

Melanoma subtypes demonstrate distinct PD-L1 expression profiles

Genevieve J Kaunitz¹, Tricia R Cottrell², Mohammed Lilo², Valliammai Muthappan³, Jessica Esandrio¹, Sneha Berry^{1,4}, Haiying Xu¹, Aleksandra Ogurtsova¹, Robert A Anders², Alexander H Fischer¹, Stefan Kraft⁵, Meg R Gerstenblith⁶, Cheryl L Thompson⁷, Kord Honda⁶, Jonathan D Cuda^{1,2}, Charles G Eberhart^{2,3,4}, James T Handa³, Evan J Lipson⁴ and Janis M Taube^{1,2,4}

PD-L1 expression in the tumor immune microenvironment is recognized as both a prognostic and predictive biomarker in patients with cutaneous melanoma, a finding closely related to its adaptive (IFN- γ -mediated) mechanism of expression. Approximately 35% of cutaneous melanomas express PD-L1, however, the expression patterns, levels, and prevalence in rarer melanoma subtypes are not well described. We performed immunohistochemistry for PD-L1 and CD8 on 200 formalin-fixed paraffin-embedded specimens from patients with acral ($n = 16$), mucosal ($n = 36$), uveal ($n = 103$), and chronic sun-damaged (CSD) ($n = 45$) melanomas (24 lentigo maligna, 13 'mixed' desmoplastic, and 8 'pure' desmoplastic melanomas). CD8+ tumor-infiltrating lymphocyte (TIL) densities were characterized as mild, moderate, or severe, and their geographic association with PD-L1 expression was evaluated. Discrete lymphoid aggregates, the presence of a spindle cell morphology, and the relationship of these features with PD-L1 expression were assessed. PD-L1 expression was observed in 31% of acral melanomas, 44% of mucosal melanomas, 10% of uveal melanomas, and 62% of CSD melanomas ($P < 0.0001$). Compared to our previously characterized cohort of cutaneous melanomas, the proportion of PD-L1(+) tumors was lower in uveal ($P = 0.0002$) and higher in CSD ($P = 0.0073$) melanomas, while PD-L1 expression in the acral and mucosal subtypes was on par. PD-L1 expression in all subtypes correlated with a moderate–severe grade of CD8+ TIL (all, $P < 0.003$), supporting an adaptive mechanism of expression induced during the host antitumor response. The tumor microenvironments observed in CSD melanomas segregated by whether they were the pure desmoplastic subtype, which showed lower levels of PD-L1 expression when compared to other CSD melanomas ($P = 0.047$). The presence of lymphoid aggregates was not associated with the level of PD-L1 expression, while PD-L1(+) cases with spindle cell morphology demonstrated higher levels of PD-L1 than those with a nested phenotype ($P < 0.0001$). Our findings may underpin the reported clinical response rates for anti-PD-1 monotherapy, which vary by subtype.

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Increased objective response rates (ORRs) to anti-PD-1/PD-L1 monotherapy as well as improved progression-free survival and overall survival have been linked to PD-L1 expression in the tumor microenvironment (TME) in some studies.^{1–4} Studies from our group and others indicate that the major mechanism driving melanoma tumor cell PD-L1 expression is an association with tumor-infiltrating lymphocytes (TIL), consistent with an endogenous antitumor

immune response that may be unleashed with anti-PD-1/PD-L1 therapies.⁵ Such adaptive PD-L1 expression by melanoma is not dependent on BRAF, NRAS, or c-KIT mutational status.^{6,7}

Most studies in melanoma patients that have examined the relationship between PD-L1 expression in the pretreatment TME and response to anti-PD-1/PD-L1 have focused on conventional cutaneous melanoma, ie, melanoma arising

¹Department of Dermatology, Johns Hopkins University School of Medicine, Bloomberg Kimmel Institute for Cancer Immunotherapy, and Sidney Kimmel Comprehensive Cancer Center, Baltimore, MD, USA; ²Department of Pathology, Johns Hopkins University School of Medicine, Bloomberg Kimmel Institute for Cancer Immunotherapy, and Sidney Kimmel Comprehensive Cancer Center, Baltimore, MD, USA; ³Department of Ophthalmology, Johns Hopkins University School of Medicine, Bloomberg Kimmel Institute for Cancer Immunotherapy, and Sidney Kimmel Comprehensive Cancer Center, Baltimore, MD, USA; ⁴Department of Oncology, Johns Hopkins University School of Medicine, Bloomberg Kimmel Institute for Cancer Immunotherapy, and Sidney Kimmel Comprehensive Cancer Center, Baltimore, MD, USA; ⁵Department of Pathology, Massachusetts General Hospital, Boston, MA, USA; ⁶Department of Dermatology, University Hospitals Cleveland Medical Center/Case Western Reserve University School of Medicine, Case Comprehensive Cancer Center, Cleveland, OH, USA and ⁷Department of Nutrition, Case Western Reserve University School of Medicine, Cleveland, OH, USA
Correspondence: Dr JM Taube, MD, Division of Dermatopathology, Johns Hopkins Hospital, Blalock 907, 600N. Wolfe Street, Baltimore, MD 21287, USA.
E-mail: jtaube1@jhmi.edu

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on hair-bearing, non-chronically sun-damaged skin. Approximately two-thirds of these melanomas harbor BRAF mutations, and are most often classified histologically as superficial spreading and nodular melanomas.⁸ Chronic sun damage (CSD) melanomas tend to occur in an older demographic on the head and neck, and include the histologic categories of lentigo maligna melanoma and desmoplastic melanoma. Desmoplastic melanoma may be further subdivided into the pure and mixed (ie, a ‘mix’ of both desmoplastic and conventional-appearing melanoma) categories, with pure desmoplastic melanomas being defined morphologically as those comprised of >90% sparse, spindled melanocytes set in a dense, fibrocollagenous stroma resembling a scar.⁹ This subtype is also often associated with intratumoral lymphoid aggregates and is more likely to be associated with mutations in tumor-suppressor genes when compared to the mixed desmoplastic type, which is more likely to be associated with BRAF and NRAS activating oncogenic driver mutations.¹⁰ Acral melanomas arise on the non-hair-bearing skin of the palms and soles; mucosal melanomas arise from melanocytes of the mucosal epithelium of the paranasal sinuses/oropharynx or anogenital region; and uveal melanomas arise from nonepithelial melanocytes in the dermis or uvea. UV radiation is not a dominant mutagen in these latter three types (for a thorough review of melanoma subtype classification see Whiteman, *et al*⁸).

A few recent studies have reported objective response rates to anti-PD-1/PD-L1 in patients with mucosal, acral, uveal, and desmoplastic melanomas (summarized in Table 1). ORRs for patients with uveal melanoma were notably lower than for patients with cutaneous melanoma, while patients with

desmoplastic melanoma demonstrated higher response rates. Most of these studies did not include immunohistochemistry (IHC) assessment of PD-L1 expression in pretreatment surgical pathology specimens, thus it is unclear how PD-L1 expression patterns in these tumor types may relate to the observed response rates. The purpose of our study was to characterize PD-L1 expression prevalence, patterns, and levels among these melanoma variants.

MATERIALS AND METHODS

Case Selection

Following IRB approval, 200 archival surgical pathology specimens from 195 distinct patients were collected from three different academic medical centers (Johns Hopkins University, Baltimore, MD; Massachusetts General Hospital, Boston, MA; and University Hospitals Cleveland Medical Center, Cleveland, OH). The cohort included 16 acral melanomas, 36 mucosal melanomas, 103 uveal melanomas, and 45 chronic sun damage (CSD) melanomas, including 24 lentigo maligna and 21 desmoplastic melanomas.¹¹ Eighty of the uveal melanomas were studied in a tissue microarray (TMA) format. A summary of specimen origin (ie, primary lesion *vs* metastasis) and histologic subtype is provided in Table 2. The histologic diagnosis for each specimen was confirmed on H&E slides by a dermatopathologist (JMT) or an ophthalmic pathologist (CE), and a single representative tissue block was chosen for additional study. Morphologic features were tabulated, including the presence of a spindled or nested morphology, lymphoid aggregates (clusters of >25 lymphocytes with or without macrophages or dendritic cells), and when relevant, the pure *vs* mixed desmoplastic features.⁹

Table 1 Response to anti-PD-1/PD-L1 therapy in melanoma subtypes

Melanoma subtype	Study	ORR (%)	Total no. patients	Oncologic agent	Reference
Acral	1	32	25	Anti-PD-1	Shoushtari <i>et al</i> ⁵²
	2	16.7	6	Anti-PD-1	Cho <i>et al</i> ⁵³
	Overall	29.0	31		
Mucosal	1	23	35 ^a	Anti-PD-1	Shoushtari <i>et al</i> ⁵²
	2	20	5 ^b	Anti-PD-L1	Herbst <i>et al</i> ⁵⁴
	3	23.3	86 ^b	Anti-PD-1	D’Angelo <i>et al</i> ⁵¹
	Overall	23.0	126		
Uveal	1	37.5	8	Anti-PD-1	Kottschade <i>et al</i> ⁵⁵
	2	8	25	Anti-PD-1	Karydis <i>et al</i> ¹⁸
	3	3.6	56	Anti-PD-1/PD-L1	Algazi <i>et al</i> ⁵⁶
	Overall	7.9	89		
CSD ^c	1	70	23	Anti-PD-1/PD-L1	Eroglu <i>et al</i> ⁴⁹

^a12 (35%) patients had anorectal disease, 14 (40%) had vulvovaginal, and 9 (26%) had head/neck. ORR was not reported for specific anatomic sites.

^bSpecific anatomic sites for the primary tumor were not delineated.

^cCSD, chronic sun damage.¹¹ Term includes desmoplastic (pure and mixed) and lentigo maligna melanoma, and subtype not-specified on sun-damaged skin. The associated study reported ORR for pure and mixed desmoplastic types, and subtype not-specified.

Table 2 Characteristics of archival surgical pathology specimens studied from patients with various melanoma subtypes

	No. specimens, <i>n</i> (%)	Primary ^a , <i>n</i>	Metastatic, <i>n</i>
Total	200 (100)	194	6
Acral	16 (8)	16	0
Mucosal	36 (18)	33	3
Anorectal	3 (1.5)	2	1
Vulvovaginal	7 (3.5)	7	0
Sinonasal	26 (13)	24	2
Uveal	103 (51.5)	101	2
CSD	45 (22.5)	44	1
Pure desmoplastic	8 (4)	8	0
Mixed desmoplastic	13 (6.5)	12	1
Lentigo maligna	24 (12)	24	0

^aFourteen primary tumor specimens (3 mucosal, 1 uveal, 8 CSD, and 2 acral) were excisions containing recurrent/residual disease, as opposed to a primary diagnostic biopsy.

Bold values indicate the overall ORR and total # patients in each category.

For the purpose of this study, the terms ‘spindled’ or ‘spindle cell’ are used as morphologic descriptors, and not as a distinct melanoma subtype.

IHC Studies and Characterization of TIL Patterns

IHC for PD-L1 expression was performed using the 5H1 antibody as previously described.⁵ A modified counterstain was used for the uveal and other heavily pigmented melanomas.¹² Specifically, Giemsa was substituted for hematoxylin to enhance differentiation between the brown chromogen signal used in the IHC method from the native melanin pigment. IHC for CD8 was also performed on each case using standard automated methods.

PD-L1 expression was observed on both tumor cells and immune cells, and was differentiated based on morphologic features and geographic distribution. PD-L1 expression on tumor cells was scored as 0%, 5%, 10%, and at increasing 10% intervals.^{5,13} Tumors were considered to be PD-L1(+) when ≥5% of tumor cells demonstrated membranous staining.^{5,13} The presence of CD8+ TIL was scored as 0 (none), mild (1, positive cells focally at advancing edge of tumor or perivascularly, <5% of tumor area), moderate (2, infiltrating tumor by extending away from intratumoral vessels or extending into tumor from advancing edge, 5–50% of tumor area), or severe (3, marked, broad infiltration by TIL or a strong immune front, >50% of tumor area). The TIL intensity grading system has been shown to correlate with TIL counts by digital image analysis in previous studies.^{14,15} For the fourteen re-excision specimens that included a primary biopsy site, PD-L1 expression and TIL densities were assessed away from the primary biopsy location. The correlations

between PD-L1 expression with CD8+ TIL infiltrates, discrete lymphoid aggregates, and spindle (fusiform) cell morphology were assessed.

Statistical Analysis

The chi-square test was used to assess differences in the proportion of PD-L1(+) tumors among all melanoma subtypes. The Kruskal-Wallis test was used to compare the levels of PD-L1 expression across all subtypes. The Fisher’s exact test was used to compare the proportion of samples with CD8+ T-cell infiltration in PD-L1(+) and PD-L1(–) samples. Comparisons between PD-L1 expression levels and morphologic characteristics (pure desmoplastic melanoma vs other CSD melanomas, presence or absence of lymphoid aggregates, and spindled vs nested morphology) were calculated using the Mann–Whitney *U*-test. Statistical significance was determined using two-tailed tests with an alpha level of 0.05 unless otherwise stated. Statistical analyses were performed with GraphPad Prism 7 (GraphPad Software) and Stata 14.1 (StataCorp, College Station, TX, USA).

RESULTS

PD-L1 Expression Patterns, Prevalence, and Expression Levels

PD-L1 tumor cell expression was observed in 30% (60/200) of specimens studied. When present, PD-L1 expression on tumor cells was predominantly observed in geographic association with CD8+ T-lymphocytes, consistent with an adaptive mechanism of expression, Figure 1a. This pattern was most apparent when moderate to severe intensities of CD8+ lymphocytes were present as compared to mild or none, and was observed in all histologic subtypes, Figure 1b. Constitutive PD-L1 expression (tumor cell PD-L1 expression with no TIL) was not observed, although PD-L1 was expressed on immune cells, including lymphocytes and macrophages, without adjacent tumor cell expression.

We previously reported PD-L1 expression in 35% of cutaneous melanoma samples from 54 patients, when a threshold of ≥5% tumor cell expression was used.⁵ Among the more rare melanoma subtypes studied here using the same method, the proportion of PD-L1(+) tumors varied significantly ($P < 0.0001$). When compared to cutaneous melanoma, uveal melanomas demonstrated the lowest proportion of PD-L1 expression, at 10% ($P = 0.0002$), while the highest proportion of PD-L1(+) specimens was seen in the CSD melanomas, at 62% ($P = 0.0073$). The proportion of acral and mucosal melanomas expressing PD-L1 was comparable to cutaneous disease, at 31 and 44% ($P = 0.77$ and 0.38, respectively), Figure 2a. We next examined whether there were differences in the median or range of PD-L1 expression levels by melanoma subtype among the PD-L1(+) specimens. We found that the median levels were similar across subtypes, although acral melanomas seemed to show a lower range of expression, Figure 2b.

Association of PD-L1 Expression with Morphologic Features

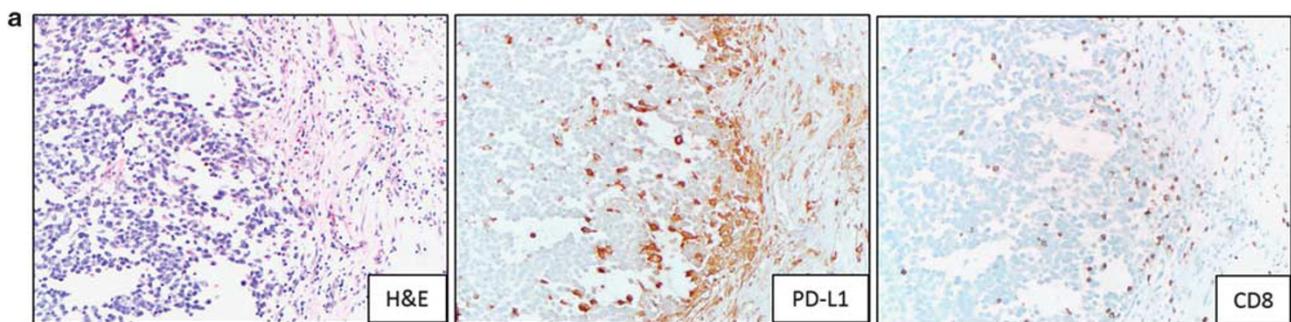
Discrete, well-formed lymphoid aggregates were observed in 75% (6/8) of pure desmoplastic melanomas, as well as in 22% (8/37) of other CSD melanoma specimens. Lymphoid aggregates were not a prominent feature in the other melanoma subtypes studied. PD-L1 was expressed by macrophages and dendritic cells as well as by lymphocytes in these aggregates, Figure 3a. In the 62% (5/8) of PD-L1(+) pure desmoplastic melanomas, PD-L1 expression was observed immediately adjacent to the lymphoid aggregates, and did not exceed a 5% tumor expression level. PD-L1(+) pure desmoplastic melanomas displayed lower median levels of PD-L1 expression as compared to other CSD melanomas ($P=0.047$), Figure 3b, Figure 4a. This is in keeping with recent results showing that PD-L1 expression is lower in pure vs mixed desmoplastic melanomas.¹⁶ PD-L1 expression levels among CSD melanomas were not influenced by the presence of lymphoid aggregates, Figure 4b.

Some of the highest PD-L1 expression levels were seen in CSD melanomas. When we examined those specimens demonstrating higher levels of expression, they were often the mixed desmoplastic subtype with a prominent spindle cell morphology, rather than the pure desmoplastic subtype.

Across all the subtypes, the only other specimen with >50% expression was a mucosal melanoma specimen, which also had a spindled configuration, Figure 2b. When the relationship among melanomas expressing PD-L1 and a spindled vs nested morphology was assessed, there was a statistically significant correlation between higher levels of PD-L1 expression and a spindled configuration (median PD-L1 expression was 50% for cases with a spindled morphology vs 5% for nested; $P<0.0001$), Figure 4c.

DISCUSSION

Cancers may express PD-L1 to form a so-called ‘molecular shield’ against antitumor immune effector cells, facilitating immune evasion.¹⁷ When TILs recognize tumors, they secrete IFN- γ and other pro-inflammatory cytokines, which can upregulate PD-L1 expression in the TME. PD-L1, in turn, may ligate PD-1 on TIL triggering their downregulation.¹⁸ Adaptive PD-L1 expression in the pretreatment melanoma microenvironment has been associated with an improved prognosis,⁵ and is also predictive of response to anti-PD-1/PD-L1 therapies in melanoma and other tumor types.^{13,19–21} Most of the early clinical trials of anti-PD-1/PD-L1 immunotherapies that evaluated PD-L1 as a biomarker of response excluded patients with non-cutaneous melanomas,



b

Melanoma subtype (n)	PD-L1(+) ^a		PD-L1(-)		p-value ^c
	CD8 TIL ^{high} ^b	CD8 TIL ^{low}	CD8 TIL ^{high}	CD8 TIL ^{low}	
acral (16)	4	1	0	11	0.0027
mucosal (36)	15	1	1	19	<0.0001
uveal (103)	10	1	1	91	<0.0001
CSD (45)	21	7	0	17	<0.0001

^a PD-L1(+) is defined as $\geq 5\%$ of tumor cells demonstrating membranous expression

^b CD8 TIL^{high} is defined as any with score of “moderate” or “severely” dense infiltrates, and CD8 TIL^{low} is defined as those with scores of “none” or “mildly” dense infiltrates as described in the Methods. Constitutive expression (PD-L1(+)) with no TIL present) was not observed in any case.

^c p-value by Fisher’s Exact test

Figure 1 All melanoma subtypes demonstrate PD-L1 expression in geographic association with CD8+ tumor-infiltrating lymphocytes (TIL), consistent with an adaptive (IFN- γ -mediated) mechanism of expression. (a) PD-L1 expression on both tumor cells and immune cells is observed at the tumor-host interface in a vulvar mucosal melanoma. 200x original magnification, all panels. (b) The adaptive pattern of expression was consistently observed across all melanoma subtypes studied. There were only two melanomas studied with moderate–severe grade of TIL present that lacked PD-L1 expression (2/54, 4%).

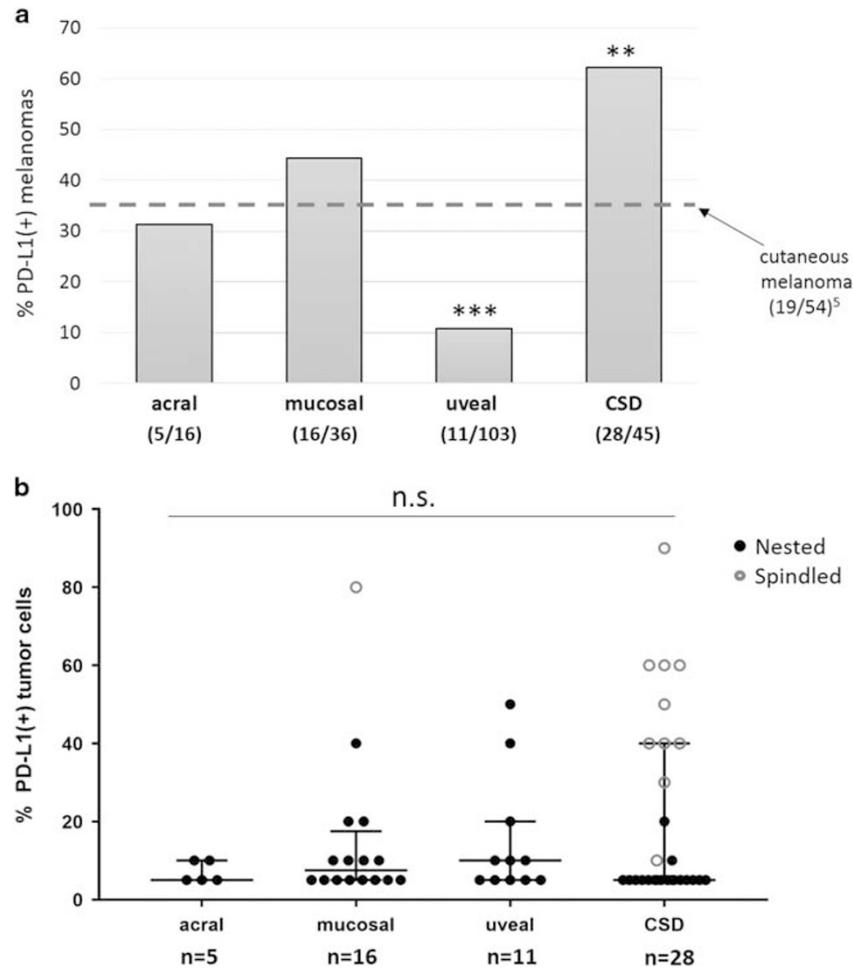


Figure 2 Prevalence and levels of PD-L1 expression vary by histologic subtype of melanoma. (a) Uveal melanomas showed the lowest proportion of cases with PD-L1 expression, while CSD melanomas showed the highest proportion. The proportion of PD-L1(+) tumors in both of these subtypes was significantly different from conventional cutaneous melanoma ($***P=0.0002$ and $**P=0.0073$, chi-square test), the latter reported previously using the same staining and scoring methods.⁵ The proportion of PD-L1(+) cases among acral and mucosal melanomas was not significantly different from that observed in cutaneous disease. (b) PD-L1 expression levels were compared by melanoma subtype in cases that were PD-L1(+). A narrower range of expression levels was observed in acral melanomas when compared to the other subtypes, but significant differences in the median levels of PD-L1 expression were not observed ($P=0.91$). n.s., not significant.

or did not distinguish between distinct histologic subtypes. Many of the more recent studies reporting response rates to anti-PD-1/PD-L1 for patients with melanoma variants did not study PD-L1 expression. We present here a detailed characterization of patterns, prevalence, and levels of PD-L1 expression in four distinct subtypes of melanoma.

We found that the proportion of PD-L1(+) cases by melanoma subtype generally tracked the reported ORRs to anti-PD-1/PD-L1 (Table 1). In our study, 62% of CSD melanomas were PD-L1(+) compared to 31% of acral melanomas, 44% of mucosal melanomas, and 10% of uveal melanomas. In a previous study, 35% of cutaneous melanomas were PD-L1(+) using a similar IHC method and scoring system. Reported overall ORRs for these subtypes range from 71% for desmoplastic (pure or mixed) melanoma to 8% for uveal melanoma, while ORRs in acral and mucosal

melanomas are intermediate, at 29 and 23%, respectively. Patients with advanced conventional cutaneous melanoma show ORR on the order of 30–40%,^{19,22–29} which varies depending on whether the agents are given in the first line or as later-line therapy. Importantly, while the proportion of PD-L1(+) tumors in these subtypes appears to generally parallel the reported ORR on a population level, PD-L1 as a biomarker has limitations on a per-patient basis. Indeed, patients whose tumors are PD-L1(+) may not respond, while ~15% of patients with PD-L1(–) tumors demonstrate an antitumor response to therapy.¹

While tumor cell PD-L1 expression varied among melanoma subtypes, we found that when present, it was geographically associated with CD8+ T-cells, similar to most conventional cutaneous melanomas. Previous studies have supported the presence of a possible IFN- γ -mediated adaptive

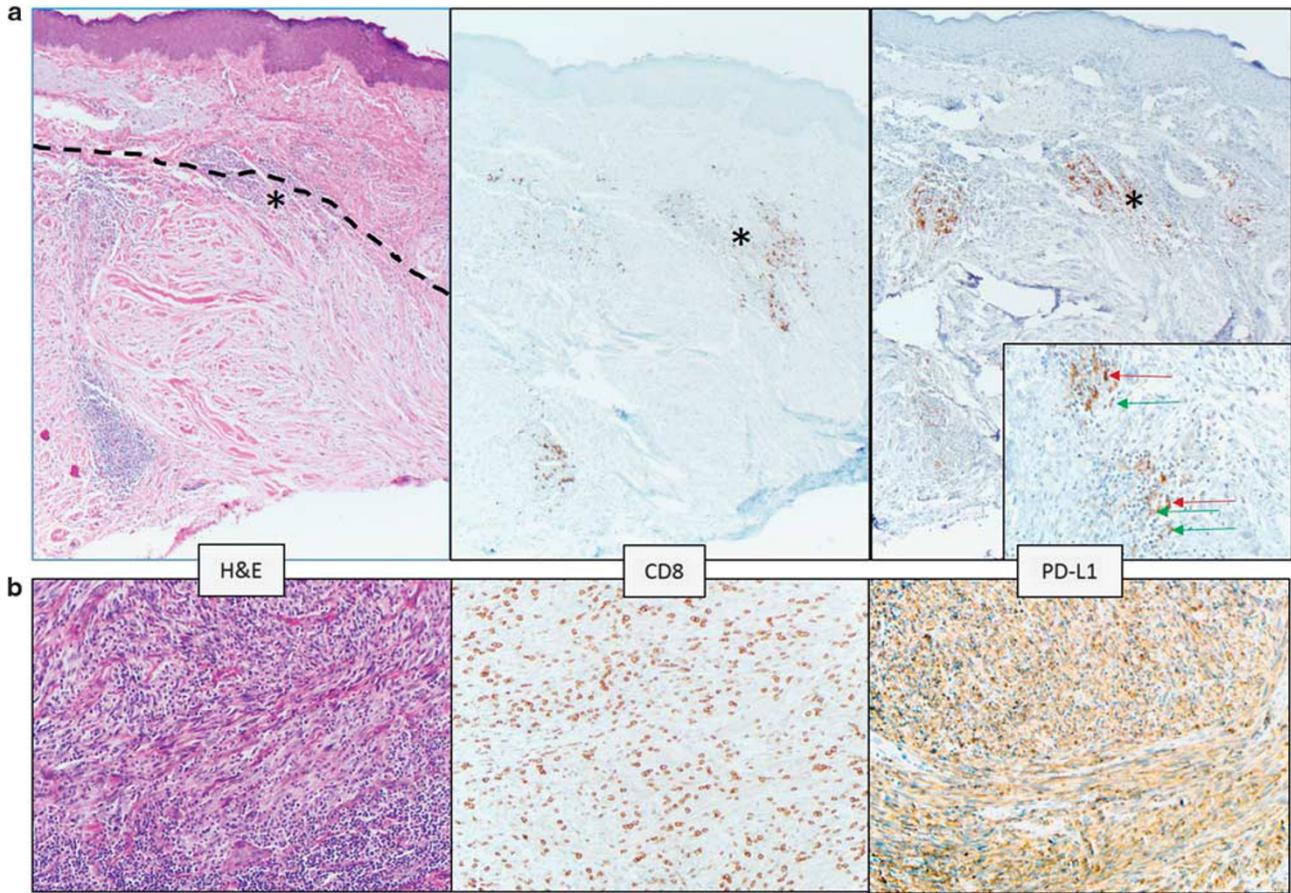


Figure 3 Pure desmoplastic melanomas demonstrate a different PD-L1 expression pattern than other CSD melanomas. (a) The dotted line in the left panel delineates the upper boundary of a desmoplastic melanoma centered in the dermis. Solar elastosis is a prominent feature in the superficial dermis. Lymphoid aggregates are present, and one at the boundary of the melanoma and normal dermis is marked with an asterisk (*) and is shown on the inset of the right panel. In the pure desmoplastic melanomas, PD-L1 expression was observed on lymphocytes (green arrow on inset) and macrophages (red arrows on inset) in lymphoid aggregates. This case did not show PD-L1 expression on tumor cells. Original magnification, $\times 100$, inset $\times 400$. (b) CSD melanoma with spindled morphology showing 60% tumor cell PD-L1 expression associated with a 'severe' grade CD8+ TIL infiltrate. 'Severe' grade is defined as a diffuse infiltrate of TIL throughout the melanoma. $\times 200$ original magnification.

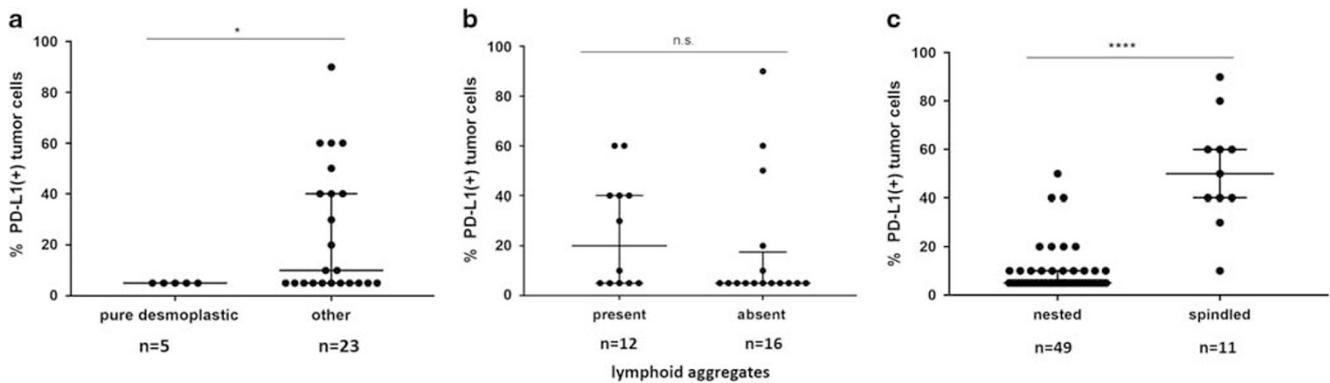


Figure 4 Association of morphologic features with levels of PD-L1 expression. (a) Among PD-L1(+) CSD melanomas, there were significantly higher median PD-L1 expression levels in cases lacking a pure desmoplastic phenotype ($*P=0.047$, Mann-Whitney U -test). (b) The presence or absence of lymphoid aggregates did not correlate with the levels of PD-L1 expression observed in PD-L1(+) CSD melanomas ($P=0.22$). (c) Among all melanoma subtypes, PD-L1(+) tumors with a spindled morphology demonstrated a higher percentage of tumor cells with PD-L1 display compared to those with a nested morphology ($****P<0.0001$). n.s., not significant.

mechanism of PD-L1 display in uveal melanoma cell lines.³⁰ The association between PD-L1 expression and TILs is also in keeping with a prior observation that the presence of TILs is associated with improved outcomes in acral melanoma³¹ and a lower risk of metastasis in mucosal melanoma,³² and that patients with PD-L1(+) mucosal melanoma experience a significantly longer recurrence-free survival.³³

CSD melanomas showed two distinct patterns of PD-L1 display. In cases of pure desmoplastic melanoma, PD-L1 expression by tumor cells was low, and in fact was only observed on tumor cells immediately surrounding the characteristic lymphoid aggregates. In contrast, those that were the mixed desmoplastic type or lentigo maligna type showed some of the highest levels of PD-L1 expression. In each of these latter cases, PD-L1 expression was associated with a diffuse CD8+ T-cell infiltrate, extending throughout the tumor. Notably, the only non-CSD melanoma case with >50% PD-L1 expression also had a spindle morphology and a moderate–severe TIL infiltrate. Spindle cell morphology in melanomas is thought to represent a more immature, dedifferentiated, mesenchymal phenotype.^{34,35} PD-L1 expression has previously been associated with an epithelial to mesenchymal transition (EMT) in other tumor types,^{36,37} though the relationship between PD-L1, EMT, and the presence of a cytotoxic T-cell infiltrate was not assessed. Our findings comparing the pure desmoplastic type vs other CSD melanomas raise the possibility that it could be a combination of EMT (as exemplified here by a spindle cell morphology) and a diffusely-arrayed antitumor immune response that may contribute to increased levels of PD-L1 expression.

Features beyond PD-L1 expression such as mutational burden have also been proposed as biomarkers of response to anti-PD-1.^{38,39} Among all melanomas, CSD melanomas have the highest mutational burden (median 62 mutations/megabase (Mb)), with UV radiation serving as the dominant mutagen.⁴⁰ Cutaneous melanoma has a mutation rate of ~15 mutations/Mb,^{41,42} while the somatic mutation rate in acral and mucosal melanomas is 5–10-fold lower.^{43,44} Uveal melanomas have the lowest mutational burden of any melanoma subtype.⁴⁵ Thus, mutational burden also generally parallels the reported ORRs for melanoma subtypes, though perhaps not as closely as PD-L1 expression. It would be anticipated that if mutational burden were the dominant factor predicting response to therapy, mucosal and acral melanomas would have a proportionately lower response rate than cutaneous melanomas, which is not the case. A recent study showed that mutational burden was a contributing factor to general prognosis for patients with melanoma, but its relative contribution was less than PD-1/PD-L1 axis molecule expression.⁷ It is possible that a similar relationship between these factors also exists when predicting therapeutic response to anti-PD-1, ie, mutational burden is a contributing factor to response, but is subdominant to PD-L1 expression. More recent studies have identified chromosomal alterations as contributing genetic factors of resistance to

immunotherapy.^{46,47} The relationship of these alterations to PD-L1 expression or specific melanoma subtypes has yet to be defined.

Pure desmoplastic melanomas tend to be characterized by the presence of lymphoid aggregates. Such intratumoral lymphoid aggregates have both prognostic and therapeutic value, as they facilitate lymphocyte recruitment and help maintain both T-cell and B-cell antitumor responses.⁴⁸ We found similar proportions of PD-L1(+) tumors in both pure desmoplastic melanoma and other CSD melanomas, though the latter showed significantly higher levels of expression. An early report studying patients with desmoplastic melanomas receiving anti-PD-1 showed similar response rates for the pure and mixed variants.⁴⁹ It is possible that responses in the pure variant may be facilitated by the lymphoid aggregates, as well as by the presence of a high mutational load. This highlights one of the limitations of this study, which is that the patients included in the analysis were not treated with anti-PD-1; the relationships proposed here between lymphoid aggregates and the levels, prevalence, and patterns of PD-L1 expression with the anti-PD-1 response are based on historical response rates.

Other potential limitations of this study include sampling and the stage of lesions studied, as well as the use of a threshold to dichotomize PD-L1 expression. PD-L1 expression may be focal and geographic. We have previously validated the use of TMAs for studying PD-L1 expression in squamous cell carcinoma and showed 85% concordance in tumor cell PD-L1 expression between tumor cores and whole-mount sections,¹⁵ but it is possible that requisite sampling ratios may vary by tumor type. Thus the reported rates of PD-L1 expression may be underestimated here in uveal melanomas, where we used TMAs to study a significant proportion of cases. We did not characterize a broad immune cell repertoire here, which represents another possible limitation. Cell types such as regulatory T-cells and myeloid-derived suppressor cells may have a suppressive effect on the host immune response. In addition, the tumors studied here were predominantly primary lesions, while most patients treated with anti-PD-1 have metastatic disease. Discordance exists in PD-L1 expression between primary and metastatic lesions from the same patient as a result of the focal and dynamic nature of this biomarker.^{3,13,19} Many of the early clinical trials that studied the relationship between PD-L1 expression in the pretreatment TME and response to anti-PD-1 did not specifically require an immediate pretreatment sample from a metastasis for the assessment of PD-L1 status. In a number of cases, primary melanomas or archival samples from metastases taken years prior to treatment were assayed.^{13,19,23,25} It remains to be determined whether a specimen from a metastasis obtained immediately prior to treatment may have improved predictive value over a primary tumor sample when determining the response to therapy in the metastatic setting. The predictive value of PD-L1 expression for the treatment of non-resectable, aggressive primary melanomas, eg, some CSD melanomas, will

also require separate, focused analysis. Lastly, PD-L1 may be considered to demonstrate a continuous range of expression, yet patients are often dichotomized into classes of PD-L1(+) vs PD-L1(-). It is likely that our reported PD-L1 expression prevalences across melanoma subtypes would change if a different threshold for PD-L1 positivity were used (ie, 1 vs 5%).

In summary, our findings highlight that PD-L1 expression varies by melanoma subtype. Patients with CSD melanomas had the highest proportion of PD-L1(+) tumors and PD-L1 expression levels. These features, when combined with other characteristics of the tumor microenvironment, such as high mutational load and the presence of lymphoid aggregates, provide a possible explanation for the observed high response rates to anti-PD-1 monotherapy in these patients. By contrast, uveal melanomas demonstrate the lowest TIL densities and PD-L1 positivity, which may be due to the fact they have the least genomic instability and that the eye is an immunologically privileged site.⁵⁰ As such, our findings also provide an explanation for low observed response rates to anti-PD-1 monotherapy in this melanoma subtype and support ongoing clinical trials exploring dual anti-CTLA-4/PD-1 blockade (NCT02626962) potentially combined with radiation (NCT02913417). Patients with acral and mucosal melanomas lacking PD-L1 expression may also benefit from dual anti-CTLA-4/PD-1 blockade or other combinatorial immunotherapeutic regimens,⁵¹ similar to patients with cutaneous melanoma.

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DISCLOSURE/CONFLICT OF INTEREST

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