Immune Cell PD-L1 Colocalizes with Macrophages and Is Associated with Outcome in PD-1 Pathway Blockade Therapy

Yuting Liu1, Jon Zugazagoitia1, Fahad Shabbir Ahmed1, Brian S. Henick2, Scott N. Gettinger3, Roy S. Herbst3, Kurt A. Schalper1,3, and David L. Rimm1,3

ABSTRACT

Purpose: Programmed death ligand 1 (PD-L1) is expressed in tumor cells and immune cells, and both have been associated with response to anti-PD-1 axis immunotherapy. Here, we examine the expression of PD-L1 to determine which cell type carries the predictive value of the test.

Experimental Design: We measured the expression of PD-L1 in multiple immune cells with two platforms and confocal microscopy on three retrospective Yale NSCLC cohorts (425 nonimmunotherapy-treated cases and 62 pembrolizumab/nivolumab/atezolizumab-treated cases). The PD-L1 level was selectively measured in different immune cell subsets using two multiplexed quantitative immunofluorescence panels, including CD56 for natural killer cells, CD68 for macrophages, and CD8 for cytotoxic T cells.

Results: PD-L1 was significantly higher in macrophages in both tumor and stromal compartment compared with other immune cells. Elevated PD-L1 in macrophages was correlated with high PD-L1 level in tumor as well as CD8 and CD68 level (P < 0.0001). High PD-L1 expression in macrophages was correlated with better overall survival (OS; P = 0.036 by cell count/P = 0.019 by molecular colocalization), while high PD-L1 expression in tumor cells was not.

Conclusions: In nearly 500 non–small cell lung cancer (NSCLC) cases, the predominant immune cell type that expresses PD-L1 is CD68+ macrophages. The level of PD-L1 in macrophages is significantly associated with the level of PD-L1 in tumor cells and infiltration by CD8+ T cells, suggesting a connection between high PD-L1 and “hot” tumors. In anti-PD-1 axis–therapy–treated patients, high levels of PD-L1 expression in macrophages are associated with longer OS and may be responsible for the predictive effect of the marker.

Introduction

Immune checkpoints are regulators of the immune system and they are crucial for maintaining the immune homeostasis by controlling the amplitude of immune responses (1). In cancer, immune checkpoints are often expressed by tumor cells to suppress the antitumor immune responses against them as an adaptive escape mechanism (2, 3). Among the discovered immune checkpoints, the interaction between programmed death 1 receptor (PD-1) on tumor infiltrating lymphocytes (TIL) and its ligand programmed death ligand 1 (PD-L1) on tumor cells has been identified as a critical immunosuppressive mechanism in cancer (4–6).

Several clinical studies have shown that antibodies targeting PD-1/ PD-L1 pathway achieved durable clinical responses with unprecedented survival rate (7, 8). However, only a fraction of patients benefit from this therapy and some of the therapies are approved with a companion diagnostic test to identify patients who are more likely to respond. The only currently FDA-approved companion diagnostic is the PD-L1 IHC test (9). A number of IHC tests are approved with varied antibody clones, staining platforms, protocols and scoring system, and the localization of PD-L1 detection. The first approved test (22c3) scored only PD-L1 expression on tumor cells while other assays, for example, the SP142 assay prescribed scoring of both tumor cells and immune cells. More recently, in tumors other than non–small cell lung cancer (NSCLC), the 22c3 test has evolved to a combined proportion score that includes both tumor cells and immune cells. The evolution toward inclusion of PD-L1 detection on immune cells in diagnostics reveals its importance to anti-PD-1 axis therapeutic efficacy as compared with PD-L1 expression by tumor cells. This evolution of diagnostic scoring coincides with recent work in mouse models that has shown the efficacy of anti-PD-1 axis blockade relies on the host expression of PD-L1 in the tumor microenvironment and draining lymph nodes rather than in tumor cells (10–12).

In this study, we follow the mouse mechanistic studies to attempt to determine the cell type in which the PD-L1 expression is associated with the drug mechanism of action. We hypothesize that the immune cells scored in the tests are predominantly macrophages and that the expression of PD-L1 in these cells is the critical target for immune therapy and therefore the best target for future assessment.

Materials and Methods

Tissue microarray and patient cohorts

Discovery cohort

We used two retrospective collections of NSCLC from Yale University, cohort A (YTMA79; n = 209) and cohort B (YTMA250; n = 291), that consist of 500 patients from 1988 to 2003 (YTMA79) and 2004 to 2011 (YTMA250) as described previously (13, 14). Detailed clinicopathologic characterization is summarized in Supplementary Table S1. The cases that were finally included in the study were fewer...
Translational Relevance

Many studies have shown that “immune cells,” without specific characterization, are a critical component of the companion diagnostic tests used for programmed death 1 receptor (PD-1) axis immunotherapy. The exact type of immune cells that express programmed death ligand 1 (PD-L1) is unclear. By quantitatively measuring PD-L1 in CD68+ macrophages, CD56+ natural killer cells, and CD8+ cytotoxic T cells we show that the vast majority of PD-L1 expression colocalizes with CD68, inferring that the key immune cell is a macrophage. It is more challenging to prove that the macrophage carries the predictive power of the test. However, in a small pilot scale, retrospective cohort, we show significant association with overall survival attributable to PD-L1 in the macrophage compartment, and not in the tumor cell compartment. This work raises the possibility that the companion diagnostic test for PD-1 axis therapies may be improved by assessing PD-L1 expression only when it is colocalized with CD68.

### Table 1. Immunotherapy Treated Cohort Characteristics.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>81</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>43 (53.1)</td>
</tr>
<tr>
<td>Female</td>
<td>38 (46.9)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>&lt;70 years old</td>
<td>42 (51.9)</td>
</tr>
<tr>
<td>≥70 years old</td>
<td>39 (48.1)</td>
</tr>
<tr>
<td>Smoking history</td>
<td></td>
</tr>
<tr>
<td>Never smoker</td>
<td>14 (17.3)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>18 (22.2)</td>
</tr>
<tr>
<td>Former smoker</td>
<td>48 (59.3)</td>
</tr>
<tr>
<td>Missing</td>
<td>1</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>61 (75.3)</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>15 (18.5)</td>
</tr>
<tr>
<td>Large cell carcinoma</td>
<td>4 (4.9)</td>
</tr>
<tr>
<td>Adenosquamous carcinoma</td>
<td>1 (1.2)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>2 (2.5)</td>
</tr>
<tr>
<td>IV (M1a)</td>
<td>21 (25.9)</td>
</tr>
<tr>
<td>IV (M1b)</td>
<td>11 (13.6)</td>
</tr>
<tr>
<td>IV (M1c)</td>
<td>47 (58)</td>
</tr>
<tr>
<td>Actionable drivers</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>37 (45.7)</td>
</tr>
<tr>
<td>EGFR mutation</td>
<td>11 (13.6)</td>
</tr>
<tr>
<td>KRAS mutation</td>
<td>21 (25.9)</td>
</tr>
<tr>
<td>MET amplification</td>
<td>1 (1.2)</td>
</tr>
<tr>
<td>RET fusion</td>
<td>1 (1.2)</td>
</tr>
<tr>
<td>HER2 mutation</td>
<td>1 (1.2)</td>
</tr>
<tr>
<td>MEK1 mutation</td>
<td>1 (1.2)</td>
</tr>
<tr>
<td>PIK3CA mutation</td>
<td>1 (1.2)</td>
</tr>
<tr>
<td>No</td>
<td>44 (54.3)</td>
</tr>
<tr>
<td>Genotype-tailored therapies</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>13 (16)</td>
</tr>
<tr>
<td>No</td>
<td>68 (84)</td>
</tr>
<tr>
<td>Site</td>
<td></td>
</tr>
<tr>
<td>Lung primary</td>
<td>41 (50.6)</td>
</tr>
<tr>
<td>Locoregional lymph node</td>
<td>14 (17.3)</td>
</tr>
<tr>
<td>Metastatic (M1) lymph node</td>
<td>4 (4.9)</td>
</tr>
<tr>
<td>Brain metastasis</td>
<td>10 (12.3)</td>
</tr>
<tr>
<td>Adrenal metastasis</td>
<td>2 (2.5)</td>
</tr>
<tr>
<td>Pleural metastasis</td>
<td>1 (1.2)</td>
</tr>
<tr>
<td>Renal metastasis</td>
<td>1 (1.2)</td>
</tr>
<tr>
<td>Skeletal soft tissue metastasis</td>
<td>4 (4.9)</td>
</tr>
<tr>
<td>Skin metastasis</td>
<td>1 (1.2)</td>
</tr>
<tr>
<td>Pericardium</td>
<td>1 (1.2)</td>
</tr>
<tr>
<td>Thoracic spine</td>
<td>1 (1.2)</td>
</tr>
<tr>
<td>Missing</td>
<td>1</td>
</tr>
<tr>
<td>N lines of therapy</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>16 (19.8)</td>
</tr>
<tr>
<td>1</td>
<td>42 (51.9)</td>
</tr>
<tr>
<td>2</td>
<td>19 (23.5)</td>
</tr>
<tr>
<td>3</td>
<td>2 (2.5)</td>
</tr>
<tr>
<td>4</td>
<td>1 (1.2)</td>
</tr>
<tr>
<td>Missing</td>
<td>1</td>
</tr>
<tr>
<td>Radiotherapy prior to immunotherapy</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>28 (34.6)</td>
</tr>
<tr>
<td>No</td>
<td>53 (65.4)</td>
</tr>
<tr>
<td>Moment of collection</td>
<td></td>
</tr>
<tr>
<td>Preimmunotherapy</td>
<td>73 (90.1)</td>
</tr>
<tr>
<td>Postimmunotherapy</td>
<td>8 (9.9)</td>
</tr>
</tbody>
</table>

Immunotherapy treated cohort

We used tumor samples from patients with NSCLC, cohort C (YTMA404; n = 81) treated with anti-PD-1 axis therapy at Yale-New Haven Hospital between 2011 and 2018. As summarized in Table 1, 62 of 81 samples were collected before immunotherapy and treated with single-agent therapy (pembrolizumab, nivolumab, or atezolizumab). All tissue specimens were obtained from FFPE biopsy or resection tissue and prepared as TMA. Response status was determined using RECIST v1.1 criteria.

Control cohort

We used a control cohort YTMA337, which is a specially selected PD-L1–specific index array NSCLC tumors (n = 30) and cell line pellets with variable levels of PD-L1 expression in 2-fold redundancy. For standardization, we stained YTMA337 alongside with each experiment as quality control to show that the YTMA337 staining pattern is quantitatively consistent between experiments.

Antibody validation

CD68 antibody (1:200; Mouse monoclonal, IgG3, clone PG-M1, DAKO) and CD8 antibody (1:250; Mouse monoclonal, IgG1, clone C8/144B, DAKO) were validated in our laboratory and published previously (17, 18). CD56 antibody (1:200; Mouse monoclonal, IgG1, clone 123C3, DAKO) was cross-validated with antibody clone 56C04 (176/209 in YTMA79; 249/291 in YTMA250) due to the loss of tissue, missing data, or poor quality of the staining as seen in other TMA studies. All tissue samples were collected with the approval from the Yale Human Investigation committee protocol #9505008219. The Yale Human Investigation Committee approved the patient consent forms or in some cases a waiver of consent all in accordance with the ethical guidelines of the U.S. Common Rule.

Many studies have shown that “immune cells,” without specific characterization, are a critical component of the companion diagnostic tests used for programmed death 1 receptor (PD-1) axis immunotherapy. The exact type of immune cells that express programmed death ligand 1 (PD-L1) is unclear. By quantitatively measuring PD-L1 in CD68+ macrophages, CD56+ natural killer cells, and CD8+ cytotoxic T cells we show that the vast majority of PD-L1 expression colocalizes with CD68, inferring that the key immune cell is a macrophage. It is more challenging to prove that the macrophage carries the predictive power of the test. However, in a small pilot scale, retrospective cohort, we show significant association with overall survival attributable to PD-L1 in the macrophage compartment, and not in the tumor cell compartment. This work raises the possibility that the companion diagnostic test for PD-1 axis therapies may be improved by assessing PD-L1 expression only when it is colocalized with CD68.
Table 1. Immunotherapy Treated Cohort Characteristics. (Cont’d)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunotherapy regimens</td>
<td></td>
</tr>
<tr>
<td>Nivolumab</td>
<td>57 (70.4)</td>
</tr>
<tr>
<td>Pembrolizumab</td>
<td>5 (6.2)</td>
</tr>
<tr>
<td>Atezolizumab</td>
<td>5 (6.2)</td>
</tr>
<tr>
<td>Others</td>
<td>14 (17.2)</td>
</tr>
</tbody>
</table>

(1:100, Mouse monoclonal, Thermo Fisher Scientific). Both CD56 antibodies were evaluated on the control cohort with immunofluorescence (IF). Comparison between two clones shows a high regression coefficient ($R^2 > 0.7$). This combined with vendor specificity validation assured to CD56 antibody validation. CD8 assays were validated in previous studies.

Quantitative immunofluorescence

TMA slides were baked at 60°C for 1 hour and then soaked in xylene twice for 20 minutes each. Rehydration was performed with two 1-minute washes in 100% ethanol followed by 1-minute wash in 70% ethanol and 5-minute rinse under streaming tap water. Antigen retrieval was then performed with pH 8 EDTA buffer for 20 minutes at 97°C in the Lab Vision PT Module (Thermo Fisher Scientific). Thirty-minute incubation in 2.5% hydrogen peroxide in methanol was performed to block endogenous peroxidases and subsequently unspecific antigens were blocked using a 0.3% BSA in Tris Base Saline Tween (TBST) for 30 minutes. For the PD-L1/CD68/CD8 multiplex panel, a cocktail of primary antibodies including PD-L1 (1:800; Rabbit monoclonal, clone SP142, Spring), CD68, and CD8 were incubated in 0.3%BSA in TBST overnight at 4°C. Horseradish peroxidases (HRP)-conjugated secondary antibodies specific to each primary antibody isotype were used sequentially (anti-mouse IgG3, 1:1,000, Abcam; anti-rabbit EnVision, DAKO; anti-mouse IgG1, 1:100, ebioscience). Note that the primary antibody used for PD-L1 here is the SP142 antibody, not the SP142 assay. In other works, the antibody has been shown to be equivalent to other cytoplasmic domain PD-L1 antibodies (19), even though the assay has proven to be of lower sensitivity (20, 21). Tyramide-bound fluorophores were added after each secondary antibody to bind to the HRPs. Specifically, biotinylated tyramide (1:50, PerkinElmer) and Alexa750-streptavidin (1:100, Life Technologies) were used for CD68, Cyanine 5 (Cy5) tyramide (1:50, PerkinElmer) was used for PD-L1, and Cy3 tyramide plus (1:100, PerkinElmer) was used for CD8. Epithelial tumor cells were detected with 1-hour incubation of anti-rabbit pan-cytokeratin (CK) (1:100, DAKO) and following 1-hour incubation of goat-anti-rabbit Alexa488 (1:100; ebioscience) at room temperature. Finally, nuclei was stained with 4,6-diamidino-2-phenyl-dole (DAPI; 1:1,000, Life Technologies) for 10 minutes at room temperature and mounted with Prolong Gold Mounting Reagent (Life Technologies). For the PD-L1/CD68/CD56 multiplex panel, a cocktail of primary antibodies including PD-L1, CD68, and CD56 were used and the rest of the protocol is the same as previously described.

Quantitative immunofluorescence (QIF) was quantified using two platforms: image collection was done on either the PM2000 (Histolux) or Vectra Polaris (Perkin Elmer) automated fluorescence microscopy platforms. The result images were analyzed and quantified using either the AQUAAnalysis Software (Navigate Biopharma) or the InForm Software (Perkin Elmer) on all tumor spots as described previously (13). Briefly, fluorescent images of DAPI, FITC (CK), Cy5 (PD-L1) Cy3 (CD56/CD8), and Cy7 (CD68) for each core were collected on either platform except for the Vectra Polaris, multipoint images were captured in five channels on all tumor spots. With the trainable feature-recognition inForm software, specific cells and tissue types were identified. The cell count for phenotype of interest (PD-L1 and CD68/CD8/CD56 double-positive phenotype) was calculated in both tumor and stromal tissue for final analysis. All acquired tumor spots were evaluated visually and spots with less than 2% of tumor or staining artifacts were excluded from the final analysis. An AQUA score of 500 AU was used to define PD-L1 positivity to reproduce a 1% TPS threshold and was determined by visualization of IF staining of PD-L1.

DNA-tagged QIF

We stained one slide from the same NSCLC TMA cohort C block with the Ultimapper Kit (Ultivue Inc). The slide was deparaffinized in an oven at 60°C (oven company) for 20 minutes followed by immediate immersion in xylene (twice) for 20 minutes each. The slide was rehydrated using graded alcohols (100%, 100%, 70% ethanol) followed by tap water. Antigen retrieval was done using EDTA (company) at pH 9 in a PT Module pressure boiler with preheating the solution to 85°C then 20 minutes at 97°C followed by cooling at 75°C, and then the module tank was kept under running tap water for 10 minutes. The slide was then washed in PBS thrice by submersion. Blocking was done using the blocking solution in the Ultimapper kit at room temperature (20°C–25°C) for 15 minutes in a humid chamber. The antibody solution was made (mixing of single-strand DNA-tagged antibodies and antibody diluent) and intubated for 60 minutes at room temperature in a humid chamber. Amplification of the DNA strand was done with preamplification solution and 15 minutes at room temperature in humid chamber followed by Amplification solution (Amplification enzyme and Amplification buffer) at 30°C in a sealed humid chamber (Slide Mount, Boekel Scientific). This was followed by nuclear staining (Hoescht) and amplification of fluorescent-tagged DNA probes for 25 minutes at room temperature. The slides where then cover slipped and scanned using the PM2000 machines and AQUA Scores were generated for each marker and studied for the impact on overall survival (OS).

Confocal microscopy

For high resolution images, the TMA slides were examined using a Leica SP5 Confocal Microscope at 100× magnification in the fluorescent mode. Image acquisition and analysis were performed on the LAS AS Software (Leica). The excitation and emission wavelengths used to detect were at 405 nm, 561 nm, 633 nm, and a multi-line Argon.

Statistical analysis

Reproducibility of the assays is measured with the control cohort with Pearson correlation coefficient ($R$). An AQUA score of 500 AU was used to stratify PD-L1 SP142 staining scores into positive or negative groups of patients for analysis. This threshold was determined with visual inspection of all NSCLC tumor cohorts and the control array. AQUA scores and inForm cell counts between responders (partial response/complete response) and nonresponders (stable disease/progressive disease) were compared with Fisher exact test two-sided. Survival analysis was carried out using Kaplan–Meier curves and significance was determined with the log-rank test. Jointpoint 4.6.0.0 was used to identify the marker score, which is an apparent change in trend that is statistically significant without referring to the outcome data (22). The univariate analyses were performed with GraphPad Prism 7, and multivariate analyses were performed using JMP11. All $P$ values were descriptive and based on two-sided tests and were not adjusted for multiple comparisons.
Results

Among the 425 NSCLC cases in discovery cohorts A and B, CD68\(^+\), CK\(^-\) cells, defined as macrophages were present in 391 of 425 (92%) cases, while CD8\(^+\), CK\(^-\) T cells and CD56\(^+\), CK\(^-\) natural killer (NK) cells were present in 388 of 425 (91.3%) and 90 of 209 (43.1%), respectively. A visually estimated threshold of 500 arbitrary units of fluorescence (au) of AQUA score was used to determine the patients into PD-L1\(^-\) positive (126/425, 29.6%) or -negative group (299/425, 70.4%). Then inForm was used to assess PD-L1 expression on both tumor tissue compartment and stromal tissue compartment where tumor was defined by a machine learning algorithm that defines CK-positive regions as tumor tissue compartment. A similar training approach was used for the stromal compartment definition using DAPI and absence of CK. Finally, a third compartment was defined/trained, where there is no tissue. PD-L1 cellular localization was then determined within the PD-L1\(^-\)positive group (determined by AQUA, \(n = 126\)) by the cell count of the following phenotype: PD-L1/CK double positive, PD-L1/CD68 double positive, PD-L1/CD56 double positive, and PD-L1/CD8 double positive in both tumor and stromal compartments. Figure 1A illustrates a series of case examples. Case 1, where tumor PD-L1 was predominantly localized with CK, also showing some expression in tumor-infiltrating macrophages. Case 2 shows PD-L1 localization with CK in the tumor compartment and with CD68 in stromal compartment. Case 3 shows PD-L1 was only localized with CD68 in both tumor and stromal. Finally, case 4 shows PD-L1 only expressed by CK in the tumor compartment. Then these data were averaged across 126 cases of PD-L1\(^-\) positive NSCLC and shown in summary pie charts in Fig. 1B. PD-L1 localization was significantly higher in CD68\(^+\) macrophages compared with CD56\(^+\) NK cells and CD8\(^+\) T cells: in the tumor compartment, PD-L1 was predominantly colocalized with CK (53%) while 41% of PD-L1 was found to be colocalized with CD68, 4% with CD8, and 2% with CD56; in the stromal compartment, the majority of PD-L1\(^-\)positive cells were CD68 positive (80%), while only 5% from CD8, and 3% from CD56.

To better illustrate the compartmentalization of PD-L1 expression, Fig. 2 shows high magnification confocal images of four examples, both combined and separated by the channel along with the same region stained with hematoxylin and eosin after capturing the fluorescent images and the chromogenic IHC image from a serial section of the same tissue region. Note that it is hard to visualize the colocalization in either the CD8 or CD56 compartments because they represent a relatively small percentage of the total PD-L1. Because the PD-L1 expression in this study was mainly found in CK and CD68\(^+\) cells, and PD-L1 in CD56 and CD8 was relatively low, the remainder of this work focuses on the expression of PD-L1 by tumor cell and CD68\(^+\) macrophage only. Correlation between PD-L1 level in CD68\(^+\) macrophages and PD-L1 level in tumor, CD8 and CD68 levels was identified.
indicating a connection between high PD-L1 level in macrophages and “hot” tumors (Supplementary Fig. S1).

Next, we assessed the association with outcome for PD-L1 expression in each compartment. The potential correlation between the prognostic value of PD-L1 level in macrophages and patients’ outcome was evaluated in a merged analysis of Yale cohorts A and B. No significant association between PD-L1 level in macrophages and major clinicopathologic variables was found. PD-L1 expression in CD68+ macrophages was defined as cases where PD-L1 expression level was above a visual threshold of 3,000 au of AQUA score (156/425, 36.7%). The remainder of the cases were PD-L1 negative in macrophage group (269/425, 63.3%). High PD-L1 expression in macrophages was significantly associated with high PD-L1 level in tumor, as well as CD8 level and CD68 level (P < 0.0001; Fig. 3A). With the median cutoff, or any discovered cut-off point, we found PD-L1 level in CD68 was not associated with patient’s survival in patients treated with standard-of-care therapy received by the patients in these retrospective cohorts gathered prior to the availability of immune therapy (Fig. 3B, Supplementary Figs. S2 and S3).

The potential predictive value of PD-L1 in macrophages was evaluated in Yale cohort C, the immunotherapy-treated cohort. The joinpoint method is independent of outcome (22), so it was used to define a cohort stratification threshold. With the normalized cell count of total PD-L1/CK double–positive phenotype and PD-L1/CD68 double–positive phenotype, a joinpoint analysis for natural population breaks identified a significant breakpoint at the 25th percentile of the PD-L1/CK cell count (n = 15; total n = 59; P = 0.00222) and the 21st percentile of the PD-L1/CD68 cell count within total cell count (n = 12; total n = 61; P = 0.00222; Supplementary Fig. S5). With these joinpoints, no predictive value was found for high cell count for the PD-L1/CK phenotype (Fig. 4A), while high cell count of PD-L1/CD68 phenotype was associated with better OS in patients treated with single therapy (pembrolizumab/nivolumab/atezolizumab; P = 0.036; Fig. 4B). Multivariate analysis indicated that the predictive value of high cell count of PD-L1/CD68 phenotype toward OS was independent of age, sex, stage smoking history, and CD8 level (Supplementary Table S3). No significance was found between the PD-L1 level in CD68+ macrophages and response to immunotherapy or progression-free survival in this small cohort.

To confirm this observation, a second independent assay method was used using DNA-based QIF on the same Yale cohort C. This method uses different antibodies and no secondary antibodies, but rather a direct labeling of primary antibodies with oligonucleotide codes. These oligonucleotides provide specificity and can be differentially amplified and assessed by multiplex QIF using either the AQUA method, inForm method, or other QIF methods. The antibodies in the
Figure 3.
A, High PD-L1 expression in macrophages was correlated with high CD8 level, high CD68 level, and high PD-L1 expression by tumor cells in 457 cases of NSCLC.
B, Error bars represent mean with 95% confidence interval. PD-L1 expression in macrophages is not prognostic in this cohort.

Figure 4.
PD-L1 level in macrophages predicts patients' OS to anti-PD-1 axis blockade therapy using two different QIF methods. A, Using InForm to count cells, double positive PD-L1 and CK cell (count \( n = 15 \)) was not associated with OS. B, Double positive PD-L1/CD68 cells (count \( n = 12 \)) were significantly associated with OS of patients with NSCLC treated with single-drug immunotherapy. Using AQUA assessment of PD-L1 in the tumor (C) or stromal (D) compartments was not associated with better outcome on monotherapy, while PD-L1 in the CD68 compartment was statistically significantly associated with better OS (E).
subtypes in 457 NSCLC patients. Expression in host cells in
sion in macrophages in mouse models and demonstrated that PD-L1
expression in the stroma, tumor, and
panel detect PD-L1, CD68, CD8, and CK. An AQUA-based analysis
was done to measure PD-L1 expression in the stroma, tumor, and
host cells in 457 NSCLC patients and showed that the majority of
PD-L1 expression is in CD68^+ macrophages, as confirmed by two
methods of QIF and confocal microscopy. Furthermore, expression of
PD-L1 in macrophages was correlated with better OS in patients
treated with immunotherapy.

PD-L1 has been found to be expressed not just by macrophages,
cytotoxic T cells, and NK cells, but rather broadly expressed by
hematopoietic and non-hematopoietic cells, including B cells, den-
dritic cells, regulatory T cells, etc (23–25). In addition, early-stage
tumor associated macrophages have been found to express both M1
and M2 markers thus the traditional M1/M2 associations with aggres-
siveness, or lack thereof, are not found (26, 27). While early-stage
TAMs are associated with increased probability of recurrence and they
have no effect on T cells (26, 28). It has been suggested that PD-L1
expression by TAMs in early-stage lung cancer does not inhibit effector
T-cell function (26).

With our current panels, we were able to demonstrate that the
majority of PD-L1 expression by nonneoplastic cells were from
CD68^+ cells that are likely to be classified as macrophages. Garris
and colleagues have recently shown that the efficacy of anti-PD-1
immunotherapy requires intratumoral dendritic cells (29). One
possible explanation is that dendritic cells can express PD-L1 and
bind to PD-1 thus resulting in downstream immune inhibition. We
have been unable to assess the percentage of PD-L1 in dendritic cells
due to the absence of a definitive, single antibody dendritic cell
marker. These findings suggest further translational studies on the
role of PD-L1 in other types of immune cells and its predictive value to
immunotherapy with treated cohort.

There are several limitations to this study. Perhaps the greatest
limitation is that this work is entirely performed on TMA s that may
over or underrepresent the biomarker expression in whole-tissue
section (WTS) because of tumor and microenvironmental hetero-
geneity. However, study has shown good concordance between
TMAs and corresponding whole-tissue section and between two
TMA cores from two paraffin blocks of the same tumor in breast
cases (30). Moreover, previous work in our laboratory assessed the
PD-L1 QIF score in TMA cases and compared it with the corre-
sponding WTS cases. We found a comparable PD-L1 detection
between TMA and WTS in NSCLC cohorts (31). The advantage of
large numbers of cases accessible by TMA is favored here. A second
limitation is the relatively small size of the immunotherapy-treated
cohort and the heterogeneity of treatment of this cohort. Access to
tissue from treated patients is limited and similarly long-term
follow-up is just now becoming available for treatment of patients
with these medications after their FDA approval and acceptance
as standard of care. As such this work should be considered
as preliminary and should be validated by larger prospective
studies.

Another concern is that the tissue used in this study was archival
tissue, not collected specifically for companion diagnostic testing. The
issue of antigen aging has recently been addressed by Herbst and
colleagues and suggests that this concern does appear to affect the
predictive value of the PD-L1 test (32). Finally, CD68 may not stain all
tumor-associated macrophages and CD36 may also capture NKT cells.
As such, this data must be considered hypothesis generating, or
discovery data and the observation that PD-L1 expression in macro-
phages as the key predictive factor requires validation in external
cohorts, or potentially future clinical trials.

In summary, we find PD-L1 expression is particularly frequent in
macrophages as compared with other immune cell types in patients
with NSCLC. Elevated level of PD-L1 in macrophages could be
evaluated in future studies as potentially contributory to the thera-
peutic efficacy of anti-PD-1 blockade therapy.

Disclosure of Potential Conflicts of Interest

J. Zugazagoitia reports receiving speakers bureau honoraria from and is an unpaid
consultant/advisory board member for Guardant Health. B.S. Henick is an employee/
paid consultant for Boehringer Ingelheim and holds ownership interest (including
patents) in Abbvie. S.N. Gettinger is an employee/paid consultant for Bristol-Myers
Squibb, R.S. Herbst is an employee/paid consultant for Abbvie Pharmaceuticals,
ARMO Biosciences, AstraZeneca, Biodexis, Bolt Biotherapeutics, Bristol-Myers
Squibb, Eli Lilly and Company, EMD Serono, Genentech/Roche, Genmab,
Halozyme, Heat Biologics, iDMAB Biopharma, Immunocore, Loxo Oncology, Merck
and Company, Midas Health Analytics, Nektar, NextCure, Novartis, Pfizer, Sanofi,
Seattle Genetics, Shire PLC, Spectrum Pharmaceuticals, Symphogen, Takeda, Tesaro,
Tocagen, and Junshi Pharmaceuticals; reports receiving commercial research grants
from AstraZeneca, Eli Lilly and Company, and Merck and Company; and is an unpaid
consultant/advisory board member for NeoS Therapeutics, Infinity Pharmaceuticals,
and NextCure. K.A. Schalper is an employee/paid consultant for Clinica Alemana
Santiago, Celgene, Moderna Therapeutics, Shattuck Labs, Pierre Fabre, AstraZeneca,
Dynamo Therapeutics, EMD Serono, and Takeda; reports receiving commercial
research grants from Navigare BP, Tesaro, Takeda, Surface Oncology, Pierre Fabre,
Merck, Bristol-Myers Squibb, AstraZeneca, and Eli Lilly; and reports receiving
speakers bureau honoraria from Merck, Bristol-Myers Squibb, Fluigend, and Takeda.
D.L. Rimm is an employee/paid consultant for AstraZeneca, Bristol-Myers
Squibb, Merck, and Roche, and reports receiving commercial research grants
from AstraZeneca, and Ultivue. No potential conflicts of interest were disclosed by the
other authors.

Disclaimer

The funding sources had no role in study design, collection, analysis, and
interpretation of data; preparation of the article, or the decision to submit for
publication.

Authors’ Contributions

Conception and design: Y. Liu, B.S. Henick, S.N. Gettinger, R.S. Herbst, D.L. Rimm
Development of methodology: Y. Liu, J. Zugazagoitia, R.S. Herbst, K.A. Schalper,
D.L. Rimm
Acquisition of data (provided animals, acquired and managed patients, provided
facilities, etc.): Y. Liu, J. Zugazagoitia, S.N. Gettinger, R.S. Herbst
Analysis and interpretation of data (e.g., statistical analysis, biostatistics,
Gettinger, R.S. Herbst, K.A. Schalper, D.L. Rimm
Writing, review, and/or revision of the manuscript: Y. Liu, J. Zugazagoitia,
Administrative, technical, or material support (i.e., reporting or organizing data,
constructing databases): Y. Liu, D.L. Rimm
Study supervision: D.L. Rimm
Other (experimentation): F.S. Ahmed

Liu et al.
Acknowledgments
This work was primarily supported by funds from Yale SPORE in Lung Cancer (to R.S. Herbst, K.A. Schalper, S.N. Gettinger, and D.L. Rimm, P50-CA196530) and also funds from the Yale Cancer Center (P30CA016359) and funds from Navigate BioPharma (Novartis subsidiary) to D.L. Rimm and K.A. Schalper.

References


**Updated version**
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-19-1040

**Supplementary Material**
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2019/10/15/1078-0432.CCR-19-1040.DC1

**Cited articles**
This article cites 30 articles, 6 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/26/4/970.full#ref-list-1

**Citing articles**
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/26/4/970.full#related-urls

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, use this link:
http://clincancerres.aacrjournals.org/content/26/4/970.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.

**Immune Cell PD-L1 Colocalizes with Macrophages and Is Associated with Outcome in PD-1 Pathway Blockade Therapy**
