

Overall Survival and PD-L1 Expression in Metastasized Malignant Melanoma

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BACKGROUND: Cancers are known to elude the immune system, for example, by MHC loss, FAS up-regulation, or increased secretion of TGF- β . Recently, ligands of coinhibitory receptors like programmed cell death ligand-1 (PD-L1, B7-H1) have come to attention for their role in tumor immune escape. Various tumors have been tested for PD-L1 expression, and conflicting results were obtained regarding its correlative impact on patient survival. This study aimed to determine the prognostic relevance of PD-L1 expression for the survival of melanoma patients. **METHODS:** Paraffin-embedded nevi, primary melanoma, and in-transit, lymph node, and distant organ metastases from a set of 63 stages III-IV melanoma patients referred to the Netherlands Cancer Institute between 2000 and 2004 for a sentinel-node procedure or systemic therapy were studied. A large effort was invested in validating specific PD-L1 staining. In addition to immunological factors such as T-cell infiltration (CD8, CD4, and regulatory T cells), TGF- β and MHC-I expression were assessed. **RESULTS:** Longitudinal analysis revealed no relevant PD-L1 expression on primary melanoma compared with metastatic disease. No significant correlations with prognosis were found regarding immunological factors, whereas known prognostic markers such as Breslow thickness and sex could be confirmed. Analyses of the overall survival of our patient cohort did not reveal a negative association with PD-L1 expression. **CONCLUSIONS:** Correlation of overall survival with PD-L1 expression by melanoma cells remains controversial, and future clinical studies should focus on antibody validation and time of analysis in respect to disease progression. *Cancer* 2011;117:2192-201. © 2010 American Cancer Society.

KEYWORDS: PD-1, PD-L1, B7-H1, melanoma.

Melanoma is a highly immunogenic malignancy. Recent efforts have focused on immunotherapeutic approaches, such as coinhibitory molecule targeting (eg, CTLA-4),¹ using vaccines,² or adoptively transferring tumor-specific T cells.³

Despite often achieving surrogate goals such as expansion and differentiation of tumor-specific immune cells, clinical responses (besides CTLA-4 blockade) based on WHO or RECIST criteria or improvement of overall survival have thus far rarely been observed or have been difficult to correlate with conventional markers used for immune monitoring.⁴

The presence of melanoma-specific T cells in the microenvironment of growing tumors⁵ suggests tumor immune escape or resistance to effector T cells. Indeed, ex vivo analysis of tumor-infiltrating lymphocytes (TILs) often indicates defective antigen-specific proliferation, cytolytic activity, and cytokine production that can be reversed by in vitro culture.⁶⁻⁸ Mechanisms thought to be involved are T-cell anergy, regulatory T cells, TGF- β , IL-10 and/or defects in FAS/FAS-L, increased indoleamine 2,3-dioxygenase, myeloid-derived suppressor cells (MDSCs), and stromal protection.^{5,9-12}

Recently, BTLA (B- and T-lymphocyte attenuator) and PD-1 (programmed cell death-1), both T-cell immune-inhibitory receptors of the CD28 family, have been added to the potential mediators of tumor immune escape.^{13,14} Two ligands of PD-1, PDL-1 (PD-1, ligand 1; B7-H1) and PD-L2 (PD-1, ligand 2; B7-DC) have been shown to mediate negative immune regulation by engaging the PD-1 receptor.¹⁵ PD-L1 is expressed on T- and B cells, dendritic cells, endothelial cells, and the fetomaternal interface, indicating a physiological role of PD-L1 in mediating peripheral tolerance.^{13,15,16}

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PD-L1/PD-1 interactions result in inhibition of T-cell growth and cytokine production. Moreover, tumor cell-expressed PD-L1 can induce inhibition or apoptosis of tumor-specific T cells.¹⁷⁻²¹ PD-L1 has been shown to be expressed in various tumors, for example, melanoma,²¹⁻²³ renal cell carcinoma (RCC),²⁴ non-small cell lung cancer,²⁵ gastric cancer,²⁶ breast cancer,²⁷ ovarian cancer,²⁸ esophageal cancer,²⁹ pancreatic cancer,³⁰ cervical cancer,³¹ and bladder cancer.³²

The prognostic relevance of PD-L1 expression, however, remains controversial so far. Although a significant negative prognostic value of PD-L1 has been found in RCC patients, opposite results were found in other malignancies.³³ The reasons for this disparate data are the small numbers of patient analyzed, the use of different antibodies to stain PD-L1, and the use of frozen versus paraffin-embedded material. Indeed, a decreased frequency of PD-L1-positive RCC was detected in a study that went from analyzing initially only frozen tissues to paraffin-embedded patient samples.^{24,34,35}

In the current work, we aimed to establish a reliable protocol for PD-L1 staining on paraffin-embedded material and addressed the following questions: 1) Does the percentage of PD-L1-positive melanomas increase with disease progression? 2) If so, does PD-L1 expression correlate with prognosis? c) Can PD-L1 expression in melanoma be identified as an independent prognostic marker?

MATERIALS AND METHODS

Patients

Patients with stages III-IV melanoma who had been referred to the Netherlands Cancer Institute—Antoni van Leeuwenhoek Hospital (NKI-AVL) between 2000 and 2004 for a sentinel-node procedure, lymph node dissection, or systemic therapy were retrospectively enrolled in this study. All living patients were asked in a letter explaining the content of the study for permission to analyze their tumor material. All tumor stages were revised at time of referral by the in-house pathologists according to the American Joint Committee on cancer staging system. This study was approved by the ethical board of the NKI-AVL in line with the Dutch regulation for research on human material (WMO).

Immunohistochemistry

Paraffin-embedded blocks of melanoma could be obtained for 63 patients (for 8 patients, inclusion was not possible because no blocks of primary tumors were

provided by the external pathologists). Twenty of the 63 primary tumors could not be analyzed because of lack of sufficient embedded material or poor-quality samples. Tissue sections were cut into 4- μ m-thick sections and dried at 70°C for 30 minutes. Dried sections were deparaffinized in xylene and dehydrated through graded alcohols to water. For all clones but 5H1, antigen retrieval was achieved by boiling the sections in citrate buffer, pH 6.0, using a microwave. The antigen retrieval for 5H1 staining was performed by EDTA boiling (according to the optimized protocol of Karim et al³¹). Endogenous peroxidase activity was blocked by incubating the sections with peroxidase-blocking reagent (DakoCytomation, Glostrup, Denmark) for 10 minutes. To reduce nonspecific binding, sections were incubated for 30 minutes at room temperature (RT) with 5% normal goat serum (Sanquin, Amsterdam, The Netherlands). Primary antibody (Ab) incubation was performed overnight at 4°C in a humidified chamber. Consequently the sections were incubated with poly-HRP-linked goat-antimouse or goat-antirabbit (corresponding to primary Ab; ImmunoVision Technologies) for 30 minutes at RT. Positive staining was visualized with a liquid DAB substrate chromogen system (DakoCytomation, Glostrup, Denmark).

Immunohistochemical staining was performed using the following antibodies: MART-1/Melan-A Ab-3 Cocktail (ThermoFisher Scientific, Waltham, Mass), GP-100 (clone HMB45; DakoCytomation, Glostrup, Denmark), TGF- β 2 (ab3649 Abcam, Cambridge, UK), MHC-I (HC-10 mAb; kind gift of Jacques Neefjes, Amsterdam, The Netherlands), CD8 (clone C8/144B; DakoCytomation, Glostrup, Denmark), CD4 (clone 4B12; MONOSAN, Uden, The Netherlands), and FOXP3 (clone 236A/E7; Abcam, Cambridge, UK).

All antihuman-PD-L1 antibodies tested were titrated in comparison with the corresponding isotype control, and specific binding was proven by competitive preincubation of the anti-PD-L1 Ab with recombinant human PD-L1/FC chimera (R&D Systems, Minneapolis, MN).

We used tonsil and placental tissue to set up the staining, as these organs have been shown to constitutively express PD-L1.³⁶

Analysis of Stained Sections of Tissues

All sections (nevi, primary melanoma, satellite or in-transit metastases, and distant metastases) were scored in a blinded manner using a light microscope. All sections stained for MART-1/Melan-A Ab-3 cocktail, GP-100,

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PD-L1, TGF- β , or MHC-I were scored for the percentage of positive tumor cells.

The melanomas were defined as MART-1, gp100, MHC-I, or TGF- β positive if at least 10% of the tumor cells (based on specific detection limit compared with the isotype control) stained positive. Infiltrating T cells (sum of CD4 and CD8) were scored as a percentage of tumor cells. Because we did not look only at primary melanoma, we could not define TIL infiltrates according to a previous definition as described by Busam et al.³⁷⁻³⁹ We defined tumors to be infiltrated with lymphocytes when the frequency of lymphocyte to tumor reached at least 5% (based on the detection limit of T-cell staining).

FoxP3-positive cells were scored as a percentage of the total number of T cells, and melanomas were defined as Treg-infiltrated if at least 10% of the total number of T-cell (CD4⁺ and CD8⁺) infiltrates were FoxP3 positive. Such a cutoff was based on FoxP3 staining of distant non-tumor tissue. Tumors were defined as PD-L1 positive if at least 1% of the melanoma cells stained positive (based on a background of isotype control staining).

Statistical Analysis

Overall survival analyses were constructed using the Kaplan-Meier technique and compared using the log-rank test. Overall survival and disease-related survival were calculated from date of first diagnosis to date of death or last follow-up (see also Table 1, status at the end of observation in October 2009).

Using Cox proportional hazard analysis, the association of PD-L1 with overall survival was adjusted for sex and Breslow thickness.

RESULTS

Patient Characteristics

In total, 71 patients were potentially eligible for this study. Paraffin-embedded tissue blocks of melanoma could be obtained for 63 patients. The clinical characteristics of the 63 patients are listed in Table 1. The average age of the patients at time of first diagnosis was 53 years (range, 18-91 years; SD, ± 18 years), and 36 (57%) were male. Most of the patients were diagnosed with superficial spreading melanoma (44%), followed by nodular melanoma (40%), metastatic melanoma without known primary tumor (6%), acral lentiginous melanoma (2%), spindle cell melanoma ($n = 1$, 2%), and unclear melanoma subtype (6%). Ulceration was described in 44% of the patients (absent in

Table 1. Patient Characteristics

	No. of Patients (%)
Sex	
Male	36 (57%)
Female	27 (43%)
Histology of first diagnosis	
Superficial spreading melanoma	28 (44%)
Nodular melanoma	25 (40%)
Unknown primary	4 (6%)
Acral lentiginous melanoma	1 (2%)
Spindle cell melanoma	1 (2%)
Unclear	4 (6%)
Ulceration of primary tumor	
No ulceration	18 (29%)
Ulcerating tumor	28 (44%)
No information available	17 (27%)
Presence of lymph angiogenesis in primary tumor	
Not present	15 (24%)
Present	9 (14%)
No information available	39 (62%)
Breslow stage	
≤ 1 mm	0 (0%)
>1 but ≤ 2 mm	12 (20%)
>2 but ≤ 4 mm	29 (48%)
>4 mm	19 (32%)
Site of most distant organ metastasis	
Skin/nodal/GI tract	21 (33%)
Lung	11 (18%)
Liver/brain/bone	31 (49%)
Status at end of study (10/2009)	
Alive with disease	1 (2%)
Alive without disease	18 (29%)
Dead without disease	2 (3%)
Dead of disease	42 (67%)

Data presented as n (%), where the percentages were rounded up to the closest full number. Mean patient age on the date of first diagnosis was 53 years (± 18 years). None of the patients had received systemic treatment prior to referral at the NKI-AVL. Two patients were treated at the NKI-AVL with an experimental therapy (1 with DC vaccination and 1 with anti-CTLA4).

29% of the patients and not reported in 27%) and vascular invasion in 14% (24%, absent; 62%, not reported). The Breslow thickness was more than 1 mm for all patients because of the referral selection (19%, 1-2 mm; 46%, 2-4 mm; 30%, >4 mm; 5%, no data). Distant metastases were skin/nodal (33%), lung (18%), and liver/brain/bone (49%). The majority of patients was treated with standard chemotherapy consisting of DTIC, and 2 patients received an experimental therapy (1 DC vaccination and 1 anti-CTLA4). At the time of analysis 67% of patients were dead of disease, 3% were dead of other reasons, 29% were alive without any sign of active disease,

Table 2. All Tested Antihuman-PD-L1 Antibodies

Clone	Provider	Species	Isotype	Stains on Paraffin	Results	
					Isotype Control	PD-L1 Fc Blocking
MIH1	eBioscience, San Diego, CA	Mouse	IgG1	No	No background	nd
5-496	O. Majdic, University of Vienna Medical School, Vienna, Austria	Mouse	IgG1	No	nd	nd
2-272	O. Majdic, University of Vienna Medical School, Vienna, Austria	Mouse	IgG1	No	No background	nd
27A2	MBL International, Woburn, MA	Mouse	IgG2b	Yes	High background	nd
16E11	Medarex, Princeton, NJ	Mouse	Unknown	No	nd	nd
9A6	Medarex, Princeton, NJ	Mouse	IgG3	No	nd	nd
16A4	Medarex, Princeton, NJ	Mouse	IgG1	No	No background	nd
6H3	Medarex, Princeton, NJ	Mouse	IgG1	No	No background	nd
ETM-79	Medarex, Princeton, NJ	Rabbit	pAb	Yes	No background	Partial block
ETM-80	Medarex, Princeton, NJ	Rabbit	pAb	Yes	No background	Partial block
1105	Medarex, Princeton, NJ	Human	IgG4	No	nd	nd
25C8E8.F8	Medarex, Princeton, NJ	Human	IgG1	No	nd	nd
24B10.G6.D7	Medarex, Princeton, NJ	Human	IgG1	No	nd	nd
5H1	L. Chen, Johns Hopkins University, Baltimore, MD	Mouse	IgG1	High background	No background	nd
#4059	ProSci, Poway, CA	Rabbit	pAb	Yes	No background	Near full block

pAb, polyclonal antibody; nd, not done; n/a, not applicable. Antibodies were tested for staining of paraffin-embedded tissues, isotype background staining, and eventually for antigen specificity.

and 2% were alive with active disease. The median time patients were followed was 207 weeks (minimum, 27 weeks; maximum, 539 weeks). One patient was lost after 2 years of follow-up.

Validation of PD-L1 Staining in Paraffin-Embedded Tissues

A major challenge for correlative studies concerning PD-L1 expression and overall survival is the available antibodies for tissue staining. Whereas frozen samples can be stained very specifically, paraffin-embedded material (which is often the only material available from pathology labs for retrospective analysis) results in staining that is not as strong and often gives a high background.^{23,34,35} Therefore, we tested all those commercially available—2 mAbs provided by Otto Majdic (University of Vienna, Vienna, Austria) and an additional 9 mAbs provided by Alan Korman (Medarex, Princeton, NJ)—for their PD-L1 reactivity in paraffin-embedded material (Table 2).

We found that the clone 27A2 (MBL), commonly used for paraffin material,²⁸ gave a high background equivalent to the isotype mAb control (provided by the same company) and could thus be falsely interpreted as positive staining, unless using proper isotype analysis (Fig. 1b). All antibodies tested, except the rabbit polyclonal anti-PD-L1 antibody from ProSci (catalogue no. 4059; Poway, CA), either did not stain paraffin-embed-

ded tissues (Fig. 1a), gave a high background staining (Fig. 1b,c), or were not blocked by PD-L1 fusion protein competition (Fig. 1d). Only the ProSci Ab was found to fulfill all requirements in an acceptable manner (Fig. 1e). The indirectly postulated loss of PD-L1-positive tumor samples when staining paraffin-embedded material instead of frozen samples³⁴ was proven by direct comparison of paraffin with frozen samples. Consistent with previous data, we found that PD-L1 staining in paraffin material underestimated the number of PD-L1-positive melanomas and the percentage of tumor cells positive for PD-L1.^{21,23,28,35} In detail, 8 of the 12 frozen tumor samples we tested were PD-L1-positive (with 10%-50% positive tumor cells), whereas only 3 of the 12 paraffin counterparts stained positive (with 1%-10% positive cells; data not shown).

Characterization of Melanoma Cohort: MART-1, gp100, and MHC-I Expression and T-Cell Infiltration

Ninety-eight percent and 95% of primary melanomas and 90% and 86% of the metastases were MART-1 and gp100 positive, respectively. MHC-I expression was observed in 45% of the primary melanomas and in 52% of metastases. In both primary and metastatic melanoma, we found 40% of tumor samples containing TIL infiltrates. Analysis for CD8, CD4, and FoxP3 revealed that

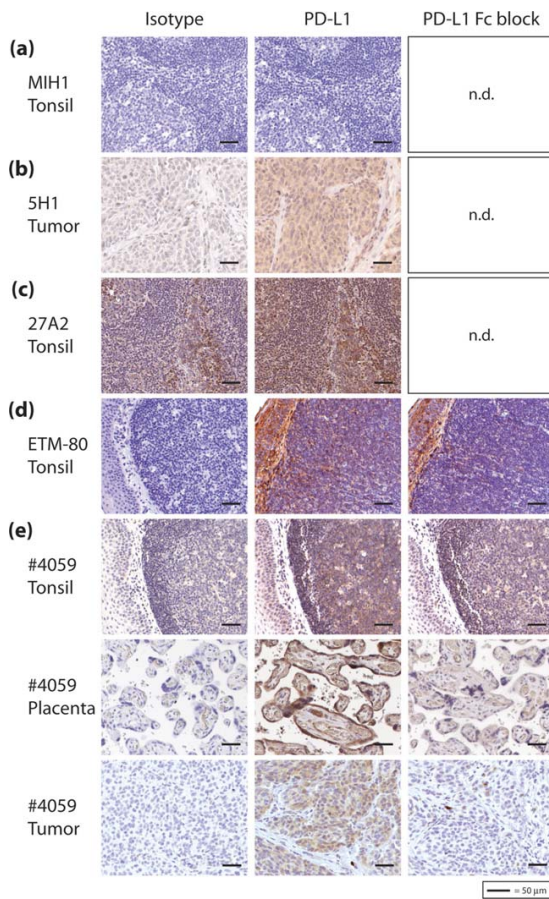


Figure 1. Testing available antihuman-PD-L1 antibodies for paraffin immunohistochemistry is shown. Antibody testing on the indicated tissue of (a) clone MIH1 (representative of staining with clones 5-496, 2-272, 16E11, 9A6, 16A4, 6H3, 1105, 25C8E8.F8, and 24B10.G6.D7), (b) clone 5H1, (c) clone 27A2, (d) ETM-80 (stained similarly to ETM-79), and (e) #4059 is shown. The inserted black bars indicate 50 μ m.

52% had a majority of CD8-positive TILs, whereas only 3% of the tumors had mainly CD4-positive TILs. The remaining 45% of tumors showed comparable CD4 and CD8 T-cell numbers. Of these TIL infiltrates, the majority had a relevant subgroup of FoxP3-positive cells (72%). Sixty-three percent of primary and 46% of metastatic melanoma were found to express TGF- β . Representative staining of samples defined as positive or negative are shown in Figure 2.

PD-L1 Expression During Tumor Progression

To address the association of PD-L1 expression with patient survival in a longitudinal way (during disease pro-

gression), PD-L1 expression was analyzed in benign nevi ($n = 10$), primary melanomas ($n = 43$), satellite metastases ($n = 8$), in-transit metastases ($n = 20$), lymph node metastases ($n = 21$), and distant organ metastases ($n = 22$). We found that 20% of the benign nevi samples were scored PD-L1 positive, whereas only 5% of primary melanomas were PD-L1 positive.

A higher frequency of PD-L1-positive staining was observed in satellite metastases (25%) and in in-transit metastases (40%). Lymph node and distant organ metastases stained PD-L1+ less frequently, in 14% and 18%, respectively (Fig. 3).

Patients who expressed PD-L1 at any time and for whom at least 2 samples were available during follow-up were analyzed for the intensity of PD-L1 expression in primary melanoma, in-transit or satellite metastasis, and lymph node or distant organ metastasis. Only 2 of 11 patients (Mel015 and Mel029) showed absent PD-L1 expression in the distant metastases compared with the earlier metastases. In all other patients, PD-L1 expression increased with disease progression (data not shown).

PD-L1 Expression and Patient Overall Survival

To allow an analysis of PD-L1 expression despite variable expression during melanoma progression (see also Fig. 3), we grouped our patient population as patients in whom PD-L1 expression could never be detected (never group) or patients in whom PD-L1 was detected at least 1 time or at 1 site (ever group) and analyzed the correlation of PD-L1 expression with overall survival from date of first diagnosis.

Although a trend toward better survival if PD-L1 was ever detected was suggested, the association of PD-L1 with overall survival was not statistically significant ($P = .2$; Fig. 4).

TIL Infiltration, Treg Infiltration, TGF- β , MHC-I Expression, and Overall Survival

Aside from PD-L1 expression, we analyzed additional immunological markers thought to correlate with a better prognosis (TIL infiltration) or worse prognosis (Treg infiltration, TGF- β expression, and MHC loss) in our melanoma patient cohort. None of these immunological parameters showed any association with patient outcome in our sample (Fig. 5).

DISCUSSION

PD-L1 (B7-H1) is expressed in various tumor types, and its interaction with PD-1 has been shown to inhibit T-cell cytokine production and proliferation.^{15,33} PD-L1/PD-1

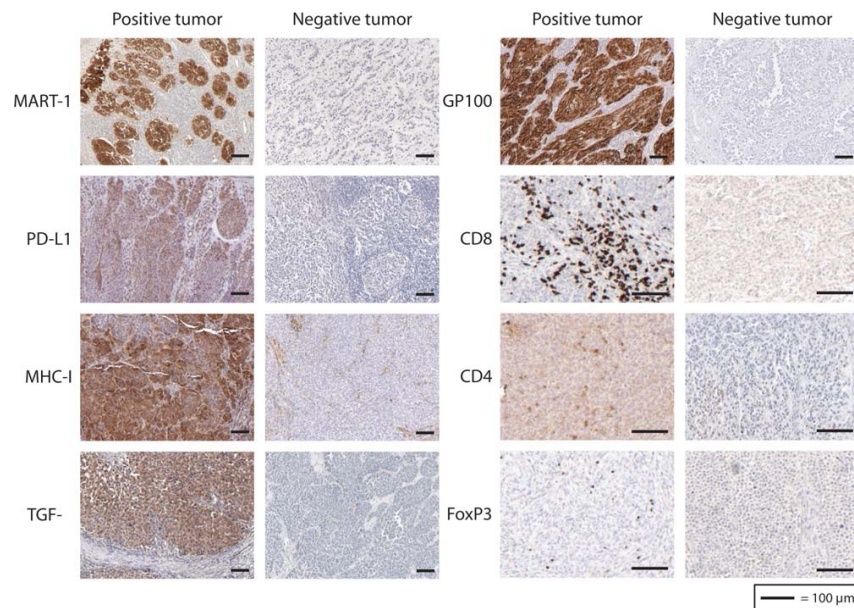


Figure 2. Histological staining of paraffin-embedded tissues is shown. Depicted are representative tissue samples that stained either positive or negative for MART-1, GP-100, PD-L1, MHC-I, TGF- β , CD8, CD4, or FoxP3. The inserted black bars indicate 100 μ m.

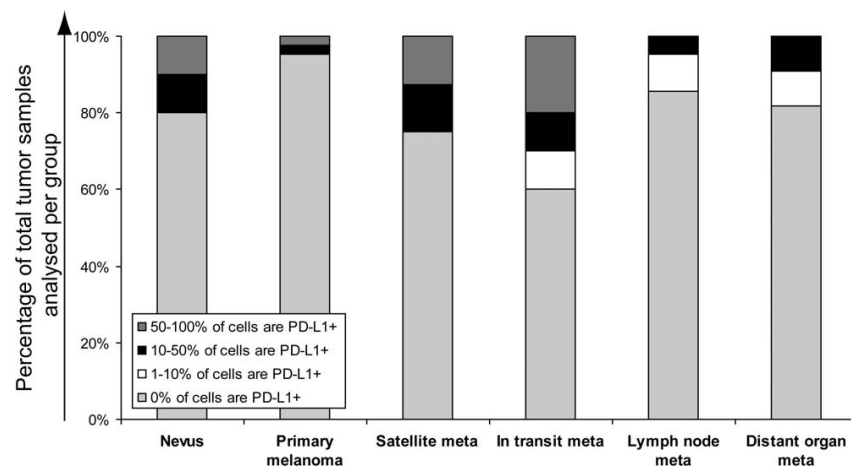


Figure 3. PD-L1 expression varied during disease progression. PD-L1 staining was scored for the percentage of positive tumor cells. (benign nevi, $n = 10$; primary tumor, $n = 43$; satellite meta, $n = 8$; in-transit meta, $n = 20$; lymph node meta, $n = 21$; distant organ meta, $n = 22$).

blockade has also been associated with improved tumor growth (reviewed in Keir et al¹⁵ and Blank et al³³). Because of its role in tumor development, the prognostic value of PD-L1 has been studied in several instances and found to be associated with poor prognosis in non-small cell lung cancer, gastric carcinoma, and renal cell carcinoma.^{25,26,35} De-

spite several reports of PD-L1 expression in melanoma,^{21-23,40} its prognostic relevance in melanoma patients has been suggested only recently.⁴¹ In line with this recent study, the current work addressed whether, in the melanoma setting, PD-L1 expression increases during disease progression and whether its expression correlates with other immunological

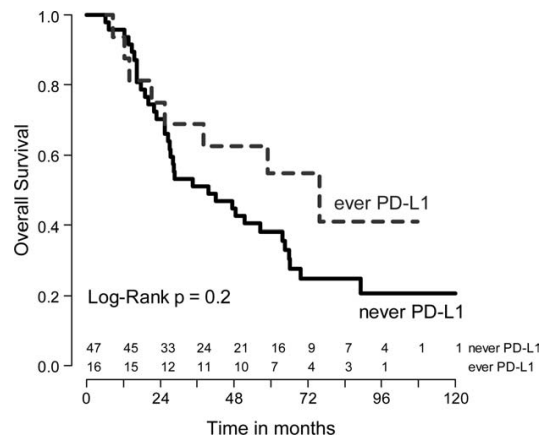


Figure 4. Patient overall survival did not correlate with PD-L1 expression. Patients were grouped based on the presence (= ever, >1% of tumor cells staining PD-L1 positive) or the absence (= never) of PD-L1 expression at any point during disease progression. Numbers at the bottom of the graph represent remaining patients.

factors thought to mediate tumor immune escape based on preclinical animal models.⁵

First, a large effort was invested to obtain reliable PD-L1 staining in paraffin-embedded material, because in our hands the antibody that has been claimed to stain best in paraffin, PD-L1,^{28,29} gave high background staining. A broad array of commercially available mAbs, as well as mAbs provided by the University of Vienna (Vienna, Austria) and clones provided by Medarex (Princeton, NJ), were tested for their staining patterns. Only 1 antibody gave no background staining (compared with its isotype control) and was competitively blocked by the addition of PD-L1Fc protein (polyclonal anti-PD-L1 Ab, #4059; ProSci, Poway, CA) in 2 PD-L1-expressing tissues: tonsil and placenta (Fig. 1, Table 2).

Our study cohort comprised a poor-prognosis population of malignant melanoma patients (stages III-IV) that had been referred to our center between 2000 and 2004. Contrary to Hino et al⁴¹ and in line with our previous data from frozen material,²³ PD-L1 expression was observed in only a minority of primary tumors, in contrast with PD-L1 expression in metastases. Interestingly, a higher percentage of PD-L1-expressing metastases was observed in satellite and in-transit metastases, compared with lymph node and distant organ metastases, suggesting a preferential kinetic of PD-L1 expression during disease progression. However, the lack of sufficient patients available for complete longitudinal analysis did not permit the

defining of a reliable pattern of PD-L1 expression (Fig. 3). Yet primary tumors consistently expressed less PD-L1 than did metastases, and PD-L1 expression was variable during disease progression, which is an important factor to take into account during future clinical studies.

The prognostic value of PD-L1 in the current study cohort was analyzed. Our observations did not support PD-L1 expression being a poor prognostic factor for malignant melanoma.⁴¹ In fact, the tumors expressing PD-L1 in our series showed an insignificant trend toward better prognosis (Fig. 4).

Such discrepancies concerning the prognostic value of PD-L1 in different studies may result from the use of different staining protocols or paraffin for embedding. Also, it is unclear whether other groups performing such studies have tested their antibodies against isotype controls and PD-L1Fc protein blockade to ensure lack of background or unspecific signals are not positive signal interpreted.

Another likely explanation for the conflicting results between the recently published data⁴¹ and our data may be that small series of fewer than 100 melanoma patients from diverse origin were analyzed. The most noticeable difference between the Asian melanoma patient population and the 2 European cohorts is tumor type. In our cohorts, superficial and nodular melanomas were predominantly diagnosed, whereas the cohort of Hino et al mainly consisted of patients with acral lentiginous melanomas. The latter have been shown to preferentially express c-kit amplifications or mutations, whereas the melanomas from the European cohort are likely to more often express *BRAF* mutations.^{42,43} Moreover, our cohort contained a majority of women, in whom PD-L1 was detected more often (63% versus 38%) than in the male counterpart. Women are known to have a better prognosis in melanoma⁴⁴ and may have thus influenced the prognostic analysis of PD-L1. Whether melanoma in female patients indeed expresses PD-L1 more often deserves further investigation. Although PD-L1/PD-1 interaction has been shown to play a role in tumor development,^{21,45,46} other immunological phenomena could explain the lack of prognostic value of PD-L1. PD-L1 is not constitutively high in antigen-presenting or tumor cells, but it is up-regulated on exposure to interferon.^{17,45} Thus, our observation of PD-L1 expression in the tumors of patients with better prognosis could indicate a preexisting tumor inflammatory environment as a consequence of IFN γ -producing tumor infiltrating lymphocytes (TILs). Although the prognostic value of TIL infiltration in

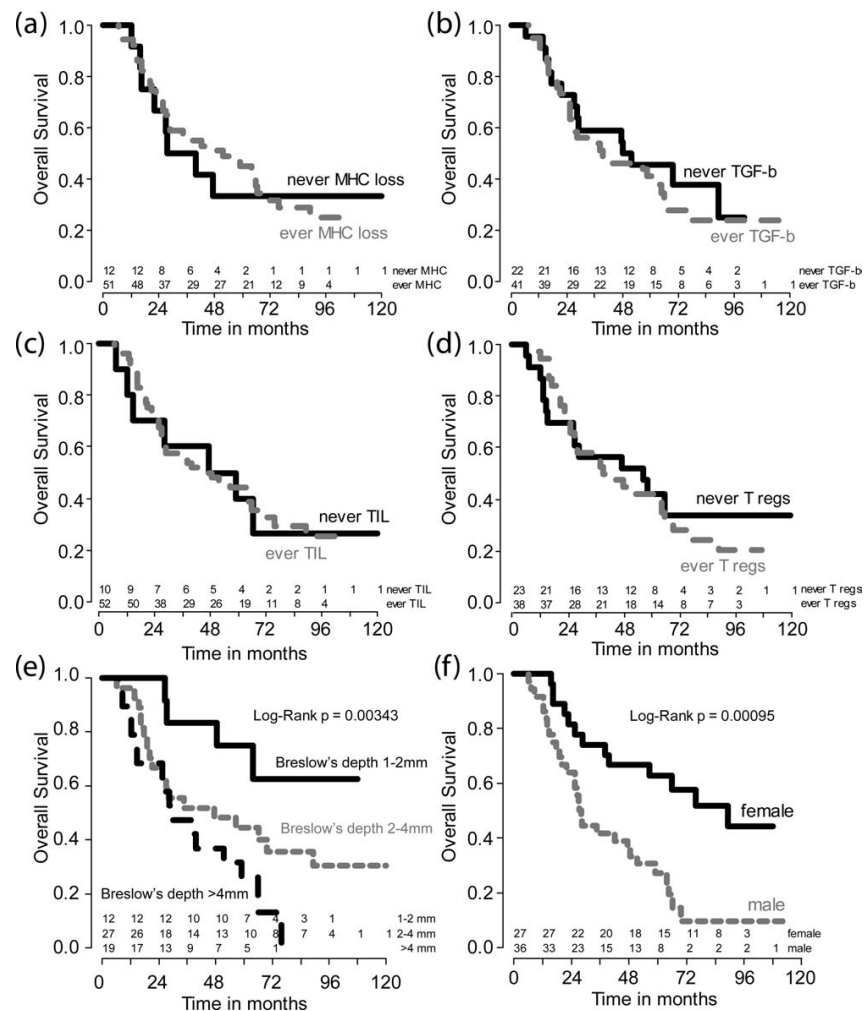


Figure 5. Patient overall survival did not correlate with immunological parameters such as MHC loss (a), TGF- β expression (b), TIL infiltration, (c) and presence of infiltrating regulatory T cells (d), but it correlated with Breslow thickness (e) and sex (f). Ever MHC loss corresponds to patients for whom >90% MHC loss was observed in at least 1 stage throughout disease progression. Ever TGF- β corresponds to the detection of >10% positive tumor cells in at least 1 stage of disease progression. Ever TIL defines patient samples for which at least 5% T-cell infiltration was detected in at least 1 stage of disease progression. Never Treg corresponds to the grouping of never TIL and ever TIL with less than 10% FoxP3 staining among total T cells at any given stage of disease progression.

melanoma is still under debate because of contradictory results,⁴⁷ the results of a recent study analyzing a group of 294 patients argues in favor of a correlation between the presence of TILs and improved survival.⁴⁸ Finally, although preclinical models show that PD-1/PD-L1 blockade improves tumor control,^{21,45,46} recent data indicate that PD-L1-mediated inhibition can occur not only during tumor-effector T-cell interaction, but also during APC-T-cell interaction and regulatory T-cell expansion.

In addition, PD-L1 is found in monocytes and myeloid-derived suppressor cells.^{15,49,50} Therefore, the positive effect of PD-L1 blockade on tumor growth control could evolve from alterations in compartments other than that of the tumor cell.

Although the well-established clinical parameters of Breslow thickness and sex did significantly correlate with overall survival ($P = .00343$ and $P = .00095$, respectively; Fig. 5e,f), in the multivariate analysis including sex,

Breslow thickness, and PD-L1 expression, we found no significant difference concerning PD-L1 expression and overall survival (hazard ratio, 0.72; $P = .45$). When studying other immunological parameters, we found that TIL infiltration, TGF- β expression, regulatory T-cell infiltration, and MHC down-regulation did not correlate with patient prognosis, possibly because of small cohort size (Fig. 5a-d).

In summary, no significant association of PD-L1 expression with outcome in melanoma patients was found. In future studies, the time of PD-L1 expression assessment should be carefully considered, and frozen material should be the primary source of data (unless new antibodies performing better in paraffin become available). While these conditions are not met, one should be careful about concluding that PD-L1 has negative clinical prognostic value in melanoma.

CONFLICT OF INTEREST DISCLOSURES

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REFERENCES

- Weber J. Review: anti-CTLA-4 antibody ipilimumab: case studies of clinical response and immune-related adverse events. *Oncologist*. 2007;12:864-872.
- Eggermont AM. Therapeutic vaccines in solid tumours: can they be harmful? *Eur J Cancer*. 2009;45:2087-2090.
- Rosenberg SA, Dudley ME. Adoptive cell therapy for the treatment of patients with metastatic melanoma. *Curr Opin Immunol*. 2009;21:233-240.
- Eggermont AM. Immunotherapy: Vaccine trials in melanoma—time for reflection. *Nat Rev Clin Oncol*. 2009;6:256-258.
- Gajewski TF. Identifying and overcoming immune resistance mechanisms in the melanoma tumor microenvironment. *Clin Cancer Res*. 2006;12:2326s-2330s.
- Guilloux Y, Viret C, Gervois N, et al. Defective lymphokine production by most CD8+ and CD4+ tumor-specific T cell clones derived from human melanoma-infiltrating lymphocytes in response to autologous tumor cells in vitro. *Eur J Immunol*. 1994;24:1966-1973.
- Radoja S, Saio M, Schaer D, Koneru M, Vukmanovic S, Frey AB. CD8(+) tumor-infiltrating T cells are deficient in perforin-mediated cytolytic activity due to defective microtubule-organizing center mobilization and lytic granule exocytosis. *J Immunol*. 2001;167:5042-5051.
- Roussel E, Gingras MC, Grimm EA, Roth JA. High expression of adhesion molecules/activation markers with little interleukin-2, interferon gamma, and tumor necrosis factor beta gene activation in fresh tumor-infiltrating lymphocytes from lung adenocarcinoma. *Cancer Immunol Immunother*. 1995;41:1-9.
- Baumgartner J, Wilson C, Palmer B, Richter D, Banerjee A, McCarter M. Melanoma induces immunosuppression by up-regulating FOXP3(+) regulatory T cells. *J Surg Res*. 2007;141:72-77.
- Mourmouras V, Fimiani M, Rubegni P, et al. Evaluation of tumour-infiltrating CD4+CD25+FOXP3+ regulatory T cells in human cutaneous benign and atypical naevi, melanomas and melanoma metastases. *Br J Dermatol*. 2007;157:531-539.
- Ilkovitch D, Lopez DM. Immune modulation by melanoma-derived factors. *Exp Dermatol*. 2008;17:977-985.
- Singh S, Ross SR, Acena M, Rowley DA, Schreiber H. Stroma is critical for preventing or permitting immunological destruction of antigenic cancer cells. *J Exp Med*. 1992;175:139-146.
- Nishimura H, Honjo T. PD-1: an inhibitory immunoreceptor involved in peripheral tolerance. *Trends Immunol*. 2001;22:265-268.
- Watanabe N, Gavrieli M, Sedy JR, et al. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. *Nat Immunol*. 2003;4:670-679.
- Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol*. 2008;26:677-704.
- Petroff MG, Chen L, Phillips TA, Hunt JS. B7 family molecules: novel immunomodulators at the maternal-fetal interface. *Placenta*. 2002;23(Suppl A):S95-S101.
- Freeman GJ, Long AJ, Iwai Y, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med*. 2000;192:1027-1034.
- Latchman Y, Wood CR, Chernova T, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol*. 2001;2:261-268.
- Tseng SY, Otsuji M, Gorski K, et al. B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells. *J Exp Med*. 2001;193:839-846.
- Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc Natl Acad Sci U S A*. 2002;99:12293-12297.
- Dong H, Strome SE, Salomao DR, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med*. 2002;8:793-800.
- Yang W, Chen PW, Li H, Alizadeh H, Niederkorn JY. PD-L1: PD-1 interaction contributes to the functional suppression of T-cell responses to human uveal melanoma cells in vitro. *Invest Ophthalmol Vis Sci*. 2008;49:2518-2525.
- Blank C, Kuball J, Voelkl S, et al. Blockade of PD-L1 (B7-H1) augments human tumor-specific T cell responses in vitro. *Int J Cancer*. 2006;119:317-327.
- Thompson RH, Gillett MD, Cheville JC, et al. Costimulatory B7-H1 in renal cell carcinoma patients: Indicator of tumor aggressiveness and potential therapeutic target. *Proc Natl Acad Sci U S A*. 2004;101:17174-17179.
- Konishi J, Yamazaki K, Azuma M, Kinoshita I, Dosaka-Akita H, Nishimura M. B7-H1 expression on non-small cell lung cancer cells and its relationship with tumor-infiltrating lymphocytes and their PD-1 expression. *Clin Cancer Res*. 2004;10:5094-5100.
- Wu C, Zhu Y, Jiang J, Zhao J, Zhang XG, Xu N. Immunohistochemical localization of programmed death-1 ligand-1 (PD-L1) in gastric carcinoma and its clinical significance. *Acta Histochem*. 2006;108:19-24.

27. Ghebeh H, Mohammed S, Al-Omair A, et al. The B7-H1 (PD-L1) T lymphocyte-inhibitory molecule is expressed in breast cancer patients with infiltrating ductal carcinoma: correlation with important high-risk prognostic factors. *Neoplasia*. 2006;8:190-198.
28. Hamanishi J, Mandai M, Iwasaki M, et al. Programmed cell death 1 ligand 1 and tumor-infiltrating CD8+ T lymphocytes are prognostic factors of human ovarian cancer. *Proc Natl Acad Sci U S A*. 2007;104:3360-3365.
29. Ohigashi Y, Sho M, Yamada Y, et al. Clinical significance of programmed death-1 ligand-1 and programmed death-1 ligand-2 expression in human esophageal cancer. *Clin Cancer Res*. 2005;11:2947-2953.
30. Nomi T, Sho M, Akahori T, et al. Clinical significance and therapeutic potential of the programmed death-1 ligand/programmed death-1 pathway in human pancreatic cancer. *Clin Cancer Res*. 2007;13:2151-2157.
31. Karim R, Jordanova ES, Piersma SJ, et al. Tumor-expressed B7-H1 and B7-DC in relation to PD-1+ T-cell infiltration and survival of patients with cervical carcinoma. *Clin Cancer Res*. 2009;15:6341-6347.
32. Inman BA, Sebo TJ, Frigola X, et al. PD-L1 (B7-H1) expression by urothelial carcinoma of the bladder and BCG-induced granulomata: associations with localized stage progression. *Cancer*. 2007;109:1499-1505.
33. Blank C, Gajewski TF, Mackensen A. Interaction of PD-L1 on tumor cells with PD-1 on tumor-specific T cells as a mechanism of immune evasion: implications for tumor immunotherapy. *Cancer Immunol Immunother*. 2005;54:307-314.
34. Thompson RH, Gillett MD, Cheville JC, et al. Costimulatory molecule B7-H1 in primary and metastatic clear cell renal cell carcinoma. *Cancer*. 2005;104:2084-2091.
35. Thompson RH, Kuntz SM, Leibovich BC, et al. Tumor B7-H1 is associated with poor prognosis in renal cell carcinoma patients with long-term follow-up. *Cancer Res*. 2006;66:3381-3385.
36. 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;68:1496-1504.
37. Busam KJ, Antonescu CR, Marghoob AA, et al. Histologic classification of tumor-infiltrating lymphocytes in primary cutaneous malignant melanoma. A study of interobserver agreement. *Am J Clin Pathol*. 2001;115:856-860.
38. Clark WH Jr, Elder DE, Guerry Dt, et al. Model predicting survival in stage I melanoma based on tumor progression. *J Natl Cancer Inst*. 1989;81:1893-1904.
39. Clark WH Jr, From L, Bernardino EA, Mihm MC. The histogenesis and biologic behavior of primary human malignant melanomas of the skin. *Cancer Res*. 1969;29:705-727.
40. Harlin H, Kuna TV, Peterson AC, Meng Y, Gajewski TF. Tumor progression despite massive influx of activated CD8(+) T cells in a patient with malignant melanoma ascites. *Cancer Immunol Immunother*. 2006;55:1185-1197.
41. Hino R, Kabashima K, Kato Y, et al. Tumor cell expression of programmed cell death-1 ligand 1 is a prognostic factor for malignant melanoma. *Cancer*. 116:1757-1766.
42. Curtin JA, Fridlyand J, Kageshita T, et al. Distinct sets of genetic alterations in melanoma. *N Engl J Med*. 2005;353:2135-2147.
43. Curtin JA, Busam K, Pinkel D, Bastian BC. Somatic activation of KIT in distinct subtypes of melanoma. *J Clin Oncol*. 2006;24:4340-4346.
44. Scoggins CR, Ross MI, Reintgen DS, et al. Gender-related differences in outcome for melanoma patients. *Ann Surg*. 2006;243:693-698.
45. Blank C, Brown I, Peterson AC, et al. PD-L1/B7H-1 inhibits the effector phase of tumor rejection by T cell receptor (TCR) transgenic CD8+ T cells. *Cancer Res*. 2004;64:1140-1145.
46. Iwai Y, Terawaki S, Honjo T. PD-1 blockade inhibits hematogenous spread of poorly immunogenic tumor cells by enhanced recruitment of effector T cells. *Int Immunol*. 2004.
47. Oble DA, Loewe R, Yu P, Mihm MC Jr. Focus on TILs: prognostic significance of tumor infiltrating lymphocytes in human melanoma. *Cancer Immun*. 2009;9:3.
48. Rao UN, Lee SJ, Luo W, Mihm MC Jr, Kirkwood JM. Presence of tumor-infiltrating lymphocytes and a dominant nodule within primary melanoma are prognostic factors for relapse-free survival of patients with thick (t4) primary melanoma: pathologic analysis of the e1690 and e1694 intergroup trials. *Am J Clin Pathol*. 2010;133:646-653.
49. Francisco LM, Salinas VH, Brown KE, et al. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J Exp Med*. 2009;206:3015-3029.
50. Youn JI, Nagaraj S, Collazo M, Gabrilovich DI. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. *J Immunol*. 2008;181:5791-5802.