Characterization of PD-L1 Expression and Associated T-cell Infiltrates in Metastatic Melanoma Samples from Variable Anatomic Sites

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Abstract

Purpose: Programmed death ligand-1 (PD-L1) tumor expression represents a mechanism of immune escape for melanoma cells. Drugs blocking PD-L1 or its receptor have shown unprecedented activity in melanoma, and our purpose was to characterize tumor PD-L1 expression and associated T-cell infiltration in metastatic melanomas.

Experimental Design: We used a tissue microarray (TMA) consisting of two cores from 95 metastatic melanomas characterized for clinical stage, outcome, and anatomic site of disease. We assessed PD-L1 expression and tumor-infiltrating lymphocyte (TIL) content (total T cells and CD4/CD8 subsets) by quantitative immunofluorescence.

Results: High PD-L1 expression was associated with improved survival (P = 0.02) and higher T-cell content (P = 0.0005). Higher T-cell content (total and CD8 cells) was independently associated with improved overall survival; PD-L1 expression was not independently prognostic. High TIL content in extracerebral metastases was associated with increased time to developing brain metastases (P = 0.03). Cerebral and dermal metastases had slightly lower PD-L1 expression than other sites, not statistically significant. Cerebral metastases had less T cells (P = 0.01).

Conclusions: T-cell–infiltrated melanomas, particularly those with high CD8 T-cell content, are more likely to be associated with PD-L1 expression in tumor cells, an improved prognosis, and increased time to development of brain metastases. Studies of T-cell content and subsets should be incorporated into trials of PD-1/PD-L1 inhibitors to determine their predictive value. Furthermore, additional studies of anatomic sites with less PD-L1 expression and T-cell infiltrate are needed to determine if discordant responses to PD-1/PD-L1 inhibitors are seen at those sites. Clin Cancer Res; 1–9. ©2015 AACR.

Introduction

Melanoma is considered an “immunogenic” tumor, responsive to immunologic manipulation. Cytokine therapies, including high-dose IL2 and IFNα, have been used to treat melanoma for decades, with responses in subsets of patients (1–3). In recent years, immune checkpoint inhibitors, including monoclonal antibodies against CTLA-4, PD-1, PD-L1, LAG-3 and others, have been studied. Antibodies that activate costimulatory molecules on T cells, such as GITR, CD-137, OX-40, CD-27, and CD-40, have entered clinical trials, as reviewed (4, 5).

Ipilimumab, the first anti-CTLA-4 antibody, was FDA-approved in 2011 based on improvement in overall survival compared with a peptide vaccine (6). Pembrolizumab was the first PD-1 inhibitor to be approved (7, 8). Nivolumab, also a PD-1 inhibitor, was approved for patients with metastatic melanoma based on unprecedented activity (9, 10), and additional inhibitors of PD-1 and PD-L1 are being evaluated in clinical trials. Notably, response rates to nivolumab and pembrolizumab are approximately 30%, significantly higher than older immune therapies, and responses can be durable.

Attempts have been made to identify predictors of response to PD-1/PD-L1 inhibitors. The overall response rate in a phase I study of nivolumab was 24%, and in patients with available tissue for assessment of PD-L1 levels in tumor cells, there was a relationship between ligand expression and response. None of the PD-L1–negative tumors responded, whereas 36% of the PD-L1–expressing tumors responded (9). Of note, not all patients had melanoma. A second phase I trial using nivolumab in solid tumors showed a similar result (11). In the phase I study of pembrolizumab in patients with advanced melanoma, the percentage of patients with PD-L1–positive tumors was higher (71%), as reviewed (12). The response rates among patients with PD-L1–positive or –negative tumors were 49% and 13%, respectively, again indicating an association between PD-L1 positivity and response. When the threshold for positivity was raised to more than 10% of tumor cells, response rates in PD-L1–positive and –negative group increased to 52% and 23%. MPDL3280A is a monoclonal antibody to PD-L1; in a phase I trial of this drug in
Translational Relevance

Given the recent success of PD-1/PD-L1 inhibitors in clinical trials for metastatic melanoma, we used a well-annotated cohort of metastatic melanoma samples to characterize Programmed death ligand-1 (PD-L1) expression and T-cell content and the association with clinical outcome, using an automated quantitative method. PD-L1–expressing tumors were more likely to have higher tumor-infiltrating lymphocytes (TIL). Both overall TIL content and the percentage of CD8-positive cells were independent predictors of improved survival and retained their independence on multivariable analysis. Patients with specimens lacking inflammation were more likely to develop early brain metastases. Moreover, the anatomic site of biopsy appears to be associated with variability in TIL content. Biomarker studies of patients treated with PD-1/PD-L1 inhibitors should include specimens from multiple sites, when possible, to determine whether these differences are clinically meaningful and whether assays combining PD-L1 expression and TIL characterization might be more predictive of response to therapy than PD-L1 alone.

A variety of staining reagents and antibodies were used in these studies, and variable cutoffs for positivity and negativity were used; some studies used >1% tumor staining as a cutpoint for positivity whereas others used >5%. Despite the variability, studies consistently show that although PD-L1–positive tumors are more likely to respond to therapy, not all do, whereas some of the patients without apparent tumor or tumor microenvironment expression of PD-L1 do respond. Additional factors therefore affect activity of this class of agents. Taube and colleagues recently studied melanoma specimens from 110 patients (56 metastatic, 54 primary specimens) for presence of T cells and PD-L1 staining patterns (14). They identified 4 patient groups; 41% had tumors that were both PD-L1–positive and had associated tumor-infiltrating lymphocytes (TIL), 1% had PD-L1–positive tumors without TILs, 13% had TILs without PD-L1 positivity, and 45% had neither. Tumor inflammation and the type of infiltrating cells might affect response to therapy, as secretion of IFNγ by T cells is known to induce PD-L1 tumor expression (15). This interaction might potentiate activity of PD-1/PD-L1 inhibiting antibodies. The location of a metastatic tumor might affect the inflammatory process. For example, Hamilton and colleagues studied 26 matched brain and extracerebral metastases for gene expression profiling and found differences in pathways associated with T-cell activity (16). Our purpose was to expand on the studies by Taube and colleagues and further characterize T-cell infiltration in PD-L1–positive and –negative metastatic melanoma specimens and to determine the association with clinical outcome using a larger cohort of metastatic melanoma specimens, well annotated for clinical outcome. Seeing that melanoma tends to metastasize to the brain frequently, we studied the association between PD-L1 positivity and/or T-cell infiltrate and time to development of brain metastases. PD-L1 expression and T-cell infiltrates were studied in metastatectomy specimens from a number of sites, including brain lesions. To obtain more accurate measures of PD-L1 expression and TILs, we used a method of quantitative immunofluorescence and characterized TILs by CD4 versus CD8 expression, which has been used in a number of prior studies (17–19).

Materials and Methods

Cell lines and Western blotting

Early-passage melanoma cell cultures and cell lines were grown in OptiMEM media (Invitrogen) supplemented with 10% heat-inactivated FBS (Invitrogen) and antibiotic–antimycotic (penicillin, streptomycin, amphotericin B; Invitrogen). Western blotting was performed by standard methods. β-Actin was used to determine sample loading.

Tissue microarray construction

We constructed a TMA of metastatic melanoma samples using methods previously described (20–22). This TMA has been previously described (23). Collection of specimens and clinical data was approved by a Yale University Institutional Review Board. Specimens were resected between 2000 and 2011. A pathologist re-examined each case and selected a representative region of invasive tumor for coring. Each tumor was represented by 2 cores from different areas of the specimen. This cohort included 95 assessable cases.

Clinical characteristics

The cohort included 62% males and 38% females. Age at diagnosis ranged from 20 to 79 (mean, 55.4). Follow-up time was up to 187.65 months (mean, 31.2 months; ref. 23). At diagnosis of stage IV disease, 13% had M1a disease, 29% M1b, and 58% M1c. Lactate dehydrogenase (LDH) was elevated in 31% and BRAF and NRAS mutations were found in 51% and 24%. Time to development of brain metastasis was up to 187.65 months (mean, 19.69). About 71% received some type of systemic immunotherapy whereas 29% did not; 3 patients received PD-1 or PD-L1 inhibitors. Of the patients treated with immune therapy, 42% had sample acquisition after administration of immune therapy (one third received ipilimumab) and 58% had sample acquisition before. Specimens included metastases from several sites: lymph nodes (26), skin (11), soft tissue (25), and visceral metastases (53 total, 40 cerebral, and 13 extracerebral). In 17 cases, more than one metastatic site was represented.

To verify antibody specificity, we used control TMAs containing placental and tonsil tissue (known to be positive for PD-L1) and pellets from MEL-624 cell lines, overexpressing or not overexpressing PD-L1, as previously described (14, 17).

Immunofluorescence and automated quantitative analysis

TMAs were heated for 30 minutes at 60°C, deparaffinized and rehydrated through xylene and serial dilutions of EtOH and H2O. Slides were boiled at 100°C for 10 minutes in antigen retrieval buffer (Tris-EDTA, pH 9 buffer, DAKO) and incubated in peroxidase blocking reagent (K1500, DAKO) for 5 minutes. For PD-L1 staining, slides were incubated at room temperature for 15 minutes in ACE blocking buffer. To block endogenous biotin, slides were first incubated in Avidin blocking solution followed by Biotin blocking reagent (Vector Laboratories) for 15 minutes at 37°C. Slides were incubated at 4°C overnight with anti-PD-L1 mouse monoclonal antibody (clone 5H1,
generated by Dr. Lieping Chen) diluted at 1:400 in ACE block (24). Simultaneously, slides were incubated overnight with rabbit anti-S100 (1:100, cat#Z0311, DAKO) to localize cell membrane/cytoplasmic compartment and to create a tumor mask. As a negative control, a second TMA slide was incubated overnight in ACE block only. All TMAs were then incubated for 45 minutes at room temperature with biotinylated anti-mouse IgG (1:500, BA-2001, Vector laboratories), followed by incubation with the ABC kit (Vector Laboratories) for 15 minutes. Slides were covered with amplification reagent (K1500, DAKO) for 4 minutes at room temperature followed by Streptavidin-HRP (K1500, DAKO) for 15 minutes and then Cyanine-5-tyramide (Perkin Elmer) for target detection. For T-cell detection, serial slide cuts were incubated at 4°C overnight with rabbit anti-CD3 (1: 3,200, cat# A0452, DAKO) and simultaneously with either mouse anti-CD4 (1:400, cat#ab7310, DAKO), mouse anti-CD8 (1:200, cat#M7103, DAKO), mouse anti-FOXP3 (1:200, cat#ab22034, Abcam), or mouse anti-S100 antibodies in 0.3% bovine serum albumin/IBS. Goat anti-rabbit horseradish peroxidase (HRP)–decorated polymer backbone (Envision, Dako) was used to amplify the CD3 signal, visualized with Cyanine-3-tyramide. Slides were incubated twice for 7 minutes with 100 mmol/L benzoic hydrazide (Sigma) and 50 mmol/L hydrogen peroxide in PBS to quench the HRP. Goat anti-mouse HRP-decorated polymer backbone (Envision, Dako) followed by Cyanine-5-tyramide were used to detect signal for CD4 and CD8. For visualization of S100, a secondary goat IgG conjugated to Alexa 546 (Molecular Probes, Inc.) was used. A nuclear mask was created by incubating the slides with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen; dilution, 1:500).

Quantitative determination of target expression

Details of image capturing and algorithms used for quantitative determination of target expression have been previously described (25). PD-L1 signal on cells within the S100 tumor mask was compartmentalized and expressed as average signal intensity within the assayed component (AQUA score), with scores on a scale of 0 to 255. To assess the degree of TIL, we used linear correlation was conducted by calculating the percentage of TILs of the total tumor area. We conducted survival curves were constructed using the Kaplan–Meier method.

Statistical analysis

JMP version 5.0 software was used for analysis (SAS Institute). Associations between continuous AQUA scores and clinical/pathologic parameters were assessed by analysis of variance. Cox proportional hazards analysis was used to test the association between expression of PD-L1 and T-cell content and survival. Survival curves were constructed using the Kaplan–Meier method.

Results

PD-L1 expression and its association with survival in patients with stage IV metastatic melanoma

Anti-PD-L1 antibody specificity was verified by Western blotting on 15 melanoma cell lines, including MEL624, which was nontransfected or transfected to overexpress PD-L1. This antibody was highly specific, as it generated a single dominant band corresponding to the correct molecular weight (Supplementary Fig. S1). As expected, PD-L1 was expressed at high levels in MEL624 PD-L1+ cells but not the nontransfected cells and was variably expressed in other melanoma cell lines; 5 had no detectable PD-L1 levels, 6 showed moderate to high PD-L1 expression, and 2 had low-level expression.

To optimize staining conditions for quantitative immunofluorescence (QIF), we constructed a TMA containing positive tissue controls, including human tonsil tissue, human placenta, and cores prepared from the MEL624 PD-L1+ or MEL624 PD-L1− cells. We further confirmed 5H1 antibody specificity by QIF (Fig. 1A).

To characterize expression of PD-L1 and T-cell infiltrates in metastatic melanoma, we used an array with 95 cases. Staining for PD-L1 was membranous and present in most cases within the tumor compartment, created by the S100 mask. Figure 1B shows an example of a PD-L1− positive case. In some cases, PD-L1 was also expressed outside the tumor mask in the tumor microenvironment. Histospots were excluded if tissue was rendered insufficient (<3% of the histospot area) and if there was anomalous staining due to either abundant necrotic tissue or high background of the specimen on the negative control slide due to high endogenous biotin.

Using the Pearson correlation test, we compared scores from the 2 cores for each specimen and found good intratumor reproducibility between matching cores for PD-L1 expression; R = 0.87 (P < 0.0001). Thus, average AQUA scores were calculated for each case. The distribution of AQUA scores for PD-L1 ranged from 2.99 to 113.6 (mean, 19.54). PD-L1 expression in tumor cells was previously shown to predict better outcome in metastatic melanoma (14). To verify this using our automated method, we analyzed the association between AQUA scores, dichotomized into “high” and “low” by the median score (15.43) and overall survival (OS). By Cox univariate analysis of dichotomized AQUA scores, high PD-L1 expression was associated with longer survival and a 29% risk reduction [HR = 0.71; confidence interval (CI), 0.52–0.95; P = 0.02]. To depict the association between high PD-L1 expression and improved survival, we used the Kaplan–Meier method; high PD-L1 is significantly associated with longer OS (Fig. 2A; median, 36.06 vs. 20.27 months; log-rank, P = 0.02). On multivariable analysis, PD-L1 did not retain independent prognostic value (P = 0.23). Other variables included in the model, age, gender, M stage, and LDH, were not independently associated with survival.

By ANOVA, we found no significant association between PD-L1 expression and other clinical variables, including age, gender, LDH levels, M stage, or presence of BRAF and NRAS mutations. About 71% of these patients received some type of systemic immunotherapy whereas 29% did not and 3 received PD-1/PD-L1 inhibitors. There was no survival difference between the 2 groups (data not shown), suggesting that the association between PD-L1 expression and improved survival is not dependent on systemic immune therapy in patients who did not receive PD-1/PD-L1 inhibitors.

Quantification of TIL and association with PD-L1 expression

Taube and colleagues previously showed that the majority of PD-L1–positive tumors also contained TILs (14). To verify this, we used AQUA to quantify the degree of TIL as the percentage area of CD3-expressing cells. Quantification was conducted by calculating the percentage of TILs of the total tumor area. We conducted further subset analysis by studying CD4- and CD8-expressing TILs. To assess heterogeneity within each case, we used linear regression and found a high degree of correlation between CD3 content in matching cores representing each tumor (R = 0.78). Correlation coefficients were somewhat lower for CD4 and CD8...
subsets ($R = 0.52$ for CD4 TILs, $R = 0.65$ for CD8 TILs). Approximately half the metastatic melanomas demonstrated presence of intratumor immune infiltrates, consistent with findings of Taube and colleagues (14). Given this reported distribution, we used the median value of CD3TIL area as a threshold for defining “high” TIL density versus “low” TIL density melanomas. The area of CD3 TILs ranged from 0% to 89.96% (mean, 7.53%; median, 1.94%). Most cases contained both CD4- and CD8-positive T-cell subsets, and only a minority of cases were only CD8-positive; 70% of the high TIL cases (CD3 TIL area above the median) had both high CD4 and CD8 TIL infiltration.

In the majority of cases, high PD-L1 expression was associated with high TIL densities as measured by the presence of CD3. High PD-L1 was similarly associated with infiltration of both CD4 and CD8 T-cell subsets. Associations measured by $\chi^2$ analysis and by two-sample $t$ test using continuous PD-L1 scores are shown...
in Table 1 and Supplementary Fig. S2. An example of a TIL dense tumor spot demonstrating high CD3, CD4, and CD8 lymphocytic infiltrations and high PD-L1 expression is shown in Fig. 1B.

Given the association between PD-L1 expression and TIL presence, we studied the association between tumor inflammation and overall survival. Using the Cox proportional hazards method, high TIL densities were associated with improved OS. The association was statistically significant when analyzing all TILs (by CD3 staining) and the CD8 subset but not CD4-positive cells (HR, 0.65; CI, 0.49–0.85; P = 0.0022 for CD3 TILs; HR, 0.75; CI, 0.57–0.98; P = 0.04 for CD8 TILs; HR, 0.87; CI, 0.65–1.14; P = 0.32 for CD4 TILs). To visualize the association between TIL density and survival, Kaplan–Meier curves were generated and P values corresponding to these curves were obtained by the Mantel–Cox log-rank method; Fig. 2B–D shows significant differences in survival between patients with high versus low CD3-positive TILs and CD8-positive subset of TILs but no differences based on CD4 expression. On multivariable analysis, high density of both CD3 cells and CD8 subset had independent prognostic value (HR, 0.57; CI, 0.34–0.94; P = 0.02 and HR, 0.62; CI, 0.40–0.99; P = 0.05, respectively; Supplementary Tables S1 and S2). PD-L1 expression did not retain its independent prognostic value in these models, likely due to its association with CD3-positive TIL. No significant association was found between TIL density (CD3-positive or CD4- and CD8-positive subsets) and other clinical variables, including age, gender, M stage, LDH levels, or presence of BRAF and NRAS mutations.

To further characterize the TIL population, we studied FOXP3 expression. Expression was somewhat variable in replicate histospots from the same patient (R = 0.48) and was slightly higher in tumors with high PD-L1 (P = 0.07 by \chi^2 analysis). FOXP3 was not associated with OS.

PD-L1 expression and TIL infiltration in melanoma brain metastases compared with extracerebral metastases

Activity of PD-1/PD-L1 inhibitors is currently being studied in patients with untreated melanoma brain metastases, but PD-L1 expression and lymphocytic infiltration have not been studied in cerebral metastases. We therefore analyzed 40 cerebral metastases included in our TMA. The distribution of AQUA scores for PD-L1 in these specimens ranged from 3.74 to 26.7 (mean, 13.89). ANOVA of PD-L1 AQUA score distribution across different metastatic sites showed that brain and skin metastases had a trend toward lower PD-L1 expression compared with lymph node, soft tissue, and other visceral sites, although differences did not reach statistical significance (P = 0.14, Fig. 3A). This difference was not significant by two-way comparison of dichotomized PD-L1 scores comparing cerebral with extracerebral sites (P = 0.31, Table 2).

The percentage area of CD3-positive TILs in brain metastatic specimens was 0% to 29.75% (mean, 4.6%; median, 1.8%). Comparing across all sites of metastasis by ANOVA, locations with lowest PD-L1 expression (brain and skin metastases) also had the least infiltration of CD3-expressing lymphocytes. The same finding was demonstrated for CD4- and CD8-expressing subsets. The association was statistically significant for CD3 TILs (P = 0.02) and trended toward significance for the CD8-expressing TILs (P = 0.08; Fig. 3B and D). By \chi^2 analysis comparing high and low T-cell–dense cases, cerebral metastases were more likely...
to have low T-cell content than extracerebral metastases (P = 0.01 for CD3 TILs). The association trended toward significance for the CD4 subset and was significant for the CD8 subset (P = 0.09 and P = 0.01, respectively; Table 2).

Patients in this cohort were closely followed with serial surveillance MRI of the brain. We studied the association between brain metastasis-free survival (BMFS), defined as the interval between diagnosis of metastatic disease and development of brain metastases, and PD-L1 expression and lymphocytic infiltration. By Cox proportional hazards, high TIL densities in extracerebral sites were associated with increased BMFS. This was statistically significant when analyzing all TILs by CD3 staining (HR, 0.76; CI, 0.58–0.99; P = 0.04). There was a trend toward significance for the CD8 subset but not for PD-L1- and CD4-positive cells (HR, 0.8; CI, 0.61–1.04; P = 0.09 for CD8 TILs; HR, 0.89; CI, 0.67–1.19; P = 0.4; for PD-L1; HR, 0.98; CI, 0.74–1.28; P = 0.9 for CD4 TILs). Kaplan–Meier curves with log-rank statistics demonstrating these associations are shown in Fig. 4. On multivariable analysis, using the Cox proportional hazards model, high TIL density was not an independent predictor of increased BMFS (P > 0.05).

**Discussion**

With the recent unprecedented success of PD-1/PD-L1 targeting antibodies for treating metastatic melanoma, our purpose was to further characterize PD-L1 expression in metastatic melanoma in the context of T-cell infiltrates. High PD-L1 expression was associated with improved OS but was not independent on multivariable analysis. Only 3 patients were treated with inhibitors of PD-1 or PD-L1 on the basis of preliminary observations among patients with melanoma treated with these drugs, the association between PD-L1 expression and TIL content and the association between PD-L1 expression and survival. PD-L1 expression is upregulated by T-cell secretion of IFNγ, and therefore patients with T-cell dense tumors expressing PD-L1 are likely to have a baseline increased immunosurveillance (24). In our cohort, the association appears to be independent of systemic immune therapy, although only 3 patients were treated with inhibitors of PD-1 or PD-L1. On the basis of preliminary observations among patients with melanoma treated with these drugs, the association between PD-L1 expression and TIL content and overall survival is likely to be stronger now that PD-1 inhibitors have been approved for this population (26).

We conducted additional studies of subsets of T cells by analyzing CD4 and CD8 cell content. Both subsets were strongly associated with PD-L1 expression. However, only CD8 infiltrates

**Table 2.** χ² analysis of PD-L1 expression and TIL density in melanoma brain metastases

<table>
<thead>
<tr>
<th>Site of metastases</th>
<th>CD3 TILs (P = 0.01)</th>
<th>CD4 TILs (P = 0.09)</th>
<th>CD8 TILs (P = 0.01)</th>
</tr>
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<tbody>
<tr>
<td>Cerebral</td>
<td>Low density</td>
<td>High density</td>
<td>Low density</td>
</tr>
<tr>
<td>Extracerebral</td>
<td>46.3% (25/54)</td>
<td>53.7% (29/54)</td>
<td>43.3% (26/60)</td>
</tr>
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**Figure 4.** Kaplan–Meier curves showing the association between PD-L1 expression (A) or TIL density (B–D) and time to development of brain metastases from the time of diagnosis of metastatic melanoma. The median PD-L1 intensity score was used to dichotomize scores into low/high, whereas the median CD3 TIL areas were used as thresholds for defining high/low TIL density. High density of CD3-positive TILs was significantly associated with a longer time to development of brain metastases.
were associated with improved OS. This is not surprising given the role of memory and effector CD8 cells in the inflammatory response to cytokine secretion and in preventing tumor invasion and metastasis (27, 28). Our study was not designed to determine the regulatory versus helper CD4+ cell components, but the former likely explains the lack of association between CD4+ tumor content and improved survival (29). However, our studies support those of Spranger and colleagues who demonstrated coexistence of CD8+ cells and CD4+ suppressive cells, including FOXP3+ regulatory T cells and PD-L1–activated T cells and suggested that this is due to compensatory mechanisms of tumor immune escape (30). Our findings also support those by Hamilton and colleagues who found that increased TIL content (both CD3 and CD8) in patients with brain metastases was associated with improved survival (16).

Published predictive correlative studies done on multi-institutional trials have focused primarily on PD-L1 expression. While this clearly correlates with T-cell content (total and subsets), the prognostic value of CD3 TILs, in particular the CD8 subset, which is independent of PD-L1 expression, suggests that this should be included in predictive biomarker studies of patients treated with PD-1/PD-L1 inhibitors. This has been shown in a small study of multiple tumor types (melanoma, renal cell carcinoma, and lung cancer) treated with nivolumab at a single institution (31). Tumor PD-L1 expression remained the strongest predictor of response to nivolumab; however, it is possible that a model incorporating both might best predict response to PD-1/PD-L1 inhibitors.

Our TMA did not include analysis of multiple sites of metastases from individual patients and did not allow for intrapatient comparison of PD-L1 expression and T-cell content. In previous studies in renal cell carcinoma, we showed that in a given patient, PD-L1 expression can vary at different anatomic sites (17). Across all metastatic sites, PD-L1 expression appears to be somewhat lower in brain and skin metastases compared with other sites, although differences were not statistically significant, and the corresponding T-cell infiltration analysis in brain and skin metastases indicated that metastases at these sites indeed have less T-cell content. This is likely due to variability in factors associated with the tumor microenvironment. For example, higher PD-L1 tumor expression in lymph node metastases might be due to cytokine secretion by surrounding lymphocytes (32). Some organs, such as the lungs, have an abundance of dendritic cells and macrophages, cells that secrete IFNγ (33, 34). This might result in increases in tumor PD-L1 in these organs.

Recent studies by Tumeh and colleagues and Taube and colleagues showed that the location of T-cell infiltrate and PD-L1–expressing cells within a tumor sample is of great importance; presence of CD8 TILs and PD-L1–expressing cells at the invasive margin is associated with a greater likelihood of response to therapy (31, 35). Our study used TMAs that were constructed using tissue from the most central location when possible. Many patients in this cohort did not have full metastasectomies, but rather incisional biopsies, and the invasive margin could not be determined. However, we believe that cores used in TMAs are reflective of clinical practice in which core or incisional biopsies are conducted at metastatic sites, often using image-guided techniques to access the center of the tumor, rather than full excisions. For predictive studies, however, given the variability in PD-L1 expression and TIL content within a tumor sample, when possible, metastasectomies should be studied rather than core needle or incisional biopsies.

Studies of the association between PD-L1 expression and response to PD-1/PD-L1 targeting drugs using variable cutpoints and reagents demonstrate that patients whose tumors express PD-L1 are more likely to respond to therapy (12). Using a more quantitative method, we demonstrated variability in the degree of PD-L1 expression at different anatomic sites. We note that we were not able to study multiple sites of metastasis in individual patients, and with the data available to us at present, we cannot determine intrapatient variability in PD-L1 expression at different anatomic sites. Taube and colleagues analyzed some patients with multiple specimens from different anatomic sites (31). They noted some differences in PD-L1 staining and in T-cell infiltrate but found that the highest scoring sample among multiple biopsies was the most likely to predict response to PD-L1 therapy. Inhibition of the PD-1/PD-L1 interaction at one site might result in increased TIL activity at that site and release of cytokines and circulating effector cells, which can result in antitumor T-cell activity at distant sites that might be PD-L1 negative. Alternatively, differences in PD-L1 expression and T-cell infiltrate at different anatomic sites might explain discordant responses seen in some patients, who might have shrinkage in one location and growth in another (7–10). The strong correlation between PD-L1 expression and T-cell content within the tumor (as represented by the S100 tumor mask) further supports studies by Tumeh and colleagues, who demonstrated close proximity between PD-L1- and PD-1-expressing cells (35). However, a limitation of our method is the inability to distinguish PD-L1 T-cell expression from PD-L1 melanocyte expression within the tumor mask and PD-L1 staining on macrophages, monocytes, and microglial cells. Methods such as flow cytometry can determine PD-L1 expression within cellular subsets, although flow cytometry cannot be interpreted within the histologic context. However, newer in situ methods that involve multiplexing of additional cellular components are being developed and might facilitate measurement of PD-L1 expression in additional cell types, such as macrophages, in the tumor microenvironment. Future studies should incorporate more quantitative measures of PD-L1 expression and T-cell content, and when possible, more than one metastatic site should be studied, with a particular focus on the site most requiring treatment, rather than previously resected tumor sites.

Brain metastases pose a particular challenge in the treatment of metastatic melanoma. The incidence of cerebral dissemination in this disease is highest of all malignancies (36). Historically, the prognosis has been poor, but with improved methods of local control of brain lesions, the prognosis is improving, and additional studies of this patient population are warranted. Our study showed increased time to brain metastases in PD-L1–positive tumors with high TIL content. However, the numbers in our study are small and validation in additional patient cohorts, particularly patients with paired brain and extracerebral samples, is warranted. Moreover, our study is inherently limited by the fact that these metastasectomy samples were obtained at variable time points after diagnosis of metastatic disease, and inflammatory content might change with time and with therapy.

Ongoing studies of PD-1 inhibitors in patients with untreated brain metastases (such as NCT02085070) will enable us to determine whether brain lesions are biologically different in terms of T-cell content and activity and whether brain metastases can be treated systemically with PD-1 inhibitors. The lower PD-L1 expression and TIL infiltrate in dermal metastases in our study provides some reassurance that the differences might not have
therapeutic implications, as clinical experience indicates that dermal metastases are not less sensitive to this class of drugs.

In summary, our studies confirm previous studies by Taube and colleagues showing an association between PD-L1 expression and improved prognosis. These studies were conducted in a larger metastatic melanoma cohort using an automated quantitative method. We further confirmed the association between PD-L1 expression and T-cell infiltrate (by CD3 positivity) and characterized the T-cell subtypes. Both overall TIL content and the percentage of CD8-positive cells were independent predictors of improved survival and retained their independence on multivariable analysis. High TIL content in extracerebral specimens was associated with increased time to developing brain metastases, suggesting that patients with higher TIL density tumors might be less likely to have tumor dissemination to the brain. Our study did not include multiple specimens from different anatomic sites in individual patients, but the anatomic site of biopsy appears to be associated with variability in PD-L1 expression and T cell content, with dermal and cerebrocerebral metastases having a lower TIL content and less PD-L1 expression. Biomarker studies of patients treated with PD-1/PD-L1 inhibitors should include specimens from multiple sites, when possible, to determine whether these differences are clinically meaningful.

Disclosures of Potential Conflicts of Interest

M. Sznl is a consultant/advisory board member for Astra-Zeneca, Bristol-Myers Squibb, Genentech, and Pfizer. D.L. Rimm is a consultant/advisory board member for Bristol-Myers Squibb. L. Chen is a consultant/advisory board member for MedImmune and Pfizer. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H.M. Kluger, C.R. Zito, L. Chen, L.B. Jilaveanu
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