

Supplemental Information

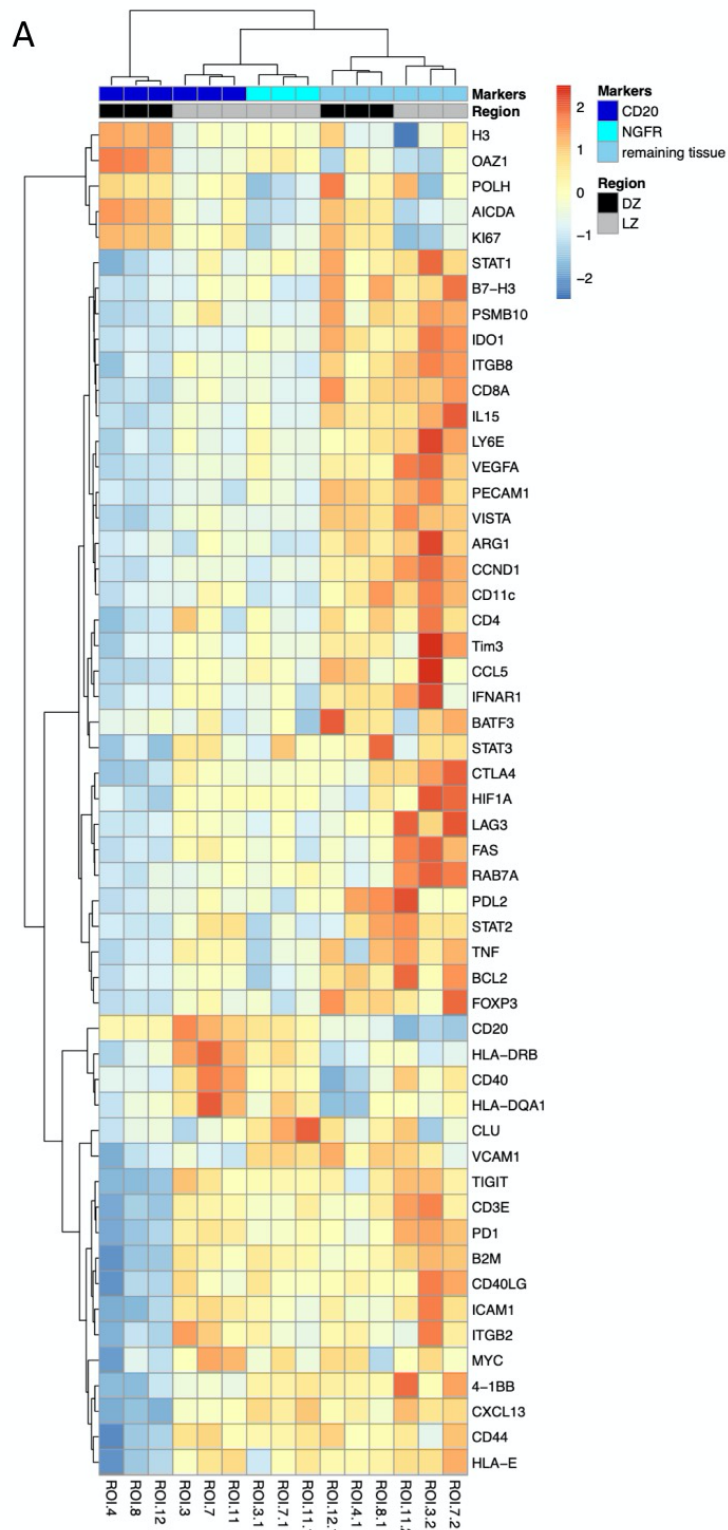
A Spatially Resolved Dark- versus Light-Zone

Microenvironment Signature Subdivides Germinal

Center-Related Aggressive B Cell Lymphomas

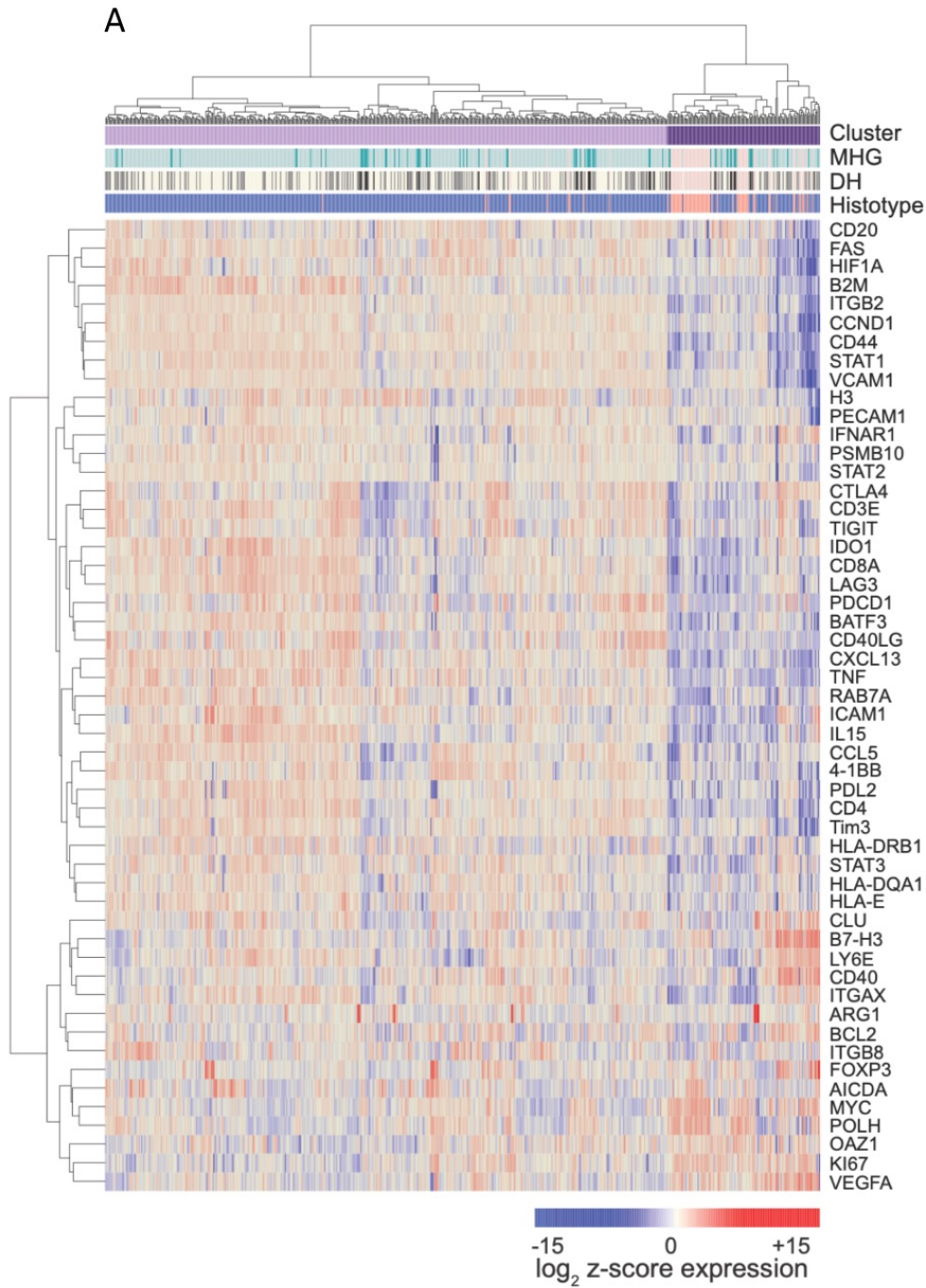
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1 Supplemental Information



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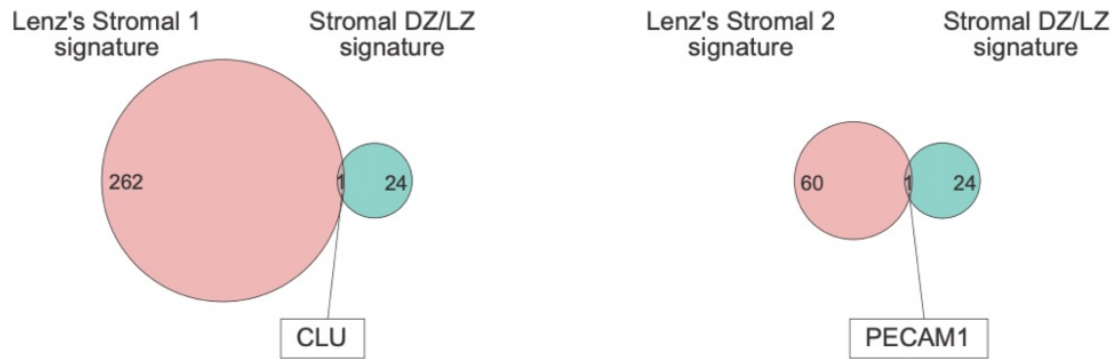
3 **Figure S1, Related to Figure 1.** Clustering of DZ and LZ ROIs according to the 53 differential genes assessed
 4 in different ROI masks as shown in Figure 1C. **A)** Clustering of the 53 genes discriminating DZ and LZ reveals
 5 different contribution of B-cell (CD20) FDC (NGFR) and remaining tissue ROI sub-compartments.



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7 **Figure S2, Related to Figure 4.** Unsupervised hierarchical clustering of Sha's dataset (GSE117556) extended
 8 to Burkitt Lymphoma (BL) cases, according to the 53 genes DZ/LZ spatial signature. **A)** The heatmap
 9 representing z-score normalized values of the 53 genes reveals that the majority of BL (56 out of 70, 80%) are
 10 classified in the DZ-like cluster with molecular high-grade GC-related DLBCL samples.

A



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12 **Figure S3, Related to Figure 5.** Analysis of the overlap between the 25 genes DZ/LZ stromal signature and
13 Lenz's stromal signatures (Lenz et al., 2008). **A)** Only one gene, *CLU*, was shared with the Stromal-1 and one
14 gene, *PECAM1*, with the Stromal-2 signature, indicating that a different level of heterogeneity is probed through
15 the 25 genes DZ/LZ stromal microenvironment signature.

16 **Transparent Methods**

17 To investigate the immune and stromal composition of two spatially-resolved GC microenvironments, namely
18 the DZ and LZ, multiplexed wide field immunofluorescence (IF) analysis was combined to ROIs definition and
19 segmentation, and in situ mRNA analysis to screen four-micrometer thick tissue sections from formalin-fixed
20 and paraffin-embedded reactive lymph nodes using a GeoMx Digital Spatial Profiler (DSP) (NanoString,
21 Seattle WA) (Merritt et al., 2020). Samples were collected according to the Helsinki Declaration and the study
22 was approved by the University of Palermo Institutional Review Board (approval number 09/2018). The
23 following antibodies were adopted for 4-plex IF tissue imaging: mouse anti-human CD20 (L26 Novocastra,
24 Leica Biosystems), mouse anti-human CD271 (NGFR, MRQ-21 Cell Marque), mouse anti-human SMA (ASM-
25 1 Novocastra, Leica Biosystems). Syto83 was used as nuclear counterstain in DSP IF-based ROI selection
26 while DAPI was adopted for validation IF stainings.

27 For the determination of the 87-plex customized TAP Human Immuno Oncology panel, mRNA binding DNA
28 probes (5' to 3' 35- to 50-nt target-complementary sequences) conjugated with UV photocleavable indexing
29 oligos were hybridized to the tissue as previously reported (Merritt et al., 2020). The UV photocleavable probes
30 were released from each ROI according to custom masks for UV illumination and digitally counted using the
31 NanoString nCounter Analysis System. For nCounter data analysis, digital counts from barcodes
32 corresponding to mRNA probes were normalized to internal spike-in controls (ERCC). Moreover, a set of six
33 internal housekeeping genes was included in the TAP Human Immuno Oncology panel to control for system
34 variation including ROI size and cellularity (Decalf et al., 2019; Merritt et al., 2020). Seven negative control
35 probes were adopted to evaluate and filter ROIs with a high degree on non-specific binding (none identified in
36 this experiment). Normalized gene expression data relative to the ROIs analyzed in this study are reported in
37 Table S4. Unsupervised hierarchical clustering of the ROIs was performed on the normalized counts using the
38 heatmap function of the homonymous R package with default parameters. Genes differentially expressed
39 between LZ and DZ ROIs were identified by applying an empirical Bayes test using the Limma R package.

40 CD20, CD44, CD54, H3, Ki-67, and MYC expression in GC DZ and LZ of the lymph nodes profiled by DSP
41 was validated at the protein level using double- or triple marker immunostainings (Figure 2). Double- and triple-
42 marker immunostainings on reactive lymph nodes were performed as follows: four micrometers-thick sections
43 from formalin fixed and paraffin-embedded lymph node biopsies were put onto positively-charged slides,
44 deparaffinized and rehydrated. Sections underwent heath-induced antigen retrieval using Novocastra Epitope
45 Retrieval Solution pH9 (Leica Biosystems). Slides underwent sequential rounds of incubation at room
46 temperature for 1h with the following primary antibodies: mouse anti-human CD20 (1:100, clone L26,

47 Novocastra, Leica Biosystems), rabbit anti-human Ki-67 (1:1000, Abcam), rabbit anti-human c-MYC (1:500,
48 clone Y69, Abcam), goat anti-human Histone-H3 (1:150, Abcam), mouse anti-human CD44 (1:40, clone
49 DF1485, Novocastra, Leica Biosystems), mouse anti-human CD54 (1:30, clone 23G12, Novocastra, Leica
50 Biosystems). Primary antibodies binding was revealed by the use of specific secondary antibodies conjugated
51 with either horseradish peroxidase (HRP) or fluorophores (Alexa 488, Alexa 568, Invitrogen; Opal 520, Opal
52 620, Akoya Biosciences). 3,3'-diaminobenzidine (DAB) was used as chromogenic substrate for HRP. Slides
53 were analyzed under a Zeiss Axioscope-A1 microscope equipped with bright field fluorescence module (Zeiss).
54 Microphotographs were collected with a Zeiss Axiocam 503 Color digital camera using the Zen 2.0 imaging
55 software (Zeiss).

56 The spatial DZ/LZ signatures were tested in the following independent transcriptomic datasets: GSE38697;
57 GSE117556. For both datasets we used normalized expression data provided by the Authors. To further
58 validate the 53 genes DZ/LZ spatial signature in *bona fide* DZ-derived aggressive lymphomas, we integrated
59 the 70 Burkitt Lymphoma samples (GSE69051) from Sha et al., with the DLBCL samples (GSE117556). Raw
60 data from these samples were processed with the Limma R package to generate normalized values applying
61 a quantile normalization. Where multiple probes represented the same gene, the gene expression was
62 summarized with the maximum value. After performing z-score normalization, hierarchical clustering based on
63 the spatially-resolved DZ/LZ 53 genes signature and/or on the 25 stromal genes signature was applied to
64 identify clusters reflecting GC heterogeneity. Hierarchical clustering analysis was performed using Ward.D2
65 and Euclidean distance. The rand-index measure was used to quantify the similarity of two clustering results.
66 For survival analysis, we used Kaplan-Meier with log-rank tests to estimate overall survival between the
67 identified clusters. Differences in patient characteristics were analyzed using the χ^2 test; a p-value <0.05 was
68 set as the threshold for significance. To visualize the overlapped genes between the different signatures we
69 applied Euler R package (<https://cran.r-project.org/package=eulerr>). All statistical analyses were performed
70 using R statistical software package (v 3.6.0) (<http://www.R-project.org>).

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72 **Supplementary References**

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74 method for in situ analysis of protein and RNA expression in tissue. *J Pathol* 247, 650-661.

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