbow plot indicating they account for a reasonable number of clusters to partition the data. Two CNTs described with this approach (TP/MacR and TP/DCR) were critical features for assigning cases to the aggregate TIME classifications as immune-deficient, Mac-enriched, and DC-enriched.

Principal component analysis (Figure 2)

The contribution of cell neighborhood and cellular phenotypes were visualized by principal component analysis (PCA) and highlighted the major contributors of variance among the first two principal components. We performed PCA on the representation of cells in each of the six CNTs and on the representation of cells of each cell lineage for each case. Before using Python scikit-learn's "decomposition. PCA" function, we transformed features via RobustScalar with default parameters.

The first and second principal components of the CNT features showed axes primarily driven by cells in TP/MacR and TP/DCR CNTs which accounted for > 66% of the variance explained by the first and second components, respectively. The eigenvector (absolute value scaled by the eigenvalue) plot showed that cells in TP/DCR accounted for less than 7% of the first eigenvector, and cells in TP/MacR accounted for less than 1% of the second eigenvector. Based on the

qualitative assessment of the dimensional reduction and the specific contributions to the variance explained in the PCA, we found CNT composition, particularly cells in TP/MacR, and TP/DCR CNTs, provided better discrimination of cases based on their TIME when contrasted with a parallel analysis using cell lineage composition for dimensional reduction. While cell lineage composition was consistent with the results observed in CNT composition, it did not contain any features that explained as much of the variance as those observed in the CNT analysis. The eigenvector plot of the first three principal components of the CNT and cell lineage features provided a visual representation of the variance explained by the CNT features (*Supplementary Figure 3A*).

Derivation of aggregate TIMEs (Figure 2)

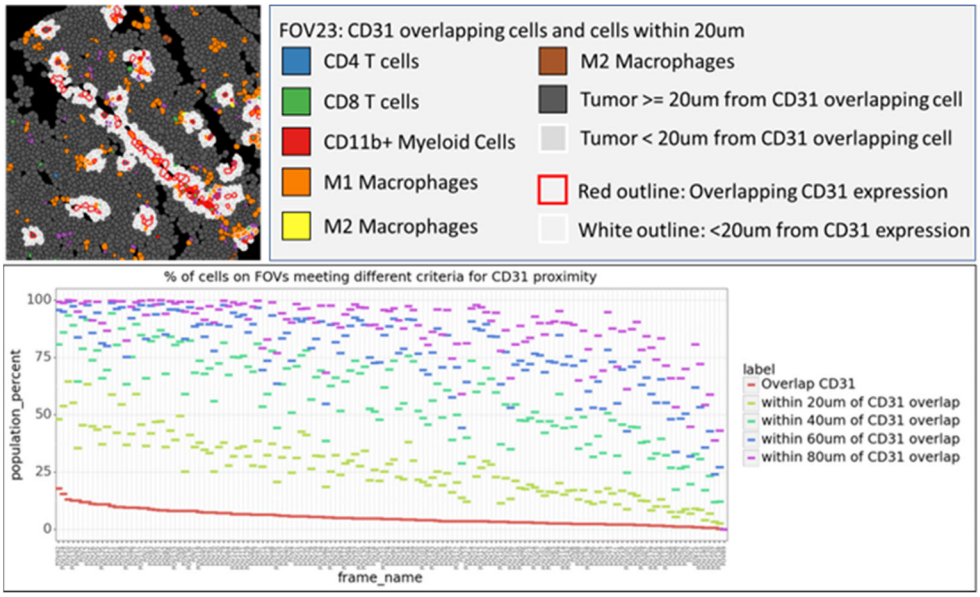
Our approach to partitioning the data into TIME classifications was informed by the observation that cells organized into TP/DCR neighborhoods and TP/MacR neighborhoods were primary contributors to the case variance by PCA and nearly mutually exclusive across cases. This observation prompted us to calculate a ratio between these features as a percentage of the cellularity. We defined a ratio between these CNT features as log2 of one plus the percent cellularity in TP/DCR neighborhoods divided by one plus the percent cellularity in TP/MacR neighborhoods, which allowed us to separate TP/DCR and TP/MacR containing cases from indistinctly defined cases. We then partitioned the scikit-learn's "StandardScaler" transformed data into three categories using k-means clustering using this standardized ratio (*Supplementary Figure 3B*).

An evaluation of these TIME category labels confirmed how mutually exclusive these neighborhoods were, and the indistinct cluster was best described as an immune-deficient category. The "DC-enriched" cluster was consistent with >3% of a TP/DCR composition, the "Mac-enriched" cluster was consistent with >3% of a TP/MacR in all cases, and the "immune-deficient" cluster was consistent with ≤3% of both TP/DCR and TP/MacR neighborhood composition in 26/27 cases. The exceptional case assigned to the "immune-deficient" cluster had a small number of TP/DCR and TP/MacR neighborhoods present. Thus, this dataset's 3% cutoff-based definition was a useful description consistent with 50 out of 51 cases (*Supplementary Figure 3C*). We used

this as a suitable simplified explanation for describing the features of these TIME classifications.

CNT enrichments near and far from CD31-positive vessels (Figure 3 A-C)

A perivascular area was defined by first annotating each cell as being in a CD31-marker rich region where the standardized CD31 expression was greater than 0.01, and second by annotating all cells with centroids < 20μm from CD31-marker cell centroids as being "proximal" to the perivascular region, versus cells ranging ≥ 20μm to < 40μm as "distal" for the statistical analysis; additional 20μm bins out to ≥60μm illustrate cell population changes across a broader range.



The distance of 20 μm was selected as a reasonable distance (the approximate distance of two lymphocytes) to survey for CNT enrichment without losing the ability to detect abrupt changes in immune cell composition as the distance from vessels increases. The 20 μm distance also ensured adequate numbers of cells outside the perivascular regions for a meaningful comparison between the proximal and distant areas (see figure above). The enrichment of CNTs within each TIME classification was tested by the pairwise difference in proximal vs. distal cellular neighborhood % of total cellularity using the two-tailed Wilcoxon Sign-Rank test.

References

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2. Baranski A, Milo I, Greenbaum S, et al. MAUI (MBI Analysis User Interface)-An image processing pipeline for Multiplexed Mass Based Imaging. *PLoS Comput Biol*. 2021;17(4):.

3. van Gassen S, Callebaut B, van Helden MJ, et al. FlowSOM: Using self-organizing maps for visualization and interpretation of cytometry data. *Cytometry A*. 2015;87(7):636–645.

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6. Jiang S, Chan CN, Rovira-Clavé X, et al. Combined protein and nucleic acid imaging reveal virus-dependent B cell and macrophage immunosuppression of tissue microenvironments. *Immunity*. 2022;

Supplementary Table 1

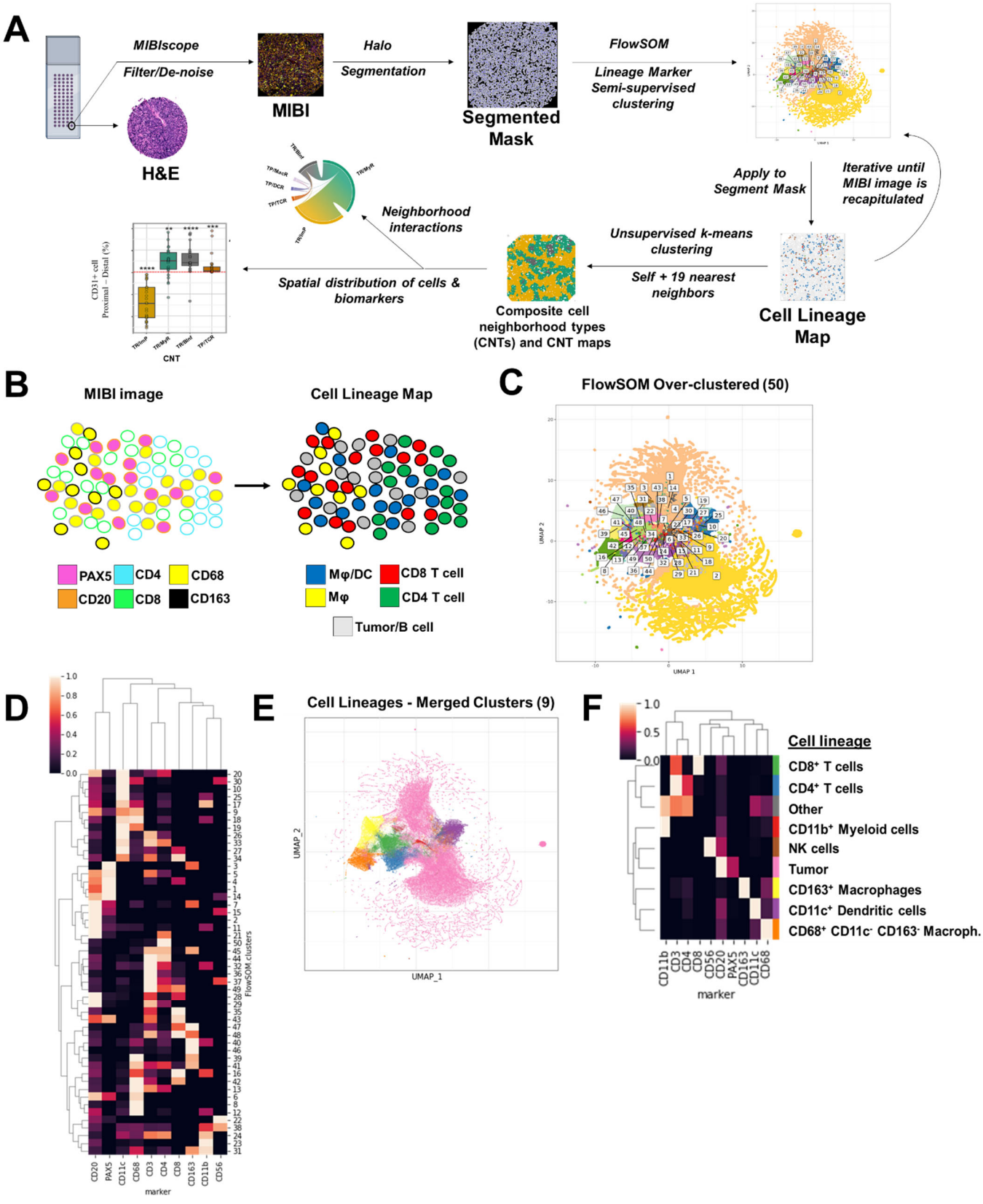
Multiplex Ion Beam Imaging (MIBI) Panel

Target	Clone	Mass	Element
dsDNA	35I9 DNA	89	Y
beta-tubulin	D3U1W	113	In
CD163	EPR14643-36	142	Nd
CD4	EPR6855	143	Nd
CD11c	EP1347Y	144	Nd
Lag3	17B4	147	Sm
PD-1	D4W2J	148	Nd
PD-L1	E1L3N	149	Sm
Arginase-1	EPR6672B	150	Nd
CD56	MRQ-42	151	Eu
CD31	EP3095	152	Sm
Ki-67	D2H10	153	Eu
CD11b	D6X1N	155	Gd
CD68	D4B9C	156	Gd
CD8	C8/144B	158	Gd
CD3	D7A6E	159	Tb
CD45RO	UCHL1	161	Dy
Vimentin	D21H3	163	Dy
Pax5	D7H5X	165	Ho
CD20	L26	167	Er
CD21	EP3093	170	Er
IDO-1	EPR20374	171	Yb
HLA-DR	EPR3692	172	Yb
DC-SIGN	DCN46	173	Yb
CD45	2B11 & PD7/26	175	Lu
HLA-I	EMR8-5	176	Yb
Na-K-ATPase	D4Y7E	176	Yb

	Lineage Assignment Markers
	Architectural/Segmentation Markers
	Functional Markers

Supplementary Table 1. Antibody panel for MIBI panel. Antibody targets, antibody clones, and metal labels are indicated.

Supplementary Figure 1

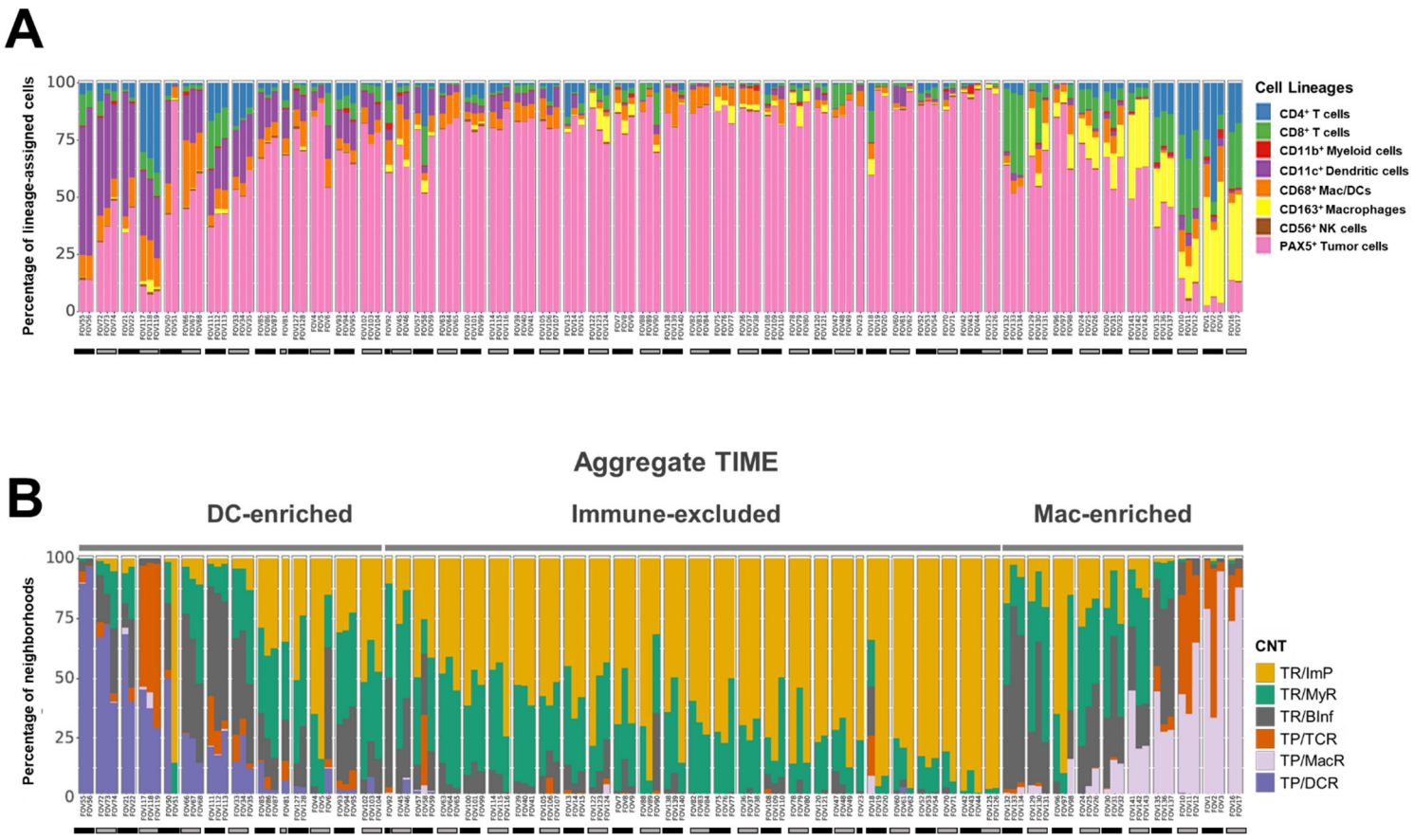


Supplementary Figure 1. Methodology for cell lineage map and cell neighborhood map generation. A. Schematic of the overall project workflow. Briefly, a tissue microarray (TMA) containing triplicate cores was serially sectioned and stained with H&E for morphologic evaluation and with the 27-plex MIBI panel for MIBIscope imaging. After filtering and denoising FOVs (one FOV per core) using custom scripts in MATLAB [1,2,5], the MIBI pseudo images were segmented using Halo v3.1; the cells were clustered using a semi-supervised approach with cell lineage-associated phenotypic markers in FlowSOM.[3,5,6] Cell phenotypes were used to generate cell lineage assignments which were subsequently mapped onto the cell segment mask to generate *cell lineage maps* for each FOV. The non-phenotyped "other cells" were omitted from the cell lineage maps and subsequent downstream analyses. This process was iterated until the cell lineage maps closely recapitulated the raw MIBI pseudo images based on an expert pathologist review (see *Figure 1A*).

Each cell, identified by its lineage, was used as an anchor cell to define a cell neighborhood to which it was assigned. Each cell neighborhood consisted of the anchor cell and its 19 nearest neighbors.[5] Individual cell neighborhoods, distinguished by their relative complement of cell lineages, were subjected to unsupervised k-means clustering.[5] Six composite cell neighborhood types (CNTs) were identified as a minimum needed to capture neighborhood heterogeneity. CNTs were mapped back onto the segment mask to generate CNT maps (*Supplementary Figures 3-5*). Subsequent analyses were performed using our "pythologist" software package (https://github.com/dfci/pythologist) to interrogate spatial relationships of relevant biomarkers and neighborhood border interactions (further details in *Materials and Methods* and *Supplemental Methods*). **B.** Conceptual schematic representation of lineage marker expression on MIBI pseudo image to cell lineage map generation. **C.** Initial FlowSOM cluster output in UMAP dimensionality reduced space. Cells were intentionally over-clustered (50) at this stage, followed by supervised assignment of the granular clusters to their appropriate cell lineage. **D.** Heatmap of relative expression of cell lineage markers in all 50 cell clusters (*white*= maximum expression; *black*= minimum expression). **E.** FlowSOM output in UMAP space after cluster annotation and merging of like clusters. Cell lineages are color-coded to indicate $CD8^+$ T cells

(*green*), $CD4^+$ T cells (*blue*), $CD11b^+$ myeloid cells (*red*), $PAX5^+$ tumor cells (*pink*), $CD163^+CD68^+CD11c^-$ macrophages (*yellow*), $CD11c^+CD68^{-/+}CD163^-$ dendritic cells (*purple*), and $CD68^+$ $CD163^+$ $CD11c^-$ macrophage/dendritic cells, not further specified (*orange*). **F.** Heatmap of relative expression of the ten cell lineage biomarkers within each of the final nine cell lineage assignments (seven immune cell lineages, malignant cells, and other cells) after annotation and merging of like clusters (*white*= maximum expression; *black*= minimum expression).

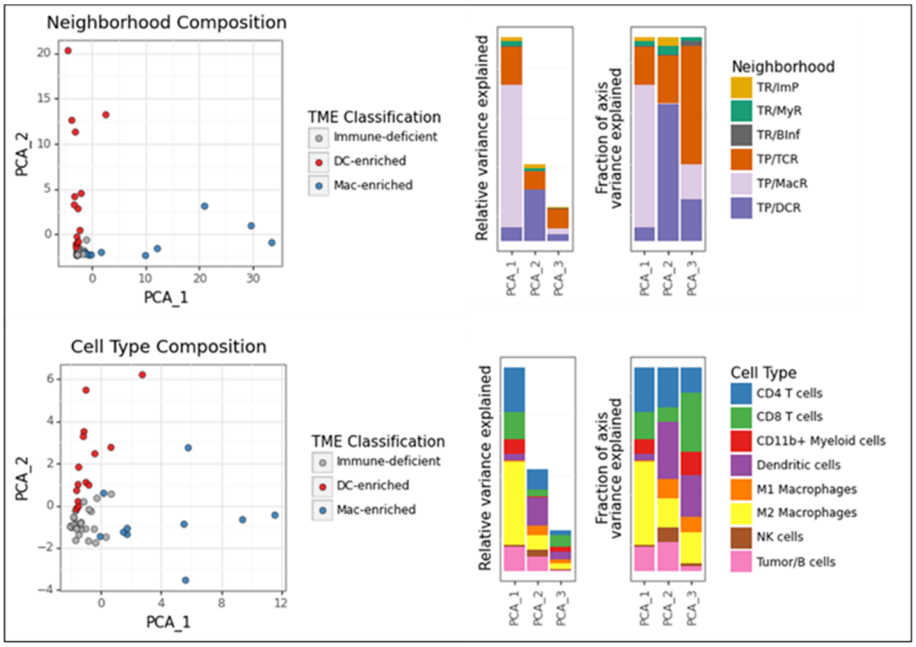
Supplementary Figure 2



Supplementary Figure 2. The distributions of cell lineages and composite cell neighborhoods are consistent across FOVs. A. Split bar graph showing cells assigned to each indicated cell lineage as a percentage of total lineage-assigned cells for each FOV, grouped according to individual cases, and demonstrating homogeneity in cell lineage distributions across FOVs by case. **B**. Split bar graph showing the cells assigned to each indicated CNT as a percentage of total CNT-assigned cells for each FOV, grouped according to individual cases, and demonstrating homogeneity in CNT designations across FOVs by case.

Supplementary Figure 3

A



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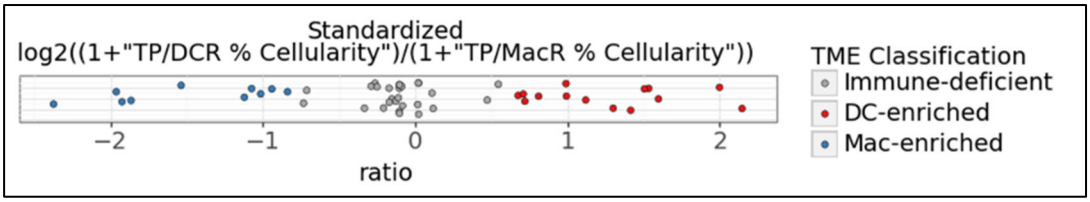

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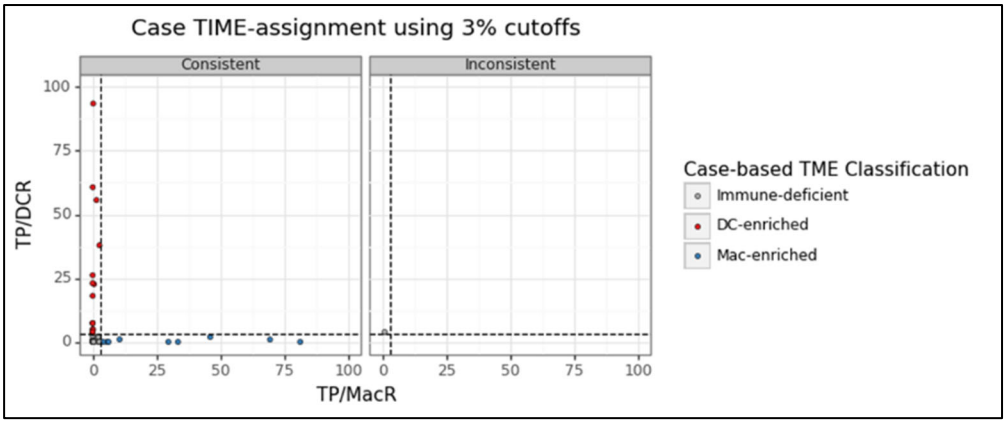
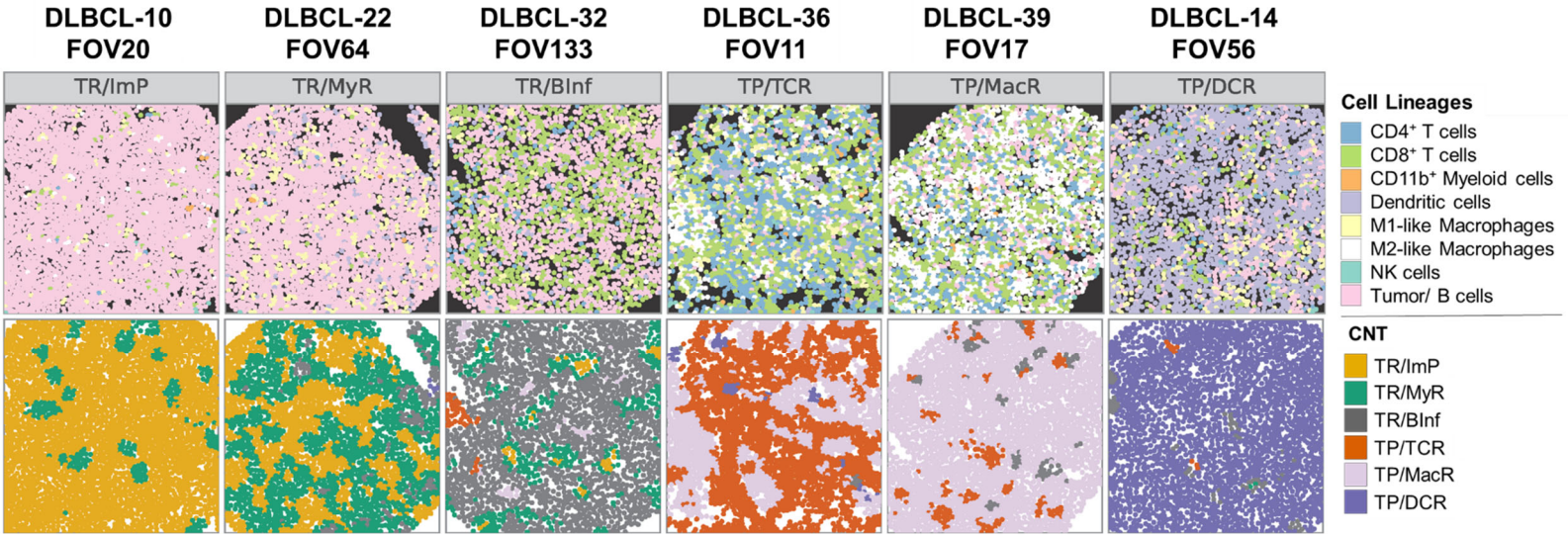


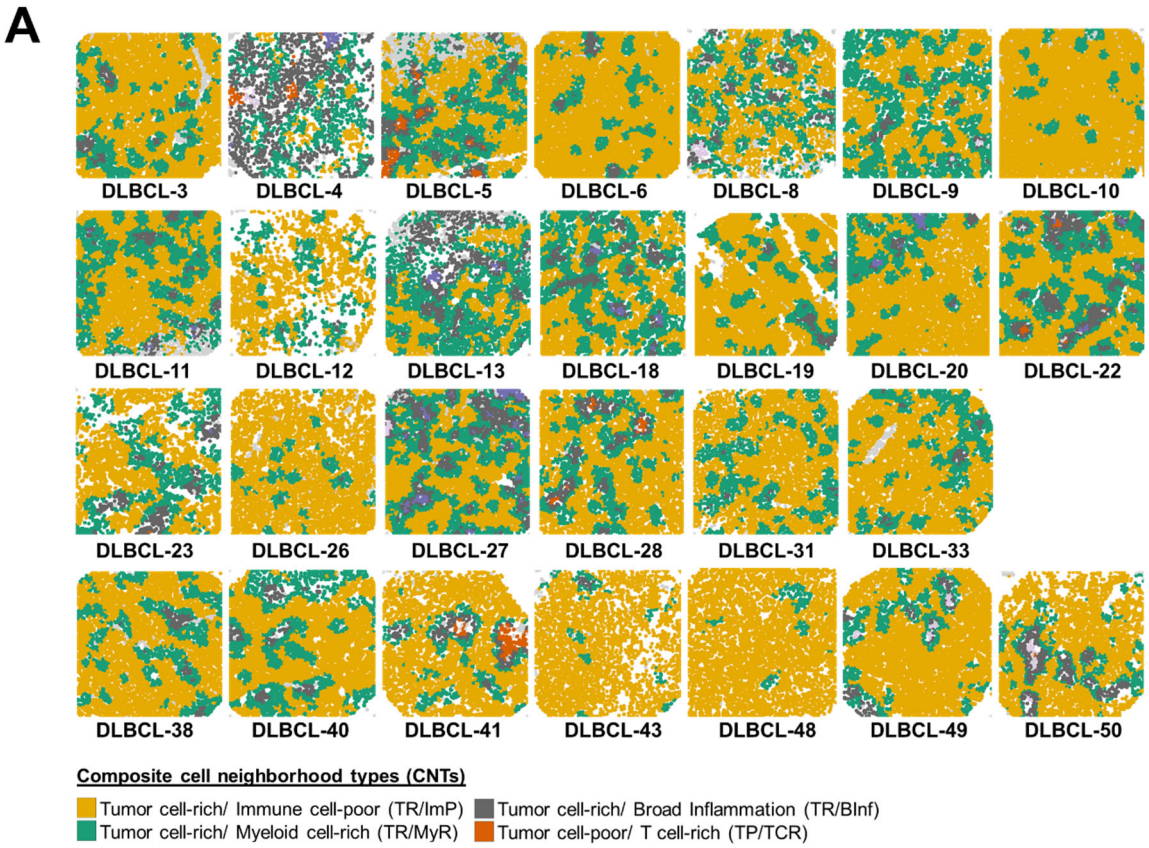
Supplementary Figure 3. The assignment of three TIME categories based on cellular neighborhood features. **A**. Principal Components analyses (PCAs) showing the relative case-to-case variance attributable to differences in the percentages of cells in each cell neighborhood type (CNT PCA, *top panels*) and the percentages of cells of each cell lineage (cell lineage PCA, *bottom panels*). The first two principal components of the CNT PCA are more clearly driven by a single microenvironmental feature (cells in TP/MacR and TP/DCR neighborhoods, respectively) than the cell lineage PCA. **B.** Case assignments to the three TIME categories using a 1-dimensional k-means clustering of the standardized log2 ratio of (1+% cellularity of TP/DCR) divided by (1+% cellularity of TP/MacR). **C**. TIME categories assigned through k-means clustering can be generalized as >3% of the TP/DCR being “DC-enriched”, >3% of TP/MacR being “Mac-enriched” and ≤3% as “immune-deficient” in 50/51 cases.

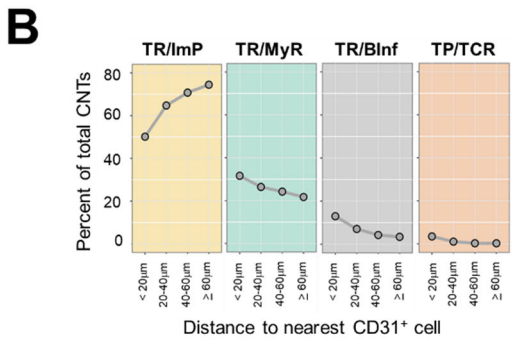
Supplementary Figure 4



Supplementary Figure 4. CNT maps capture local cell lineage heterogeneity while maintaining spatial information. Individual fields of view (FOVs) from selected cases in which large proportions of cells reside in local cell neighborhoods of a single composite cell neighborhood type (CNT). The cell lineage maps (*top panels*) and their respective CNT maps (*bottom panels*) are indicated. Note that subtle differences in local cell compositions are captured by the different CNTs while maintaining spatial resolution. TR/IP= tumor cell-rich/ immune cell-poor, TR/MyR= tumor cell-rich/ myeloid cell-rich, TR/BInf= tumor cell-rich/ broad inflammation, TP/TCR= tumor cell-poor/ T cell-rich, TP/MacR= tumor cell-poor/ macrophage-rich, TP/DCR= tumor cell-poor/ dendritic cell-rich.

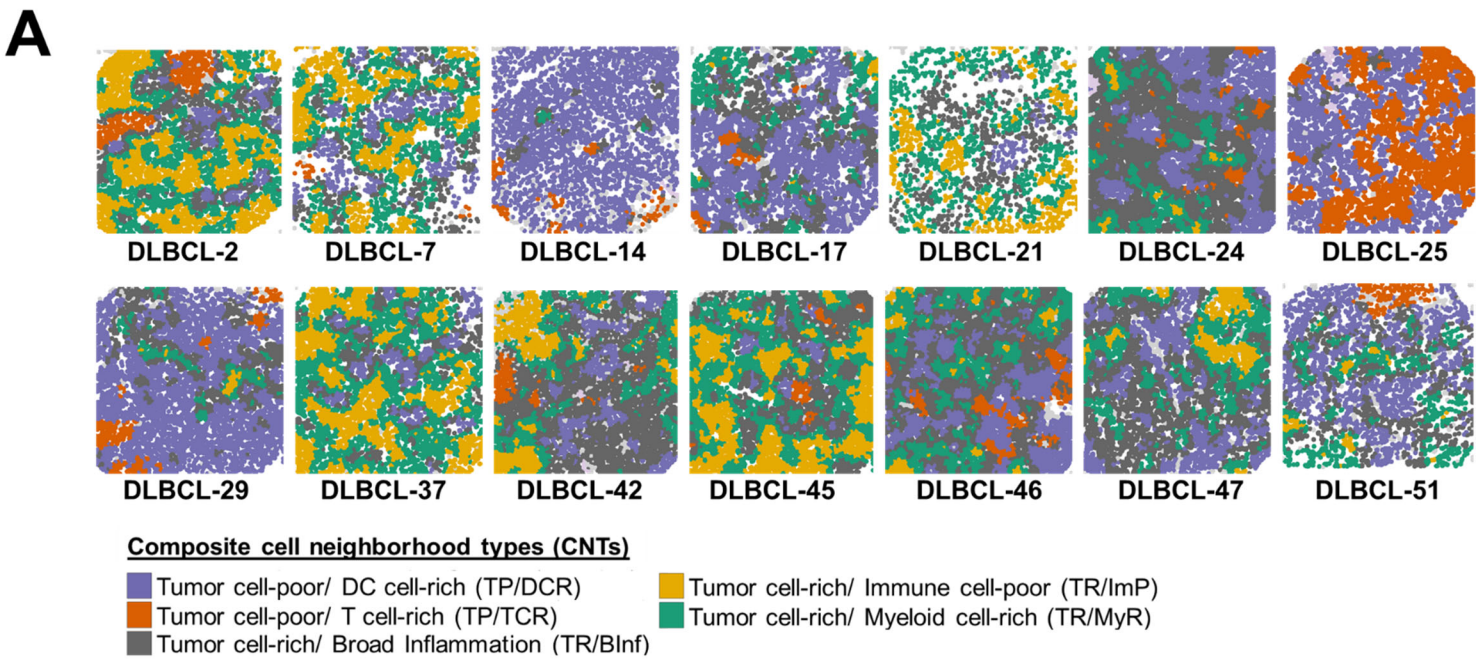
Supplementary Figure 5

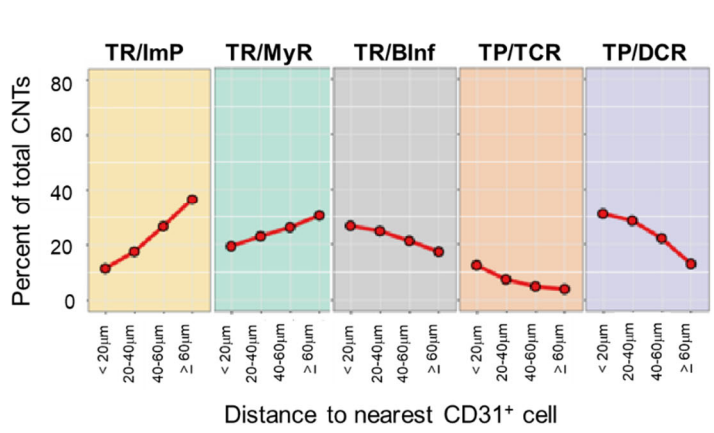




Supplementary Figure 5. Representative CNT maps from DLBCLs with an immune-deficient TIME and relationships of CNTs to CD31-positive vessels. A. Representative CNT map from a single FOV from each case with an immune-deficient aggregate TIME. CNT assignments are color-coded as indicated. **B.** The percentage of total cells assigned to the indicated CNT at the indicated distances from the nearest CD31-positive cell. Data are summative of all cases with an immune-deficient aggregate TIME.

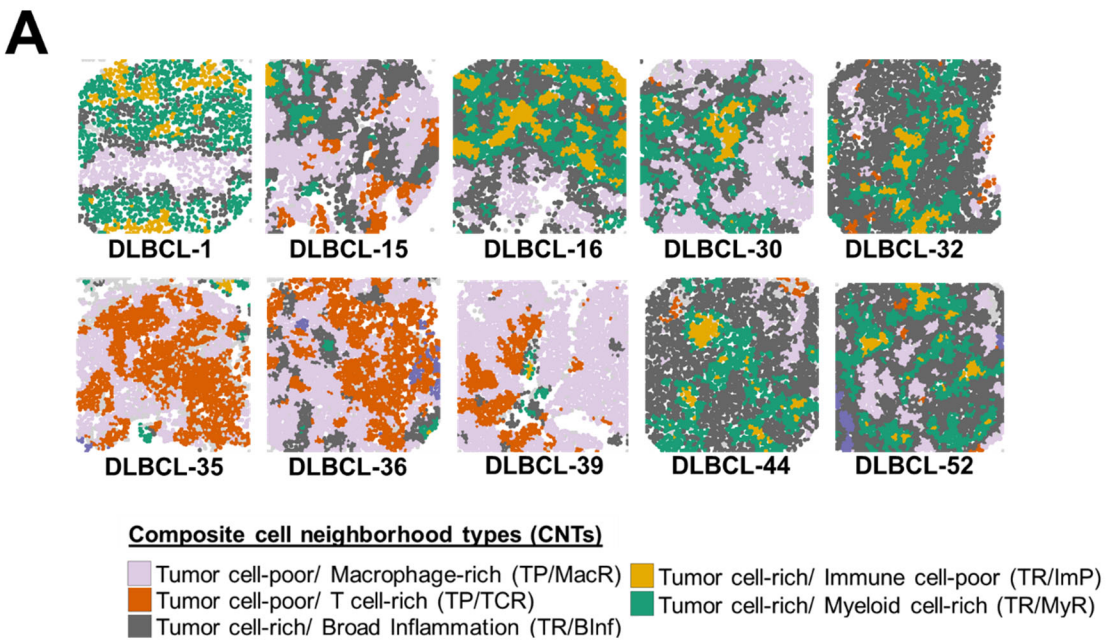
Supplementary Figure 6

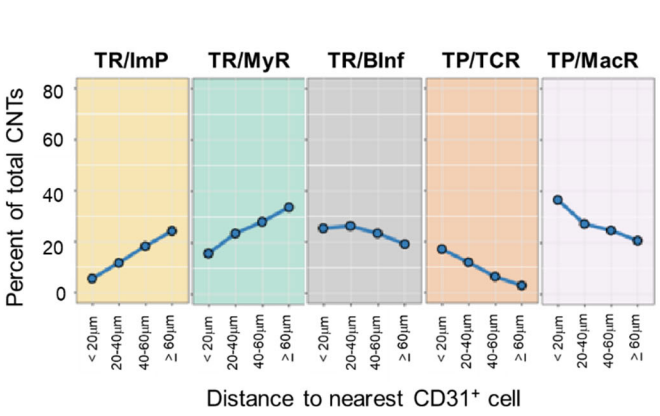




Supplementary Figure 6. Representative CNT maps from DLBCLs with a DC-enriched TIME and relationships of CNTs to CD31-positive vessels. A. Representative CNT map from a single FOV from each case with a DC-enriched aggregate TIME. CNT assignments are color-coded as indicated. **B.** The percentage of total cells assigned to the indicated CNT at the indicated distances from the nearest CD31-positive cell. Data are summative of all cases with a DC-enriched aggregate TIME.

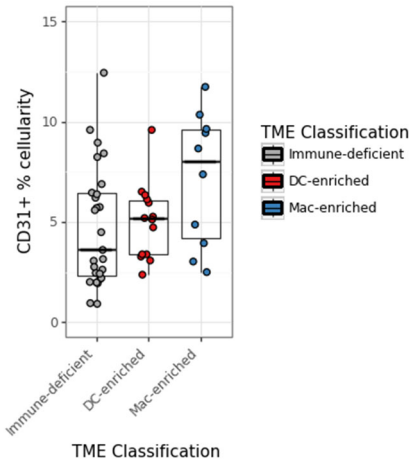
Supplementary Figure 7





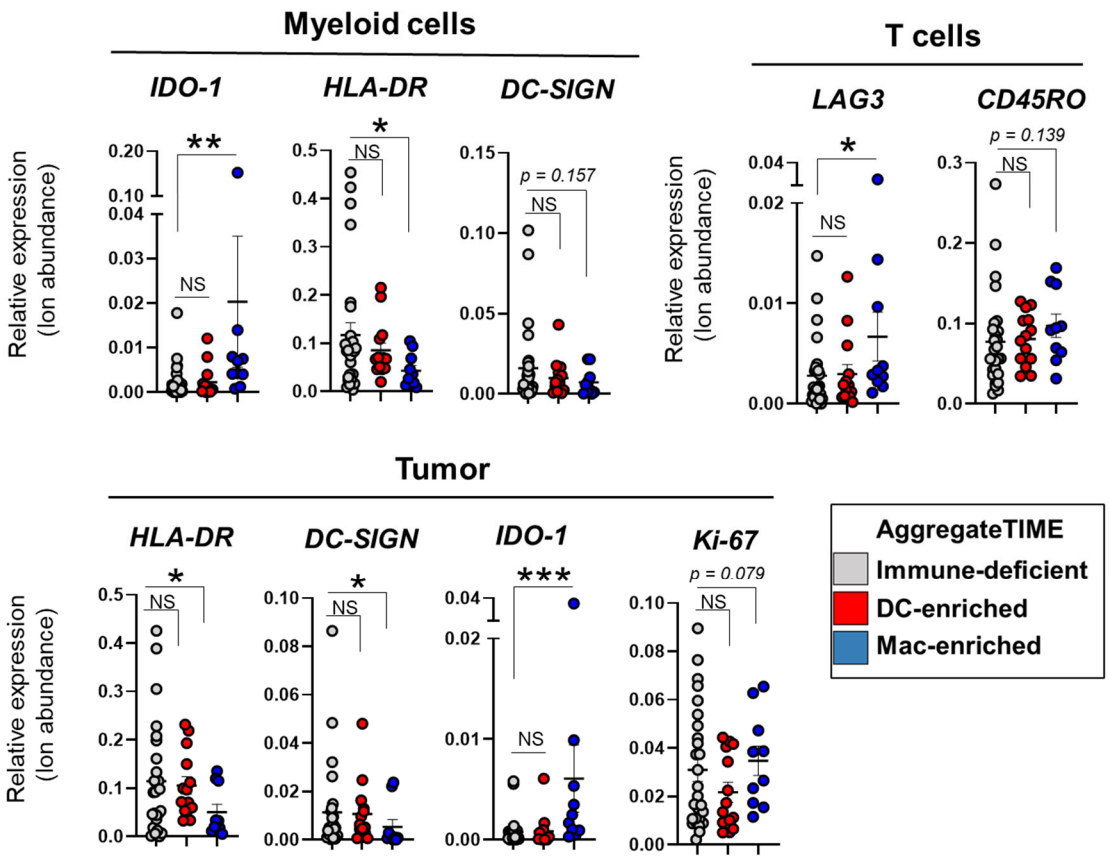
Supplementary Figure 7. Representative CNT maps from DLBCLs with a Mac-enriched TIME and relationships of CNTs to CD31-positive vessels. A. Representative CNT map from a single FOV from each case with a Mac-enriched aggregate TIME. CNT assignments are color-coded as indicated. **B.** The percentage of total cells assigned to the indicated CNT at the indicated distances from the nearest CD31-positive cell. Data are summative of all cases with a Mac-enriched aggregate TIME.

Supplementary Figure 8



Supplementary Figure 8. CD31-positive cells as a percentage of overall cellularity. Box and whiskers plot showing the median (horizontal lines), standard error (boxes), and extreme (vertical lines) values for CD31-positive cells as a percentage of the overall cellularity for each case (circles) and divided according to aggregate TIME category. There is no significant difference in the abundance of CD31+ areas between TIME classifications ($p = 0.09$ Kruskal-Wallis test).

Supplementary Figure 9



Supplementary Figure 9. Differential expression of functional biomarkers. The mean (horizontal lines), standard errors (whiskers), and individual case values (circles) for the relative expression (ion count abundance) of the indicated functional markers as determined by MIBI. Cases are colored by aggregate TIME category, as indicated. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.01$, NS= not significant.