

Association of PD-1, PD-1 Ligands, and Other Features of the Tumor Immune Microenvironment with Response to Anti-PD-1 Therapy

Janis M. Taube^{1,2,3}, Alison Klein^{2,3,5}, Julie R. Brahmer³, Haiying Xu¹, Xiaoyu Pan³, Jung H. Kim¹, Lieping Chen⁶, Drew M. Pardoll³, Suzanne L. Topalian⁴, and Robert A. Anders²

Abstract

Purpose: Immunomodulatory drugs differ in mechanism-of-action from directly cytotoxic cancer therapies. Identifying factors predicting clinical response could guide patient selection and therapeutic optimization.

Experimental Design: Patients ($N = 41$) with melanoma, non-small cell lung carcinoma (NSCLC), renal cell carcinoma (RCC), colorectal carcinoma, or castration-resistant prostate cancer were treated on an early-phase trial of anti-PD-1 (nivolumab) at one institution and had evaluable pretreatment tumor specimens. Immunoarchitectural features, including PD-1, PD-L1, and PD-L2 expression, patterns of immune cell infiltration, and lymphocyte subpopulations, were assessed for interrelationships and potential correlations with clinical outcomes.

Results: Membranous (cell surface) PD-L1 expression by tumor cells and immune infiltrates varied significantly by tumor type and was most abundant in melanoma, NSCLC, and RCC. In the overall cohort, PD-L1 expression was geographically associated with infiltrating immune cells ($P < 0.001$), although lymphocyte-rich regions were not always associated with PD-L1 expression. Expression of PD-L1 by tumor cells and immune infiltrates was significantly associated with expression of PD-1 on lymphocytes. PD-L2, the second ligand for PD-1, was associated with PD-L1 expression. Tumor cell PD-L1 expression correlated with objective response to anti-PD-1 therapy, when analyzing either the specimen obtained closest to therapy or the highest scoring sample among multiple biopsies from individual patients. These correlations were stronger than borderline associations of PD-1 expression or the presence of intratumoral immune cell infiltrates with response.

Conclusions: Tumor PD-L1 expression reflects an immune-active microenvironment and, while associated other immunosuppressive molecules, including PD-1 and PD-L2, is the single factor most closely correlated with response to anti-PD-1 blockade. *Clin Cancer Res*; 20(19); 5064–74. ©2014 AACR.

Introduction

Although there is abundant *in vitro* evidence for human immune reactivity against solid tumors, such responses are often ineffectual *in situ*, as the tumor exerts locally immunosuppressive effects. Current cancer immu-

notherapies focus on overcoming this inhibition, either by global activation of the immune system or by local manipulation of immunoregulatory molecules in the tumor microenvironment, including so-called immune checkpoints. Ipilimumab, the prototype drug directed against an immune checkpoint, is a mAb that blocks the coinhibitory CTLA-4 receptor on T cells from interacting with its ligands, B7-1 and B7-2, expressed on antigen-presenting cells but not on solid tumors. Ipilimumab was the first drug to demonstrate increased overall survival in patients with advanced melanoma (1), and there is ongoing investigation to identify biomarkers to optimize patient selection for this therapy.

Following proof-of-principle studies with anti-CTLA-4, attention has turned to the PD-1:PD-L1/PD-L2 immunologic synapse. Programmed death-1 (PD-1) is expressed on activated T and B cells (2). Its major ligand PD-L1 (B7-H1) is typically expressed on a subset of macrophages, but can be induced by inflammatory cytokines in a variety of tissue types (3–7). When activated T cells expressing PD-1

Departments of ¹Dermatology, ²Pathology, ³Oncology, and ⁴Surgery, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine; ⁵Department of Epidemiology, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland; and ⁶Department of Immunobiology, Yale University, New Haven, Connecticut

Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org>).

Corresponding Authors: Janis M. Taube, The Johns Hopkins School of Medicine, 401 N. Wolfe St., Baltimore, MD 21287. Phone: 410-955-3484; Fax: 410-955-2445; E-mail: jtaube1@jhmi.edu; and Suzanne L. Topalian, Department of Surgery, 1550 Orleans St., CRB2 Room 508, Baltimore, MD 21287. Phone: 410-502-8218; Fax: 410-502-1958; E-mail: stopali1@jhmi.edu

doi: 10.1158/1078-0432.CCR-13-3271

©2014 American Association for Cancer Research.

Translational Relevance

One of the most intriguing findings from early clinical trials of PD-1 pathway blockade for advanced solid tumors has been the correlation between pretreatment tumor PD-L1 expression and treatment response. The current study expands on this observation by exploring PD-1, PD-L1, and PD-L2 expression by tumor cells and infiltrating immune cell subsets, and their relationships to each other and to clinical response to anti-PD-1 (nivolumab). Significant associations were found among tumor cell PD-L1 expression, the presence of intratumoral immune cell infiltrates, and PD-1 receptor expression by tumor-infiltrating lymphocytes, suggesting that PD-L1 reflects an immune-reactive milieu. However, among these parameters, tumor cell PD-L1 expression was most closely associated with response to anti-PD-1 therapy. PD-L1 expression was also significantly associated with tumor types responding to anti-PD-1 (melanoma, lung, and kidney cancer), suggesting that it could provide a means for identifying additional tumor types which may respond to PD-1 pathway blockade.

encounter PD-L1, T-cell effector functions are diminished. PD-1 also binds PD-L2 (B7-DC), which is expressed selectively on macrophages and dendritic cells (7–9). These unique expression patterns suggest that PD-L1 promotes self-tolerance in peripheral tissues, whereas PD-L2 may function in lymphoid organs, although the role of PD-L2 in immunomodulation is not as well understood (10). Multiple tumor types have been shown to express PD-L1 and PD-L2, effectively co-opting a native tolerance mechanism (11–13). The more selective expression pattern of the ligands for PD-1, relative to those for CTLA-4, has important treatment implications. First, it suggests that more focal immune-related side effects may be encountered with PD-1 blockade compared with CTLA-4 blockade, which is also predicted by the phenotypes of murine genetic knockout models (14, 15). Second, it suggests that the local tumor microenvironment may be the key site to yield evidence of molecular markers predicting clinical response to PD-1 pathway blockade.

Clinical trials of blocking mAbs against PD-1 and PD-L1 are currently under way for patients with treatment-refractory metastatic melanoma and a variety of epithelial malignancies, and have validated this pathway as a therapeutic target (16–19). We have previously reported that approximately 30% of patients with treatment-refractory advanced melanoma and renal cell carcinoma (RCC) receiving anti-PD-1 (nivolumab) experienced durable objective tumor regressions (16). Objective tumor responses were also observed in 17% of patients with non-small cell lung carcinoma (NSCLC). Preliminary analysis of pretreatment tumor specimens from a subset of 42 among 306 patients treated on this study identified tumor cell surface PD-L1 expression as one factor associated with the clinical activity

of anti-PD-1 therapy (16, 17). The purpose of the current study is to expand on this initial observation by exploring additional components of the pretreatment tumor microenvironment in patients receiving nivolumab therapy, including infiltrating immune cell subsets, the expression of PD-1, PD-L1, and PD-L2 by immune cells and by tumor cells, and the potential interrelationship of these factors to each other and to clinical response following PD-1 blockade.

Materials and Methods

Case selection

Tumor specimens were derived from 41 patients with advanced, treatment-refractory solid tumors, including NSCLC, melanoma, kidney, castration-resistant prostate cancer (CRPC), and colorectal cancer who were treated on a clinical trial of nivolumab (anti-PD-1; BMS-936558, MDX-1106, ONO-4538) at the Johns Hopkins Kimmel Cancer Center (NCT00730639; ref. 16). All patients signed informed consent. Patients received at least 3 of 4 planned biweekly doses of anti-PD1 in the first treatment cycle, had treatment responses that were evaluable, and had formalin-fixed paraffin-embedded (FFPE) archival or newly obtained pretreatment tumor specimens available for study. Responses were classified by the investigators according to RECIST version 1.0 with modifications (20). Tumor specimen characteristics, including primary or metastatic lesion, anatomic site (lymph node, lung, or other), specimen size, and the time interval from specimen acquisition to treatment initiation, were noted.

Histopathologic analysis

The pathologic diagnosis was confirmed by one of two board-certified pathologists (J.M. Taube or R.A. Anders) who reviewed FFPE tissue sections stained with hematoxylin & eosin (H&E), and a representative paraffin block from each specimen was chosen for IHC analysis. The size of the specimen and the presence of necrosis and lymphoid aggregates (collections of approximately 100 lymphocytes without germinal center formation, Supplementary Fig. S1) were noted. Specimens were considered "small" if they were core needle biopsies or tissue blocks made from fine needle aspirate specimens containing tissue fragments. Cytology specimens where tissue architecture was not represented were not included in this study. IHC for PD-L1 and PD-1 was performed using the mAbs 5H1 and M3, respectively, as previously described (12, 21). Methods for PD-L2 IHC with the mAb MIH18 (BioLegend) are described in Supplementary Material. Positive control specimens for PD-L1 IHC were created by transfecting cultured human melanoma 624-mel cells with a recombinant plasmid encoding full-length human PD-L1 (12). PD-L1 expression was sometimes observed in the background native tissue, e.g., alveolar macrophages in lung specimens and dendritic cells in nonneoplastic lymph node parenchyma from lymph node dissections, providing an internal positive staining control. An isotype

control was used as a negative control for each case stained for PD-L1, to control for potential false-positive staining. Tonsil tissue served as both a positive and negative control for PD-L2, and PD-1 staining, due to cell-type-specific endogenous expression of these molecules (dendritic cells and activated lymphocytes, respectively). IHC for CD3, CD4, CD8, CD20, and CD68 was performed according to standard automated protocols.

Cell surface ("membranous") PD-L1 and PD-L2 expression by tumor cells and tumor-infiltrating immune cells, including lymphocytes (TIL) and histiocytes, was independently scored by two pathologists blinded to clinical outcomes, as previously described (12). Specifically, cases were scored at 5% intervals. Specimens with $\geq 5\%$ membranous expression were considered "positive". CD3 and CD68 immunostains were performed on each case and were used to interpret which cell type demonstrated expression of a given ligand. The intensity of immune infiltrates was assigned a semiquantitative score from 0 to 3: 0 = "none" (no immune infiltrates), 1 = "focal" (mostly perivascular in tumor with some intratumoral extension), 2 = "moderate" (prominent extension of immune infiltrates away from perivascular areas and amongst tumor cells), or 3 = "severe" (immune infiltrates obscuring tumor). The presence of CD20⁺ TILs (B cells) was scored according to the same criteria.

Intratumoral CD4:CD8 T-cell subset ratios were determined as 1:1, 1:2, 1:3, or 1:4. The geographic association of immune infiltrates with tumor PD-L1 expression was noted. The proportion of TILs expressing PD-1 was scored as "focal" (isolated, $<5\%$ of lymphocytes), "moderate" (5%–50% of TILs expressing PD-1), or "severe" ($>50\%$ of TILs PD-1⁺).

Statistical analysis

The geographic associations of immune cell infiltrates and tumor characteristics were evaluated using the Fisher exact test and χ^2 test. We examined the correlation between proportion of tumor cells expressing PD-L1 and the intensity of immune cell infiltration using the Kruskal–Wallis rank test. For individual patients with multiple tumor specimens, each specimen was treated as an independent variable. When analyzing the association of pathologic features with treatment outcomes in patients with multiple specimens, the analysis was conducted in two different ways: first, by considering only the specimen that was procured closest to the date of treatment initiation, and second, by selecting the highest expression of each variable across all specimens from that patient. Statistical analyses were performed with the STATA V11 software package. All tests were two-sided except as indicated, and *P* values of <0.05 were considered significant.

Results

Patient characteristics

Among 306 response-evaluable patients on this multi-institutional trial, 60 were treated in the Kimmel Cancer Center at Johns Hopkins. From this group of 60 patients,

98 potential pretreatment tumor specimens were identified for study. However, 14 specimens were not available (outside hospitals did not participate in research or did not send sufficient material for study, or material could not be located), 14 were exhausted (no definitive invasive tumor remaining, not diagnostic without tumor-specific IHC, or tissue architecture not preserved), and 2 had technical difficulties (material not suitable for IHC, or excessive isotype control background staining). The final study cohort consisted of 68 pretreatment tumor specimens that were archival or newly obtained from 41 different patients, as summarized in Table 1. Specimen acquisition dates ranged from December 1997 to June 2011, with a mean time from acquisition to IHC staining of 3.1 years, and a median of 2 years (range, 0–13 years). The time interval between biopsy acquisition and first dose of anti-PD-1 ranged from 1 day to 12 years. Sixteen of 41 patients (39%) had multiple specimens available for analysis.

PD-L1 expression by tumor and infiltrating immune cells

Tumor PD-L1 expression varied significantly by tumor type, with the majority of 56 melanoma, NSCLC, and

Table 1. Characteristics of 68 pretreatment tumor specimens derived from 41 patients receiving anti-PD-1 therapy

Characteristic	Number
Patient response to treatment (<i>n</i> = 41)	
Complete	2
Partial	8
Stable disease \geq 6 months	2
No response	29
Patient diagnosis (<i>n</i> = 41)	
Melanoma	16
NSCLC ^a	12
Kidney cancer	6
Colorectal cancer	5
CRPC	2
Number of tumor specimens analyzed per patient (<i>n</i> = 41)	
1	25
2	9
3	5
4	0
5	2
Primary vs. metastatic tumor (<i>n</i> = 68)	
Primary	29
Metastasis	39
Anatomic location (<i>n</i> = 68)	
Lymph node (metastasis)	17
Lung (primary or metastasis)	15
Other	36

^aTwelve cases include 9 adenocarcinomas and 3 squamous cell carcinomas of the lung.

Table 2. Association of tumor microenvironmental features with PD-L1 expression by tumor cells^a or infiltrating immune cells

Parameter (number of specimens examined)	Specimens ⁿ (%)	Tumor cells			Infiltrating immune cells		
		PD-L1(-) ⁿ (%)	PD-L1(+) ^b ⁿ (%)	<i>P</i> value ^c	PD-L1(-) ⁿ (%)	PD-L1(+) ^b ⁿ (%)	<i>P</i> value ^c
Tumor type (<i>n</i> = 68)							
Melanoma	30 (44)	14 (47)	16 (53)	0.005	15 (50)	15 (50)	0.007
NSCLC	17 (25)	8 (47)	9 (53)		8 (47)	9 (53)	
Kidney cancer	9 (13)	1 (11)	8 (89)		0 (0)	9 (100)	
Colorectal cancer	8 (12)	7 (87)	1 (13)		4 (50)	4 (50)	
CRPC	4 (6)	4 (100)	0 (0)		4 (100)	0 (0)	
Tumor site (<i>n</i> = 68)							
Lymph node	17 (25)	11 (65)	6 (35)	0.219	10 (59)	7 (41)	0.339
Lung	15 (22)	5 (33)	10 (67)		5 (33)	10 (67)	
Other	36 (53)	18 (50)	18 (50)		16 (44)	20 (56)	
Primary vs. metastatic tumor (<i>n</i> = 68)							
Primary	29 (43)	17 (59)	12 (41)	0.327	15 (52)	14 (48)	0.463
Metastasis	39 (57)	17 (44)	22 (56)		16 (41)	23 (59)	
Immune infiltrate score ^d (<i>n</i> = 68)							
0	14 (21)	13 (93)	1 (7)	0.001	N/A	N/A	0.69
1	35 (51)	16 (46)	19 (54)		12 (34)	23 (66)	
2	16 (24)	5 (31)	11 (69)		5 (31)	11 (69)	
3	3 (4)	0 (0)	3 (100)		0 (0)	3 (100)	
Proportion of TILs expressing PD-1 ^e (<i>n</i> = 63)							
0	38 (60)	25 (66)	13 (34)	0.001	22 (58)	16 (42)	0.005
1	13 (21)	5 (38)	8 (62)		4 (31)	9 (69)	
2	12 (19)	1 (8)	11 (92)		1 (8)	11 (92)	
3	0 (0)	0 (0)	0 (0)		0 (0)	0 (0)	
CD4:CD8 ratio (<i>n</i> = 50)							
CD4 ≥ CD8	22 (44)	9 (41)	13 (59)	0.565	7 (32)	15 (68)	1.000
CD4 < CD8	28 (56)	9 (32)	19 (68)		10 (36)	18 (64)	
CD20 ⁺ B cells (<i>n</i> = 51)							
Absent	34 (67)	23 (68)	11 (32)	0.006	21 (62)	13 (38)	0.017
Present ^f	17 (33)	4 (24)	13 (76)		4 (24)	13 (76)	
Lymphoid aggregates (<i>n</i> = 68)							
Absent	58 (85)	32 (55)	26 (45)	0.083	30 (52)	28 (48)	0.017
Present	10 (15)	2 (20)	8 (80)		1 (10)	9 (90)	
Necrosis (<i>n</i> = 68)							
Absent	50 (74)	27 (54)	23 (46)	0.41	25 (50)	25 (50)	0.276
Present	18 (26)	7 (39)	11 (61)		6 (33)	12 (67)	
Small sample (<i>n</i> = 68)							
No	44 (65)	19 (43)	25 (57)	0.204	17 (39)	27 (61)	0.135
Yes	24 (35)	15 (62)	9 (38)		14 (58)	10 (42)	

Abbreviation: N/A, not applicable.

^aTumor cell PD-L1 expression was previously reported for 53 of these 68 specimens (16).

^bTumor or infiltrating immune cells were considered PD-L1(+) if ≥5% of cells had membranous (cell surface) PD-L1 expression.

^cFisher exact test, two-sided, comparing PD-L1(+) versus (-) specimens for the indicated parameter.

^dImmune infiltrates include TILs and accompanying histiocytes. Infiltrates were scored as "none" (0), "focal" (1), "moderate" (2), or "severe" (3; see Materials and Methods).

^eProportion of TILs expressing PD-1 was graded as "none" (0), "focal" (1), "moderate" (2), or "severe" (3). A score of "0" includes cases where there were no TILs, or where TILs were present but did not express PD-1.

^fCD20 "present" refers to any grade ("focal," "moderate," or "severe").

RCC specimens demonstrating expression, in contrast to only one among a total of 12 colorectal cancer and CRPC specimens ($P = 0.005$; Table 2). When tumor cell PD-L1

expression was observed, it was associated with infiltrating immune cells, including lymphocytes and histiocytes, in 33 of 34 cases ($P = 0.001$; Table 2, Fig. 1A). The

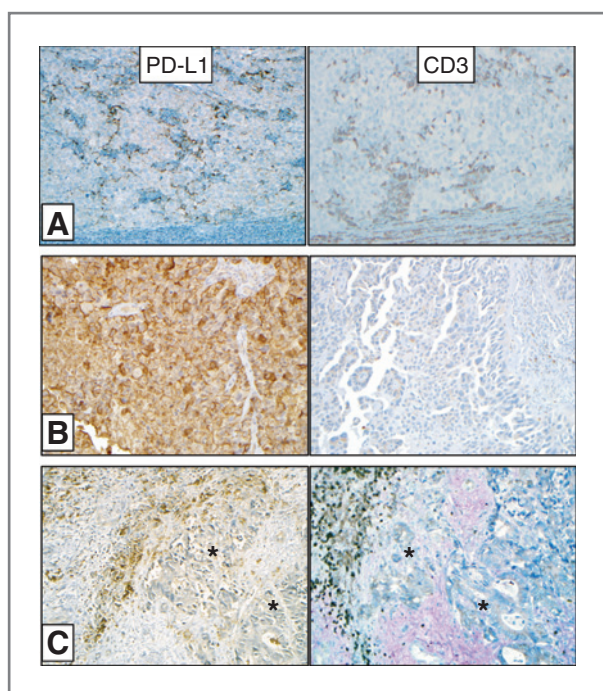


Figure 1. Association of tumor PD-L1 expression with immune infiltrates. Left, IHC for PD-L1. Right, IHC for CD3⁺ TILs. A, representative specimen from a subcutaneous melanoma metastasis demonstrating focal PD-L1 expression by tumor cells geographically associated with TILs. B, diffuse membranous tumor cell PD-L1 expression in a NSCLC brain metastasis, not associated with TILs. C, colorectal carcinoma metastasis to liver with membranous PD-L1 expression on infiltrating immune cells (brown stain), but not on tumor cells (asterisks). In all panels, original magnification, $\times 200$. Higher power images and additional IHC studies of the representative melanoma and colorectal carcinoma case are shown in Supplementary Figs. S2 and S3.

proportion of tumor cells expressing PD-L1 correlated with the intensity of immune cell infiltration (Kruskal–Wallis test, $P = 0.003$). A single case of NSCLC with broad tumor PD-L1 expression and no immune infiltrates was also observed (Fig. 1B).

We found that PD-L1 was expressed not only on tumor cells, but also on immune-infiltrating cells, including TILs and associated histiocytes/macrophages. PD-L1 expression by infiltrating immune cells varied by tumor type ($P = 0.007$), as shown in Table 2. Of interest, although only one among 8 cases of colorectal cancer displayed PD-L1⁺ tumor cells, 4 of 8 cases demonstrated PD-L1⁺ immune infiltrates (Fig. 1C, Supplementary Fig. S3). This was in contrast to findings in melanoma, NSCLC, and RCC specimens, where PD-L1 was often expressed coordinately on tumor cells and associated immune infiltrates. PD-L1 was also seen in the native tissue stroma, e.g., alveolar macrophages in lung specimens and dendritic cells in nonneoplastic lymph node parenchyma from lymph node dissections. Neither tumor cell nor infiltrating immune cell PD-L1 expression correlated with the anatomic site of the tumor specimen (lymph node vs. lung vs. elsewhere), or with specimens derived from primary versus metastatic lesions.

Association of PD-L1 in the tumor microenvironment with characteristics of immune cell infiltrates and other histologic features

To better characterize the microenvironment of PD-L1⁺ cancers, TILs were assessed for the presence and proportion of CD4⁺ and CD8⁺ T cells, and for the presence of CD20⁺ B cells (Supplementary Fig. S4). PD-1 receptor expression by these cells was also assessed. We found that TIL PD-1 expression was significantly associated with PD-L1 expression by tumor cells and by immune cell infiltrates (Table 2, $P = 0.001$ and 0.005 , respectively), reflecting a potentially immunosuppressive environment. PD-1 expression was also associated with increasing intensity of immune infiltrates and the presence of lymphoid aggregates (Kruskal–Wallis test, $P = 0.002$ and Fisher exact test $P = 0.010$, respectively), but did not vary significantly by CD4:CD8 ratio. Both the presence of B cells and lymphoid aggregates were associated with PD-L1 expression on tumor and infiltrating immune cells, but neither the CD4:CD8 ratio nor the presence of tumor necrosis correlated with PD-L1 expression in the tumor microenvironment (Table 2).

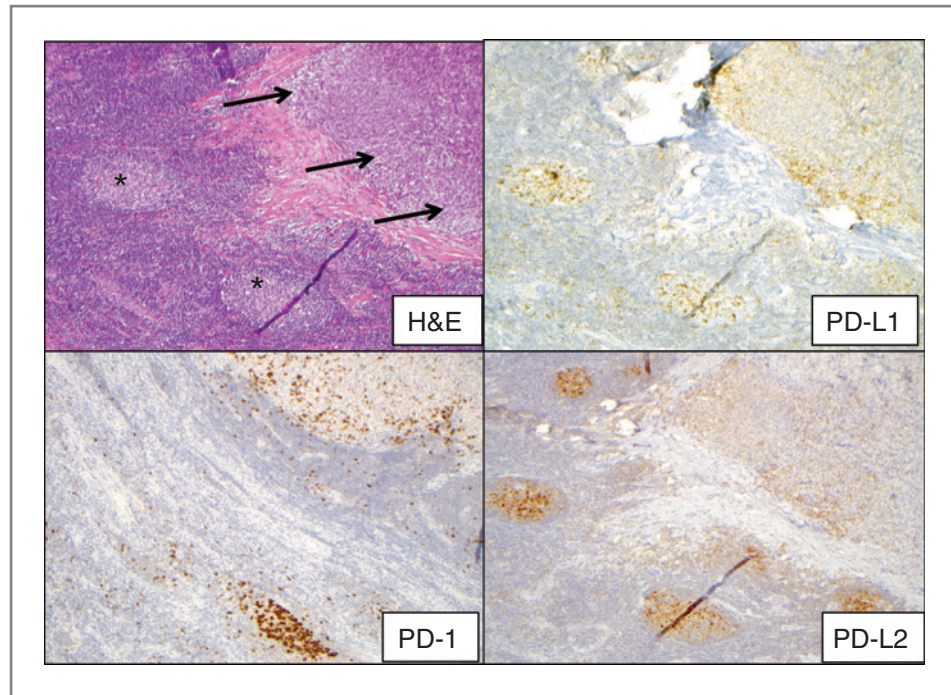
PD-L2 expression

PD-L2 is the second known ligand for the PD-1 T-cell coreceptor. Its binding to PD-1 is blocked by nivolumab, but its potential role in mediating immunosuppression in the human tumor microenvironment, and as a marker associated with clinical outcomes to anti-PD-1 therapy, has not been established. To explore this, 38 tumor specimens from 27 patients were examined for PD-L2 protein expression using IHC. PD-L2 expression by either tumor or infiltrating immune cells was observed in only 8 of 38 specimens (21%), including 1 RCC, 5 melanoma, and 2 NSCLC specimens. Four specimens demonstrated tumor cell expression of PD-L2, and in all of these cases, areas of tumor cell PD-L2 expression were adjacent to immune infiltrates (Fig. 2). In three of these four cases, PD-L2 expression by immune cell infiltrates was also observed. The four other cases demonstrated focal membranous PD-L2 expression by immune-infiltrating cells but not by tumor cells. A diffuse pattern of PD-L2 expression was not observed. PD-L2 expression was geographically associated with PD-L1 expression ($P = 0.053$), and there was only one case in which PD-L2 expression was observed at the interface of tumor and immune cells in a PD-L1 "negative" tumor (Fig. 3). In this instance, PD-L1 expression was present on singular infiltrating immune cells, but not on tumor cells, and thus failed to reach the 5% threshold to be scored "positive" in either cell population.

Additional immune microenvironmental features

Potential interrelationships of several tumor microenvironmental parameters listed in Table 2 were examined independently from PD-L1 expression. Among 51 tumor specimens assessed for the presence of CD20⁺ B cells, there was a borderline association with tumor type ($P = 0.072$), with B cells most likely to be seen in RCC (3/4, 75%), followed by melanoma (9/21, 42%), CRPC (1/3, 33%), and

Figure 2. Immunoarchitecture within a melanoma lymph node metastasis. On a section stained with H&E (upper left), a tumor deposit is indicated by arrows and lymph node germinal centers by asterisks. Expression of PD-1, PD-L1, and PD-L2 was observed in the lymph node germinal centers, providing an internal positive control for staining. Within the tumor deposit, PD-L1 and PD-L2 were expressed by both tumor and infiltrating immune cells, associated geographically with PD-1 expression. Additional characterization of the immune infiltrate is provided in Supplementary Fig. S4. In all panels, original magnification, $\times 100$.



NSCLC (4/16, 25%); B cells were not found in 7 colorectal cancer specimens examined. The presence of B cells correlated with both increasing immune infiltrate grade and PD-1 expression ($P = 0.017$ and 0.001 , respectively). A borderline association between TIL PD-1 expression and tumor location was observed ($P = 0.067$): among 63 specimens examined for PD-1 expression, 46% (6/13) of lung lesions (primary or metastatic), 20% (3/15) of lymph node metastases, and 9% (3/35) of tumors located elsewhere had $\geq 5\%$ of TILs expressing PD-1, suggesting that the native tissue stroma may influence the expression of PD-1.

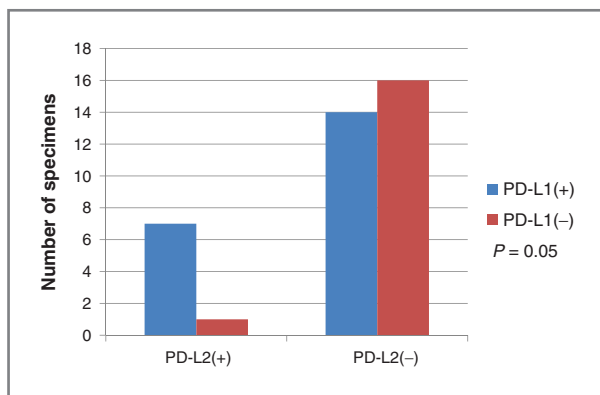


Figure 3. Association of PD-L2 expression in the tumor microenvironment with PD-L1 expression by tumor cells. PD-L2 expression as assessed by IHC was positive in 8 of 38 tumor specimens, and was expressed on tumor cells and/or immune-infiltrating cells. Although PD-L2 was observed less frequently than PD-L1 expression, when present, it was almost always geographically associated with tumor cell PD-L1 expression.

Biopsy characteristics

Because of the focal, geographic expression of many of the immunologic markers studied here, we queried whether the size of the biopsy sample, i.e., core needle biopsy or fine needle aspirate containing tissue fragments ($n = 24$), versus larger excisional specimen ($n = 44$), affected our assessment; dispersed single-cell cytology specimens were not included in this analysis. There was no significant relationship between biopsy size and PD-L1 expression (Table 2), PD-1 expression by TIL, or intensity score of immune infiltrates. In 30 melanoma specimens ranging in age from 2 months to 13 years, we also queried whether our ability to detect PD-L1 expression by IHC was related to the age of the specimen, defined as the time from specimen acquisition to staining for PD-L1, which might indicate epitope degradation over time. There was no significant relationship between specimen age and tumor PD-L1 expression based on the IHC technique used in this analysis.

Pathologic parameters associated with clinical response to anti-PD-1 therapy

We previously reported a preliminary correlation between tumor cell surface PD-L1 expression in pretreatment biopsies and objective response to nivolumab therapy (16). In that study, if multiple specimens were assessed from individual patients, a patient was defined as "PD-L1 positive" if any specimen contained $\geq 5\%$ PD-L1⁺ tumor cells. In the current study, among 41 patients, 16 had multiple pretreatment tumor specimens for study (2–5 specimens per patient, Table 1). We assessed a panel of pathologic parameters in the single tumor specimen obtained closest to the initiation of anti-PD-1 therapy (Table 3), reflecting anticipated clinical practice, or in the specimen with maximum expression in the case

Downloaded from <http://aacrjournals.org/clinoncres/article-pdf/20/19/5064/2021025/5064.pdf> by guest on 13 April 2024

Table 3. Relationship between pretreatment microenvironmental parameters in the tumor specimen obtained closest to treatment initiation, and clinical response to anti-PD-1^a

Pathologic parameter (number of patients analyzed)	All patients ⁿ (%)	Objective response ^b			Clinical benefit ^c		
		Non (%)	Yes ⁿ (%)	<i>P</i> value ^d	Non (%)	Yes ⁿ (%)	<i>P</i> value ^d
Tumor PD-L1 expression (<i>n</i> = 41) ^e							
Absent	18 (44)	17 (94)	1 (6)	0.025	17 (94)	1 (6)	0.005
Present	23 (56)	14 (61)	9 (39)		12 (52)	11 (48)	
Immune cell infiltrate PD-L1 expression (<i>n</i> = 41) ^e							
Absent	18 (44)	16 (89)	2 (11)	0.142	16 (89)	2 (11)	0.038
Present	23 (56)	15 (65)	8 (35)		13 (57)	10 (43)	
Immune infiltrate score (<i>n</i> = 41) ^f							
Absent	9 (22)	8 (89)	1 (11)	0.410	8 (89)	1 (11)	0.240
Present	32 (78)	23 (72)	9 (28)		21 (66)	11 (34)	
TIL PD-1 expression (<i>n</i> = 37) ^g							
Absent	21 (57)	18 (86)	3 (14)	0.067	17 (81)	4 (19)	0.077
Present	16 (43)	9 (56)	7 (44)		8 (50)	8 (50)	
Immune cell or tumor cell PD-L2 expression (<i>n</i> = 13) ^e							
Absent	9 (69)	7 (78)	2 (22)	1.000	6 (67)	3 (33)	0.497
Present	4 (31)	4 (100)	0 (0)		4 (100)	0 (0)	
CD4:CD8 (<i>n</i> = 29)							
CD4 ≥ CD8	13 (45)	10 (77)	3 (23)	0.454	9 (69)	4 (31)	0.702
CD4 < CD8	16 (55)	10 (63)	6 (37)		9 (56)	7 (44)	
CD20 ⁺ B cells (<i>n</i> = 30)							
Absent	19 (63)	16 (84)	3 (16)	1.000	16 (84)	3 (16)	0.372
Present ^f	11 (37)	9 (82)	2 (18)		7 (64)	4 (36)	
Lymphoid aggregates (<i>n</i> = 41)							
Absent	35 (85)	26 (74)	9 (26)	1.000	25 (71)	10 (29)	1.000
Present	6 (15)	5 (83)	1 (17)		4 (67)	2 (33)	
Necrosis (<i>n</i> = 41)							
Absent	27 (66)	19 (70)	8 (30)	0.447	19 (70)	8 (30)	1.000
Present	14 (34)	12 (86)	2 (14)		10 (71)	4 (29)	
Small sample (<i>n</i> = 41)							
No	27 (66)	20 (74)	7 (26)	1.000	18 (67)	9 (33)	0.494
Yes	14 (34)	11 (79)	3 (21)		11 (79)	3 (21)	
Interval between specimen procurement and treatment initiation (<i>n</i> = 41)							
<1 year	16 (39)	10 (63)	6 (37)	0.15	9 (56)	7 (44)	0.16
≥1 year	25 (61)	21 (84)	4 (16)		20 (80)	5 (20)	

^aCorrelation of tumor cell PD-L1 expression with objective response was previously reported for 34 of 41 patients included in this series, using the "highest ever" value in the case of multiple specimens from individual patients (16).

^bObjective response is defined as complete or partial tumor regression, RECIST 1.0 with modifications.

^cClinical benefit is defined as objective response or stable disease lasting ≥6 months.

^dFisher exact test, comparing responders with nonresponders for the pathologic parameter analyzed.

^eTumor or infiltrating immune cells were considered PD-L1 or PD-L2 (+) if ≥5% of cells had membranous (cell surface) expression detected by IHC.

^fImmune infiltrates (lymphocytes and histiocytes) and CD20⁺TIL were graded as "none," "focal," "moderate," or "severe" (see Materials and Methods). TIL or CD20 "present" indicates grades "focal," "moderate," and "severe."

^gAbsent TIL PD-1 expression includes both cases where there were no TILs, and cases where TILs were present, but did not express PD-1.

of multiple samples from a given patient (Supplementary Table S1). Pathologic findings were correlated with objective tumor regression (complete or partial response, RECIST 1.0 with modification), as well as with "clinical benefit" (objec-

tive response, or stable disease lasting at least 6 months), versus no response to therapy.

Similar to our previous analysis based on the "highest ever" expression in individual patients with multiple tumor

specimens (16), PD-L1 expression by tumor cells correlated significantly with objective response and with clinical benefit, when analyzed in the single specimen procured closest to the start of therapy ($P = 0.025$ and 0.005 , respectively, Table 3). Of interest, correlation of PD-L1 expression by infiltrating immune cells with objective clinical response did not reach statistical significance ($P = 0.14$), although there was a significant correlation with clinical benefit ($P = 0.038$). Furthermore, expression of the PD-1 receptor on TILs had only a borderline association with clinical response, even though PD-1 is the immediate target of nivolumab. Importantly, the presence of TILs when analyzed as an independent factor did not correlate with clinical outcomes. Additional microenvironmental factors, including PD-L2 expression by tumor cells or TILs, the CD4:CD8 ratio, the presence of CD20⁺ B cells, tumor necrosis, and lymphoid aggregates, did not correlate with treatment response when either the sample closest to therapy or the specimen with the "highest ever" value were assessed. However, because some of these factors were analyzed in fewer than 30 patients, more extensive study is needed to confirm these observations.

The range of time from tumor specimen procurement to treatment initiation was broad in our study (1 day to 12 years). The vast majority of specimens analyzed were archival, and obtaining a new tumor biopsy before treatment initiation was not a requirement of this clinical trial. Given the known spatial and temporal heterogeneity of PD-L1 expression (12, 16), we queried whether the time between tumor biopsy and the initiation of anti-PD-1 therapy independently correlated with clinical outcomes. Despite the wide range of specimen age, we did not observe such an association. As shown in Table 3, responding patients were evenly distributed between <1 year versus ≥ 1 year biopsy interval groups, and 9 of the 10 responders demonstrated PD-L1 tumor expression, suggesting that the correlation of PD-L1 expression with clinical outcomes is not related to the timing of tissue acquisition. Although consideration of sampling interval warrants additional investigation, our results do not provide direct support for the idea that tumor PD-L1 expression must be determined immediately before anti-PD-1 treatment initiation, to be predictive of clinical outcome. Realistically, such an approach will not always be possible, and is not uniformly practiced in current clinical trials of drugs blocking the PD-1 pathway.

Discussion

Cancer immunotherapy, which targets and modulates antitumor immune cells, differs mechanistically from cytotoxic therapies and kinase inhibitors, which directly mediate tumor cell death. Accordingly, these treatment approaches differ in their profiles of clinical activity as well as safety (22–24). The identification of factors predicting response to immunotherapy is highly desirable, to preselect patients most likely to benefit and spare others from unnecessary exposure to potential side effects. However, this is challenging due to the dynamic nature of the antitumor

immune response and its heterogeneity across space (anatomic location) and time (progression from primary to metastatic cancer). We previously reported a correlation between pretreatment tumoral PD-L1 expression and response to anti-PD-1 therapy (nivolumab) in a subset of patients on an expanded phase I trial (16). In the current study, we have reexamined PD-L1 as a marker associated with anti-PD-1 response, and have extended our investigations to evaluate other factors in the tumor microenvironment potentially associated with the clinical activity of anti-PD-1.

Recent studies associate an inflammatory tumor microenvironment with responsiveness to certain forms of immunotherapy such as cancer vaccines and ipilimumab (25, 26), and our observations suggest that this may also be true for PD-1 pathway blockade. In the current study, patients whose tumors expressed PD-L1 were more likely to respond to anti-PD-1 therapy. Although PD-L1 is generally regarded as an immunosuppressive molecule, its expression is not necessarily synonymous with tumor immune evasion and may reflect an ongoing antitumor immune response that includes the production of IFN γ and other inflammatory factors (12). This is consistent with retrospective studies in select tumor types, such as melanoma, Merkel cell carcinoma, mismatch repair–proficient colorectal cancer, and NSCLC where tumor PD-L1 expression has been shown to be a positive prognostic factor (12, 13, 27, 28). We observed tumor cell surface PD-L1 expression in distinct patterns, which generally correlated with tumor type. Tumor cell surface PD-L1 expression was associated with immune cell infiltrates in some cases (mainly melanoma and RCC), whereas in others it was constitutive or out of proportion to infiltrating immune cells (NSCLC). We also observed instances of PD-L1 membranous expression on infiltrating immune cells but not on tumor cells, particularly in colorectal cancer (21). Although the biologic significance of these distinct expression patterns is currently unclear, they likely reflect the combined effects of innate and adaptive cellular and soluble factors that shape the tumor microenvironment, as well as the type of malignancy and composition of other components of the tumor stroma. For example, neoantigens associated with infection by tumor-promoting viruses or somatic mutational events in malignant cells may trigger inflammatory responses leading to local PD-L1 expression (13, 21, 27), while PD-L1 expression in non-virus-associated head and neck squamous cell cancers, glioblastoma multiforme, and anaplastic lymphoma kinase (ALK)–positive T-cell lymphomas has been associated with PTEN and ALK/STAT3 oncogenic signaling pathways (29–31).

In this study, we examined a potential relationship between TIL expression of PD-1, the direct target of nivolumab, with clinical outcomes but found only a borderline association. Because the intensity of immune cell infiltrates was significantly associated with tumor cell PD-L1 expression, we also explored the possibility that simply the presence of immune cell infiltrates might predict favorable clinical outcomes to anti-PD-1 therapy. The presence of

TIL has been correlated with improved outcomes in retrospective studies of different tumor types, including melanoma and colorectal carcinoma (32–35). In addition, HER2-positive breast cancer patients with TIL in their pretreatment specimens have shown improved benefit from certain chemotherapeutic regimens (36). Further, increased numbers of TIL in posttreatment biopsies have been shown to correlate with the activity of ipilimumab in patients with melanoma (37). However, the current study is the first to examine the relationship of the presence of TIL in pretreatment tumor specimens to anti-PD-1 response, and a significant relationship between these factors was not observed. These findings suggest that the functional profile of TILs is a key factor determining PD-L1 expression (12). That is, TILs may be necessary to drive PD-L1 expression in some tumors, but their presence alone is not sufficient to induce PD-L1 and was not an independent factor correlating with clinical response in this relatively limited cohort. Because preclinical evidence suggests that anti-PD-1 can restore dampened B-cell functions (38), we also examined whether the presence and intensity of B-cell infiltrates correlated with clinical outcomes. Similar to our findings with CD3⁺ TILs, CD20⁺ B cells were significantly associated with PD-L1 expression by tumor and infiltrating immune cells, but their presence alone did not correlate with clinical outcomes following PD-1 blockade, suggesting the importance of defining cellular functional profiles. Other immune cell types, including suppressive cells (regulatory T cells and myeloid-derived suppressor cells), remain to be explored in the context of PD-1 pathway blockade (39, 40).

Recent work by others to analyze a potential association between pretreatment tumor PD-L1 expression and response to PD-1 pathway blockade—anti-PD-1 (41) or anti-PD-L1 (42)—has confirmed our original observation (16) linking PD-L1⁺ tumors with the likelihood of treatment response. However, in these new studies, some PD-L1-negative patients also responded to treatment, raising concerns that excluding the "marker negative" patient population from treatment might exclude potential responders. It is important to note that these three studies differ in the anti-PD-L1 mAbs used for IHC, staining techniques (manual versus automated), definitions of PD-L1 "positive" tumor (cell surface versus cytoplasmic expression, by tumor cells only or by other cells in the tumor milieu, threshold of "positivity"), scoring increments, and definitions of PD-L1 "positive" patients (based on a single tumor biopsy, or on maximal expression in the case of multiple biopsies from an individual patient). Also, because of the focal nature of PD-L1 expression within many tumors and emerging information about intratumoral genetic heterogeneity (43), if very small needle biopsies or dispersed single-cell cytology specimens are evaluated, a false-negative evaluation could potentially result. Another potential explanation for PD-L1(–) responders includes yet unidentified factors contributing to response. Despite these methodologic differences, the overall conclusions of these reports are remarkably similar, highlighting a robust association between the PD-L1 marker and mechanism-of-action for this class of drugs.

Although response rates are enhanced in the PD-L1⁺ patient population, it is currently unknown why the majority of PD-L1⁺ patients do not respond to PD-1 pathway blocking drugs. One possibility is that PD-L1⁺ tumors from nonresponders express additional dominant or codominant immune checkpoints supporting treatment resistance. To address this, we examined PD-L2, the second known ligand for PD-1, for possible associations with PD-L1 expression and clinical outcomes. PD-L2 protein detected by IHC was found almost exclusively in geographic association with PD-L1 protein, consistent with its known upregulation by inflammatory cytokines, including IFN γ which also drives PD-L1 expression (44). However, PD-L2 expression was seen less frequently than PD-L1 in our series (in only 8 of 38 specimens examined), and no significant correlation with clinical outcomes was observed. Although the results of our series should be considered preliminary, similar conclusions were drawn in a recent report of PD-L2 expression detected by quantitative molecular techniques, in patients receiving anti-PD-L1 therapy (45). Studies aimed at identifying additional positive or negative predictive markers of response to anti-PD-1 treatment, and potential interactions among multiple factors in the tumor microenvironment, are currently under way in our laboratories.

In summary, this in-depth analysis of multiple factors in pretreatment tumor specimens from patients with advanced cancers receiving anti-PD-1 therapy prioritizes tumor cell PD-L1 expression as being most closely associated with objective tumor regression. It reveals other microenvironmental features, such as TIL PD-1 expression and the intensity of T-cell and B-cell infiltrates, as being associated with PD-L1 expression by tumor cells or immune-infiltrating cells, but not independently associated with treatment response. Thus, PD-L1 expression reflects an immune-active tumor milieu, and may illuminate additional tumor types that should be targeted for clinical testing with PD-1 pathway blockade. These results should still be considered preliminary, and ongoing phase II and III clinical trials of PD-1 pathway blockade are broadening the assessment PD-L1 expression as it relates to clinical outcomes including survival, in larger cohorts of patients. Additional investigations will be necessary to confirm these findings and will address whether multicomponent panels of pretreatment tumor markers may have more powerful associations with clinical outcomes, compared with individual factors. Assessment of on-treatment alterations in tumor molecular profiles will also be necessary to reveal whether tumors lacking PD-L1 expression and TILs may convert to PD-L1-expressing tumors following "priming" with combinatorial treatment regimens designed to incite an immune response, followed by PD-1 pathway blockade to liberate antitumor immunity.

Disclosure of Potential Conflicts of Interest

J.M. Taube and J.R. Brahmer report receiving commercial research grants from and are consultants/advisory board members for Bristol-Myers Squibb. H. Xu is a consultant/advisory board member for Amplimmune. D. Pardoll reports receiving a commercial research grant from Bristol-Myers Squibb and is a consultant/advisory board member for Aduro, ImmuneXcite, and Jounce

Therapeutics. S.L. Topalian reports receiving a commercial research grant from Bristol-Myers Squibb and is a consultant/advisory board member for Amplimmune, Bristol-Myers Squibb, Jounce Therapeutics, and Sanofi. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: J.M. Taube, H. Xu, L. Chen, S.L. Topalian, R.A. Anders

Development of methodology: J.M. Taube, H. Xu, L. Chen

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.M. Taube, J.R. Brahmer, X. Pan, J.H. Kim

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.M. Taube, A. Klein, L. Chen, S.L. Topalian, R.A. Anders

Writing, review, and/or revision of the manuscript: J.M. Taube, A. Klein, J.R. Brahmer, H. Xu, J.H. Kim, L. Chen, D.M. Pardoll, S.L. Topalian, R.A. Anders

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.M. Taube, X. Pan, J.H. Kim

Study supervision: J.M. Taube, J.R. Brahmer, S.L. Topalian, R.A. Anders

Acknowledgments

The authors thank Evan Lipson, William Sharfman, and Charles Drake (Johns Hopkins University School of Medicine) for providing outstanding clinical care to the patients on this trial, Gulsun Erdag for helpful discussions on PD-L2 assay development, and Cheryllann Carr-Cozier, Marina Laiko, and Deborah Roberts for regulatory and administrative assistance (all from Johns

Hopkins University School of Medicine). They also thank Mark Salvati, Joseph Grosso, and John Cogswell (Bristol-Myers Squibb) for helpful comments on this article.

Grant Support

This study was supported by Bristol-Myers Squibb (to J.M. Taube, S.L. Topalian, D.M. Pardoll, J.R. Brahmer, and R.A. Anders), NIH (R01 CA142779; to J.M. Taube, S.L. Topalian, D.M. Pardoll, and L. Chen), the Melanoma Research Alliance (to S.L. Topalian, D.M. Pardoll, L. Chen, and J.M. Taube), the Barney Family Foundation (to J.M. Taube and S.L. Topalian), the Laverna Hahn Charitable Trust (to S.L. Topalian and J.M. Taube), the Dermatology Foundation (to J.M. Taube), the Commonwealth Foundation (to J.M. Taube and D.M. Pardoll), and Moving for Melanoma of Delaware (to J.M. Taube, D.M. Pardoll, and S.L. Topalian). J.M. Taube, S.L. Topalian, D.M. Pardoll, J.R. Brahmer, and R.A. Anders were also supported by a Stand Up To Cancer—Cancer Research Institute Cancer Immunology Translational Cancer Research Grant (SU2C-AACR-DT1012). Stand Up To Cancer is a program of the Entertainment Industry Foundation administered by the American Association for Cancer Research.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 2, 2013; revised March 27, 2014; accepted March 29, 2014; published OnlineFirst April 8, 2014.

References

- Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 2010;19:363:711–23.
- Agata Y, Kawasaki A, Nishimura H, Ishida Y, Tsubata T, Yagita H, et al. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int Immunol* 1996;8:765–72.
- Dong H, Zhu G, Tamada K, Chen L. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat Med* 1999;5:1365–9.
- Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* 2002;8:793–800.
- Mazanet MM, Hughes CC. B7-H1 is expressed by human endothelial cells and suppresses T cell cytokine synthesis. *J Immunol* 2002;169:3581–8.
- Petroff MG, Chen L, Phillips TA, Azzola D, Sedlmayr P, Hunt JS. B7 family molecules are favorably positioned at the human maternal-fetal interface. *Biol Reprod* 2003;68:1496–504.
- Ishida M, Iwai Y, Tanaka Y, Okazaki T, Freeman GJ, Minato N, et al. Differential expression of PD-L1 and PD-L2, ligands for an inhibitory receptor PD-1, in the cells of lymphohematopoietic tissues. *Immunol Lett* 2002;84:57–62.
- Yamazaki T, Akiba H, Iwai H, Matsuda H, Aoki M, Tanno Y, et al. Expression of programmed death 1 ligands by murine T cells and APC. *J Immunol* 2002;169:5538–45.
- Tseng SY, Otsuji M, Gorski K, Huang X, Slansky JE, Pai SI, et al. B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells. *J Exp Med* 2001;193:839–46.
- Flies DB, Chen L. The new B7s: playing a pivotal role in tumor immunity. *J Immunother* 2007;30:251–60.
- Zou W, Chen L. Inhibitory B7-family molecules in the tumour micro-environment. *Nat Rev Immunol* 2008;8:467–77.
- Taube JM, Anders RA, Young GD, Xu H, Sharma R, McMiller TL, et al. Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. *Sci Transl Med* 2012;4:127ra37.
- Lipson EJ, Vincent JG, Loyo M, Kagohara LT, Lubner BS, Wang H, et al. PD-L1 expression in the merkel cell carcinoma microenvironment: association with inflammation, merkel cell polyomavirus and overall survival. *Cancer Immunol Res* 2013;1:54–63.
- Nishimura H, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* 1999;11:141–51.
- Nishimura H, Okazaki T, Tanaka Y, Nakatani K, Hara M, Matsumori A, et al. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science* 2001;291:319–22.
- Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* 2012;366:2443–54.
- Brahmer JR, Drake CG, Wollner I, Powderly JD, Picus J, Sharfman WH, et al. Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *J Clin Oncol* 2010;28:3167–75.
- Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med* 2012;366:2455–65.
- Hamid O, Robert C, Daud A, Hodi FS, Hwu WJ, Kefford R, et al. Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. *N Engl J Med* 2013;369:134–44.
- Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, et al. New guidelines to evaluate the response to treatment in solid tumors. European organization for research and treatment of cancer, national cancer institute of the United States, national cancer institute of Canada. *J Natl Cancer Inst* 2000;92:205–16.
- Lipson EJ, Sharfman WH, Drake CG, Wollner I, Taube JM, Anders RA, et al. Durable cancer regression off-treatment and effective reinduction therapy with an anti-PD-1 antibody. *Clin Cancer Res* 2013;19:462–8.
- Blansfield JA, Beck KE, Tran K, Yang JC, Hughes MS, Kammula US, et al. Cytotoxic T-lymphocyte-associated antigen-4 blockade can induce autoimmune hypophysitis in patients with metastatic melanoma and renal cancer. *J Immunother* 2005;28:593–8.
- Downey SG, Klapper JA, Smith FO, Yang JC, Sherry RM, Royal RE, et al. Prognostic factors related to clinical response in patients with metastatic melanoma treated by CTL-associated antigen-4 blockade. *Clin Cancer Res* 2007;13:6681–8.
- Beck KE, Blansfield JA, Tran KQ, Feldman AL, Hughes MS, Royal RE, et al. Enterocolitis in patients with cancer after antibody blockade of cytotoxic T-lymphocyte-associated antigen 4. *J Clin Oncol* 2006;24:2283–9.

25. Gajewski TF, Fuertes M, Spaapen R, Zheng Y, Kline J. Molecular profiling to identify relevant immune resistance mechanisms in the tumor microenvironment. *Curr Opin Immunol* 2011;23:286–92.
26. Ji RR, Chasalow SD, Wang L, Hamid O, Schmidt H, Cogswell J, et al. An immune-active tumor microenvironment favors clinical response to ipilimumab. *Cancer Immunol Immunother* 2012;61:1019–31.
27. Droezer RA, Hirt C, Viehl CT, Frey DM, Nebiker C, Huber X, et al. Clinical impact of programmed cell death ligand 1 expression in colorectal cancer. *Eur J Cancer* 2013;49:2233–42.
28. Velcheti V, Schalper KA, Carvajal DE, Anagnostou VK, Syrigos KN, Sznol M, et al. Programmed death ligand-1 expression in non-small cell lung cancer. *Lab Invest* 2014;94:107–16.
29. Lyford-Pike S, Peng S, Young GD, Taube JM, Westra WH, Akpeng B, et al. Evidence for a role of the PD-1:PD-L1 pathway in immune resistance of HPV-associated head and neck squamous cell carcinoma. *Cancer Res* 2013;73:1733–41.
30. Parsa AT, Waldron JS, Panner A, Crane CA, Parney IF, Barry JJ, et al. Loss of tumor suppressor PTEN function increases B7-H1 expression and immunoresistance in glioma. *Nat Med* 2007;13:84–8.
31. Marzec M, Zhang Q, Goradia A, Raghunath PN, Liu X, Paessler M, et al. Oncogenic kinase NPM/ALK induces through STAT3 expression of immunosuppressive protein CD274 (PD-L1, B7-H1). *Proc Natl Acad Sci U S A* 2008;105:20852–7.
32. Azimi F, Scolyer RA, Rumcheva P, Moncrieff M, Murali R, McCarthy SW, et al. Tumor-infiltrating lymphocyte grade is an independent predictor of sentinel lymph node status and survival in patients with cutaneous melanoma. *J Clin Oncol* 2012;30:2678–83.
33. Ogino S, Noshio K, Irahara N, Meyerhardt JA, Baba Y, Shima K, et al. Lymphocytic reaction to colorectal cancer is associated with longer survival, independent of lymph node count, microsatellite instability, and CpG island methylator phenotype. *Clin Cancer Res* 2009;15:6412–20.
34. Erdag G, Schaefer JT, Smolkin ME, Deacon DH, Shea SM, Dengel LT, et al. Immunotype and immunohistologic characteristics of tumor-infiltrating immune cells are associated with clinical outcome in metastatic melanoma. *Cancer Res* 2012;72:1070–80.
35. Thomas NE, Busam KJ, From L, Krickler A, Armstrong BK, Anton-Culver H, et al. Tumor-infiltrating lymphocyte grade in primary melanomas is independently associated with melanoma-specific survival in the population-based genes, environment and melanoma study. *J Clin Oncol* 2013;31:4252–9.
36. Loi S, Sirtaine N, Piette F, Salgado R, Viale G, Van Eenoo F, et al. Prognostic and predictive value of tumor-infiltrating lymphocytes in a phase III randomized adjuvant breast cancer trial in node-positive breast cancer comparing the addition of docetaxel to doxorubicin with doxorubicin-based chemotherapy: BIG 02-98. *J Clin Oncol* 2013;31:860–7.
37. Hamid O, Schmidt H, Nissan A, Ridolfi L, Aamdal S, Hansson J, et al. A prospective phase II trial exploring the association between tumor microenvironment biomarkers and clinical activity of ipilimumab in advanced melanoma. *J Transl Med* 2011;9:204–19.
38. Velu V, Titanji K, Zhu B, Husain S, Pladevega A, Lai L, et al. Enhancing SIV-specific immunity in vivo by PD-1 blockade. *Nature* 2009;458:206–10.
39. Mohos A, Sebestyen T, Liszky G, Plotar V, Horvath S, Gaudi I, et al. Immune cell profile of sentinel lymph nodes in patients with malignant melanoma - FOXP3+ cell density in cases with positive sentinel node status is associated with unfavorable clinical outcome. *J Transl Med* 2013;11:43–53.
40. Taube JM. Emerging immunologic biomarkers: setting the [TNM-immune] stage. *Clin Cancer Res* 2014;20:2023–5.
41. Grosso J, Horak C, Inzunza D, Cardona D, Simon J, Gupta A, et al. Association of tumor PD-L1 expression and immune biomarkers with clinical activity in patients with advanced solid tumors treated with nivolumab. *J Clin Oncol* 31, 2013 (suppl; abstr 3016).
42. Herbst R, Gordon M, Fine G, Sosman J, Charles J, Hamid O, et al. A study of MPDL3280A, an engineered PD-L1 antibody in patients with locally advanced or metastatic tumors. *J Clin Oncol* 31, 2013 (suppl; abstr 3000).
43. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med* 2012;366:883–92.
44. Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol* 2001;2:261–8.
45. Hodi FS, Powles T, Cassier P, Kowanetz M, Herbst RS, Soria JC, et al. MPDL3280A (anti-PDL1): clinical activity, safety and biomarkers of an engineered PD-L1 antibody in patients with locally advanced or metastatic tumors. *Eur Cancer Congr* 2013;abstr 879.