Low T-cell proportion in the tumor microenvironment is associated with immune escape and poor survival in diffuse large B-cell lymphoma

Joo Y. Song,[1] Mary Nwangwu,[2] Ting-Fang He,[2] Weiwei Zhang,[3] Hany Meawad,[1] Victoria Bedell,[1] Joyce Murata-Collins,[1] Pamela Skrabek,[4] Michel R. Nasr,[5] David Scott,[6] James Godfrey,[7] Peter Lee,[2] Wing C. Chan,[1] Dennis D. Weisenburger,[1,3] Anamarija M. Perry[8] and Alex F. Herrera[7]

[1]Department of Pathology, City of Hope Medical Center, Duarte, CA, USA; [2]Immuno-Oncology, Beckman Research Institute, City of Hope Medical Center, Duarte, CA, USA; [3]Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE, USA; [4]Department of Hematology, CancerCare Manitoba, Manitoba, Canada; [5]Department of Pathology, SUNY Upstate Medical University, Syracuse, NY, USA; [6]British Columbia Research Center, Vancouver, British Columbia, Canada; [7]Department of Hematology & Hematopoietic Cell Transplantation, City of Hope Medical Center, Duarte, CA, USA and [8]Department of Pathology, University of Michigan, Ann Arbor, MI, USA

Correspondence: Joo Y. Song
josong@coh.org

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Abstract

The tumor microenvironment (TME) is important in the pathogenesis and prognosis of lymphoma. Previous studies have demonstrated that features of the diffuse large B-cell lymphoma (DLBCL) TME can be associated with prognosis, but questions remain about the mechanisms underlying these TME features, and the interplay between tumor cells and the local TME. Therefore, we performed multispectral immunofluorescence (mIF) using two 6-color panels to interrogate the cellular proportions of T-cell subsets, macrophages, and natural killer cells in 57 cases of *de novo* DLBCL treated with R-CHOP chemotherapy. We found that very low $CD3^+$ T-cell proportion and low $CD4^+PD1^+$ and $CD8^+PD1^+$ T cells have poor survival compared to those with a high T-cell proportion. Also, cases with concurrently low TIM3 and PD1 have a poor prognosis. This poor prognosis with low T-cell proportion was validated using immune deconvolution of gene expression profiling data from 351 cases of DLBCL and an additional cohort of 53 cases of DLBCL using routine immunohistochemistry. In addition, cases with loss of B2M, HLA I and/or HLA II protein expression on the tumor cells also had a low T-cell proportion, providing evidence that lack of these proteins allows for immune evasion. Overall, our results show that patients with DLBCL with a low T-cell proportion in the TME have a poor survival when treated with R-CHOP and exhibit mechanisms of immune escape.

Introduction

The tumor microenvironment (TME) plays an important role in the pathogenesis and outcome of lymphomas.[1-3] In diffuse large B-cell lymphoma (DLBCL), the majority of the cellular content is usually tumor cells with a paucity of non-malignant cells in the TME. Malignant B cells typically efface the architecture and few cells remain except for scattered histiocytes, natural killer (NK) cells, stromal cells, and T cells.[2] Immune cells in the TME may express inhibitory receptors such as programmed cell death protein 1 (PD1), lymphocyte-activation gene 3 (LAG3), and T-cell immunoglobulin and mucin-domain containing 3 (TIM3), which may inhibit anti-tumor immune surveillance and facilitate the proliferation and survival of neoplastic cells.[4] Whereas a number of studies[5-7] have comprehensively evaluated the genomic profile of DLBCL, and others[8-14] have studied the relationship between immune cell proportions in the TME (particularly of T-cell subsets) and outcomes in DLBCL, comprehensive studies of the intersection between the tumor microenvironment and genomics of DLBCL are limited. Therefore, we performed a comprehensive analysis of the TME in DLBCL using multispectral immunofluorescence in conjunction with genomic analysis in a clinically-annotated cohort of patients with DLBCL treated with R-CHOP.

Methods

We identified 57 diagnostic cases of *de novo* DLBCL with ample material for tissue microarray (TMA) construction (2 mm cores) who were treated with R-CHOP at the City of Hope Medical Center, Duarte. Patients with primary

mediastinal B-cell lymphoma, Epstein-Barr virus (EBV)-positive DLBCL, T-cell/histiocyte-rich large B-cell lymphoma, prior low-grade B-cell lymphoma, or an immunocompromised state (including human immunodeficiency virus infection) were excluded. This study was approved by the Institutional Review Boards at the City of Hope Medical Center and the University of Manitoba.

Immunohistochemistry and fluorescence *in situ* hybridization cytogenetic analysis on tissue microarrays

Cases were re-reviewed to confirm the diagnosis of DLBCL, not otherwise specified (NOS). Immunohistochemistry (IHC) was performed using formalin-fixed, paraffin-embedded tissue microarrays and antibodies to CD20, CD3, CD10, BCL6, MUM1, MYC, BCL2, B2M, HLA I, HLA II, and TIM3. FISH cytogenetic analysis for *MYC, BCL2* and *BCL6* gene rearrangements was performed.

Mutation analysis and copy number analysis

We used a custom targeted panel of 334 genes, which includes the most frequently mutated genes in B-cell lymphoma, and performed DNA sequencing on an Illumina HiSeq 2500 (Illumina, San Diego, CA, USA) as previously described.[15] The Oncoscan Copy Number Variation (CNV) assay (ThermoFisher, Waltham, MA, USA) was performed.

Gene expression analysis for classification

We used extracted RNA on the nCounter platform (NanoString Technologies, Seattle, WA, USA) to determine the cell of origin (COO) using the Lymphoma/Leukemia Molecular Profiling Project (LLMPP) code set Lymph2Cx[16] as well as the DLBCL90 double-hit gene expression (DHIT) signature[17] (DHITsig) on the nSolver as previously described.[15]

Multispectral immunofluorescence

For multispectral immunofluorescence (mIF) analysis, staining was performed using the Opal 7 kit (PerkinElmer, Waltham, MA, USA). Two panels were used (Panel 1: CD3, CD8, CD4, PD1, PAX5, DAPI; Panel 2: PAX5, CD163, CD79a, PD-L1, CD56, DAPI), and the stains were scanned on the Vectra spectral imaging system and analyzed using the InForm software.

Validation cohorts

Immune cellular proportion determination using gene expression profiling and CIBERSORTx - We used the gene expression profiling (GEP) data from 351 cases in the study of Lenz *et al*. of *de novo* DLBCL: 184 germinal center phenotype (GCB) DLBCL, 167 activated B-cell phenotype (ABC) DLBCL treated with R-CHOP.[18] We used the CIBERSORTx method[19] to determine the relative cell proportions (e.g., total T cells, follicular helper T cells, regulatory T cells, NK cells, and macrophages) in this independent cohort to confirm the relative cellular proportions by GEP.

Immunohistochemical staining for T cells and digital scanning - To determine whether routine IHC would correlate with the findings by mIF, we used another independent cohort of 54 cases of *de novo* DLBCL (37 GCB DLBCL, 17 ABC DLBCL) treated with R-CHOP as well as the discovery cohort of DLBCL. Cases on a TMA were stained with CD3 and PD1, and analyzed using the QuPath v0.3.0 qualitative pathology and bioimage analysis software.

For further methodological details, including statistical approaches correlating molecular and clinicopathological findings, please see the *Online Supplementary Appendix*.

Results

Of the 57 cases included in the original cohort, there were 42 cases of GCB DLBCL (74%), 12 cases of ABC DLBCL (21%), and 3 cases of unclassified DLBCL (5%) as determined by Nanostring. There were 5 cases that were double- or triple-hit lymphoma for *MYC/BCL2* (n=4) or *MYC/BCL2/BCL6* (n=1) by FISH analysis, and 8 cases that were DHITsig-positive (pos) and 32 were DHITsig-negative (neg) by DLBCL90 analysis. The LymphGen tool (https://llmpp.nih.gov/lymphgen/index.php)[5] was also employed and the cases were defined as follows: 11 cases (19%) were EZB, 5 cases (9%) BN2, 2 cases (4%) ST2, 3 cases (5%) MCD, 3 (5%) A53, and 33 cases (58%) as other.

Tumor microenvironment evaluation by multispectral immunofluorescence

Among the 57 DLBCL cases evaluated in our discovery cohort, the median proportions of each cell type observed were: 17% for $CD3^+$ T cells (quartiles 1-3, 7-27%), 5.2% for $CD4^+$ T cells (quartiles 1-3, 1.6-8.2%), 10.1% for $CD8^+$ T cells (quartiles 1-3, 2.7-15.3%), 51% for B cells (quartiles 1-3, 36-72%), 1.8% for plasma cells (quartiles 1-3, 0.6-6.2%), 2% for macrophages (quartiles 1-3, 0.8-9%), 10% for $CD8^+$ T cells (quartiles 1-3, 3-15%), 0.006% for NK cells (quartiles 1-3, 0.003-0.2%), and 7% for $PD1^+$ T cells (quartiles 1-3, 3-14%). Using logistic regression analysis, five cellular proportions in the TME were associated with a poor overall survival (OS) and progression-free survival (PFS) (*Online Supplementary Figure S1*). These were: 1) low total T-cell proportion by CD3 (Figure 1A and B, Figure 2A and B; ROC cutoff, 6.95%); 2) low $CD4^+$ $PD1^+$ T-cell proportion ($CD3^+CD4^+PD1^+$/total cells) (Figure 2C and D; ROC cutoff, 2.55%); 3) low $CD8^+$ $PD1^+$ T cells ($CD8^+PD1^+$/total cells) (Figures 1C and D, 2E and F; ROC cutoff, 1.2%); 4) high B-cell/tumor cell proportion (ROC cutoff, 74%); and 5) high plasma cell proportion (ROC cutoff, 3.5%). The 5-year OS for patients with low (15/57 cases) and high (42/57 cases) total $CD3^+$ T-cell proportions was 48% (95% confidence interval [CI]: 21-71%) *versus* 86% (95%CI: 72-99%), respectively (P=0.0036) (Figure 2A); for low (13/57 cases) *versus*

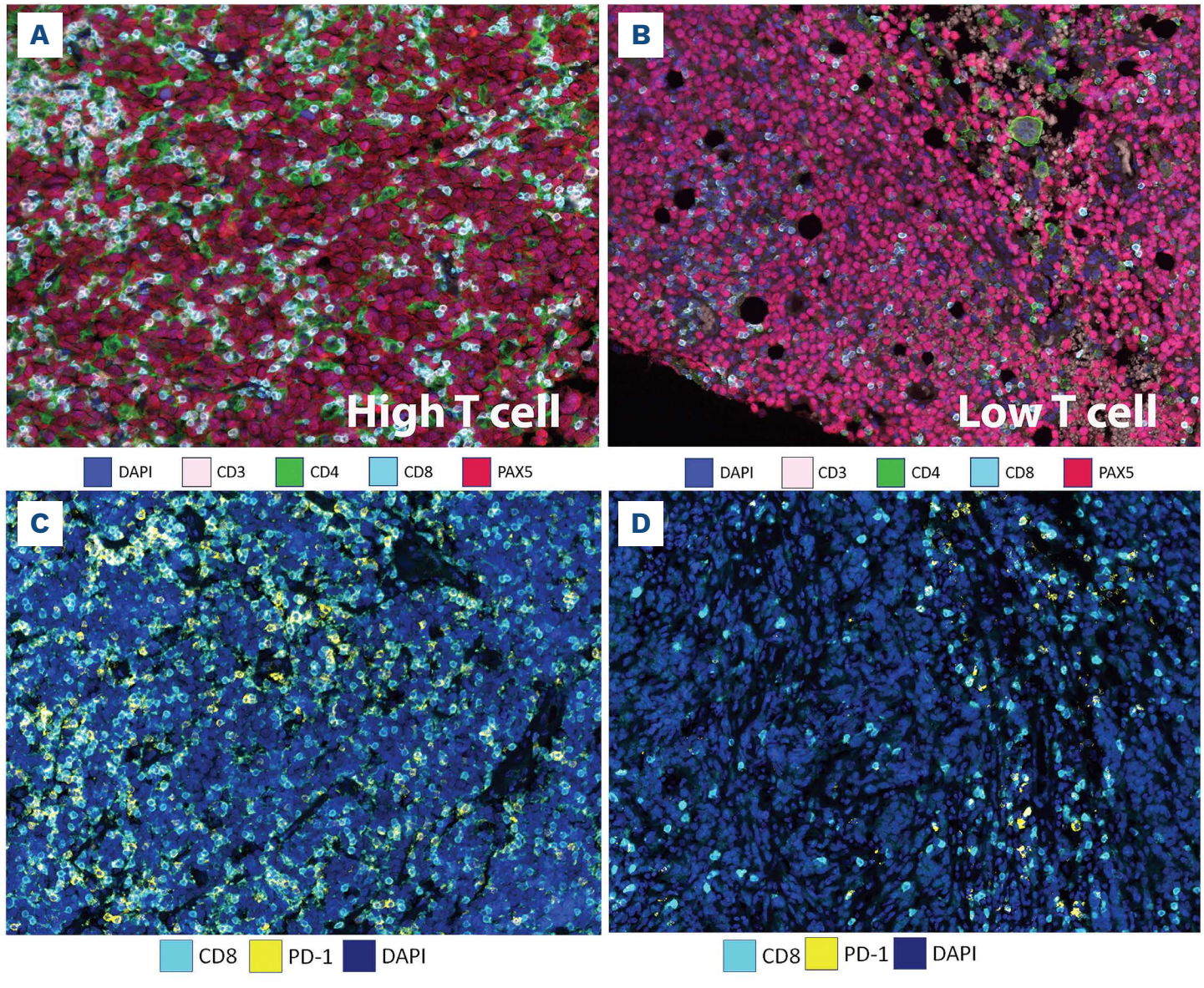


Figure 1. Multispectral immunofluorescence stains of diffuse large B-cell lymphoma performed on the Vectra system. (A) High T-cell proportion *versus* (B) low T-cell proportion. (C) An example of a case with high $CD8^+PD1^+$ T cells and (D) a comparison case with low $CD8^+PD1^+$ T cells.

high (44/57 cases) $CD4^+PD1^+$ T cells, 39% (95%CI: 11-65%) *versus* 87% (95%CI: 75-98%), respectively (*P*=0.0004) (Figure 2C); and for low (11/57 cases) *versus* high (46/57 cases) $CD8^+PD1^+$ T cells, 32% (95%CI: 1-62%) *versus* 85% (95%CI: 73-98%), respectively (*P*=0.0004) (Figure 2E). The PFS curves showed similar findings (Figure 2B, D and F). Total T-cell and T-cell subset proportions remained significantly associated with survival irrespective of the COO (*Online Supplementary Figure S2*). No other immune cell subtype was associated with outcome. The clinical characteristics of patients with low and high total $CD3^+$ T-cell proportions are shown in Table 1. The clinical characteristics, COO and DHITsig frequencies were similar between the two groups. Multivariate analysis adjusted for age and International Prognostic Index (IPI) (low=IPI 1,2; high=IPI 3,4), demonstrated that low $CD3^+$ T-cell proportion, low $CD3^+CD4^+PD1^+$, and low $CD3^+CD8^+PD1^+$ T cells, and low $CD4^+PD1^+$ T cells with low TIM3 were all associated with OS, particularly in the high IPI groups (*Online Supplementary Figure S3*).

High-grade B-cell lymphomas, particularly cases with a double-hit signature, have been shown to be low in T cells.[17] Since DHITsig-pos cases have a poor prognosis with

Table 1. Clinical characteristics of patients with diffuse large B-cell lymphoma according to T-cell proportion.

	Low T cell (N=15)	High T cell (N=42)	*P*
Age >60 years	27%	33%	ns
Male sex	67%	57%	ns
Cell of origin			
ABC	27%	19%	ns
GCB	73%	74%	ns
Unclassified	0%	7%	ns
Advanced stage	60%	60%	ns
Elevated LDH	67%	53%	ns
Extranodal sites >1	33%	29%	ns
High or high-intermediate IPI	53%	34%	ns
DHITsig-positive	30%	17%	ns

ns: not significant (*P*>0.05); ABC: activated B-cell type diffuse large B-cell lymphoma (DLBCL); GCB: germinal center B-cell type DLBCL; advanced stage: stages III/IV; LDH: lactate dehydrogenase; IPI: International Prognostic Index; DHITsig-positive: double-hit gene expression signature-positive; N: number. Low and high T-cell content cutoff is 6.95%.

standard therapy, we investigated whether DHITsig-pos cases exhibited a low T-cell proportion.[15,17] However, we found that the DHITsig-pos cases (n=8) had a median $CD3^+$ T-cell percentage of 19% compared to 16% for the DHITsig-neg cases (n=32) (*P*=0.95) (*Online Supplementary Figure S4*), although this may be due to the low number of DHITsig-pos cases analyzed.

We also evaluated the proportion of PD-L1 expression on the histiocytes and tumor cells, and we see a moderate positive correlation with total T-cell proportion and PD-L1-positive histiocytes (r=0.40, 95%CI: 0.16 to 0.60; *P*=0.002) but no correlation was seen with PD-L1-positive tumor cells (r=0.17, 95%CI: -0.098 to 0.41; *P*=0.21). A low correlation was seen with $CD4^+PD1^+$ T cells and PD-L1-positive histiocytes (r=0.28, 95%CI: 0.023 to 0.51; *P*=0.033) and PD-L1-positive tumor cells (r=0.28, 95%CI: 0.023 to 0.51; *P*=0.034).

In addition, nearest neighbor analysis was performed to determine if a particular cell type in the TME (e.g., $CD4^+$ or $CD8^+$ T cells, histiocytes, NK cells) within close proximity to the malignant B cells correlated with survival. Just as a lower proportion of T cells correlated with poor survival, we found that low numbers of $CD3^+CD4^+PD1^+$ T cells and $CD3^+CD8^+PD1^+$ T cells from the tumor cells (50 pixels) were both associated with poor OS (*P*=0.0095 and *P*=0.0022, respectively) (*Online Supplementary Figure S5*).

Mutation and copy number analysis

We also evaluated the 57 cases by mutational and copy number analysis. We found that cases with a low total T-cell proportion had more frequent abnormalities of immune-related genes such as *B2M, TNFRSF14, CD58*, and *FAS* (12/15, 80%) compared to those with high total T-cell

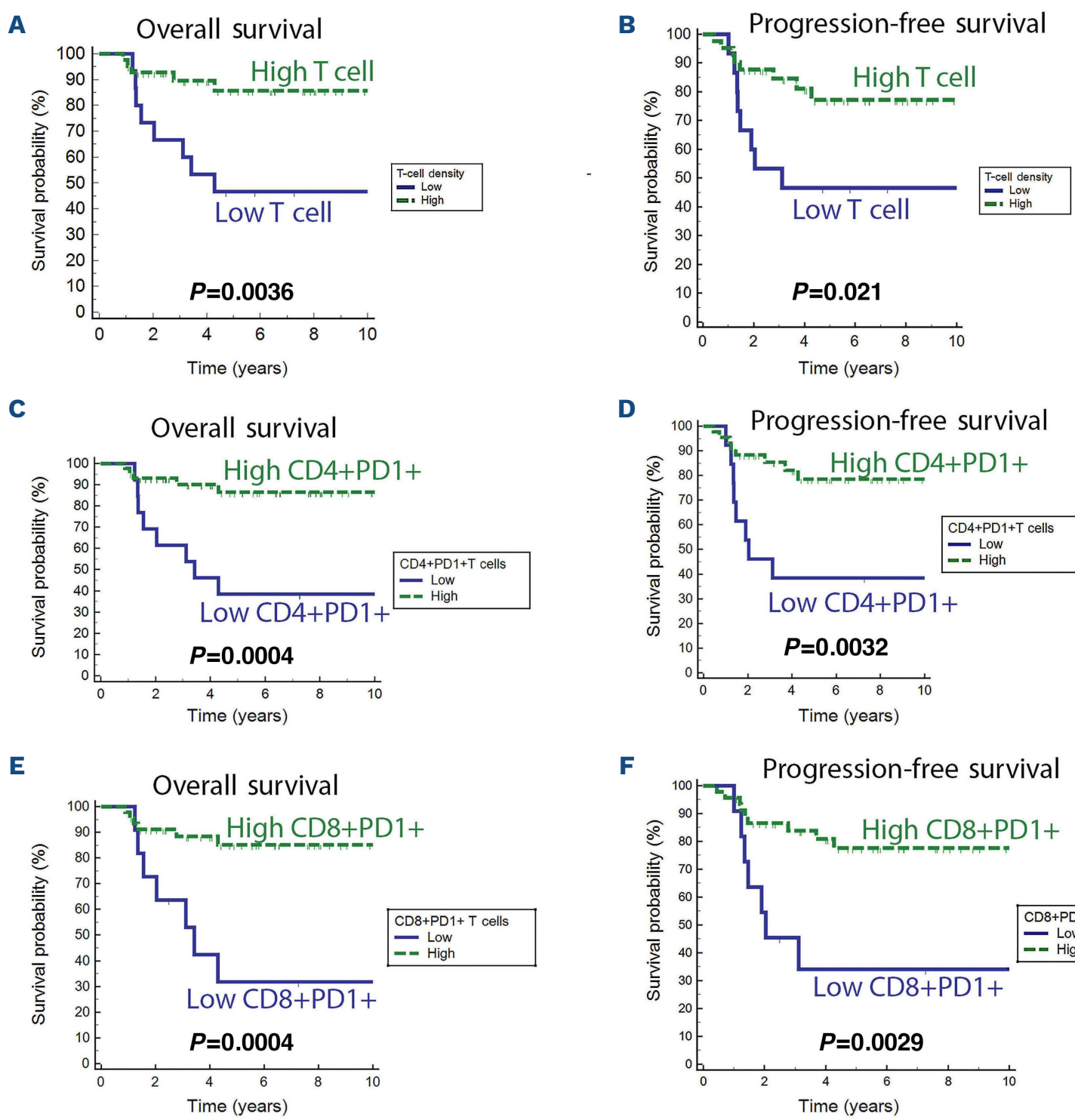


Figure 2. Overall survival and progression-free survival of diffuse large B-cell lymphoma patients based on T-cell proportions. (A and B) Overall T-cell proportion (low/high cutoff 6.95%; median 17%). (C and D) $CD4^+PD1^+$ T cells (low/high cutoff 2.55%; median 1.2%). (E and F) $CD8^+PD1^+$ T cells (low/high cutoff 1.2%; median 4.5%).

proportion (15/42, 36%; *P*=0.006) (Figure 3). There was no correlation between PD-L1 copy number gain and T-cell proportion (*P*=0.49).

B2M, HLA I, HLA II, and TIM3 protein expression by immunohistochemistry

We found that the cases of DLBCL were positive for B2M, HLA I, and HLA II in 40%, 47%, and 33% by IHC, respectively (Figure 4). Loss of expression for both B2M and HLA I was frequently seen in DLBCL (50%, 29/57). Interestingly, cases that showed low T-cell proportion by mIF showed frequent loss of B2M, HLA I, and/or HLA II by IHC compared to cases that had a higher T-cell proportion (*Online Supplementary Table S1*). Cases that had loss of all three proteins (B2M, HLA I, and HLA II) were more frequent in the low T-cell proportion group compared to the high T-cell proportion group (73% *vs*. 26%; *P*=0.002). Median T-cell proportions were also lower in cases that were negative for B2M, HLA I, and HLA II (*Online Supplementary Table S3, Online Supplementary Figure S6*). We also saw a lower percentage of NK cells ($CD56^+$) in cases with loss of HLA II compared to HLA II positive cases (0.04% *vs*. 0.18%; *P*=0.0007), but this was not seen with B2M or HLA I. Using a targeted mutational panel, we observed that cases with B2M IHC loss had a higher number of mutations compared to B2M-positive cases (median 12.5 mutations *vs*. 8 mutations; *P*=0.048), but this was not significant for HLA I/II. Among the total of 8 cases with *EZH2*, 5 cases lacked expression of B2M, HLA I and HLA II (63%), but there was no significant difference in median T-cell proportions between *EZH2* mutated and *EZH2* wild-type cases (10% *vs*. 17%, respectively; *P*=0.44). We also found that loss of expression of *B2M* or HLA I by IHC correlated with B2M mutations (*P*=0.04 and *P*=0.013, respectively) but not with copy number loss (*P*=0.45 and *P*=0.054, respectively).

The median percentage of cells expressing TIM3 was 2.2% (range, 0-65.5%) (Figure 3). We observed that cases with low T-cell content had a lower median proportion of cells with TIM3 expression (0.56%, 95%CI: 0.11-1.67%) compared to cases with high T-cell content (2.77%, 95%CI: 2.02-7.1%; *P*=0.0011). There was also a correlation between TIM3 expression and B2M (P=0.03) as well as HLA I (*P*=0.013) expression, but not with HLA II expression (*P*=0.61). There was no association between TIM3 proportion and OS or PFS (ROC cutoff 2.6%; *P*=0.086 and *P*=0.46, respectively) (Figure 5A and B). But, when combining PD1 ($CD4^+PD1^+$ T cells) proportion with TIM3 proportion, we observed that cases with both low TIM3 and low PD1 proportion were associated with poor OS and PFS compared to cases with a high proportion of TIM3 and/or PD1 (*P*<0.0001 and *P*=0.0002, respectively) (Figure 5C and D).

Validation of the tumor microenvironment findings with CIBERSORTx analysis

Using data from Lenz *et al.*,[18] we analyzed the cellular proportions in 351 cases of *de novo* DLBCL (184 GCB DLBCL, 167 ABC DLBCL) treated with R-CHOP. We used the CIBERSORTx method[19] to infer cell-type gene expression profiles and found that the signatures of cases with a low T-cell content and low $PD1^+$ T cells were associated with a poor OS (*P*=0.012 and *P*=0.0005, respectively) (Figure 6A and B). Cases with a low expression of TIM3 (HAVCR2) and low $PD1^+$ T cells were associated with a poor OS (*P*=0.0003) (Figure 6C). We also observed a trend within the COO subtypes (Figure 6D and E).

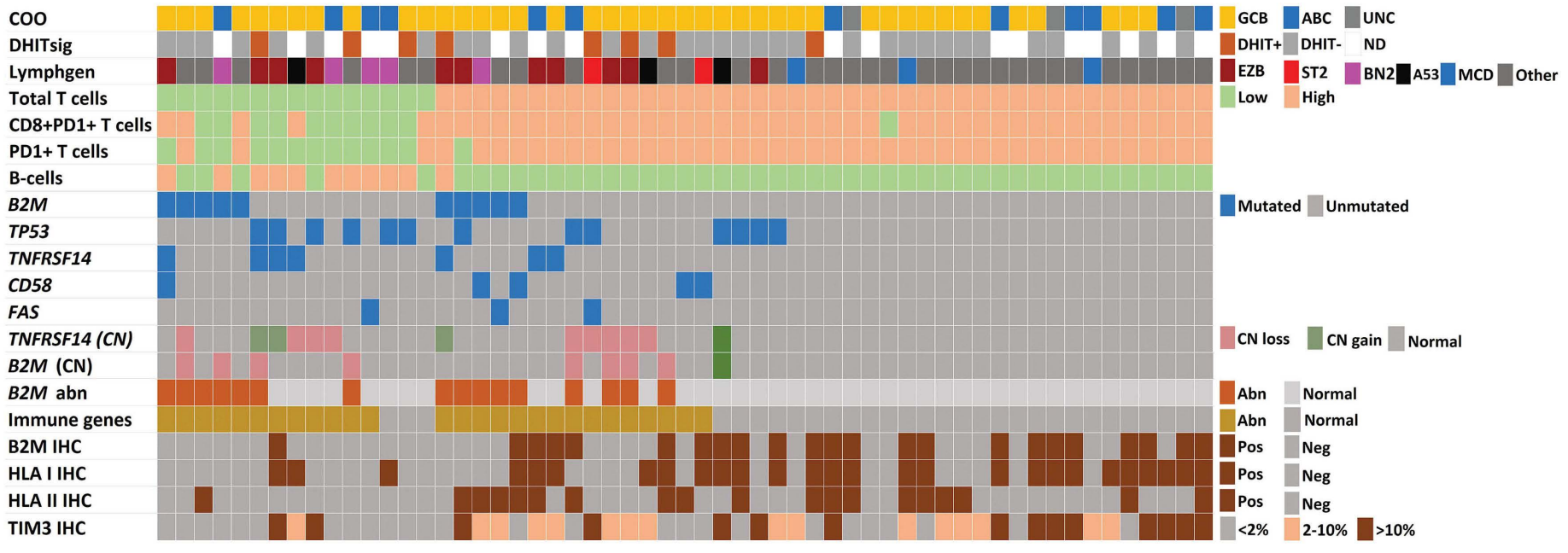


Figure 3. Mutational spectra of cases with low and high T-cell content. Total T cells: low (green), high (salmon). There are more abnormalities in genes related to immune surveillance in the cases with low T-cell content compared to cases with high T-cell content (80% *vs*. 36%; *P*=0.006). Cases with low T-cell content also showed loss of B2M, HLA I, and HLA II by IHC. COO: cell-of-origin; DHITsig: double hit signature; CN: copy number; abn: abnormal; UNC: unclassified; GCB: germinal center type; ABC: activated B-cell type; IHC: immunohistochemistry.

Validation of the tumor microenvironment findings using routine immunohistochemical stains and bioimage analysis

An independent cohort of 54 cases of DLBCL was also stained for CD3 and PD1 by IHC, scanned using digital whole slide imaging and analyzed using bioimage analysis software to determine $CD3^+$ and $PD1^+$ T-cell proportions (Figure 7A and B). We found that the total T-cell proportion was associated with poor OS and PFS (P=0.03 and P=0.045, respectively; ROC cutoff 4.26%) (Figure 7C and D), with similar trends for PD1 (ROC cutoff 18.6%) (Figure 7E and F). Staining of the original discovery cohort with chromogenic IHC of 49 cases of DLBCL showed good correlation between the CD3 proportion by mIF and by IHC (r=0.78, 95%CI: 0.63-0.87; P<0.0001) and still showed prognostic significance with OS (see *Online Supplementary Figure S8*).

Discussion

The TME plays a critical role in the pathobiology of solid tumors and lymphomas, particularly classical Hodgkin lymphoma[3] where the TME cells outnumber the tumor cells. We found that "hot" or inflamed tumors by mIF, GEP, and IHC had good outcomes whereas "cold" tumors had poor outcome. These "cold" tumors had low proportions of T cells, particularly T cells with an exhausted phenotype expressing PD1 and TIM3. We also found genetic differences that were associated with these "hot" and "cold" tumors. "Cold" tumors had more frequent abnormalities related to immune surveillance such as *B2M* and *TNFRSF14*, as well as loss of the surface proteins B2M, HLA I and II.

In our study, similar to others,[14,20] we found that "hot" tumors, specifically those with increased T-cell proportion and expressing PD1 and TIM3, were associated with an improved prognosis. We sought to investigate the TME in DLBCL using a multiparameter approach incorporating mIF and routine IHC, in conjunction with genomic analysis, to elucidate the major cellular subsets in tissue sections and determine the mechanisms of immune escape. We have correlated our findings with the clinical outcome and have confirmed our findings using two independent validation cohorts that similarly demonstrated T-cell proportion to be associated with outcome. In our study, we found that "hot" DLBCL tumors with a high T-cell content, high $CD4^+PD1^+$ T cells, and high $CD8^+PD1^+$ T cells had better prognosis when treated with R-CHOP (Figure 2). We used standard IHC stains for CD3 and PD1 in an independent cohort of DLBCL treated with R-CHOP and found that CD3 proportion predicted for survival, with a trend for PD1 as well. In addition, we re-evaluated the original discovery cohort with chromogenic IHC and found good correlation between the CD3 mIF and IHC proportions with continued significance related to OS. Our study confirms the findings of others that T-cell proportion is associated with outcomes[14,20] and is a proof of principle that routine IHC, in conjunction with bioimaging, may be a viable and low-cost option to determine T-cell proportions when more

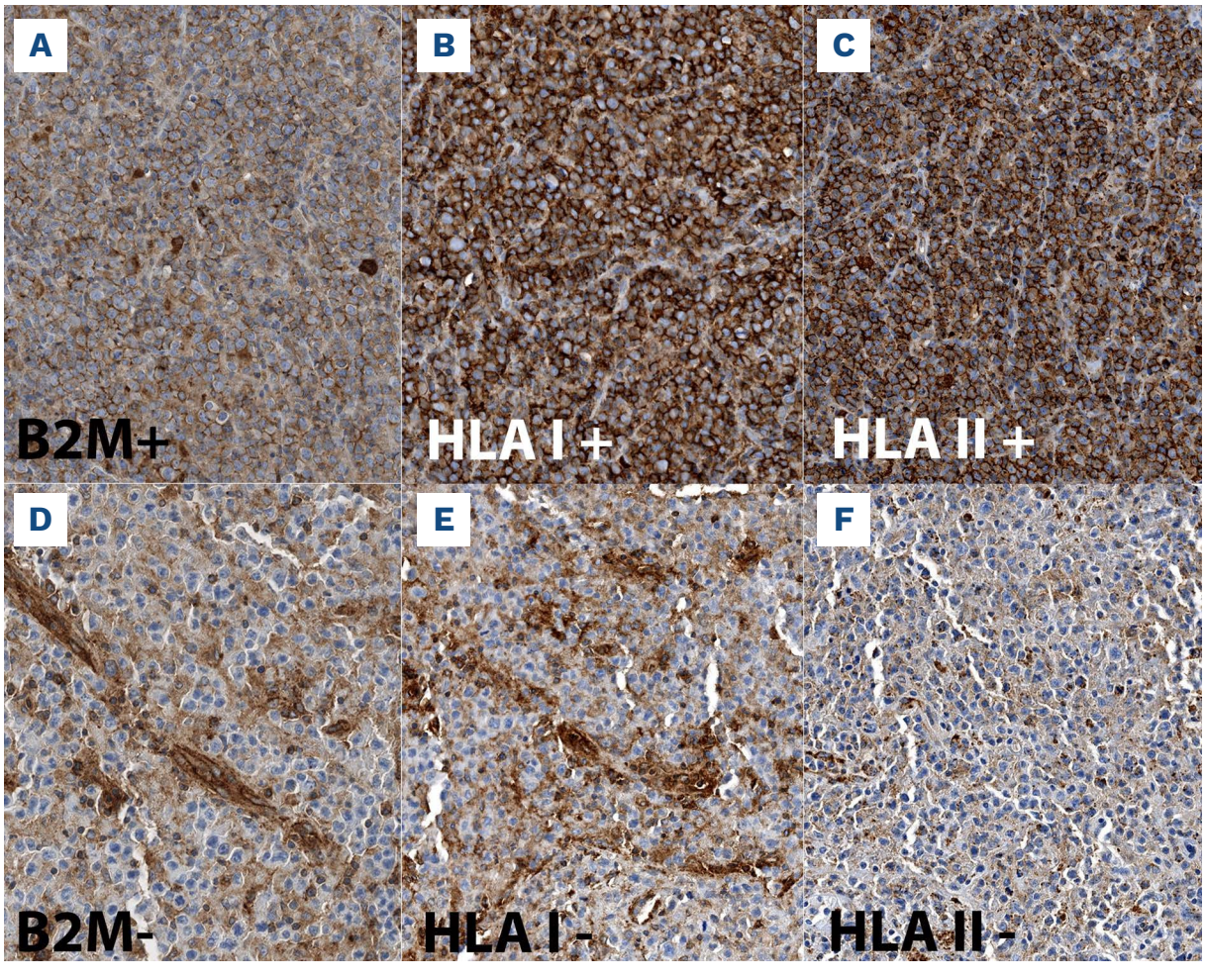


Figure 4. Diffuse large B-cell lymphoma staining with B2M, HLA I, and HLA II immunohistochemistry. A case of diffuse large B-cell lymphoma (DLBCL) with expression of (A) B2M, (B) HLA I, and (C) HLA II by immunohistochemistry (IHC). (D-F) A separate case of DLBCL that is negative for (D) B2M, (E) HLA I, and (F) HLA II by IHC.

advanced technology such as mIF is not available. Combining TIM3 proportion with PD1 expression also showed that cases with both low PD1 and low TIM3 have a very poor OS and PFS, which was confirmed using deconvoluted GEP data[19] from an independent cohort of DLBCL.[18] In addition, cases with low T-cell content exhibited mechanisms of immune escape such as decreased expression of HLA proteins as well as genomic abnormalities related to immune surveillance.

TIM3 is an inhibitory receptor that is expressed on exhausted T cells, and co-blockade of TIM3 and PD1 has resulted in greater restoration of the T-cell response compared to PD1 alone in various cancers.[21,22] TIM3 also marks the most dysfunctional subset among tumor-infiltrating $CD8^+PD1^+$ T cells.[23] Roussel *et al.*[20] recently found that tumor-infiltrating lymphocytes expressing PD1 and TIM3 are expanded in DLBCL, particularly ABC DLBCL, and exhibit a transcriptomic signature related to T-cell exhaustion. Their ABC DLBCL cases had a poor survival in cases with low expression of PD1 and TIM3 by gene expression profile.[20] Similarly, our cases with low expression of PD1 and TIM3 were associated with poor OS (Figure 5), and this was further confirmed with the GEP data from Lenz *et al.*[18] We also found that low/absent TIM3 expression with low $CD4^+PD1^+$ T cells was associated with the absence of B2M and HLA I proteins. It may be that cases with low PD1 and low TIM3 expression represent particularly "cold" tumors that utilize loss of class I HLA, thus escaping recognition by cytotoxic T cells.

In solid tumors, it is well known that increased tumor infiltrating lymphocytes (TIL) are associated with better survival.[24] "Cold" tumors with a suppressive TME prevent the anti-tumor function of cytotoxic T cells. One suppressive mechanism used by the tumor cells is to down-regulate their HLA class I proteins due to genetic abnormalities (e.g., *B2M* or *HLA I*), thereby avoiding immune surveillance. There are also suppressive cytokines and other signals released by the tumor or the TME cells that cause the T cells to be in an 'exhausted' state. These exhausted T cells have high levels of inhibitory receptors such as PD1, LAG-3, TIM-3, cytotoxic T lymphocyte antigen-4 (CTLA-4), band T lymphocyte attenuator (BTLA), and T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT). One possible reason why cases of DLBCL rich

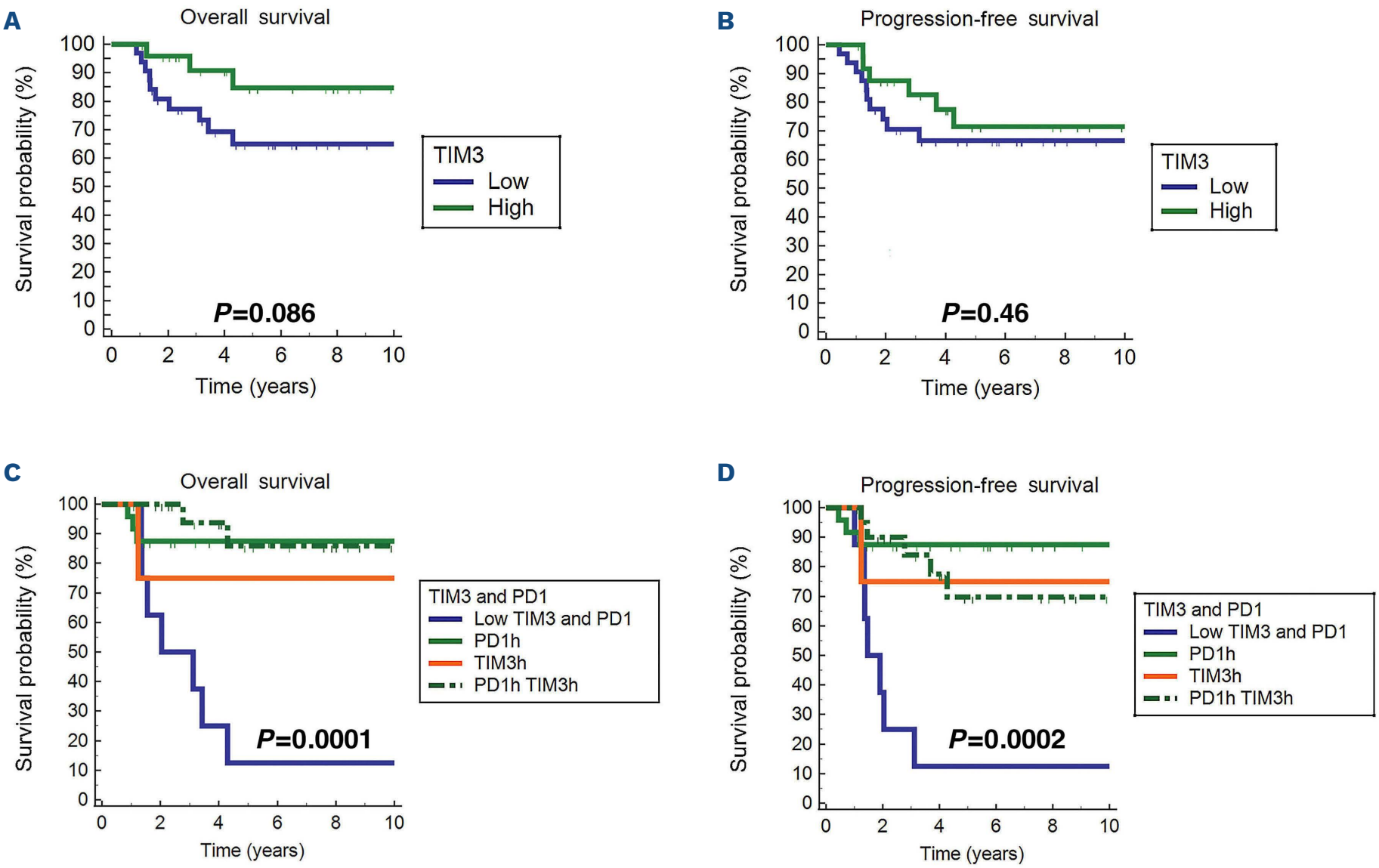


Figure 5. Survival based on TIM3 and PD1 staining in diffuse large B-cell lymphoma. (A) Overall survival (OS) and (B) progression-free survival (PFS) in relation to TIM3 proportion in the tumor microenvironment. Combining $CD4^+PD1^+$ T-cell proportion (PD1) with TIM3 identifies a group of patients with poor (C) OS and (D) PFS having low expression of these two markers compared to cases with high PD1 (PD1h) and/or high TIM3 (TIM3h).

in T cells, particularly high $CD8^+PD1^+$ T cells, respond to immunochemotherapy like R-CHOP may be that the therapy reduces the tumor burden, results in tissue damage, releases tumor antigens, and allows these T cells to recover their anti-tumor properties, similar to observations in patients with solid tumors.[25] Moreover, R-CHOP therapy may re-invigorate the "hot", inflamed tumors since anthracycline-based chemotherapy can induce immunogenic death of lymphoma cells.[26] Based on our findings, it may be worthwhile studying immunotherapies or immunomodulating therapies prior to or in combination with chemotherapy specifically in patients who have tumors with low T-cell content in order to reinvigorate the TME and potentially allow for more robust responses to treatment. Likewise, "hot" or T-cell inflamed tumors may be predisposed to benefit from immunotherapies or combined chemotherapy with immunotherapy/immunomodulatory approaches.[27,28]

We sought to evaluate potential underlying genomic mechanisms for our observations regarding the DLBCL TME. We evaluated whether the cases that were high-grade B-cell lymphomas (DHITsig-pos) were inadvertently selected for in the low T-cell group, as double-hit lymphoma are found to have a paucity of T cells.[17] However, in our cases, we found no difference in the proportion of T cells between the DHITsig-pos and DHITsig-neg cases. This lack of association may be due to the low number of DHITsig-pos cases in the cohort. We did observe that cases of DLBCL with a low T-cell content had a higher frequency of genomic abnormalities related to immune surveillance genes such as (*B2M, TNFRSF14, CD58*, and *FAS*; 80% *vs.* 36%) (Figure 3). These mutations likely resulted in a loss of the ability to present antigen by the tumor cells and their lack of recognition by T cells, resulting in fewer T cells homing into the TME. Recent studies[29,30] have demonstrated that patients who progress following chimeric antigen receptor (CAR) T cells exhibit *FAS* mutations, suggesting that there may be common antigen-independent mechanisms of resistance to chemotherapy and immunotherapy approaches.

In our study, we found that many cases that had low T-

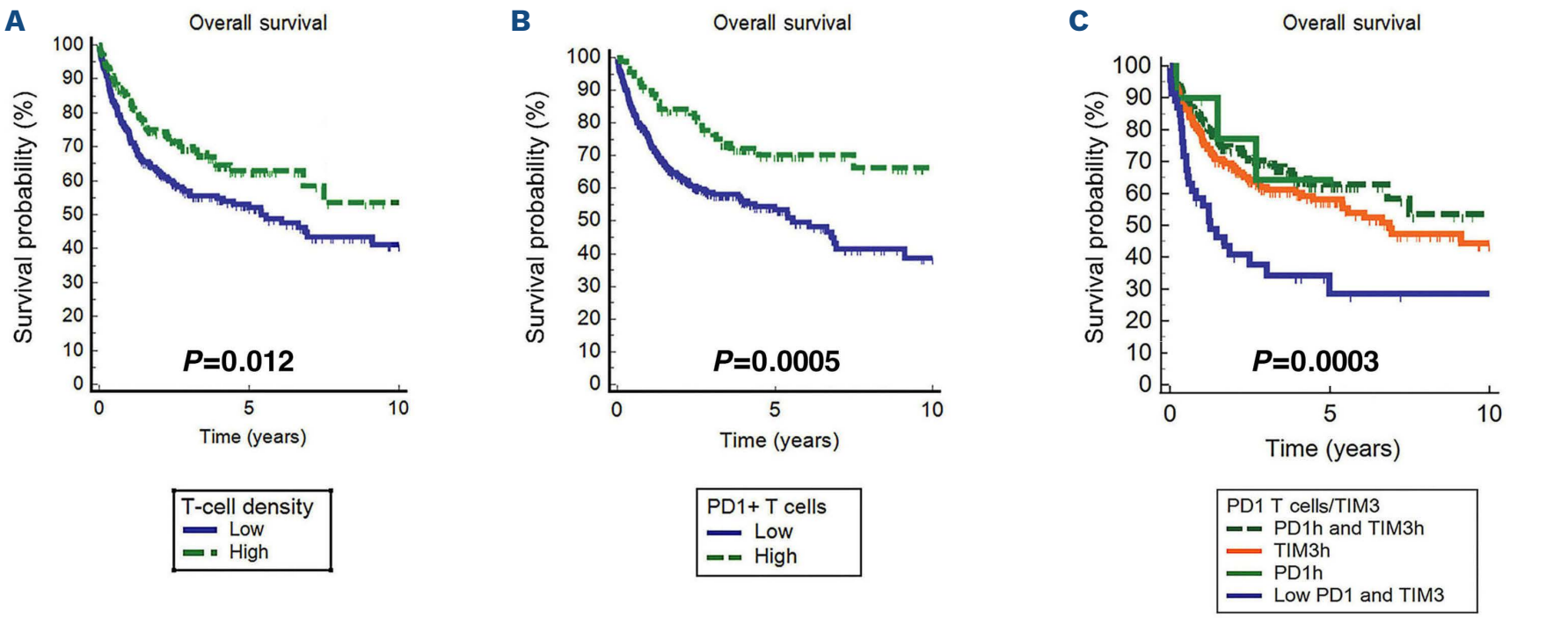
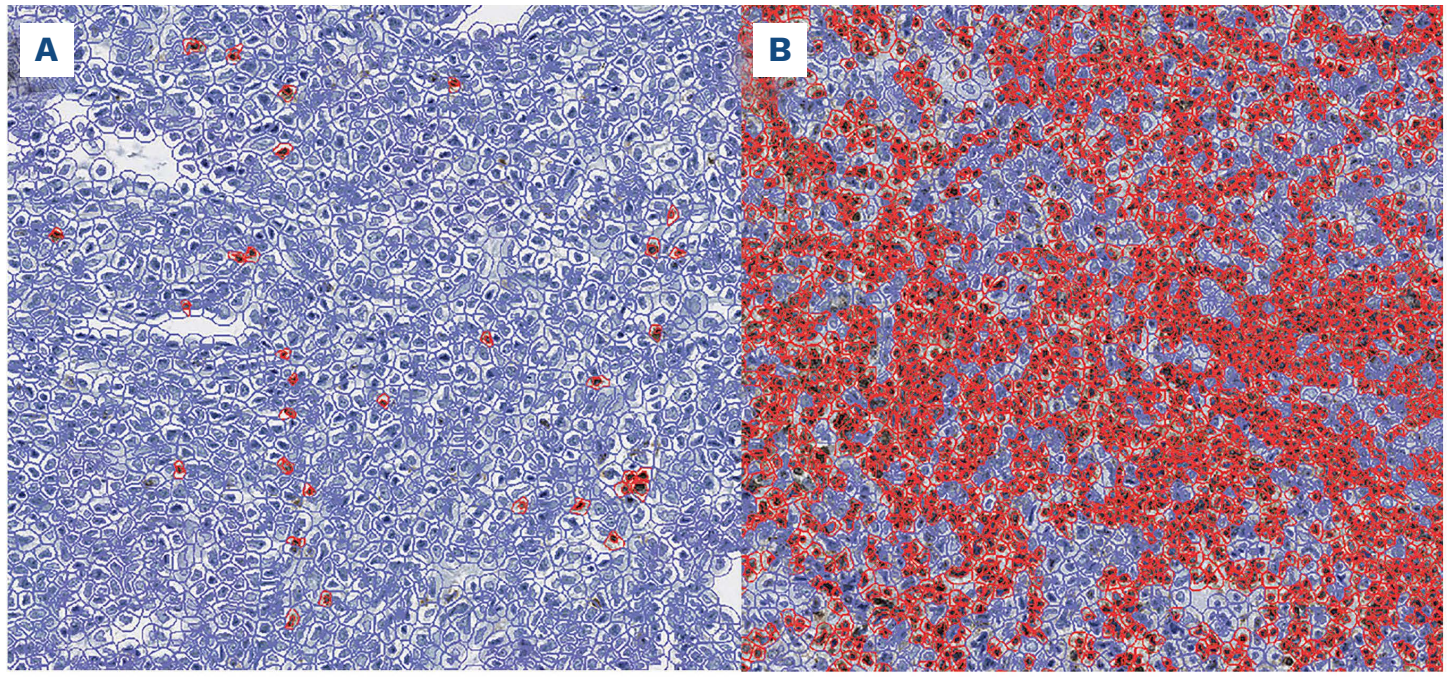
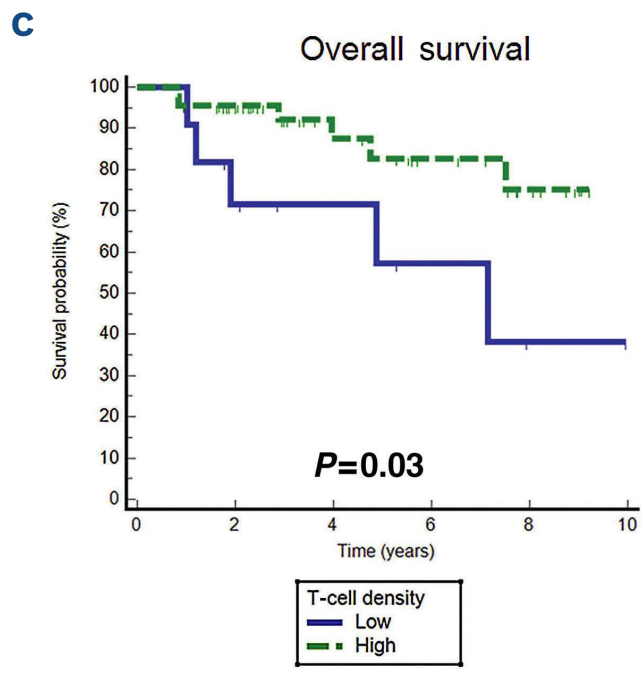
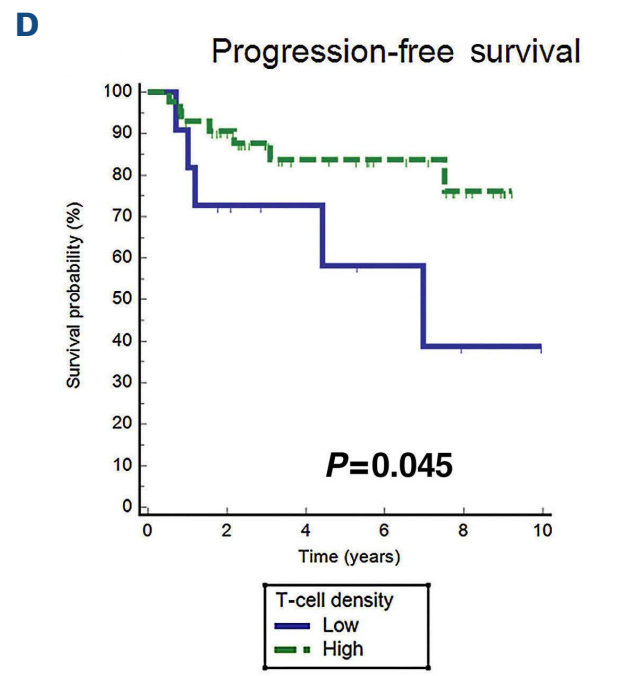
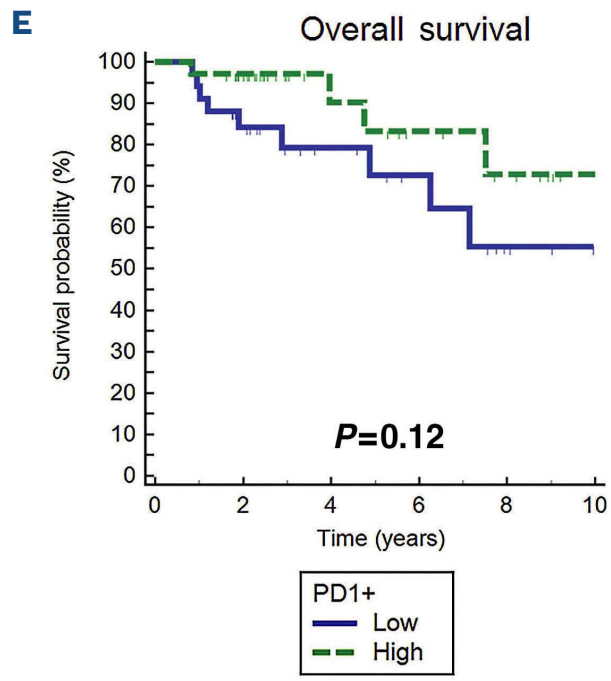


Figure 6. Validation of TIM3 and PD1 by gene expression profiling in an independent cohort of diffuse large B-cell lymphoma. Utilizing CIBERSORTx and the diffuse large B-cell lymphoma (DLBCL) dataset from Lenz *et al.*,[18] we confirm that low total T-cell and low $PD1^+$ T-cell proportions are associated with poor overall survival OS (A and B). Cases with low $PD1^+$ T cells and TIM3 (HAVCR2 expression) had poor (OS) compared to cases with high TIM3 (TIM3h) and/or high PD1 T cells (PD1h) (C). A similar trend was also seen based on cell-of-origin for germinal center type (GCB) but not activated B-cell type (ABC) DLBCL (D and E).









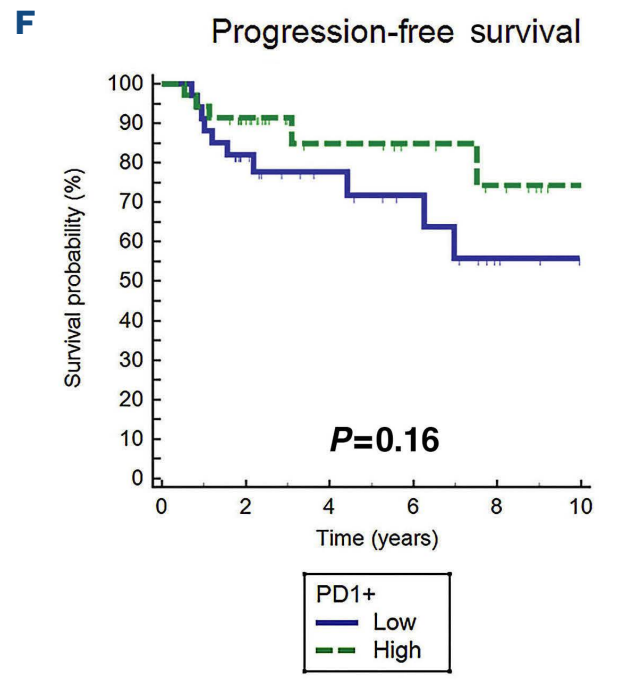


Figure 7. Validation of total T-cell content and PD1 staining in an independent cohort of diffuse large B-cell lymphoma. Using single stain immunohistochemistry for CD3 and PD1, we scanned the slides and used bioimage software to objectively enumerate the positive cells: CD3 (red), tumor cells (blue). Low total $CD3^+$ T-cell content (A) compared to high T-cell content (B) was associated with poor overall survival and progression-free survival (C and D) (ROC cutoff 4.26%), with similar trends for PD1 staining (E and F) (ROC cutoff 18.6%).

cell proportion and decreased CD4 and CD8 T cells also showed absence of B2M, HLA I, and/or HLA II proteins (Figure 4). A study by Rimsza *et al.*[31] similarly showed that cases of DLBCL with loss of HLA II protein also had exceedingly few $CD8^+$ T cells. A recent study showed an association between *B2M* mutations and absence of HLA I expression in DLBCL, resulting in decreased presentation of neoantigens on the tumor surface and leading to evasion of immune surveillance.[32] Similarly, we see in our study that cases with *B2M* mutations showed an association with loss of B2M and HLA I protein expression. Unlike a recent study,[27] we did not find a correlation between *PD-L1* copy number gains and T-cell proportion or association with inferior PFS. In that study, there was a high proportion of non-GCB DLBCL cases with high PD-L1 protein expression which correlates with *PD-L1* alterations and inferior PFS. We may not have observed these findings due to the small number of cases of ABC DLBCL in our cohort, and we also did not evaluate for *PD-L1* translocation in our study.

Ennishi *et al.*[33] described cases of GCB DLBCL with *EZH2* mutations and showed lower expression of MHC II and a significantly lower number of TIL in the TME. There were 8 cases in our study with an *EZH2* mutation, and 5 of these showed lack of B2M, HLA I, and HLA II protein expression (63%). Cases with mutations in *EZH2* also showed lower T-cell content, but we had too few cases with *EZH2* mutation to adequately evaluate this for statistical significance.

Limitations to this study are that we used a TMA with representative cores rather than whole tissue sections. As a result, we cannot exclude the possibility that the cores may not completely represent the TME within the whole tumor. In addition, we validated our mIF findings using different approaches from mIF (GEP and routine IHC), but there were consistent findings across methodologies, which supports our hypothesis that "cold" tumors show a poor prognosis regardless of what method is used for analysis and that our findings are not method-dependent. It is unclear which method provides optimal evaluation of the T-cell proportion, but other studies have also shown correlation with mIF and deconvoluted GEP analysis,[14,20] and we also saw good correlation between mIF and IHC in our discovery cohort.

In conclusion, our findings demonstrate that analysis of the TME can play an important prognostic role in DLBCL, with "cold" tumors containing a low proportion of exhausted T cells with inhibitory molecules such as PD1 and TIM3 associated with a poor prognosis. We identified underlying genomic abnormalities associated with these low T-cell content DLBCL along with absence of HLA molecules (B2M, HLA I, and HLA II) on the tumor cells that allow for evasion of immune surveillance. These findings further our understanding of the role of the TME in DLBCL and the mechanisms that tumor cells utilize for immune escape.

Disclosures

No conflicts of interest to disclose.

Contributions

JYS and AFH are responsible for study conception and design. All authors collected and assembled the data. WZ, HM, VB, JC, PS, MRN, DS, PL, WCC, DDW and AMP performed data analysis. JYS prepared the first draft of the paper and all authors helped with revisions.

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Data-sharing agreement

Original data are available from the corresponding author.

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