Immune Profiling and Quantitative Analysis Decipher the Clinical Role of Immune-Checkpoint Expression in the Tumor Immune Microenvironment of DLBCL

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Abstract

PD-1/L1 and CTLA-4 blockade immunotherapies have been approved for 13 types of cancers and are being studied in diffuse large B-cell lymphoma (DLBCL), the most common aggressive B-cell lymphoma. However, whether both PD-1 and CTLA-4 checkpoints are active and clinically significant in DLBCL is unknown. Whether PD-1 ligands expressed by tumor cells or by the microenvironment of DLBCL are critical for the PD-1 immune checkpoint is unclear. We performed immunophenotypic profiling for 405 patients with de novo DLBCL using a MultiOmyx immunofluorescence platform and simultaneously quantitated expression/coexpression of 13 immune markers to identify prognostic determinants. In both training and validation cohorts, results demonstrated a central role of the tumor immune microenvironment, and when its functionality was impaired by deficiency in tumor-infiltrating T cells and/or natural killer cells, high PD-1 expression (but not CTLA-4) on CD8+ T cells, or PD-L1 expression on T cells and macrophages, patients had significantly poorer survival after rituximab–CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) immunotherapy. In contrast, tumor-cell PD-L2 expression was associated with superior survival, as well as PD-L1 CD20 cells proximal (indicates interaction) to PD-1+ CD8+ T cells in patients with low PD-1+ percentage of CD8+ T cells. Gene-expression profiling results suggested the reversibility of T-cell exhaustion in PD-1+/PD-L1+ patients with unfavorable prognosis and implication of LILRA/B, IDO1, CHI3L1, and SOD2 upregulation in the microenvironment dysfunction with PD-L1 expression. This study comprehensively characterized the DLBCL immune landscape, deciphered the differential roles of various checkpoint components in rituximab–CHOP resistance in DLBCL patients, and suggests targets for PD-1/PD-L1 blockade and combination immunotherapies.
Introduction

Antitumor T-cell response is critical for the immune surveillance of cancer but is often dampened by immune checkpoints. The checkpoint receptor CTLA-4 inhibits T-cell activation at the priming phase by competing with CD28 to bind CD80/CD86 (1–3). PD-1 suppresses the function of activated T cells through ligation of PD-1 ligands PD-L1 (4, 5) or PD-L2 (6), mainly at the effector phase (7). PD-L1 can also interact with CD80 on activated T cells, and this interaction is required for T-cell anergy induction and maintenance (8). The CTLA-4/PD-1 immune checkpoint can be reversed by CTLA-4/PD-1/PD-L1–blocking antibodies. However, the mechanisms of action are not completely understood (9, 10), and therapeutic effects of these blocking antibodies differ between CD4+ and CD8+ T cells (11, 12), between effector T and regulatory T cells (Tregs; refs. 13, 14), and between naïve T and memory T (Tm) cells (15). In addition, whether PD-L1 expressed by tumor cells or by host cells is essential for the immunotherapeutic efficacy of PD-1/L1 blockade remains controversial (9).

Diffuse large B-cell lymphoma (DLBCL) is the most common aggressive B-cell lymphoma, including two major molecular subtypes classified by gene-expression profiling (GEP): germinal center B-cell–like (GCB) and activated B-cell–like (ABC) DLBCL. The standard treatment for DLBCL, combination immunochemotherapy R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone), is associated with a high complete response rate of ~80%. However, 10% to 15% of DLBCLs are refractory, and 20% to 25% of patients experience relapse after an initial response posing clinical challenges (16). PD-1 and CTLA-4 blockade monotherapies showed promising efficacy in treating relapsed/refractory DLBCL in phase I clinical trials (17, 18). However, in a phase II study, the objective response rate of anti–PD-1 monotherapy was low in patients with relapsed/refractory DLBCL who were ineligible for, or failed with, autologous hematopoietic cell transplantation (10% and 3%, respectively; ref. 19). Phase II results of mono/combination immunotherapies in other clinical settings are currently unavailable.

Although PD-1/PD-L1 expression in DLBCL has been studied by different assessment methods, different cell sources of PD-1/PD-L1 expression and functional dependency on ligand–receptor interaction obscure the role of the PD-1 check- point in DLBCL (9, 10). Some studies found that high PD-L1 expression in tumor cells and soluble PD-L1 in the peripheral blood were associated with poorer survival in DLBCL (20–23) but other studies found no correlation between tumor-cell PD-L1 expression and clinical outcome (23–25). ‘Microenvironmental’ PD-L1 expression was inconsistently associated with nonsignificantly poorer or better survival (20, 23); increased tumor-infiltrating PD-1+ lymphocytes were more often associated with favorable than with unfavorable prognostic effects in DLBCL (10). Low PD-1 expression in DLBCL cells was also found (26–28), but its prognostic effect is unknown.

Cell-specific and topological analysis of immune-checkpoint expression in patients has become feasible with the fluorescent multiplex immunohistochemistry (IHC) and automated quantitation technology (29, 30). In this study, we used a MultiOmyx platform to simultaneously quantify the expression of 13 immune markers (CTLA-4, PD-1, PD-L1, PD-L2, CD20, Pax5, Cd3, Cd4, Cd8, Foxp3, Cd45R0, Cd56, and Cd68) in situ in a large cohort of de novo DLBCL cases. Checkpoint expression (PD-1, CTLA-4, and PD-L1/L2) in tumors and microenvironment components analyzed by three different methods (percentage within a cell type, cell density, and tissue architecture) and PD-L1/PD-L2 genetic alterations evaluated by fluorescence in situ hybridization (FISH) were correlated to the survival and gene-expression profiles of patients, in order to decipher the clinical role of immune-checkpoints and identify prognostic determinants in DLBCL.

Materials and Methods

Patients and molecular characterization

The multicenter cohort study included 405 patients with de novo DLBCL treated with R-CHOP from the International DLBCL R-CHOP Consortium Program (31, 32). Patients with Epstein-Barr virus (EBV) infection, primary mediastinal large B-cell lymphoma, primary central nervous system DLBCL, primary cutaneous DLBCL, HIV infection, or transformed DLBCL have been excluded. The median age was 63 years; the median follow-up was 47.5 months. Based on GEP (deposited in Gene-Expression Omnibus GSE31312) of total RNA by Affymetrix GeneChips Human Genome U133 Plus 2.0 (32, 33), 176 and 148 cases were determined as GCB and ABC subtype, respectively. This study was conducted in accordance with the Declaration of Helsinki. Data collection protocols were approved as being of minimal to no risk or as exempt by the institutional review board of each participating institution.

Antibodies for MultiOmyx fluorescent multiplex IHC

Formalin-fixed, paraffin-embedded (FFPE) tissue microarrays were stained with 13 antibodies conjugated to either cyanine 3 (cy3) or cyanine 5 (cy5) and 4’,6-diamidino-2-phenylindole (DAPI) via eight staining rounds. The antibodies used, by staining order, were mouse anti–CTLA-4 (sc376016; Santa Cruz Biotechnology), rabbit anti-CDS5 (156R-95; Cell Marque), anti–PD-L2 (clone 36C6.9E5; provided by G.J. Freeman), rabbit anti-Pax5 (AC-0158; Cell Marque), mouse anti-CDS45RO (304202; BioLegend), mouse anti-CDS8 (M7103; Dako), mouse anti-FoxP3 (290113; BioLegend), mouse anti-CDS3 (M7254; Dako), rabbit anti–PD-L1 (M4420; Spring Bioscience), rabbit anti–CDS4 (ab181724; Abcam), rabbit anti–PD-1 (ab186928; Abcam), rabbit anti–CDS20 (ab166865; Abcam), and mouse anti–CDS68 (MS-397-PABX; Thermo Fisher Scientific). Fluorescently labeled secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc.

MultiOmyx staining

FFPE tissue arrays were baked at 65°C for 1 hour. Slides were deparaffinized with xylene, rehydrated by decreasing ethanol concentration washes, and then processed for antigen retrieval. A two-step antigen retrieval was adopted to allow antibodies with different antigen retrieval conditions to be used together on the same samples (34, 35). Samples were then blocked against nonspecific binding with 10% (wt/vol) donkey serum and 3% (wt/vol) bovine serum albumin (BSA) in phosphate-buffered solution (PBS) for 1 hour at room temperature and stained with DAPI for 15 minutes. Directly conjugated primary antibodies were diluted in PBS supplied with 3% (wt/vol) BSA to optimized concentrations and applied for 1 hour at room temperature on a Leica Bond III Stainer. In the case of primary–
secondary antibody staining, samples were incubated with primary antibody, followed by incubation with species-specific secondary antibodies conjugated to either cyanine 3 (cy3) or cyanine 5 (cy5).

A total of 8 rounds of antibody staining were performed in sequence on the FFPE slides. CTLA-4 and CD56 antibody cocktail was used in round 1, followed by PD-L2, PAX5, and CD45RO staining as single antibody conjugation in round 2, 3, and 4, respectively. CD8 and FOXP3 antibody cocktail was used in round 5, followed by CD3 and PD-L1 staining in round 6, CD4 and PD-1 staining in round 7, and CD20 and CD68 staining in round 8.

Microscopy and image acquisition

Stained images were collected on INCell analyzer 2200 microscope (GE Healthcare Life Sciences) equipped with high-efficiency fluorochrome-specific filter sets for DAPI, cy3, and cy5. For multiplexed staining where colocalization was desired, the regions of interest (~0.4–0.6 mm² tissue area) were imaged, and stage coordinates were saved. The coordinates of each image region were then recalled for each subsequent round after minor readjustment using reference points from the first-round DAPI image and determining the appropriate offset. The exposure times were set at a fixed value for all images of a given marker. For image analyses, microscopy images were exported as full-resolution TIFF images in grayscale for each individual channel collected.

Dye inactivation in tissue

For the dye inactivation process, following staining round image acquisition, slides were decoverslipped, and dye inactivation was performed as previously described in US patent 7,741,045 (36). Briefly, the slides were immersed in alkaline solution containing H₂O₂ for 15 minutes with gentle agitation at room temperature. After 15 minutes, the slides were washed with PBS. The samples were imaged to check the efficacy of the dye inactivation, and then subjected to another round of staining.

MultiOmyx image analytics

The acquired images from sequential rounds were registered using DAPI images acquired in the first round of staining via a rigid registration algorithm for each region of interest. The parameters of transformation were then applied to the subsequent rounds, which ensured that the pixel coordinates across all the imaging rounds corresponded to the same physical locations on the tissue. Classification and coexpression analysis were performed in multiple stages. First, a nuclear segmentation algorithm was applied on the DAPI image to delineate and identify individual cells. Location information and expression of all the markers were computed for every cell identified. Then, morphologic image analysis and shape detection were performed using proprietary algorithms. (NeoGenomics Laboratories; https://neogenomics.com/pharma-services/lab-services/multiomyx). These algorithms detect and classify cells as positive or negative for each marker depending on their subcellular localization and morphology. A tissue-quality algorithm was also applied to the images to ensure image artifacts that arose owing to tissue folding or tear did not affect cell classification. Coexpression analysis and phenotype identification were performed by combining individual marker classification results.

For spatial analysis, two phenotypes of interest were defined: the anchor phenotype and the target phenotype. For each case, every cell of the anchor phenotype (PD-1⁺CD4⁺CD3⁺ or PD-1⁺CD8⁺CD3⁺), the average distance of K-nearest neighbor (KNN; K = 10) cells of the target phenotype (PD-L1⁺CD20⁻, PD-L1⁻CD68⁺, or PD-L1⁻CD3⁻). was computed and plotted. The mean KNN distance in the study cohort was 128 μm. Based on the findings from previous studies (30, 37) and considering our use of a greater K value compared with K = 1 in a previous study (30), the cutoff for distant/no interaction was determined as >100 μm.

PD-L1/L2 FISH

Two sets of FISH probes were used to study the PD-L1 and PD-L2 genes individually. The RP11-590H20– and CH17-432N17–specific bacterial artificial chromosomes, which map to the PD-L1 and PD-L2 genes (9p24.1 and cyto band, respectively), were purchased from the human BAC clone library at Children’s Hospital Oakland Research Institute, and labeled by Nick translation assay with TexasRed to generate locus-specific FISH probes (red). The RP11-145H11 clone (9q21) was labeled with fluorescein isothiocyanate fluorescence to generate a control probe to enumerate chromosome 9 (green). FISH analyses were performed according to the manufacturers’ instructions as previously described (38) on 5-mm tissue microarray sections mounted on positively charged slides (Superfrost, Thermo Fisher Scientific). Briefly, the FFPE slides were deparaffinized in xylene and then rehydrated gradually in a graded series of ethanol. The Histology FISH Accessory Kit (Dako, Agilent) was used following the manufacturer’s instructions. Briefly, the method consists of pretreatment in 2-(N-morpholino)ethanesulfonic acid, followed by protein digestion performed on pepsin solution. After dehydrieration, the samples were denatured in the presence of the specific probe at 66°C for 10 minutes and left overnight for hybridization at 45°C in a Dako hybridizer machine. Finally, the slides were washed with 20 × saline-sodium citrate buffer with Tween-20 detergent at 63°C and mounted on fluorescent mounting medium (DAPI). The FISH signals within nuclei in all the cells in tissue were manually enumerated by two investigators working independently. FISH images were also captured using charge-coupled device camera (SenSys camera; Photometrics) connected to a PC running the CytoVision image analysis system (Applied Imaging) with focus motor and Z-stack software.

Four patterns were defined: no alteration, with two green control signals and two red PD-L1/PD-L2 signals; polyploidy, with more than two green control signals and the same number of red PD-L1/PD-L2 signals; gain, with the number of PD-L1/PD-L2 signals between three to five copies greater than that of the two control probe signals; and amplification, with the number of PD-L1/PD-L2 signals at least six copies greater than that of the control probe signals.

Statistical analysis

Clinical features were compared by Fisher’s exact test. Expression between groups was compared by the unpaired Student t test (two-tailed). Survival of two groups of patients with different expression status using cutoffs determined by the X-Tile software (version 3.6.1, Yale School of Medicine, New Haven, CT) was compared by the Kaplan–Meier method and log-rank
test. Cox proportional hazards regression model was used for multivariate analyses. \( P \leq 0.05 \) was considered significant. Multiple testing corrections were performed using the Benjamini–Hochberg procedure. GEP data were analyzed to identify differentially expressed genes between two groups by multiple Student \( t \) tests via control of the false discovery rate as described previously (31–33).

**Results**

**Lack of T-cell and/or NK cell infiltration predicts poor survival**

To characterize the tumor immune microenvironment composition, numbers of DLBCL cells (CD20+/PAX5+), tumor-infiltrating T cells (CD3+), macrophages (CD3+CD68+), and natural killer (NK) cells (CD56+CD3-) were quantified. Cell densities (cell counts/mm²) in 405 patients with EBV-DLBCL were plotted in Fig. 1A. ABC-DLBCL compared with GCB-DLBCL had higher macrophage \((P = 0.0006)\) and CD8+ T-cell density \((P = 0.034; \text{Supplementary Fig. S1A})\). T cells were further subtyped by CD4, CD8, FOXP3 (Treg), and CD45RO (Tm) markers. The median CD4+ T-cell:CD8+ T-cell ratio was 1.53; the median Treg/CD4+ T-cell percentage was 25.5%; and the median Tm cell percentages were 63.1% and 70.7% for CD4+ T cells and CD8+ T cells, respectively.

Immune cell infiltration was evaluated by dividing the number of tumor-infiltrating immune cells by the total number of DLBCL cells, T cells, macrophages, and NK cells in each sample, resulting in a percentage that was slightly higher than the total tissue cellularity, which used the number of all nucleated cells (DAPI+) as the denominator. Very low T-cell infiltration (0%–2.6%) referred to as a CD3− “cold” tumor immune microenvironment, was associated with unfavorable clinical parameters including high international prognostic index (Supplementary Table S1) and decreased progression-free survival (PFS) and overall survival (OS) rates (Fig. 1A). Similarly, deficiency in NK cell, CD4+ T-cell, and CD8+ T-cell infiltration (cutoff: 3.0%, 1.6%, and 1.5%, respectively), but not macrophage deficiency or CD4+ T-cell: CD8+ T-cell, Treg:CD4+ T-cell, and Treg:CD8+ T-cell ratios, showed a significant adverse prognostic impact (Supplementary Table S2). Patients with high (>20%) CD4+ T-cell infiltration had better survival compared with those with low CD4+ T-cell infiltration; in contrast, high infiltration of CD8+ T cells and CD8+ Tm cells (cutoff ≥17.4% and ≥16.3%, respectively) was associated with poorer survival among CD8+ T-cell patients (Supplementary Fig. S1B).

**High PD-1, but not CTLA-4, expression on T cells is associated with adverse prognosis**

To assess immune-checkpoint expression in each patient with DLBCL, numbers of PD-1−, PD-L1−, PD-L2−, and CTLA-4− cells of different cell types were quantitated, and cell type-specific percentages of expression were calculated by dividing the number of positive cells by the total number of cells within each cell population. PD-1−, PD-L1−, PD-L2−, and CTLA-4− cell densities and percentages of different cell types in the study cohort are plotted in Fig. 1B. The ABC subtype, compared with the GCB
subtype, had significantly higher PD-L1 expression in CD20+ B cells and CD68+ macrophages (representative images in Fig. 1C) and PD-1 expression in CD20+ B cells (Fig. 1D).

A representative image of PD-1 expression in CD4+ T cells and CD8+ T cells is shown in Fig. 2A. A high PD-1+ percentage (PD-1hi; cutoff: ≥54.8%) in CD8+ T cells was associated with significantly poorer survival in overall DLBCL (prevalence: 29%; Fig. 2B) and the CD8+ T-cell subcohort (Table 1). PD-1+ expression in CD4+ T cells (cutoff: ≥3%; prevalence: 95%) was associated with elderly age, high international prognostic index (Supplementary Table S1), and significantly poorer survival (Table 1). However, higher PD-1+ percentage of CD4+ T cells did not show further adverse prognostic effect; paradoxically, PD-1+ expression in CD4+ T cells (cutoff: ≥75%) showed trends toward better OS (P = 0.067) and PFS (P = 0.056) but the case number was small (n = 25; Supplementary Fig. S1C).

As PD-1 signaling is known to affect T-cell proliferation and effector functions, we examined T-cell infiltration and GEP in DLBCL samples. Patients with PD-1hiCD8+ T cells compared with patients with PD-1loCD8+ T cells had increased CD8+ Tm cell infiltration (P = 0.0004), CD4+ Tm cell infiltration (P < 0.0001), and a gene signature including CXCL9, GZMK, PRF1, HLA-A, TRBC1 (also included in the CD3+ T-cell infiltration gene signature), CIQC, and CD5 upregulation (Fig. 2C; Supplementary Table S3), indicating a correlation between PD-1 expression and T-cell activation. In contrast, patients with PD-1 expression in conventional helper T cells (Th, CD3+CD4+FOXP3+) cells compared with those with PD-1- Th cells had decreased infiltration of naïve CD4+ T cells (P < 0.0001) and downregulation of BCL2L1 (encoding antiapoptotic Bcl-XL), CCL17, IL13RA1, and CD200 expression (Supplementary Table S3).

In approximately one third of the cohort, PD-1 was also expressed in CD20+ DLBCL cells (Fig. 2D), but at lower expression than in T cells (Fig. 1B; median expression, 1% vs. 34%). Although high PD-1+ percentage in DLBCL cells (cutoff: ≥65%) showed adverse prognostic effects (Supplementary Table S2), PD-1hiCD20+ patients were rare (n = 4) and had concurrent PD-1hi expression in CD8+ T cells and PD-1 expression in CD4+ T cells. Using the mean percentage (≥8%) as the PD-1+ CD20+ cutoff, PD-1 expression in DLBCL cells (prevalence: 25%) was not prognostic but showed a distinct gene signature that had overlap with the gene signature for PD-1 expression (≥8%) in Tregs (Fig. 2C), including upregulation of MAF, which may increase the susceptibility of T cells to apoptosis, and GMAPs, which are critical for the differentiation and survival of T cells and B cells. PDCD1 was not shown in any of the PD-1 expression gene signatures (Supplementary Table S3).

Unlike the PD-1 receptor, CTLA-4 expression was very low, infrequent (Fig. 1B), mainly in the cytoplasm, and also found in NK cells and macrophages. CTLA-4 expression in T cells was associated with significantly better OS in DLBCL (Fig. 2B; Table 1) and upregulation of CTLA4 and ICOS. Tregs had the highest CTLA-4+ percentage (mean: 6.3%; median: 2.3%) among T-cell subsets, and the expression in Tregs (representative image
PD-1/L1 expression in immune (but not DLBCL) cells confers poor outcome.

High PD-L1/PD-L2 expression in DLBCL cells is associated with favorable prognosis.

Table 1. Univariate and multivariate survival analyses for immune-checkpoint expression in DLBCL evaluated by percentage within a cell type

<table>
<thead>
<tr>
<th>PD-L1 expression in a T-cell subtype</th>
<th>Univariate analysis</th>
<th>Multivariate analysis for OS</th>
<th>Multivariate analysis for PFS</th>
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<tr>
<td>PD-L1 in CD8+ T cells</td>
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<tr>
<td>PD-L1 hi</td>
<td>Poorer OS: $P = 0.0052$; poorer PFS: $P = 0.024$</td>
<td>Better OS: $P = 0.0037$; better PFS: $P = 0.018$ in CD8+ patients</td>
<td>1.93 (1.25-2.98) 0.003 1.67 (1.11-2.52) 0.013</td>
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<tr>
<td>PD-L1 in CD4+ T cells</td>
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<td></td>
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<tr>
<td>PD-L1 hi</td>
<td>Poorer OS: $P = 0.0025$; poorer PFS: $P = 0.0079$</td>
<td>Better OS: $P = 0.0016$; better PFS: $P = 0.0033$ in CD4+ patients</td>
<td>2.98 (1.20-7.39) 0.018 2.97 (1.09-8.09) 0.033</td>
</tr>
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</table>

PD-L1 expression in a specific cell type

| B cells | | | |
| PD-L1 hi | Better OS: $P = 0.0034$ (CD3+ cells; $P = 0.067$ for better PFS) | Better OS: $P = 0.037$ (CD3+ patients) | 0.38 (0.15-0.93) 0.035 0.55 (0.27-1.12) 0.099 |
| PD-L1 in CD8+ T cells | | | |
| PD-L1 hi | Poorer OS: $P = 0.0052$; poorer PFS: $P = 0.024$ | Better OS: $P = 0.0037$; better PFS: $P = 0.018$ in CD8+ patients | 1.93 (1.25-2.98) 0.003 1.67 (1.11-2.52) 0.013 |
| PD-L1 in CD4+ T cells | | | |
| PD-L1 hi | Better OS: $P = 0.0034$ (CD3+ cells; $P = 0.067$ for better PFS) | Better OS: $P = 0.037$ (CD3+ patients) | 0.38 (0.15-0.93) 0.035 0.55 (0.27-1.12) 0.099 |

PD-L2 expression in a specific cell type

| B cells | | | |
| PD-L2 hi | Better OS: $P = 0.0018$; better PFS: $P = 0.0018$ | Better OS: $P = 0.0039$; better PFS: $P = 0.0046$ in CD3+ patients | | | | |
| CTLA-4 expression in a T-cell subtype

| Tregs | Better OS: $P = 0.018$; better PFS: $P = 0.008$ | Better OS: $P = 0.023$; better PFS: $P = 0.018$ in CD4+ patients | 0.45 (0.25-0.82) 0.009 0.49 (0.27-0.88) 0.018 |
| Th cells | Better OS: $P = 0.023$; better PFS: $P = 0.039$ in CD4+ patients | Better OS: $P = 0.02$; better PFS: $P = 0.036$ in CD4+ patients | 0.58 (0.36-0.95) 0.031 0.58 (0.35-0.94) 0.027 |

NOTE: For multivariate survival analyses, Cox models were used and the factors included high international Prognostic Index score, sex, B-symptoms, >5-cm tumor size, and individual immune-checkpoint expression. For PD-L1/PD-L2 expression in T cells, analyses were performed in patients with T-cell infiltration. For expression in other cell types, analyses were performed in the overall DLBCL cohort.

Significant $P$ values are in bold.

Abbreviations: HR, hazard ratio; CI, confidence interval; NK, natural killer, CD56+/CD3-; Th, helper T cells, CD3+/FOXP3+; Tregs, regulatory T cells, CD3+CD4+FOXP3+.
percentage, respectively (Fig. 1B and C). The case distribution of PD-L1 expression with respect to PD-1hiCD8+ T-cell expression is shown in Fig. 3A.

Tumor-cell PD-L1 expression (PD-L1+; cutoff: ≥3.0%; prevalence: 84%) was associated with poorer OS in patients with PD-1hiCD8+ T cells (P = 0.034; Fig. 3B) but not in overall DLBCL. However, this adverse impact lost significance in multivariate analysis (Supplementary Table S2). PD-L1+ percentage of CD20+ cells did not show a dose-dependent unfavorable prognostic effect among PD-1hiCD8+ T-cell patients, and patients with intermediate PD-L1 expression (15%–32%) had a good prognosis (Supplementary Fig. S1D).

High PD-L1+ percentage of CD20+ cells (PD-L1hi; cutoff: ≥40%; prevalence: 8.3%) was associated with better survival in patients with PD-1loCD8+ T cells (P = 0.032 for OS, Fig. 3B; P = 0.04 for PFS) and in overall DLBCL (P = 0.034 for OS; marginal P = 0.067 for PFS; Table 1) without association with any favorable clinical parameter. With a lower cutoff, >30% (20, 21), PD-L1hi expression (prevalence: 12%) was associated with favorable PFS (P = 0.037) and OS (P = 0.06) only in patients with PD-1hiCD8+ T cells but not in overall DLBCL (Supplementary Fig. S1E; Supplementary Table S2). We also evaluated PD-L1+ percentage in double-positive CD20+ PAX5+ B cells and found that B-cell PD-L1hi expression (cutoff: ≥36%) remained to have favorable prognostic effect in overall DLBCL (P = 0.05) and in patients with PD-1hiCD8+ T cells (P = 0.06). GEP analysis showed that PD-L1hi expression in DLBCL cells was associated with upregulation of CD274, JAK2, STAT1, APOBEC3A (cytidine deaminase), KLRC4 (NK lectin-like receptor C4), HAVCR2 (TIM3), and DRAM and downregulation of IGL@ (Supplementary Table S3).

The mean PD-L1+CD20+ expression in PD-1hiCD8+ T-cell patients was similar to that in PD-1loCD8+ T-cell patients. Regardless of the PD-1hi/PD-1lo status, PD-L1+CD20+ expression was associated with increased CD8+CD4+ Tm cell infiltration and higher PD-1+CD8+ T-cell densities, and PD-L1hiCD20+ expression was associated with increased CD8+ Tm and CD68+ cell infiltration. However, only in patients with PD-1hiCD8+ T cells,
PD-L1hi CD20+ expression was associated with higher PD-1hi CD8+ T-cell densities (P = 0.002). In contrast, only in patients with PD-L1lo CD8+ T cells, PD-L1hi patients compared with PD-L1lo DLBCL patients had a lower mean PD-1 percentage in CD8+ T cells (P = 0.0023; Supplementary Table S4).

To determine whether the presence of the PD-L1–PD-1 interaction underlies the opposing prognostic effects of PD-L1 expression in the PD-1hiCD8+ and PD-1loCD8+ T-cell groups, we quantified the KNN distance from PD-L1+CD20+ B cells to PD-1+CD4+ T cells or to PD-1+CD8+ T cells. We found that almost all patients with PD-1hi CD8+ T cells, who had poorer survival compared with patients with PD-1lo CD8+ T cells, had PD-L1–PD-1 proximity (representative image in Fig. 1C). In contrast, patients with minimal PD-1 expression in CD8+ T cells (PD-1+ percentage >0% but <11%; n = 50) had favorable prognosis and significantly lower prevalence of proximity between PD-L1+CD20+ B cells and PD-1+CD8+ T cells compared with other DLBCL patients (28% vs. 79%), even though these patients had higher mean PD-L1+ percentage in DLBCL cells (P = 0.0002). However, patients without any PD-1+CD8+ T-cell expression (PD-1+ percentage: 0%) and PD-L1–PD-1 interaction (n = 14) had low PD-L1 expression and poor prognosis (Supplementary Fig. S1F and S1G). After exclusion of these cases, the rest of PD-1hiCD8+ cases (referred to as PD-1low CD8+ cases, n = 221) also had a high prevalence (72%) of PD-L1+CD20+ B-cell/PD-1+CD8+ T-cell proximity (representative image in Fig. 3C). Contrary to expectations, PD-1low CD8+ cases with proximal PD-L1 to PD-1+CD8+ T cells (but not PD-1+CD8+ T cells) compared with those with distant PD-L1 had similar survival in univariate analysis (OS, Fig. 3B; PFS, P < 0.0001) and multivariate analysis adjusting for clinical parameters (Table 1). Supplementary Figure S2A shows two representative KNN distance plots for each cell in PD-1low CD8+ cases with or without PD-L1–PD-1 proximity.

Regardless of the PD-1hi/PD-1lo status, PD-L1+CD20+ B-cell/PD-1+CD8+ T-cell proximity was associated with higher CD8+/CD4+ T-cell infiltration. Only in patients with PD-1low CD8+ T cells, was PD-L1–PD-1 proximity associated with a significantly higher mean PD-1+ percentage in CD8+ T cells (P < 0.0001) and CD4+ T cells (P = 0.0022) and a higher PD-1+ percentage in DLBCL cells (P = 0.033; Supplementary Table S4), whereas cases without PD-L1–PD-1 proximity had a significantly higher frequency of MYC/BCL2 double expression (31.7%, P = 0.007). Further multivariate analysis in patients with PD-1low CD8+ T cells found that PD-L1+CD20+ B-cell/PD-1+CD8+ T-cell proximity was a significant favorable prognostic factor independent of MYC/BCL2 double or single expression (P = 0.005 to 0.02). PD-L1–PD-1 proximity in PD-1low CD8+ cases had a distinct gene signature (Supplementary Table S3), which essentially is part of the CD3+ T-cell infiltration gene signature (Supplementary Fig. S2B), including upregulation of genes involved in T-cell receptor signaling (CD3E, CD3D, and TRBC1) and cytolytic function (GZMK, GZMA, and PRF1).

Another PD-1 ligand, PD-L2, was expressed infrequently. PD-L2+ expression in DLBCL cells (major source, Fig. 1B) and T cells was associated with significantly better survival by univariate but not multivariate analysis (Fig. 3D; Table 1). In line with this, PD-L2+ expression (regardless of source) was associated with low PD-1 expression in CD4+ T cells and PD-L1hi expression in DLBCL cells (Supplementary Table S4). Despite the low case numbers, PD-L2+ expression in DLBCL cells showed a prominent GEP signature, including upregulation of PD-1, CD28, CC1L17, IL13RA1 (IL-13 is a typical Th2 cytokine), and SLAMF1 (which promotes Th2 but inhibits Th1 cytokines), and downregulation of CD1C, PRKC8, and FOXP1 (Supplementary Table S3).

PD-L1/PD-1 genetic alterations were analyzed by FISH using either a PD-L1 or PD-L2 probe (Fig. 4A). PD-L1 and PD-L2 gene amplification was observed in 3.0% and 3.2% of the cohort, respectively, and associated with higher expression of PD-L1/PD-L2 protein (Fig. 4A) and PD-L1 mRNA. In addition, 7.0% to 7.8% of cases had polyplody or PD-L1/PD-L2 gain. None of these PD-L1/PD-L2 copy-number alterations showed prognostic effects.

PD-L1 expression in tumor-infiltrating immune cells has adverse prognostic effects

Microenvironment PD-L1 expression in macrophages (prevalence: 73%), T cells (lowest mean PD-L1hi percentage), and NK cells (lowest mean PD-L1 cell density; Fig. 1B) was associated with significantly poorer survival in DLBCL patients (Fig. 4B; Table 1), although PD-L1 expression in T cells and NK cells was associated with increased CD8+ Tm cell and macrophage infiltration. PD-L1 expression in T cells was also associated with increased CD4+ Tm and naive CD8+ T-cell infiltration, and PD-L1 expression in CD4+ T cells was associated with a decreased mean Treg:CD4+ T-cell ratio (all P < 0.0001; ref. 24). In contrast, PD-L1 expression in macrophages and NK cells was associated with decreased infiltration of naïve CD4+ T cells (P = 0.0006 and P < 0.0001, respectively; Supplementary Table S4).

Multivariate analyses adjusting for clinical parameters indicated that the adverse prognostic impact of PD-L1 expression in macrophages and NK cells was significant only in patients with PD-1hiCD8+ T cells (Fig. 4B). In patients with PD-1low CD8+ T cells, spatial analysis showed that close distance from PD-L1+ macrophages or PD-L1+ CD8+ Tm cells to PD-1+ CD8+ T cells was associated with significantly better survival (for OS, P < 0.0001; Supplementary Fig. S2C; for PFS, P = 0.0043 and P = 0.0049, respectively).

Among the PD-L1+ GEP signatures, the signature for PD-L1 expression in T cells was most prominent, which overlaps with the signatures for CD3+ T-cell infiltration and PD-1hi CD8+ expression (Fig. 4C). Shared immunosuppressive signatures by PD-L1 expression in T cells, macrophages, and NK cells included upregulation of IDO1, SOD2, CHISL1, and LILRBs (Fig. 4C). The gene signature of PD-L1 expression in macrophages also included IL10, IL18BP, and genes involved in metabolism and signaling (such as EGFR and RAS, Supplementary Table S3).

Increased PD-1+ CD8+ T cells and PD-L1+ T cells and macrophages are associated with poor survival

Compared with cell percentages, cell density analysis found fewer significant prognostic factors to stratify patients (Fig. 5A; Supplementary Fig. S2D). High densities of PD-1+ CD8+ T cells, PD-L1+ T cells, and PD-L1+ macrophages showed adverse prognostic effects (Fig. 5B), whereas high densities of PD-L2+ CD20+ cells, PD-L2+ T cells, and PD-L1+ CD20+ cells showed favorable effects (Table 2; Supplementary Table S2).

Compared with PD-1hi/PD-1l- cell densities, high PD-1+/PD-L1+ cell densities showed more prominent gene signatures but with larger overlaps (Fig. 4C), in line with the significant associations between high densities of PD-1+ CD8+ T cells, PD-L1+ T cells, and PD-L1+ CD68+ cells (all P < 0.0001), but not between PD-1+ percentage in CD8+ T cells and PD-L1+ percentage in T cells/CD68+ cells (Supplementary Table S4).
PD-1+/PD-L1+ T-cell and PD-L1+ macrophage cellularity is associated with inferior survival

Tissue cellularity analysis showed additional prognostic factors including the favorable high PD-L1 CD56+, PD-L2 CD68+, and PD-L1+CD20+ tissue cellularity (Table 2; Supplementary Table S2). However, the adverse effects of high PD-1+/PD-L1+ T-cell cellularity on OS were only signiﬁcant after exclusion of patients with CD3+ cold tumors. In contrast, the favorable effects of high tissue cellularity of PD-L1+CD20+ cells, PD-1+CD20+ cells, PD-L2+CD3+ T-cell cells, and PD-L2+CD68+ cells on OS were signiﬁcant in overall DLBCL, but not after exclusion of CD3+ patients (Table 2).

There were signiﬁcant positive associations between different PD-1+ and PD-L1+ immune cellularity (Supplementary Fig. S2E) and between PD-1+CD4+ and CTLA-4+ T-cell cellularity, whereas negative associations were shown between PD-1+ T-cell cellularity and PD-L1+/PD-L2+ B-cell cellularity (Supplementary Table S4). Patients with high PD-1+/PD-L1+ T-cell or PD-L1+ macrophage tissue cellularity showed distinct gene signatures but with large overlaps (Fig. 5C). The case distribution of expression by high tissue cellularity versus by percentage in a cell type is shown in Fig. 5A.

For the multiple comparisons performed in the DLBCL cohort, prognostic signiﬁcance was adjusted by the Benjamini–Hochberg procedure (Supplementary Table S5), and further interrogated by randomly assigning 18 consortium centers into two independent cohorts. As shown in Supplementary Fig. S3, in both training and validation cohorts, CD3+/CD8+ T cells, PD-1 expression in CD4+ T cells, PD-L1 expression in Th cells, and PD-L1 expression in macrophages in patients with PD-1hiCD8+ T cells had signiﬁcant adverse prognostic effects, whereas PD-L2+ expression in CD20+ B cells predicted superior survival, as well as proximity/interaction between PD-L1+CD20+ B cells and PD-1+CD8+ T cells in patients with PD-1low+CD8+ T cells. The adverse effect of PD-L1 expression in NK cells, the favorable effect of CTLA-4+ expression in T cells, and the context-dependent prognostic effect of PD-L1hi and PD-L1+ expression in CD20+ B cells did not reach statistical signiﬁcance in one cohort but did in the other. Compared with evaluation of expression by percentage within a cell type,
assessment of cell abundance by cell density or cellularity showed less consistent significance in two independent cohorts. However, in both training and validation cohorts, low T-cell/NK cell cellularity and high PD-1/PD-L1 expression in CD8⁺/CD4⁺ T cells by either cell density or cellularity showed significantly unfavorable prognostic effects, whereas high PD-L2⁺CD20⁺ B-cell cellularity showed significantly favorable prognostic effect.

**Discussion**

Dysfunction of antitumor responses by immune-checkpoint expression in the tumor microenvironment (rather than expression in T cells infiltrating into normal tissues or peripheral blood; refs. 39–41) needs to be better characterized by modern immunopathology. Using a 13-marker/14-color MultiOmyx immunofluorescent platform, this study comprehensively analyzed the immunelandscape of DLBCL biopsies and deciphered the clinical role of PD-1/CTLA-4 immune checkpoints in a large number of DLBCL patients. To minimize effects of tissue heterogeneity, small sampling, variable tissue fixation, and limitation of digital imaging field, we selected immune-infiltrated tumor regions on the tissue microarray and used the percentage of positive cells in a specific cell type as a method to evaluate intratumoral checkpoint expression. This method showed higher sensitivity to indicate the unfavorable role of checkpoint expression and enhanced ability to identify immunologic determinants than the traditional methods evaluating immune cell abundance, because numbers of different types of immune cells, but not percentage of specific cells with immune-checkpoint expression, often increased simultaneously in DLBCL samples. Cell abundance assessment by cell density showed better specificity in prognostication, but the cellularity method is more able to identify immune desert/cold cases. For cold CD3⁺/CD56⁺/CD57 cells and high CD8⁺CD45RO⁺ T-cell infiltration, our method using a different denominator to evaluate the tumor immune microenvironment composition showed better prognostic results compared with tissue cellularity. The results in this study provide valuable insights for future application of multiplex IHC and digital quantitative analysis.

In two independent cohorts, we demonstrate that high PD-1 expression in CD8⁺ T cells and PD-L1 expression in T cells and CD68⁺ cells had significant adverse prognostic effects in patients with de novo DLBCL. In contrast, high PD-L2 expression in DLBCL cells was favorable and associated with lower PD-1 expression in CD4⁺/CD8⁺ T cells and CD80 upregulation. Controversially in earlier studies, PD-L2 costimulated T-cell proliferation and cytokine production through unknown receptors (42, 43). Proximity (interaction) between PD-L1⁺CD20⁺ B cells and PD-1⁺CD8⁺ T cells was associated with significantly improved survival in patients with low PD-1⁺ percentage in CD8⁺ T cells, suggesting that in this scenario, PD-1–PD-L1 interaction indicates T-cell activation and nonexclusion rather than exhaustion (9), or that the suppressive effect of PD-L1–
Table 2. Univariate and multivariate survival analyses for immune-checkpoint expression in DLBCL evaluated by cell density and tissue cellularity

<table>
<thead>
<tr>
<th>Cell density of expression-positive cells</th>
<th>Univariate analysis</th>
<th>Multivariate analysis for OS</th>
<th>Multivariate analysis for PFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>High PD-1 CD8+ T-cell density</td>
<td>Poorer PFS: P = 0.014; poorer OS: P = 0.0096</td>
<td>1.80 (1.14-2.85) 0.011</td>
<td>2.00 (1.31-3.05) 0.001</td>
</tr>
<tr>
<td>High PD-L1 T-cell density</td>
<td>Poorer OS: P = 0.0075; poorer PFS: P = 0.02</td>
<td>1.93 (1.23-3.03) 0.004</td>
<td>1.84 (1.20-2.83) 0.006</td>
</tr>
<tr>
<td>High PD-L1 Th cell density</td>
<td>Poorer OS: P = 0.0018</td>
<td>1.68 (1.10-2.58) 0.017</td>
<td>1.59 (1.06-2.38) 0.025</td>
</tr>
<tr>
<td>High PD-L1 CD68+ cell density</td>
<td>Poorer OS: P = 0.0092; poorer PFS: P = 0.023</td>
<td>1.50 (0.86-2.63) 0.15</td>
<td>1.43 (0.84-2.46) 0.19</td>
</tr>
<tr>
<td>High PD-L1 CD20+ B-cell density</td>
<td>Better PFS: P = 0.047</td>
<td>0.50 (0.27-0.95) 0.034</td>
<td>0.51 (0.28-0.92) 0.204</td>
</tr>
<tr>
<td>High PD-L2 CD20+ B-cell density</td>
<td>Better OS: P = 0.01; better PFS: P = 0.0048</td>
<td>1.16 (1.08-2.56) 0.02</td>
<td>1.75 (1.16-2.58) 0.007</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tissue cellularity of expression-positive cells</th>
<th>Univariate analysis</th>
<th>Multivariate analysis for OS</th>
<th>Multivariate analysis for PFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>High tissue cellularity of PD-1 CD8+ T cells</td>
<td>Poorer PFS: P = 0.014; poorer PFS: P = 0.0008</td>
<td>1.66 (1.08-2.56) 0.02</td>
<td>1.75 (1.16-2.58) 0.007</td>
</tr>
<tr>
<td>High tissue cellularity of PD-1 CD4+ T cells</td>
<td>Poorer PFS: P = 0.01</td>
<td>1.56 (0.98-2.48) 0.059</td>
<td>1.55 (1.01-2.38) 0.046</td>
</tr>
<tr>
<td>High tissue cellularity of PD-L1 T cells</td>
<td>Marginal P for poorer OS: P = 0.05</td>
<td>1.71 (1.12-2.61) 0.012</td>
<td>1.65 (1.10-2.46) 0.014</td>
</tr>
<tr>
<td>High tissue cellularity of PD-L1 CD68+ cells</td>
<td>Poorer OS: P = 0.012; poorer PFS: P = 0.0035</td>
<td>0.58 (0.32-1.03) 0.061</td>
<td>0.71 (0.43-1.19) 0.19</td>
</tr>
<tr>
<td>Positive tissue cellularity of PD-1 CD20+ B cells</td>
<td>Not significant (P = 0.082 for OS and P = 0.17 for PFS in CD3+ patients)</td>
<td>0.14 (0.09-0.99) 0.049</td>
<td>0.25 (0.061-1.01) 0.051</td>
</tr>
<tr>
<td>High tissue cellularity of PD-L1 CD56+ cells</td>
<td>Poorer PFS: P = 0.02</td>
<td>0.39 (0.18-0.84) 0.017</td>
<td>0.52 (0.27-0.99) 0.048</td>
</tr>
<tr>
<td>High tissue cellularity of PD-L2 CD20+ B cells</td>
<td>Not significant (P = 0.071 for better OS and P = 0.12 for PFS in CD3+ patients)</td>
<td>0.12 (0.017-0.90) 0.039</td>
<td>0.10 (0.014-0.75) 0.025</td>
</tr>
<tr>
<td>High tissue cellularity of CTLA-4 CD3+ cells</td>
<td>Poorer PFS: P = 0.017; better PFS: P = 0.007</td>
<td>0.53 (0.29-0.98) 0.044</td>
<td>0.79 (0.48-1.30) 0.35</td>
</tr>
</tbody>
</table>

NOTE: For multivariate survival analyses, Cox models were used and the factors included high International Prognostic Index score, sex, B-symptoms, >5-cm tumor size, and individual immune-checkpoint expression. For PD-1/PD-L1 expression in T cells, analyses were performed in patients with T-cell infiltration. For expression in other cell types (except for PD-L1 CD68+ cellularity as indicated), analyses were performed in the overall DLBCL cohort. Significant P values are in bold. Abbreviations: HR, hazard ratio; CI, confidence interval; Th, helper T cells, CD3+ CD4+ FOXP3+ cells.
PD-1 interaction is exerted only in a minority of T cells, therefore not significantly affecting overall T-cell function. Our results support two studies’ findings in murine tumor models, which showed that PD-L1 expression by host antigen-presenting cells, but not by tumor cells, is indispensable for PD-L1 blockade–mediated tumor regression (44, 45).

These results are opposite to those by Kiyasu and colleagues (20), that tumor-cell PD-L1hi (≥30%) expression, but not microenvironmental PD-L1/PD-1 expression, was associated with significantly poorer OS in 273 DLBCL patients. The different results could be attributable to the inclusion of various DLBCL entities (such as EBV+ DLBCL of the elderly with a higher PD-L1hi frequency than de novo DLBCL not otherwise specified) and limitations in the PD-L1/PAX5 double-immunostaining method and prognostic analysis in the study by Kiyasu and colleagues. Cell density of PD-1+ tumor-infiltrating lymphocytes was used to analyze OS (but not PFS) in overall DLBCL without exclusion of CD3+ cases. For microenvironmental PD-L1 analysis, patients with tumor-cell (and microenvironmental) PD-L1hi expression were excluded.

Our GEP analysis showed that GZMs, PRF1, CD5, and CD44, but not IFNG or PDCD1, were upregulated in PD-1hiCD8+ T-cell patients, which may indicate a “plastic” dysfunctional state of CD8+ T cells, possibly reversible by PD-1/L1 blockade (9, 46, 47). However, T-cell deficiency was found in 64% of DLBCL patients with poor prognoses, and LILRs, CH3L1, IDO1, SOD2, IL10, FCGR3B, and CD163 upregulation were associated with prevalent PD-L1 expression, which could explain the low response rate to anti-PD-1 monotherapy (close to the frequency of PD-L1hiCD20+ B-cell expression and that of 9p24.1 genetic alterations in this study cohort) in relapsed/refractory DLBCL after a median of three prior lines of therapies in a phase II clinical trial (19). Also, PD-1 expression was found in B cells, which may contribute to resistance to PD-1 blockade therapy. One previous study showed elevated PD-1 expression in circulating NK cells in DLBCL patients (48), although in our study PD-1 expression was not found in intratumoral NK cells or macrophages in any patient. Whether PD-1 expression in non-T cells can contribute to therapeutic resistance of PD-1 blockade needs to be clarified by future studies.

Another finding of this study is the association of CTLA-4 expression with favorable prognosis, increased Treg cellularity and Treg:CD4+ T-cell ratio, ICOS, CD40LG, and CD28 upregulation, and decreased PD-1 expression in CD4+ T cells. Although germline Ctila1 knockout resulted in the rapid development of lethal lymphoproliferative disorder, Ctila1 deletion in adult mice conferred protection from autoimmune disease and did not enhance antitumor immunity (49, 50), which were attributable to compensatory upregulation of IL-10, LAG-3, and PD-1 expression upon Ctila1 deletion and to a lack of tumor-infiltrating CD8+ T-cell expansion (49).

In conclusion, high intratumoral expression of PD-1 on CD8+ T cells and PD-L1 on macrophages and T cells, but not expression of PD-L1/PD-L2 on DLBCL cells or CTLA-4 on T cells, are resistance mechanisms for standard immunochemotherapy in DLBCL and hence are therapeutic targets for PD-1/PD-L1 blockade in patients with refractory/refractory DLBCL. Combination therapies are needed for DLBCL patients lacking activated tumor-infiltrating T/NK cells or having additional immunosuppressive mechanisms in the tumor microenvironment. Further studies are necessary to establish prognostic and predictive biomarkers based on immune infiltration and PD-1/PD-L1/PD-L2/CTLA-4 expression in DLBCL samples.

Disclosure of Potential Conflicts of Interest
E.D. Hsi reports receiving commercial research funding from Eli Lilly and AbbVie and is a consultant/advisory board member for Celgene, Seattle Genetics, and Jazz Pharmaceuticals. J.H. van Krieken reports receiving a commercial research grant from Amgen. G.J. Freeman reports receiving commercial research funding from Bristol-Myers Squibb, Roche/Genentech, Novartis, and Ipsi; has ownership interest in Novartis, Roche/Genentech, Bristol-Myers Squibb, m pplimmune/AstraZeneca, Merck, EMD Serono, Boehringer Ingelheim, and Dako; and is a consultant/advisory board member for Novartis, Lilly, Roche/Genentech, Bristol-Myers Squibb, Bethyl Laboratories, Xios Therapeutics, Quiet Therapeutics, and Seattle Genetics. No potential conflicts of interest were disclosed by the other authors.

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Study supervision: B. Xu, K.H. Young
Other (organized all collaboration medical centers and support for IRB and MTA approval): K.H. Young
Other (provision of study thought, materials, key reagents, and technology): H. You

Acknowledgments
This work is supported by the Sister Institution Network Fund at The University of Texas MD Anderson Cancer Center.

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Published OnlineFirst February 11, 2019; DOI: 10.1158/2326-6066.CIR-18-0439

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doi:10.1158/2326-6066.CIR-18-0439

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