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PD-L1 Studies Across Tumor Types, its Differential Expression and Predictive Value in Patients Treated with Immune Checkpoint Inhibitors

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Abstract

Purpose—With recent approval of inhibitors of PD-1 in melanoma, non-small cell lung cancer (NSCLC) and renal cell carcinoma (RCC), extensive efforts are underway to develop biomarkers predictive of response. PD-L1 expression has been most widely studied, and is more predictive in NSCLC than RCC or melanoma. We therefore studied differences in expression patterns across tumor types.

Experimental Design—We employed tissue microarrays with tumors from NSCLC, RCC or melanoma and a panel of cell lines to study differences between tumor types. Predictive studies were conducted on samples from 65 melanoma patients treated with PD-1 inhibitors alone or with CTLA-4 inhibitors, characterized for outcome. PD-L1 expression was studied by quantitative immunofluorescence using two well-validated antibodies.

Results—PD-L1 expression was higher in NSCLC specimens than RCC, and lowest in melanoma ($P=0.001$), and this finding was confirmed in a panel of cell lines. In melanoma tumors,

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PD-L1 was expressed either on tumor cells or immune-infiltrating cells. The association between PD-L1 expression in immune-infiltrating cells and progression-free or overall-survival in melanoma patients treated with ipilimumab and nivolumab was stronger than PD-L1 expression in tumor cells, and remained significant on multi-variable analysis.

Conclusions—PD-L1 expression in melanoma tumor cells is lower than NSCLC or RCC cells. The higher response rate in melanoma patients treated with PD-1 inhibitors is likely related to PD-L1 in tumor-associated inflammatory cells. Further studies are warranted to validate the predictive role of inflammatory cell PD-L1 expression in melanoma and determine its biological significance.

Keywords

PD-L1; PD-1 inhibitors; melanoma; lung cancer; renal cell carcinoma

INTRODUCTION

Immune checkpoint inhibitors have become the mainstay of treatment for melanoma and other tumor types. The first immune checkpoint inhibitor to gain approval, ipilimumab, inhibits CTLA-4 on cytotoxic T cells, resulting in durable responses in 11–19% of patients with advanced melanoma and prolonging overall survival (1–3). Treatment with ipilimumab, however, causes grade 3–4 immune-related adverse events in approximately 30% of patients at the FDA-approved dose of 3 mg/kg, diminishing the risk/benefit ratio of this drug.

Inhibitors of PD-1 or its ligand, PD-L1, have similarly been studied in advanced melanoma and other tumor types, and have now been approved for a number of diseases including melanoma, renal cell carcinoma (RCC), bladder cancer, non-small cell lung cancer (NSCLC), head and neck cancer and Hodgkin's lymphoma (4–12). Response rates to PD-1 and PD-L1 inhibitors in melanoma were higher than those of ipilimumab, and the toxicity profile more favorable, with response rates in the range of 30–40% and approximately 15% of patients having grade 3–4 immune related adverse events (1, 4–6).

The combination of ipilimumab and nivolumab has been studied in a number of diseases, and is now approved for advanced melanoma. The response rate with the combination was superior to that of either drug alone (57.6% in the first line setting), and the rate of grade 3–4 adverse events was 55%, more than double that of monotherapy (1, 13, 14). Biomarkers predictive of response or resistance are therefore needed to improve patient selection, and given that this is a relatively new regimen with limited patient follow-up, predictive biomarkers have barely been studied.

To date, despite a number of attempts to identify biomarkers predictive of response to ipilimumab monotherapy, no biomarker has consistently been shown to be associated with response or clinical benefit (15, 16). Given the broader use of inhibitors of PD-1 or PD-L1 in multiple tumor types, intense efforts are underway to identify predictors of response. Expression of PD-L1 on tumor cells has been the most widely studied predictive biomarker, and has been shown to correlate with response to therapy in multiple tumor types, although the correlation is insufficient in most tumor types, including melanoma and renal cell

carcinoma, for clinical use. Other predictive biomarkers that have been studied in melanoma tumors include tumor mutation burden, T cell receptor repertoire, T cell infiltrate, gene expression profiles and presence of MHC molecules. Inflammatory gene expression signatures within the tumor, particularly those associated with interferon- γ secretion, are associated with response to PD-1 inhibitors (17). Tumors with a greater mutation load might be more sensitive, particularly BRCA2 mutations (18). Presence of CD8⁺ T cells at the periphery of the melanoma tumor bed is associated with a greater likelihood of response to PD-1 inhibitors, as is presence of tumor specific MHC class II molecules (19, 20). PD-L1 expression, however, is the one biomarker that has consistently been shown to be associated with response in multiple trials and clinical settings, albeit insufficiently correlated to be broadly used alone as a companion diagnostic.

Most predictive biomarker studies involving PD-L1 expression have employed standard immunohistochemistry (IHC), as reviewed (21–23). These studies have used a variety of antibodies and cutpoints for positivity (24). For example, in the randomized trial of nivolumab versus chemotherapy in the second line setting, 43.6% of patients with > 5% tumor cell staining for PD-L1 had a response, compared to 20.3% of those with < 5% tumor cell staining (6). This study used an antibody made by DAKO. The phase I trial of nivolumab in multiple tumor types used the 5H1 antibody, and no responses were seen in PD-L1 negative tumors, defined once again as < 5% tumor staining (25). Herbst et. al. used a third antibody to PD-L1 (Clone SP-142, Ventana) that demonstrated expression on both tumor and immune infiltrating cells, and showed that the percent of positive cells by IHC, particularly on tumor infiltrating cells, was associated with response, and was more predictive than tumor cell staining, using a scoring system ranging from 0–3, with multiple cutpoints (<1%, 1–5%, 5–10% or >10%, respectively) (26). Of note, in this study, as in the study by Topalian et al, the fraction of tumors positive for PD-L1 expression varied by tumor type.

PD-L1 expression as a companion diagnostic has been approved for use in NSCLC, but not in melanoma. Cut-points for positivity as high as 50% have been used in some lung cancer studies (11). In our previous studies of PD-L1 expression in melanoma and RCC, (27–29) we rarely observed tumors with >50% positive PD-L1 staining, yet response rates to PD-1 inhibitor monotherapy are higher in melanoma, and possibly also in RCC. Our first purpose was therefore to compare PD-L1 expression across tumor types using a uniform platform. Our second goal was to determine whether PD-L1 in tumor versus immune-infiltrating cells might be predictive of response to PD-1 inhibitors or dual immune checkpoint inhibitors (ipilimumab and nivolumab in combination), as published data on PD-L1 expression and response to these regimens are limited to expression on tumor cells.

MATERIALS AND METHODS

Cell lines and Western blotting

Early passage melanoma cell cultures were derived from tumors of patients treated at Yale University. Cells in culture were tested for mutations by sequencing and mutation patterns were compared to the tumors from which they were derived for authentication. NSCLC and RCC cell lines were purchased from American Type Tissue Culture (ATCC). Cells

underwent less than 20 passages prior to use in these studies. After 20 passages, cells were discarded and a vial from an early passage from the cell bank was used. Cells were grown in OptiMEM or RPMI media (Invitrogen) supplemented with 10% heat-inactivated FBS (Invitrogen) and antibiotic-antimycotic drugs (penicillin, streptomycin, amphotericin B; Invitrogen). Western blotting was performed by standard methods. β -Actin was utilized to verify equal loading and protein content among samples and for normalization of PD-L1 signal. ImageJ software was used to quantitatively assess PD-L1 levels in each sample lysate.

Tissue microarray (TMA) construction

A TMA was created from specimens from a cohort of sixty-five patients treated with either anti-PD-1 monotherapy or combination therapy (ipilimumab and nivolumab) at our institution between 2009 and 2015. Collection of specimens and clinical data was approved by a Yale University Institutional Review Board. A pathologist (AA) re-examined FFPE tissue sections from each case and selected representative regions of invasive tumor for coring. Three punches from each specimen were obtained and placed on the paraffin block, using methods previously described (30–32). Patient tumor characteristics and clinical information were collected from pathology reports and clinical records. The demographics for the sixty-five patients evaluable for response are summarized in Supplementary Table S1. The cohort included 62% males, 38% females. Age at diagnosis ranged from 37 to 92 (mean-63). At start of therapy, 12% had M1a disease, 20%-M1b and 68%-M1c. LDH was elevated in 34% of patients; B-RAF and N-RAS mutations were found in 29% and 19% of cases, respectively; 3% of patients had mutated C-KIT while 9% were unknown. Forty eight percent received ipilimumab and nivolumab and 52% received anti-PD-1 monotherapy. RECIST 1.1 criteria were used for clinical assessment and classification of response.

To assess antibody specificity and for comparison of staining across diseases we used control arrays containing placental and tonsil tissue (known to be positive for PD-L1 expression), pellets from MEL-624 cell lines, overexpressing or not overexpressing PD-L1, and cases of metastatic melanoma, NSCLC and clear cell RCC, as previously described (27).

Immunofluorescence and Automated Quantitative Analysis

Staining was performed for automated analysis, as previously described (27, 29, 33). Briefly, TMA slides were deparaffinized and hydrated followed by antigen retrieval. For the E1L3N antibody (cat#13684, Cell Signaling Technology), slides were boiled for 20 minutes in a pressure cooker containing 6.5mM sodium citrate (pH 6.0). Endogenous peroxidase activity was blocked using hydrogen peroxide solution. Unspecific staining was blocked in 0.3% bovine serum albumin solution before overnight incubation with anti-PDL1 antibody diluted in TBS supplemented with 0.05% Tween 20 (1:250, at 4°C). To detect the cell membrane/cytoplasmic compartment and to create a tumor mask, we simultaneously used a cocktail of mouse anti-S100 and anti-HMB45 antibodies, diluted at 1:100 in the overnight solution (BioGenex) for melanoma samples. The tumor mask for NSCLC and RCC was generated using mouse anti-cytokeratin (cat#M3515, DAKO) or antibody cocktail of anti-cytokeratin, CAIX (gift from Jan Zavada), CD10 (cat#M7308, DAKO) and Streptavidin HRP (cat#S2438, Sigma-Aldrich), respectively. Slides were then incubated with goat anti-mouse

IgG conjugated to Alexa 546 diluted at 1:200 (Molecular Probes, Inc.) in anti-rabbit amplification reagent (Envision; cat#K4003, Dako).

For clone 5H1 (generated by Dr. Lieping Chen), antigen retrieval was performed in Tris-EDTA buffer (pH 9.0, DAKO) supplemented with 0.3% Tween 20 (34). Following peroxidase blocking, slides were incubated at room temperature for 15 minutes in ACE blocking buffer. To block endogenous biotin, slides were first incubated in Avidin solution followed by Biotin blocking reagent (Vector Laboratories) for 15 minutes at 37°C. Slides were incubated overnight with clone 5H1 (1:400 at 4°C) and rabbit anti-S100 (1:100, cat#Z0311, DAKO) in ACE block. A second TMA control slide, incubated overnight in ACE block only was used to verify lack of false-positive staining. A biotinylated anti-mouse IgG (Vector laboratories) was used as a secondary reagent (1:500 for 45 minutes at RT), followed by incubation with the ABC kit (Vector Laboratories) for 15 minutes. Amplification reagent and Streptavidin-HRP were used for signal amplification as recommended (DAKO). For visualization of S100 slides were incubated with Alexa 546-conjugated anti-rabbit IgG.

For T cell detection, the following primary antibodies were utilized: rabbit anti-CD3 (1:3200, cat#A0452, DAKO), mouse anti-CD4 (1:400, cat#M7310, DAKO), mouse anti-CD8 (1:200, cat#M7103, DAKO) and mouse anti-FOXP3 (1:200, cat#ab20034, Abcam). Staining was carried out using our standard protocol. To amplify the signal goat anti-rabbit and/or anti-mouse Envision were used followed by fluorophore tyramides. Slides were incubated with benzoic hydrazide solution (100mM in PBS) containing 50mM hydrogen peroxide before second amplification to quench residual HRP.

Target staining was visualized with Cyanine-5-tyramide (PD-L1, CD4 and CD8 or Cyanine-3-tyramide (CD4 and FOXP3) (Perkin-Elmer). A nuclear mask was created by incubating the slides with 4,6-diamidine-2-phenylindole (DAPI) (1:500, Invitrogen). Coverslips were mounted with ProLong Gold antifade medium (Invitrogen/Life Technologies).

Quantitative Determination of Target Expression

Image capturing and quantitative measurements were conducted using methods and algorithms previously described (33). Tumor was distinguished from the surrounding stromal elements by S-100 signal for melanoma and cytokeratin for NSCLC or RCC. The tumor mask was created by converting the anti-S100 image via automated processing and thresholding. DAPI signal defining the nuclear compartment, was utilized to create a total tissue mask (tumor and stroma). The stromal compartment was obtained by subtracting the tumor mask from the total tissue mask. Quantification of PD-L1 signal for each antibody in the tumor or stromal compartment (total signal intensity / area of the compartment) was performed, and output obtained on a scale of 0–255. To assess the degree of tumor-infiltrating lymphocytes (TIL), we used the percentage of either CD3-, CD4- or CD8-, FOXP3-positive T-cell area within the histospot. Tumor spots were excluded if they contained insufficient tissue (<3% of the histospot area) or abundant necrotic tissue and if excessive background staining was present on the negative control slide.

Statistical Analysis

JMP version 5.0 software was used (SAS Institute, Cary, NC). Data was analyzed using either continuous immunofluorescence scores or variables dichotomized at the median. The Pearson correlation test was used to compare PD-L1 measurements (maximum and mean scores) within compartments and concordance between antibodies. The association with objective response status (complete response (CR) and partial response (PR) vs. stable disease (SD) and progressive disease (PD)) or other clinical/pathological parameters (AJCC version 8 M stage, LDH level, gender and age) was assessed by the two-sample t test (analysis of variance) for continuous measurements or the Chi-square test for dichotomized variables. The prognostic significance of parameters was assessed using univariate and multivariable Cox proportional hazards models with progression free survival and overall survival as end points. Survival curves were constructed using the Kaplan-Meier method.

RESULTS

PD-L1 expression in tumor cells and in immune-infiltrating cells in various tumor types

Various studies suggest that it is not the intracellular fraction but the cell surface PD-L1 (membrane-expressed PD-L), which includes the PD-1-binding domain, that is biologically significant and associated with a higher likelihood of response to PD-1 blockade (21, 35). Most antibodies to PD-L1 bind to its extracellular domain, which comprises the greater part of the protein and stain tumor tissue with cytoplasmic and membranous patterns. Antibodies directed against the intra-cellular domain, a short 31 amino acid region, have also been shown to be selective for membranous PD-L1.(36) For our study we employed two different anti-PD-L1 antibodies; a mouse monoclonal antibody (clone 5H1, generated by Dr. Lieping Chen and validated in previous publications,(25, 27, 29) which binds the extracellular domain of PD-L1, and a rabbit monoclonal antibody (clone E1L3N from Cell Signaling), which recognizes the intracellular domain or total endogenous level of the protein. To verify antibody specificity and sensitivity, we first used quantitative immunofluorescent (QIF) analysis of control TMAs containing placenta, tonsil tissue (known to be positive for PD-L1 expression), and pellets from MEL-624 cell lines, overexpressing or not overexpressing PD-L1, as previously described (27). Control arrays also included 12 cases of metastatic melanoma, 12 NSCLC and 21 RCC specimens, which allowed comparison of staining patterns and intensity across the three diseases. Staining was confined either to the cell surface (membranous staining) and in some cases to the cytoplasm and was specific for both antibodies as judged by our positive /negative cell line controls (Figure 1). As expected, PD-L1 was expressed at high levels in MEL624 PD-L1+ cells but not in the parental MEL624 cells. In melanoma specimens, PD-L1 was expressed either on tumor cells or in the tumor microenvironment on immune-infiltrating cells. In contrast, in NSCLC and RCC specimens from our control arrays, PD-L1 expression was predominantly on tumor cells. An example of a PD-L1 positive case for each tumor type is shown in Figure 1, corresponding to the strongest expressing histospots for each of the three diseases. For comparative studies across tumor types we quantified the fluorescent signal (either maximum or mean intensity values) within the entire histospot. Expression levels in the corresponding specimens by the two antibodies (5H1 and E1L3N) was highly correlated ($R = 0.89$). The expression of PD-L1 in NSCLC tumors was significantly higher by ANOVA when compared to RCC and melanoma

specimens (mean AQUA score of 21.5 vs. 16 vs. 4, respectively for clone 5H1, $P = 0.001$; 21.5 vs. 12.2, vs. 9.6, respectively for clone E1L3N, $P = 0.0008$, Supplementary Figure S1 A,B).

To further verify differential PD-L1 expression in tumor cells from the three histologies as opposed to stromal and immune infiltrating cells, we probed lysates from a panel of tumor-derived melanoma cultures, and NSCLC or RCC cell lines by Western blot (Figure 2). Both antibodies were highly specific for their target, demonstrating a band that corresponded to a molecular weight of 48 kDa. Equal loading and protein content among samples was confirmed by β -Actin signal. PD-L1 levels varied significantly by tumor cell type. Expression levels were high or moderately high in six of ten NSCLC and two of six RCC lysates tested. By comparison, only two of the nine melanoma cell lines demonstrated PD-L1 expression and levels in these samples were much lower than in NSCLC and RCC lysates. For semi-quantitative assessment of protein levels, signal was normalized to β -Actin and fold increase above background signal was determined for each sample using ImageJ software. Expression levels in the corresponding cell lines by the two antibodies (5H1 and E1L3N) was highly correlated ($R = 0.94$). NSCLC cell lines had significantly higher PD-L1 levels than RCC or melanoma lysates by ANOVA (mean levels: 14,162 vs. 5,905 vs. 3,032, respectively for clone 5H1, $P = 0.047$; 21,612 vs. 11,158, vs. 1,828, respectively for clone E1L3N, $P = 0.014$, Supplementary Figure S1 C,D).

Association between PD-L1 expression in melanoma tumor versus tumor microenvironment cells and response to PD-1 based therapy

To assess the association between PD-L1 expression on the cell surface of either tumor cells (melanoma cells) or infiltrating immune cells (stromal cells) and the likelihood of response to PD-1 blockade (alone or in combination with ipilimumab), we constructed a TMA containing 3 cores from different areas of pre-treatment tumors from 65 melanoma patients. The diameter of the histocores is similar to that of biopsies, rendering TMAs a useful surrogate for clinical evaluation of metastatic samples, which is often based on biopsy rather than metastatectomy. The TMA was stained with either 5H1 or E1L3N antibodies and immunofluorescence scores were generated using methods previously described (29). Each variable was measured separately in either the tumor or stromal compartment for the two antibodies.

For patients who had two or three assessable histospots, a composite score was formed by calculating the mean value. The mean value was compared to the maximum score in these cases to determine whether there were significant differences, and we found a very strong correlation between mean and maximum values across measurements, with correlation coefficients ranging from 0.94 to 0.98; Supplementary Figure S2). Therefore, the rest of the analyses are based on mean values only. For patients who had only one histospot, a single value was used.

To assess the variability of PD-L1 staining between clones 5H1 and E1L3N, the mean QIF scores (mean intensity either within the tumor compartment or stroma) for each case from the corresponding TMAs were compared and found to be highly correlated ($R = 0.86$ and $R = 0.78$, respectively; Supplementary Figure S3).

To assess the association between PD-L1 expression and response to therapy, we compared PD-L1 expression in two patient groups: responders (complete response and partial response) and nonresponders (stable disease and progressive disease). Our analysis in the group of patients receiving monotherapy had a number of limitations; several were treated with pembrolizumab in an expanded access trial, some had received prior nivolumab and most had received multiple other lines of therapy, and the response rate was therefore lower than expected. We note that 11 of 31 cases with PD-L1 scores available for analysis, were treated with ipilimumab within three months of receiving anti-PD-1 monotherapy and were excluded from the analysis due to the potential interaction between the drugs. In the smaller group of 20 remaining patients treated with PD-1 inhibitor monotherapy, high PD-L1 expression in the stromal compartment was weakly associated with response to therapy, but this did not reach statistical significance ($P=0.07$ by Chi-square analysis of scores dichotomized by the median value, data not shown). Given the limitations of this cohort, both in terms of the lower than expected response rate and the small cohort size, the remainder of the analysis focuses on patients treated with the combination of ipilimumab and nivolumab.

In patients treated with combination therapy (ipilimumab and nivolumab), the time between tissue acquisition and initiation of immune checkpoint inhibitors ranged from 1–34 months (median-10, mean-11). Twenty-two patients received no intercurrent systemic therapies, six received one line of prior therapy and three received two prior regimens. Prior therapies included interleukin-2 (five patients), interferon (one patient), vemurafenib (three patients), dacarbazine, carboplatin plus paclitaxel and IL-21 plus nivolumab (one each). Unpaired t -tests showed that expression of PD-L1 was significantly higher in patients who achieved an objective response (complete or partial) than in nonresponding patients (Figure 3). PD-L1 expression in the stromal compartment, however, better discriminated between responders and non-responders than expression in tumor cells, with either the 5H1 or E1L3N antibodies.

We next assessed the association between continuous PD-L1 scores and progression free survival (PFS) or overall survival (OS) by Cox univariate analysis. High PD-L1 expression was associated with improved PFS and OS and this result was consistent between the two antibodies, particularly when PD-L1 was analyzed in the stromal compartment (Table 1). Kaplan-Meier survival curves for PD-L1 scores dichotomized by the median and PFS or OS are shown in Figures 4 and 5. With either antibody, PD-L1 expression in non-tumor cells was more strongly associated with outcome than expression in the S100 positive cells. In addition, we note that the percent area of PD-L1-expressing cells in this cohort ranged from 0–27.88% (median-1.93%, mean-4.72%). Tumors with PD-L1 intensity above the median had a corresponding mean PD-L1-positive percent area of 8.47 compared to 0.44 in low expressers.

On multivariable analysis, which included age, gender, LDH and American Joint Committee on Cancer (AJCC) M stage, PD-L1 expression was an independent predictor of prolonged PFS or OS (Supplementary Tables S2 and S3, respectively). All other variables included in the model were not associated with PFS or OS. To assess the association between PD-L1

expression and other clinical parameters, we used the Student *t*-test. There was no significant association between PD-L1 and age, gender, LDH or AJCC M stage (data not shown).

Quantification and characterization of TIL and association with PD-L1 expression

To characterize T cell infiltrates in the pretreatment specimens and assess correlations with response to therapy, the TMAs were stained with antibodies to CD3, CD4, CD8 and Foxp3. Staining was membranous for CD3, CD8 and CD4 while FOXP3 showed exclusively nuclear staining. TIL subpopulations were characterized by the percent area of each, CD3, CD4, CD8 and Foxp3 expressing cells within the total area of the histospot, as done in previous studies (28). Our group and others have previously shown that PDL1 expression correlates with the overall T cell infiltrate (29). In this cohort we further confirmed the association between high PD-L1 expression in tumor cells or stroma and high TIL densities as measured by CD3 positivity (Supplementary Figure S4). TIL populations were split in two groups with high and low TIL content determined by the median % area value. No association was found between the density of TIL subtypes or TIL ratios and response to therapy, PFS or OS.

DISCUSSION

PD-L1 expression is being used as a predictive biomarker for immune checkpoint inhibitors or incorporated into multi-parametric predictive biomarker models in numerous diseases. Here we show that expression of PD-L1 varies across tumor types. The expression was globally markedly higher in NSCLC tumors than RCC tumors, and expression was lower in melanomas. Moreover, the pattern of expression appears to differ between the three tumor types, with more expression on non-tumor cells within the tumor microenvironment in melanoma samples than in NSCLC or RCC. Most clinically used assays assess the overall percent of positively staining cells within the entire tumor to develop predictive biomarker models, and we therefore compared expression of PD-L1 in the tumor microenvironment and in tumor cells in pre-treatment samples from patients treated with immune checkpoint inhibitors. Expression in the tumor microenvironment appears to better predict response to therapy.

The confusion related to PD-L1 as a biomarker is multi-faceted, and due to technical issues of location and size of biopsy specimen, antibody variability and interpretation of results. Clinical trials using PD-L1 or PD-1 inhibitors have used a variety cut-points to determine “positive” versus “negative” expression, ranging from as low as 1% in the phase III trial of nivolumab versus everolimus in RCC, in which 75% of patients had < 1% tumor expression to as high as 50% in the phase III trial of pembrolizumab in NSCLC (7, 11). Most melanoma trials have used a cut-point of 5%. Variability in cut-points for positivity in the different studies might also be due to use of non-uniform antibodies and staining procedures. Our first goal was to determine whether the vast range of reported PD-L1 positivity in different studies was due to differences across tumor types versus differences in staining reagents. We utilized two antibodies, one that binds to the extracellular domain of PD-L1 (5H1) and the other than binds to the intracellular domain, E1L3N. Both antibodies were shown to be highly specific for the target, and staining patterns between the two did correlate in general.

When comparing the three tumor types for differences in global PD-L1 expression using uniform conditions with either antibody, we found that PD-L1 expression was significantly higher in NSCLC than RCC or melanoma, and somewhat higher in RCC than melanoma. These differences might account for the higher cut-points used in some NSCLC trials compared to melanoma, as our data indicate that a cutpoint of 50% in melanoma, would render all patients “negative” for PD-L1 expression.

Expression of PD-L1 was stronger in a panel of NSCLC cell lines, intermediate in RCC cell lines, while melanoma cell lines were least likely to express PD-L1, and PD-L1 positive cell lines had lower expression intensity than NSCLC or RCC, further indicating that activity of PD-1 or PD-L1 inhibitors in melanoma might be due to the interaction between PD-L1 in non-tumor cells and PD-1 in tumor infiltrating lymphocytes.

Our second purpose was to study the clinical significance of PD-L1 expression in the tumor versus the tumor microenvironment in melanoma samples. We employed TMAs to determine PD-L1 expression in up to three locations in the tumor, as the diameter of TMA histocores is similar to that of biopsies, rendering them a useful surrogate for clinical evaluation of metastatic samples, which is often based on biopsy rather than metastatectomy. Our studies were therefore not designed to determine location of PD-L1 positive cells within the tumor bed, and cannot be compared to findings of Taube and colleagues who have shown that an abundance of PD-L1 positive cells at the tumor edge, rather than deep with the tumor, is associated with response to therapy (35, 37).

We examined PD-L1 expression in either melanoma cells or non-tumor cells separately for each antibody. Our studies included only 34 patients who received anti-PD-1 monotherapy, of whom only 20 had not received ipilimumab within three months prior to initiation of PD-1 inhibitors. In this small group of 20, PD-L1 expression was weakly associated with response to therapy, with a P value that only trended towards significance. Given the small sample size and the relatively low response rate to therapy (<30% in this heterogeneous population that included many heavily pre-treated patients), these results are consistent with published data. This includes the recently published data by Daud et al. on PD-L1 expression on samples collected prior to pembrolizumab; in 451 pre-treatment specimens, PD-L1 tumor cell expression (defined as 1% staining as a cut-off) was associated with a greater likelihood of response to pembrolizumab (38). Here, we intentionally avoided using an arbitrary predetermined cut-point and binarized expression by the median – this might also explain the weak association between response and PD-L1 expression. In the group of 31 patients treated with concurrent CTLA-4/PD-1 checkpoint inhibition, PD-L1 expression was associated with response and improved progression-free and overall survival. Our purpose was not to generate a predictive biomarker, but rather to better understand the importance of the tumor compartment containing PD-L1 expressing cells in responding versus non-responding patients, and we found that expression in non-tumor cells was associated with response to a greater degree than expression in tumor cells, irrespective of the binding site of the antibody. This is supported by our cell line data which show that PD-L1 expression in melanoma tumor cells is weak, and by data reported by Herbst et al in multiple tumor types (26).

The biological significance of stromal PD-L1 expression has not been fully determined, however, PD-L1 has been shown to be expressed by macrophages and activated lymphocytes, presumably to decrease inflammation (34, 39). It is therefore plausible that PD-L1 in stromal cells in melanoma samples interacts with PD-1 on effector T cells, inhibiting their function. Moreover, Topalian et al have proposed that PD-L1 expression in the tumor microenvironment might be induced by interferon-gamma secretion by effector T cells (23). Response to immune checkpoint inhibitors, at least in melanoma, might therefore be dependent on active interaction between PD-1 on CD8 cells and PD-L1 in stromal cells which is inhibited by these drugs, rather than expression on tumor cells by mechanisms such as PTEN loss, as described in other tumor types (40).

PD-L1 is known to be an inducible protein; for example, interferon- γ upregulates PD-L1 expression, as does treatment with multiple drugs, including B-RAF inhibitors. Some of these patients received other systemic therapies between the time of tissue acquisition and initiation of treatment with immune checkpoint inhibitors, and with the small sample size used for this study combined with the lack of serial tumor sampling, we cannot determine the effects of prior therapies of PD-L1 tumor expression and response to therapy (41). In addition, PD-L1 is not only a predictive marker, but also a prognostic marker. In previous studies done by our group using the same quantitative immunofluorescence method, we showed that high PD-L1 expression in metastatic melanoma samples is associated with improved survival in patients who did not receive PD-1 inhibitors (29).

In summary, conflicting data in the literature regarding PD-L1 as a predictive biomarker for PD-1 inhibitors are due to use of inconsistent cutpoints for determining positivity, variable reagents and staining methods and variation in interpreting staining results. Here we show that tumor type and location of PD-L1 expression are additional variables that need to be considered; PD-L1 expression in melanoma cells and RCC cells is significantly lower than in NSCLC cells, and PD-L1 expression in stromal cells in melanoma samples is more predictive of response to PD-1 inhibitors than expression in tumor cells. These findings, combined with the higher response rate to PD-1 inhibitors in melanoma than NSCLC, suggest that mechanisms of immune evasion might differ between these two tumor types. Further assessment of PD-L1 expression in stromal cells as a predictive biomarker in melanoma patients treated with PD-1 inhibitors is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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STATEMENT OF TRANSLATIONAL RELEVANCE

Given the increasing use of PD-1/PD-L1 inhibitors in solid tumors, extensive efforts are underway to develop predictive biomarkers. PD-L1 expression by immunohistochemistry is the most widely-studied biomarker and is FDA approved for non-small cell lung cancer (NSCLC). However, PD-L1 status has been less useful in predicting treatment response in patients with melanoma or renal cell carcinoma (RCC). Using a uniform quantitative immunofluorescence staining method, we show that expression in NSCLC cells is markedly higher than in RCC, and lowest in melanoma cells. Conversely, PD-L1 staining in stromal inflammatory cells in melanomas can be higher than in tumor cells, and staining outside of the tumor cells better predicts tumor shrinkage in pre-treatment samples from melanoma patients treated with ipilimumab and nivolumab, currently the most active approved immunotherapy regimen. Further predictive biomarker studies of PD-L1 expression in stromal cells, alone or in combination with other biomarkers, are warranted to improve patient selection.

Figure 1.

Examples of strong PD-L1 fluorescent staining. Staining is shown in paraffin embedded pellets of MEL624 transfected to overexpress PD-L1 or parental cells, histospots non-small cell lung cancer (NSCLC), renal cell carcinoma (RCC) and melanoma specimens. Either an anti-S100 antibody or anti-cytokeratin (CK) antibody and fluorophore tyramide were used to distinguish tumor cells (green) from the surrounding stroma and leukocytes. 5H1 antibody (upper panels) or E1L3N antibody (lower panels) conjugated to Cyanine-5-tyramide (red) was used to visualize PD-L1. Overlaid images show PD-L1 expression within the entire histospot comprising both the tumor (orange/yellow) and stroma (red), at 10× magnification.

Figure 2.

PD-L1 levels in a panel of tumor-derived metastatic melanoma, non-small cell lung cancer (NSCLC) or renal cell carcinoma (RCC) cell lines. MEL-624 cell lines, overexpressing or not overexpressing PD-L1 were used as controls (boxed). Protein was extracted from NSCLC, RCC and melanoma cell lines and subjected to SDS-PAGE and Western blot analysis to detect PD-L1 with either the 5H1 or E1L3N antibody. The level of PD-L1 normalized to the β-actin level in each sample was measured by ImageJ software.

Figure 3.

Association of PD-L1 expression in the tumor or stromal compartments and response to therapy (ipilimumab and nivolumab concurrent therapy). Unpaired t-tests were utilized to compare PD-L1 expression and response to therapy; PD-L1 expression was higher in responders (CR+PR) compared to non-responders (SD+PD).

Figure 4.

Kaplan-Meier curves showing the association between PD-L1 expression and progression-free survival (PFS). The median PD-L1 intensity score, measured either in tumor cells or stroma by the 5H1 or E1L3N antibodies, was utilized to dichotomize our cohort into low/high categories. Patients with high PD-L1 levels in the stromal compartment had a longer median PFS compared to low expressers.

Figure 5. Kaplan–Meier curves showing the association between PD-L1 expression and overall survival (OS). PD-L1 scores were dichotomized by the median score in the tumor or stromal compartment. High PD-L1 level in the stromal compartment was significantly associated with prolonged OS.

Table 1

Univariate Cox regression analysis of continuous QIF scores for PFS and OS

Antibody	PD-L1 Scores	Association with PFS		Association with OS	
		Hazard Ratio (95% CI)	P	Hazard Ratio (95% CI)	P
Clone 5H1	Tumor Compartment	0.91 (0.82–0.99)	0.027	0.9 (0.79–1.00)	0.072
	Stromal Compartment	0.89 (0.79–0.97)	0.006	0.85 (0.71–0.96)	0.007
Clone E1L3N	Tumor Compartment	0.91 (0.75–1.07)	0.318	0.91 (0.72–1.11)	0.434
	Stromal Compartment	0.74 (0.55–0.95)	0.019	0.67 (0.44–0.93)	0.016