Clinical Cancer Research

Dynamic Changes in PD-L1 Expression and Immune Infiltrates Early During Treatment Predict Response to PD-1 Blockade in Melanoma

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Abstract

Purpose: Disruption of PD-L1/cytotoxic T-cell PD-1 signaling by immune checkpoint inhibitors improves survival in cancer patients. This study sought to identify changes in tumoral PD-L1 expression and tumor-associated immune cell flux with anti-PD-1 therapies in patients with melanoma, particularly early during treatment, and correlate them with treatment response.

Experimental Design: Forty-six tumor biopsies from 23 patients with unresectable AJCC stage III/IV melanoma receiving pembrolizumab/nivolumab were analyzed. Biopsies were collected prior to (PRE, $n = 21$), within 2 months of commencing treatment (EDT, $n = 20$) and on disease progression after previous response (PROG, $n = 5$). Thirteen patients responded (defined as CR, PR, or durable SD by RECIST/irRC criteria), and 10 did not respond.

Results: PRE intratumoral and peritumoral PD-1^+ T-cell densities were sevenfold ($P = 0.006$) and fivefold higher ($P = 0.011$), respectively, in responders compared with nonresponders and correlated with degree of radiologic tumor response ($r = -0.729$, $P = 0.001$ and $r = -0.725$, $P = 0.001$, respectively). PRE PD-L1 expression on tumor and macrophages was not significantly different between the patient groups, but tumoral PD-L1 and macrophage PD-L1 expression was higher in the EDT of responders versus nonresponders ($P = 0.025$ and $P = 0.033$). Responder EDT biopsies (compared with PRE) also showed significant increases in intratumoral $CD8^+$ lymphocytes ($P = 0.046$) and intratumoral $CD68^+$ macrophages ($P = 0.046$).

Conclusions: Higher PRE PD-1^+ T cells in responders suggest active suppression of an engaged immune system that is disinhibited by anti-PD-1 therapies. Furthermore, immunoprofiling of EDT biopsies for increased PD-L1 expression and immune cell infiltration showed greater predictive utility than PRE biopsies and may allow better selection of patients most likely to benefit from anti-PD-1 therapies and warrants further evaluation. *Clin Cancer Res; 23(17); 5024–33. ©2017 AACR.*

Introduction

Immune checkpoint inhibitor drugs represent a revolutionary approach in the immunotherapeutic treatment of patients with advanced cancer. Pembrolizumab and nivolumab are the first FDA-approved humanized mAbs engineered to disrupt the tumor PD-L1/ cytotoxic T-cell PD-1 signaling axis and thereby obstruct one mechanism by which a tumor may suppress cytotoxic T-cell activity. These agents generate durable clinical responses in most cancer types, including in approximately 30% to 40% of patients with advanced-stage melanoma. Unlike mutation testing that accurately selects patients most likely to respond to targeted therapies, there are currently no sufficiently reliable biomarkers predictive of response to PD-1 inhibition that may be used in the clinic to select patients for treatment, especially in melanoma. Tumor expression of PD-L1 is known to be associated with a favorable response to PD-1 inhibition, but samples from a minority of patients who respond can be devoid of tumoral PD-L1 expression (1, 2). This, combined with the well-characterized geographic and temporal heterogeneity of PD-L1 expression (3), highlights its limitations as a predictive biomarker. Our current understanding of what determines a patient's response to this new class of drugs is thus incomplete. Likewise, mechanisms by which a previously responsive tumor develops tolerance to enhanced T-cell antitumor function remain to be elucidated.

Tumeh and colleagues analyzed 26 patients with advanced-stage metastatic melanoma with matching pre- and during PD-1 inhibition biopsies (4) and showed that a high intratumoral and peritumoral density of PD-1^+ and $CD8^+$ T cells in pretreatment

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Translational Relevance

The immune checkpoint inhibitors that disrupt PD-L1/cytotoxic T-cell PD-1 signaling improve survival in patients with cancer and are most active in those with melanoma. Unlike mutation testing, which accurately predicts response to targeted therapies, predictive biomarkers of response/resistance to anti-PD-1 therapies (including PD-L1 testing) have limited clinical utility. Furthermore, clinical evidence of response/resistance may not be apparent until months after therapy commencement, potentially delaying a switch to alternative treatment. We demonstrate that biopsies taken early during treatment (median, 11 days) from patients with melanoma responding to anti-PD-1 therapies show increased cytotoxic T cells, PD-1$^+$ cells, and macrophages, in addition to the acquisition or elevation of PD-L1 expression within tumor and/or macrophage cells, and had greater predictive utility of response than baseline samples, taken prior to treatment start. Pathologic immune profiling of early-during-treatment biopsies represents a potential opportunity to better select patients with the highest likelihood of responding to anti-PD-1 therapies and warrants further evaluation.

biopsies, as well as tumor PD-L1 expression, were predictive of a response to PD-1 inhibition. The during-treatment tumor biopsies of responders, as opposed to nonresponders, included a proliferating population of activated CD8$^+$ lymphocytes that produced IFNγ and were associated with enhanced expression of PD-L1 on tumor cells. More restricted CD8$^+$ clonality was also evident in the pretreatment biopsies of responders and was enhanced after PD-1 inhibition.

In this study, we present a detailed characterization of the immune microenvironment before and during anti-PD-1 therapy in patients with metastatic melanoma. We demonstrate that the successful activation of an adaptive immune response, as evident by an increase in intratumoral cytotoxic T-cell and macrophage populations as well as a dramatic upregulation of tumoral and macrophage PD-L1 expression, can occur within days of commencing treatment and are powerful predictors of eventual treatment response and improved progression-free survival (PFS). The latter findings open up a novel approach to the prediction of response to anti-PD-1 mAbs, whereby assessment of biopsies taken early during treatment may provide the best opportunity to predict treatment response. Furthermore, we also present analysis of PD-L1 expression and the immune microenvironment in tumor samples of patients who have acquired PD-1 inhibitor resistance after previously responding to this therapy and demonstrate immune escape in this setting.

Materials and Methods

Study design

Patients with unresectable AJCC stage IIIC or IV melanoma partaking in pembrolizumab or nivolumab phase I, II, or III clinical trials or the pembrolizumab/nivolumab access programs were recruited into the study under the auspices of the Treat Excise Analyze for Melanoma (TEAM) study at the Melanoma Institute Australia (New South Wales, Australia) in accordance with the International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS), with Human Ethics Review Committee approval (Royal Prince Alfred Hospital Research Ethics Committee protocol no. X10-0305 and HREC/10/RPAH) and patient consent. Patient selection was dependent on the availability of multiple tumor biopsies: before treatment (PRE biopsy), within 60 days of commencing treatment (early during treatment, EDT biopsy), and/or on tumor progression while on treatment and after a prior response (PROG biopsy). Six patients were treated with nivolumab 3 mg/kg every 2 weeks; 2 of these were also randomized to blinded coadministration of ipilimumab or placebo (patients R6 and R7). Patients on pembrolizumab received of 2 or 10 mg/kg every 2 or 3 weeks (Table 1). Tumor response to treatment was evaluated under the RECIST V1.1 (5) or immune-related response criteria (irRC; ref. 6) assessed at 6 to 12 weekly intervals as per clinical trial protocols. Patients were classified as responders if they obtained any clinical benefit with durable stable disease (SD, greater than 6 months), partial response (PR), or complete response (CR) to anti-PD-1 antibodies as their best response by RECIST or irRC criteria. Patients who obtained progressive disease (PD) or SD for less than 6 months as their best response were classified as "nonresponders." Overall survival (OS) and PFS were evaluated from the commencement of treatment until last follow-up or patient death or tumor progression, respectively.

Assessment of tumor histopathologic characteristics and immune cell infiltrate

Whole-tumor excision biopsies underwent overnight fixation in 10% phosphate-buffed formalin prior to processing and embedding in paraffin blocks. Four-micron-thick sections were cut and stained with hematoxylin and eosin. Melanoma histomorphology was qualitatively and semiquantitatively assessed across the whole section for the following features: the percentage of viable tumor and percentage of tumor undergoing coagulative necrosis, cell morphology, growth pattern, cytoplasmic color and consistency, chromatin pattern, nucleolar prominence, melanin content, tumor mitotic rate, and quantity of apoptotic debris.

IHC

IHC staining for CD3, CD4, CD8, CD20, CD68, PD-1, and PD-L1 was conducted on an Autostainer Plus (Dako; Agilent Technologies) using 4-μm-thick tissue sections. Sections were dehydrated for 1 hour at 60°C, and heat-induced epitope retrieval was performed using EnVision FLEX target retrieval solution for 20 minutes at 97°C. The sections were then cooled to room temperature in TBST wash buffer for 5 minutes. Antibody dilutions were as follows: CD4 (Cell Marque- SP35) 1:100, CD8 (Cell Marque- SP16) 1:200, CD20 (Cell Marque- L26) 1:100, CD68 (Cell Marque- KP-1) 1:1,000, PD-1 (Cell Marque- MRQ- 22/NAT105) 1:100, PD-L1 (Cell Signaling Technology- E13LN) 1:200.

PD-1 and PD-L1 staining underwent signal amplification using the (K8022). All antibody detection utilized the Envision FLEX kit (K8023) with a DAB chromogen (Dako; Agilent technologies) prior to counterstaining with hematoxylin.

The following antibody panel was used to calculate the density of peritumoral and intratumoral lymphocytes (CD3, CD4, CD8, CD20, PD-1) and macrophages (CD68). For calculation of intratumoral cell density, 4 representative high-power fields were

Table 1. Clinicopathologic, treatment, and follow-up characteristics of the patient cohort

Patient ID	Gender	Age (y)	Baseline LDH	Baseline ECOG	Anti-PD-1 antibody	BRAF mutation	Best % change by RECIST or irRC	RECIST/iRC response ([e] = irRC)	Biopsies analyzed	PFS (months)	OS (months)
NR1	M	36	Elevated	2	Pembro	Yes	40	PD	PRE, EDT	3.2[b]	8.5
NR2	M	51	Elevated	0	Pembro	Yes	1[a]	PD[a]	PRE, EDT	2.7	6.5
NR3	F	56	Normal	0	Pembro	Yes	153.8	PD[a]	PRE	2.5	>33.9
NR4	F	51	Elevated	0	Pembro	Yes	N/A[a]	PD[a]	PRE, EDT	2.2	5.5
NR5	M	68	Elevated	1	Pembro	No	72	PD	PRE, EDT	2.7	3.2
NR6	M	79	Normal	0	Pembro	No	75	PD[a]	PRE, EDT	2.7	32.6
NR7	F	56	Normal	0	Nivo	No	40	PD	PRE	1.5	2.8
NR8	M	49	Normal	1	Nivo	Yes	26	PD	PRE, EDT	0.8	1.9
NR9	M	65	Elevated	1	Pembro	No	11	SD	PRE, EDT[d]	3.5	5.6
NR10	M	46	Normal	1	Nivo	No	−18	SD	PRE, EDT	3.4	>27.2
R1	F	51	Elevated	0	Pembro	Yes	−48	SD[a]	PRE, PROG	6.9	13.4
R2	M	36	Normal	0	Pembro	Yes	−87	PR[a]	PRE, EDT	>37.2[b]	>37.2
R3	F	57	Elevated	1	Pembro	No	−86	PR	PRE, EDT[d]	>26.1[b]	>26.1
R4	M	51	Elevated	0	Pembro	No	−5	SD[a]	PRE, EDT	8.3	27.1
R5	M	60	Normal	0	Pembro	No	−97	PR[a]	PRE[d], EDT	29.6	>55.1
R6	M	54	Elevated	0	Nivo[c]	No	−69	PR	PRE, EDT[d]	>35.5[b]	>35.5
R7	F	68	Elevated	1	Nivo	No	−92	CR	PRE, EDT	>41.0[b]	>41.0
R8	M	75	Normal	1	Nivo[c]	No	−31	SD	PRE, EDT	10.3[b]	>34.8
R9	M	50	Elevated	1	Pembro	No	−18	SD	EDT, PROG[d]	9.6	25.5
R10	F	56	Normal	1	Pembro	No	−20	SD	PRE, EDT	7.1[b]	10.5
R11	F	38	Elevated	2	Pembro	No	−36	PR	PRE[d], EDT, PROG	2.0[b]	>27.9
R12	M	59	Normal	1	Pembro	No	−29	SD[a]	PRE, EDT, PROG[d]	>37.1[b]	>37.1
R13	F	54	Normal	0	Pembro	No	−70	CR	EDT	>27.9[b]	>27.9

Abbreviations: ECOG, Eastern Cooperative Oncology Group; F, female; LDH, lactate dehydrogenase; M, male; N/A, not available; Nivo, nivolumab; NR, nonresponder; PD-1, programmed cell death protein-1; Pembro, pembrolizumab; R, responder.

[a]Clinical progression.

[b]Patient on anti-PD-1 at the time of data analysis.

[c]Patient randomized to combined nivolumab and ipilimumab or nivolumab monotherapy (Checkmate 067).

[d]No residual invasive melanoma identified.

[e]irRC.

assessed. For peritumoral cell density, the number of positive cells bounded within an area 4.0 mm × 0.25 mm immediately adjacent to the invasive tumor margin was calculated in at least one region featuring the highest density of immune cells, and whenever possible, the average score of 2 peritumoral regions was calculated.

Scoring of PD-L1 staining was determined as described previously (3, 7). Briefly, the percentage of tumor cells and macrophages showing positive membrane staining was determined, and the intensity of staining was judged on a semi-quantitative scale of 0 to 3+: no staining (0), weakly positive staining (1+), moderately positive staining (2+), and strongly positive staining (3+). The PD-L1 immunoreactive score (IRS) was derived as the product of the percentage of positive cells and the staining intensity to produce a score out of 300. A threshold of $\geq$1% tumor cell PD-L1 expression defined overall tumor positivity. All scoring was conducted blinded to patient outcome and timing of the biopsy (PRE, EDT, or PROG). Heavily pigmented tumors underwent overnight bleaching in a solution of 10% hydrogen peroxide. No change in the immunoreactivity for any of the antibodies used was identified in control nonpigmented tumor sections.

Statistical analysis

Correlation between the various immune markers and response (RECIST criteria) was assessed using Spearman ρ test. Paired t test was used to test for significant changes in PD-L1 IRSs and immune markers according to biopsy time points. The Mann–Whitney U test was used to test for differences between the expression of markers in responders and nonresponders. To determine differences in histologic features and clinical characteristics of melanoma samples across responder and nonresponder subgroups, χ^2 test and Fisher exact test were used where appropriate. Cox regression analysis utilizing above and below median values was used to assess predictive factors for PFS. All statistical analysis was performed with JMP 11 (SAS Institute Inc.).

Results

Patients, biopsies, and treatment

Forty-six excisional biopsies from 23 patients were studied. Tumor biopsies were collected PRE ($n = 21$) and EDT (median, 11 days, range 1–68 days, $n = 20$), and again on disease progression after initial treatment response (i.e., acquired resistance, PROG, $n = 5$). Five biopsies did not contain viable melanoma tumor for assessment; 1 PRE, 3 EDT, and 2 PROG (Supplementary Results).

Thirteen patients obtained durable SD ($n = 6$), PR ($n = 5$), or CR ($n = 2$) to anti-PD-1 antibodies as their best response on RECIST or irRC criteria and were classified as responders for the analysis (Table 1). Of the 7 patients who had a PR or CR to anti-PD-1 inhibitor therapy, 4 had elevated LDH levels. Ten patients showed PD or only a brief period of SD on RECIST or irRC criteria and were classified as nonresponders (Table 1). The majority of patients received pembrolizumab. Characteristics of the patient cohort are shown in Table 1.

The median follow-up time was 27.1 months (range, 1.9–55.1 months), and the median PFS was 2.7 months for the nonresponder group and 29.5 months for the responder group (log-rank $P = \leq 0.0001$). Median OS from the commencement of anti-PD-1 antibody was 6.1 months for nonresponders, while there were 4 deaths within the 13 patients in the responder group after a median follow-up of 27.9 months (log rank $P = 0.0037$) (Table 1).

PD-L1 expression in pretreatment biopsies and treatment response

Within the PRE biopsies, the incidence of PD-L1 positivity (i.e., greater than 1%) was higher within both tumor cells (66.7% vs. 30%) and tumor-associated $CD68^+$ macrophages (66.7% vs. 50.0%) in responders compared with nonresponders, although these differences did not reach statistical significance ($P = 0.179$, $P = 0.650$, respectively; Table 2). The tumor and macrophage PD-L1 IRSs between the response groups at PRE did not differ significantly ($P = 0.171$ and $P = 0.175$, respectively; Table 2; Fig. 1). The PRE tumor and macrophage PD-L1 IRS did not correlate with the degree of tumor shrinkage on radiology or overall patient response classification ($r = 0.003$, $P = 0.812$ and 0.077, $P = 0.264$, respectively).

$PD\text{-}1^+$ lymphocyte counts are higher in PRE biopsies of responders and correlate with greater tumor shrinkage and longer PFS

PRE biopsies of patients who responded to anti-PD-1 antibodies showed a higher intratumoral $PD\text{-}1^+$ count than nonresponders (256 vs. 40, $P = 0.006$; (Table 2; Figs. 1 and 2). Higher numbers of $PD\text{-}1^+$ lymphocytes were also observed in the peritumoral microenvironment in responders compared with nonresponders (313 vs. 64.8; $P = 0.011$). Consistent with these findings, there was a positive correlation between the degree of tumor shrinkage on radiological assessment and greater numbers of PRE $PD\text{-}1^+$ lymphocytes in both the intratumoral and in the peritumoral areas ($r = -0.729$, $P = 0.001$ and $r = -0.725$, $P = 0.001$, respectively; Fig. 2A and B).

A similar positive correlation was also observed between higher numbers of intratumoral and peritumoral $PD\text{-}1^+$ lymphocytes and longer PFS [HR = 0.996; 95% confidence interval (CI), 0.991–1; $P = 0.062$ and HR = 0.996; 95% CI, 0.992–1; $P = 0.055$, respectively]. Enhanced PFS and OS were also demonstrated in patients with PRE tumor samples exhibiting higher than median intratumoral (>46 cells, PFS 10.3 months vs. 2.7 months, log-rank $P = 0.015$ and OS not reached months vs. 6 months, log-rank $P = 0.021$) or peritumoral (>81 cells, PFS 10.2 months vs. 2.7

Table 2. PD-L1 expression and immune cell characteristics of tumor samples taken prior to treatment (PRE), early during treatment (EDT), and on disease progression (PROG) in patients with melanoma receiving anti-PD-1 therapy

	PRE scores (Std Dev)		EDT scores (Std Dev)		PROG scores (Std Dev)	Difference between responders vs. non-responders (*P*)		Changes at PROG in responders (*P*)	
Variable	Non-responders ($n = 10$)	Responders ($n = 9$)	Non-responders ($n = 7$)	Responders ($n = 10$)	Responders ($n = 2$)	At PRE	At EDT	PRE vs. PROG	EDT vs. PROG
Tumor PD-L1 % positive[a]	30	66.7	42.9	90	100	0.179	0.101	1.000	1.000
Tumor PD-L1 IRS	14.6 (44.1)	8.1 (11.2)	4 (6)	49 (61)	3.0 (2.8)	0.171	**0.025**	0.811	0.389
Macrophage PD-L1 positive[a]	50.0	66.7	57.1	90	100	0.650	0.250	1.000	1.000
Macrophage PD-L1 IRS	12.3 (25.6)	63.9 (81.5)	46 (100)	150 (91)	75 (63.6)	0.175	**0.033**	0.634	0.235
CD3 density									
Intratumoral	595 (704)	536 (708)	600 (681)	1,198 (986)	223 (12.7)	0.825	0.157	0.814	0.197
Peritumoral	477 (544)	854 (834)	325 (308)	925 (813)	426 (283)	0.266	**0.041**	0.410	0.505
CD8 density									
Intratumoral	332 (452)	416 (452)	462 (548)	929 (592)	198 (17)	0.514	0.097	1.000	0.197
Peritumoral	448 (563)	552 (516)	236 (268)	768 (831)	373 (269)	0.638	0.055	0.814	0.739
PD-1 density									
Intratumoral	40 (56)	256 (231)	93 (164)	496 (787)	170 (220)	**0.006**	0.071	0.480	0.667
Peritumoral	64.8 (74)	313 (261)	49 (74)	267 (240)	37 (46)	**0.011**	**0.022**	0.099	0.143
CD4 density									
Intratumoral	182 (290)	171 (167)	155 (232)	222 (300)	222 (300)	0.513	0.7170	0.814	0.1.00
Peritumoral	108 (66)	195 (173)	194 (136)	383 (332)	384 (333)	0.294	0.2623	0.296	0.046
CD20 density									
Intratumoral	78 (189)	54.8 (67)	6 (10)	114 (250)	3 (4)	0.412	0.117	0.237	0.280
Peritumoral	18.9 (14)	244 (393)	9 (11)	236 (461)	0.3 (0.4)	0.083	**0.017**	0.037	0.053
CD68 density									
Intratumoral	300 (310)	299 (257)	433 (405)	709.7 (432)	532 (365)	0.860	0.157	0.346	0.519
Peritumoral	259 (249)	295 (222)	196 (115)	389 (123)	541 (403)	0.643	**0.017**	0.192	0.558
CD3:CD4 density									
Intratumoral	4.0 (3.4)	3.8 (3.1)	12.5 (25.5)	16.8 (30.2)	2.8 (1.8)	0.870	0.064	0.814	0.099
Peritumoral	4.2 (2.8)	7.7 (12.0)	1.9 (0.9)	3.0 (1.5)	2.8 (1.8)	0.817	0.150	0.296	1.000
CD3:CD8 density									
Intratumoral	1.6 (0.9)	1.2 (0.2)	1.9 (1.3)	1.3 (0.5)	1.1 (0.03)	0.072	0.283	1.000	1.000
Peritumoral	1.6 (0.7)	1.8 (0.7)	1.8 (0.8)	1.4 (0.5)	1.1 (0.03)	0.555	0.262	0.090	1.000
CD8:PD-1 density									
Intratumoral	12.2 (23.6)	2.1 (2.0)	11.4 (18.6)	15.4 (37.4)	7.8 (10.2)	0.064	0.410	1.000	1.000
Peritumoral	16.5 (30.6)	1.9 (0.7)	22.7 (23.9)	5.0 (4.7)	7.8 (10.2)	**0.045**	0.200	1.000	1.000

NOTE: *P* values <0.05 are in bold.
[a]>1% staining.

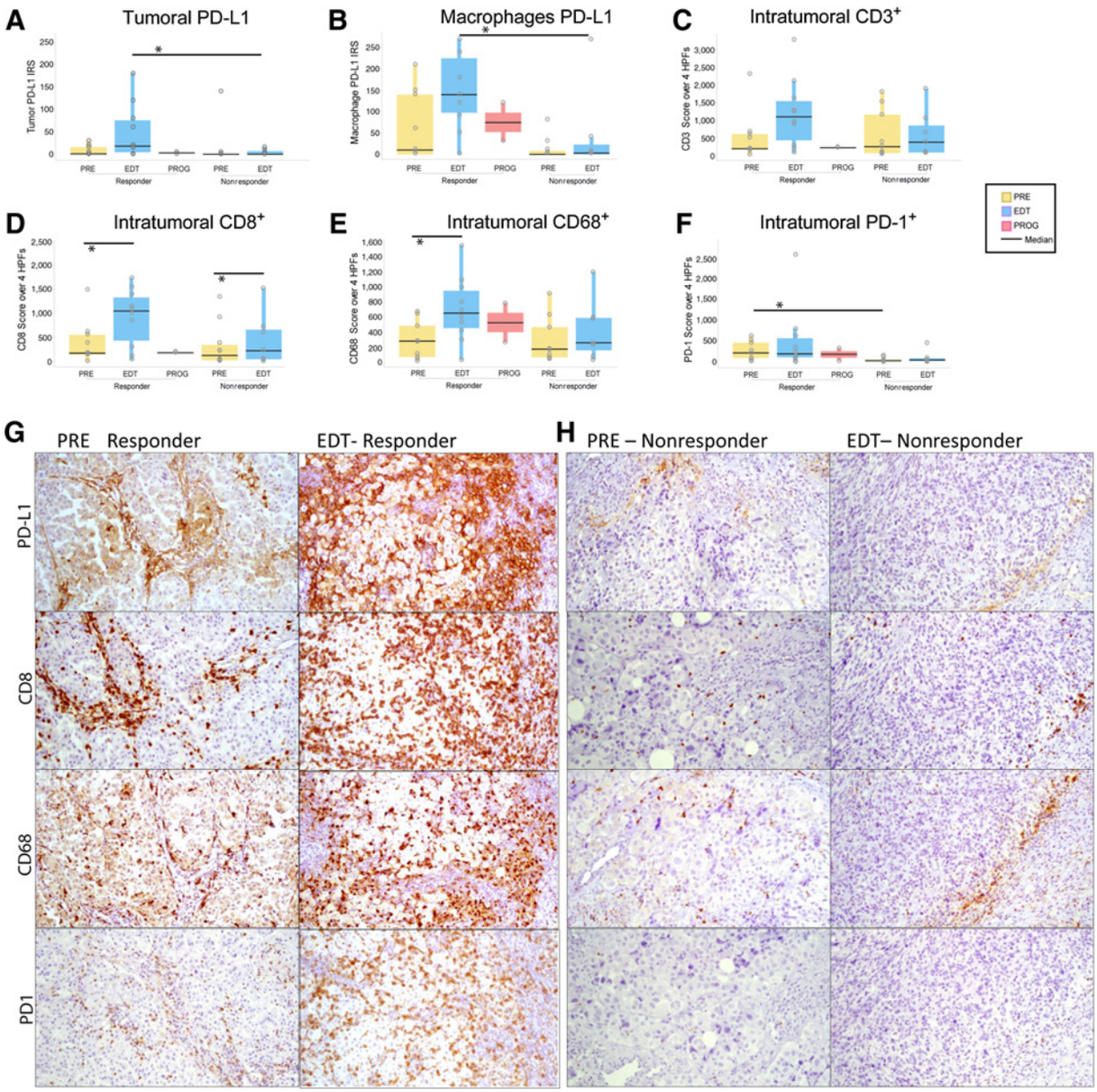

Figure 1.

Characteristics of tumor tissue sampled pretreatment (PRE), early during treatment (EDT), and on acquisition of PD-1 inhibitor resistance (PROG) assessed as whole group mean values in responders and nonresponders: tumor PD-L1 IRS (**A**), macrophage PD-L1 IRS (**B**), $CD3^+$ cells (**C**), $CD8^+$ cells, (**D**) and $CD68^+$ cells (**E**), and $PD\text{-}1^+$ cells (**F**). Errors bars, 95% CI. *, $P = \leq 0.05$. Immunohistochemically stained sections of a responding (patient R02, **G**) and a nonresponding (patient NR02, **H**) patient's tumor are depicted in the bottom. HPF, high-power field. Magnification, ×200.

months, log-rank $P = 0.039$ and median OS not reached vs. 6 months, log-rank $P = 0.009$) $PD\text{-}1^+$ cell counts (Fig. 2C–F).

Immune cells in pretreatment biopsies and their correlates with treatment responses

No significant differences between $CD3^+$, $CD4^+$, $CD68^+$, $CD20^+$, and $CD8^+$ intratumoral and peritumoral T-cell counts in PRE samples were observed in responders than nonresponders (Table 2).

Tumor cell and macrophage PD-L1 expression is enhanced early during treatment with PD-1 inhibition in responders

Tumor PD-L1 expression in EDT biopsies was detectable in 9 of 10 responders and in 4 of 7 nonresponders ($P = 0.343$). The mean tumor PD-L1 IRS at EDT was significantly greater for responders (PD-L1 IRS: 49) than nonresponders (PD-L1 IRS: 4; $P = 0.025$; Table 2; Fig. 1). Two of the 3 responders in whom tumor PD-L1 expression was not previously detected in their PRE biopsy gained tumor PD-L1 expression in their matched EDT sample. In

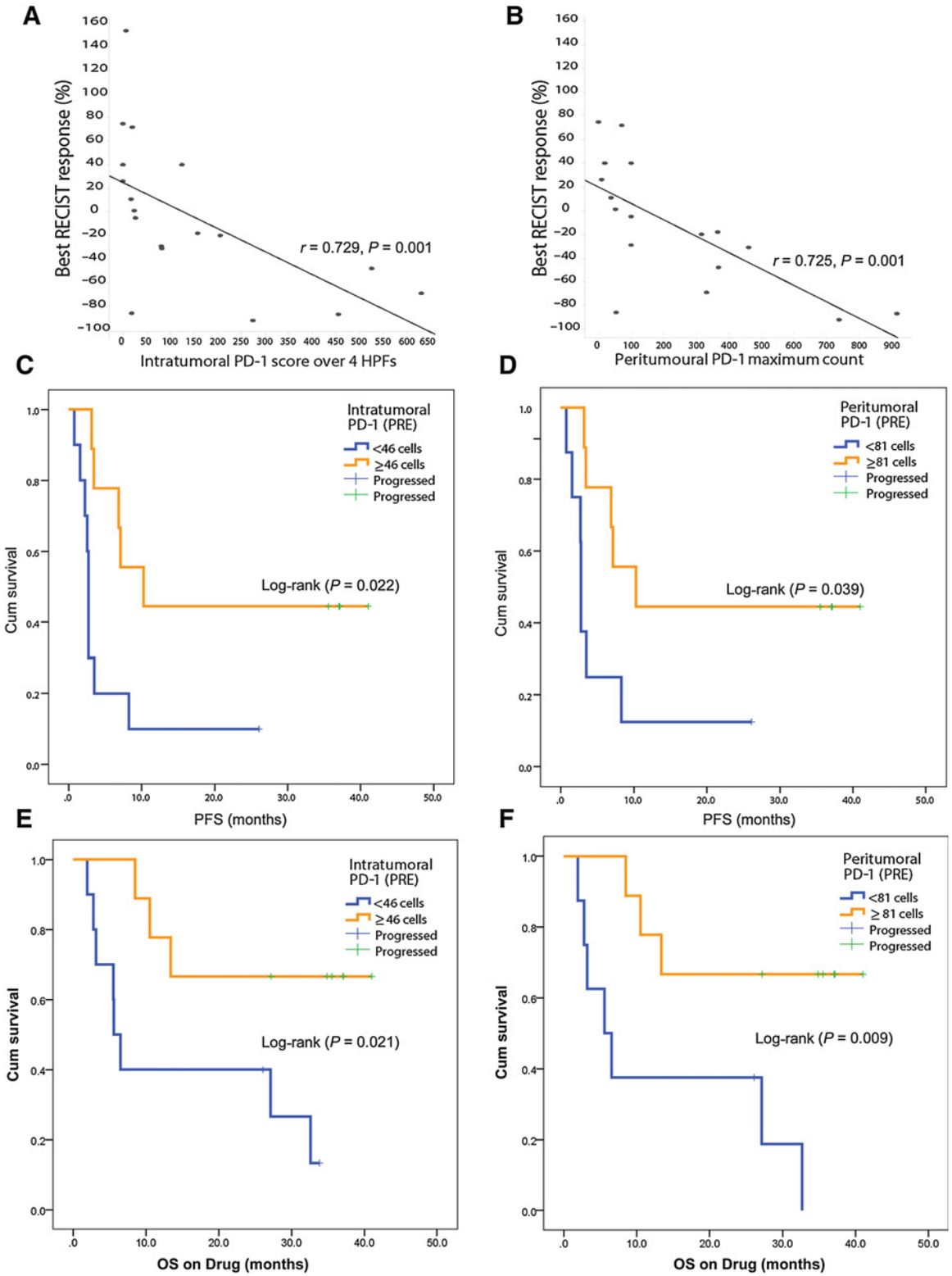

Figure 2.

Clinical correlates of response with PD-1$^+$ cell density. Higher densities of intratumoral and peritumoral PD-1$^+$ cells significantly correlated with a greater reduction in tumor volume (RECIST; **A** and **B**). Greater densities of intratumoral and peritumoral PD-1$^+$ cells significantly associated with longer PFS and OS (**C–F**).

contrast, one nonresponder acquired tumoral PD-L1 expression was observed in the 5 PRE PD-L1$^-$ tumors of nonresponders (Fisher exact test $P = 0.4643$).

Macrophage PD-L1 expression in EDT biopsies was detectable in 9 of 10 responders and in 4 of 7 nonresponders ($P = 0.65$). The only responder with a previously PRE PD-L1$^-$ macrophage component acquired PD-L1 expression within the EDT biopsy. In contrast, there was no acquisition of macrophage PD-L1 expression at EDT in the 3 nonresponders exhibiting PD-L1$^-$ macrophages at PRE (Fisher exact test $P = 0.2500$). Importantly, the macrophage PD-L1 IRS at EDT was also higher in responders than nonresponders ($P = 0.033$; Table 2; Fig. 1A and B).

There were no statistically significant elevations between paired PRE versus EDT tumor PD-L1 IRS (PRE: 8.1 vs. EDT: 49, $P = 0.206$) and macrophage PD-L1 IRS (PRE: 63.9 vs. EDT: 150, $P = 0.059$) in responders. No consistent change was observed in the nonresponders PD-L1 PRE and EDT values (tumor PD-L1 PRE: 15 vs. EDT 4.0, $P = 0.167$ and macrophage PD-L1 PRE: 12 vs. EDT: 46, $P = 0.286$).

Immune cell infiltrates in EDT biopsies: higher tumor-infiltrating lymphocytes and macrophages correlate with response to anti-PD-1 antibody treatment

In responders, EDT biopsies when compared with their matched PRE biopsy from the same patient ($n = 6$ patients) showed an increase in the intratumoral density of CD8$^+$ T cells and CD68$^+$ macrophages ($P = 0.026$ and $P = 0.05$, respectively; Fig. 1; Supplementary Figs. S1 and S2), and a borderline increase in CD3$^+$ T cells ($P = 0.077$; Supplementary Fig. S2). Nonresponders ($n = 7$ patients, 14 biopsies) showed a significant increase in the intratumoral density of CD8$^+$ T cells ($P = 0.043$; Supplementary Fig. S1). Nonstatistically significant increases in intratumoral PD-1$^+$ T cells were seen both in responders and nonresponders ($P = 0.474$ and $P = 0.082$, respectively; Supplementary Fig. S1). No consistent changes were observed in CD4$^+$, CD20$^+$, and plasma cells in either group.

However, when analyzed as whole groups, the responders, in comparison with nonresponders, showed greater intratumoral PD-1$^+$ cells in their pretreatment biopsies ($P = 0.006$; Table 2; Fig. 1). EDT biopsies showed significantly higher tumoral and macrophage PD-L1 IRS in responders than nonresponders ($P = 0.025$ and 0.034, respectively). Peritumoral density of PD1$^+$ cells was significantly higher in responders PRE biopsies ($P = 0.011$) and CD20, CD68, PD1, and CD3$^+$ cells in EDT biopsies ($P = 0.017$, $P = 0.017$, $P = 0.022$, and $P = 0.041$, respectively).

Characteristics of tumor immune microenvironment in acquired resistance to PD-1$^+$ inhibition (PROG biopsies)

Two PROG specimens (tumor from patients who had responded, then progressed) were available for analysis; 2 of the initial 5 PROG specimens had no viable tumor (see Supplementary Results). The PFS for the 2 patients was 6.9 months (patient R1) and 2 months (patient R11) days.

The PROG specimens displayed positive PD-L1 expression within the tumor and macrophage infiltrates. The mean PD-L1 IRS at PROG in the tumor (PD-L1 IRS: 3) and macrophages (PD-L1 IRS: 75) was lower than at EDT (tumor PD-L1 IRS: 49, macrophages PD-L1 IRS: 150; Table 2). The PROG biopsies showed lower cellular infiltrates within the intratumoral and peritumoral compartments for CD3$^+$, CD4$^+$, CD8$^+$, and PD1$^+$ T cells, CD20$^+$ B cells, and CD68$^+$ macrophages and in the intratumoral density of plasma cells. However, these are low numbers, and none of these differences reached statistical significance (Table 2).

Tumor histomorphology prior to and in response to PD-1 inhibition

Tumor cytomorphology (growth pattern, chromatin appearance, nucleolar prominence, cell shape, cytoplasm color and consistency, cell border characteristics, cytoplasmic pigment) was not predictive of treatment response and neither was there a tumor cytomorphologic pattern characteristic of response at EDT (Supplementary Table S1). However, apoptotic debris were more frequently encountered within the PRE biopsies of responders ($P = 0.008$; Supplementary Table S1), and statistically significant trends were observed between 2 histologic parameters at PRE: (i) higher percentage of viable tumor ($r = 0.2101$, $P = 0.0484$); and (ii) higher mitotic rate ($r = 0.2238$ $P = 0.0408$) and a greater degree of tumor shrinkage on radiology.

Discussion

This study presents a detailed analysis of the changes that occur in patient tumor biopsies following treatment with anti-PD-1 antibodies and highlights the potential clinical utility of utilizing early during treatment biopsies to inform clinical decision making. Currently available predictive biomarkers of response/resistance to anti-PD-1 therapies, such as PD-L1 testing of pretreatment biopsies, have only limited clinical utility (8). Furthermore, clinical evidence of response or resistance may not be apparent until months after therapy commencement, potentially delaying a switch to alternative more effective treatment. In this study, we demonstrate that biopsies taken early during treatment (median, 11 days) from melanoma patients responding to anti-PD-1 therapies show increased cytotoxic T cells and macrophages, in addition to the acquisition or elevation of PD-L1 expression within tumor and/or macrophage cells. This increase in the immune cell infiltrate early during treatment correlated with treatment response. This suggests that pathologic immune profiling of early-during-treatment biopsies may better select patients with the highest likelihood of responding to anti-PD-1.

There are also other important novel findings of our study. We demonstrated that although tumoral and macrophage PD-L1 expression at baseline was more commonly observed in patients who respond to anti-PD-1 inhibition, it was the density of intratumoral and peritumoral PD-1$^+$ T cells that more strongly correlated with tumor shrinkage and PFS. Finally, we highlight that, upon the development of acquired resistance to anti-PD-1 therapy in patients who previously responded, there is a reduction in tumoral and macrophage PD-L1 expression as well as a diminution of T cells, and to a lesser degree macrophages, within the intratumoral and peritumoral microenvironment, thus demonstrating evidence of treatment-related immune escape.

Baseline tumoral PD-L1 expression has been studied as a potential predictive biomarker of PD-1 antibodies (8–13). Most studies have identified a positive association between PD-L1 expression and response to PD-1 inhibition. However, the strength of this association is not absolute, as patients with pretreatment biopsies exhibiting no (or low) PD-L1 expression

may respond to treatment (14), and not every patient with demonstrable PD-L1 expression responds to anti-PD-1 antibodies (1, 2). Furthermore, intertumoral and intratumoral PD-L1 expression is highly heterogeneous (3), the cutoff for considering positive staining is not agreed upon, and numerous antibodies exhibiting various sensitivities are in use. The use of PD-L1 expression as the sole predictive biomarker of response to PD-1 inhibition response therefore is not appropriate for clinical use.

We have shown that patients with tumors exhibiting high concentrations of the antibodies' target, that is, PD-1^+ T cells within the tumor microenvironment at baseline (PRE biopsies), are most likely to show a clinical benefit from anti-PD-1 antibodies, and this may represent a superior predictive biomarker of anti-PD-1 response than PD-L1 expression. The presence of PD-1^+ T cells within or surrounding tumors indicates a population of T cells that have been stimulated by tumor-specific antigens (4, 15–18). These activated and terminally differentiated cytotoxic T cells are susceptible to be induced into a state of anergy by the binding of their PD-1 receptor by PD-L1–expressing tumor or other PD-L1–expressing antigen-presenting cells (APC), such as macrophages and dendritic cells (19). The abundance of intratumoral and peritumoral PD-1^+ T cells in the PRE biopsies of responders (but not in nonresponders) is consistent with the notion that their presence, in conjunction with tumor and macrophage PD-L1 expression, indicates a degree of PD-L1–mediated T-cell inactivation, which upon being disinhibited by humanized anti-PD-1 mAbs, reactivates the tumoricidal function of these tumor-specific cytotoxic T cells. Indeed, as we have shown, the greater the intratumoral and peritumoral density of PD-1^+ T cells at PRE, the greater the degree of tumor shrinkage on radiological assessment.

The potential assessment of EDT biopsies for immune cell infiltrates and increased PD-L1 expression may serve as a more useful predictive tool for treatment efficacy than PRE biopsies. Indeed, Chan and colleagues (20) recently published striking increases in T-cell infiltrates and PD-L1 expression in the early-during-treatment biopsies of responders versus nonresponders. If confirmed in additional studies, EDT biopsies may be utilized to determine whether this expensive form of therapy should be continued, ceased (potentially sparing patients from prolonged therapy who may be deriving no benefit), or potentially whether alternative agents should be added to the anti-PD-1 therapy.

Recently, Hugo and colleagues (21) employed whole-genome sequencing and transcriptomic analyses to identify a set of signatures that correlated with response and innate resistance to anti-PD-1 therapy. The notable absence of an IFNγ signature or increased expression of T-cell–specific markers in responders that we and others (4) have identified may be the product of the analytic platform employed, with RNA-seq's requirement for higher tumor content, making it less sensitive for the presence of genes expressed by immune cell and stromal components. The genes Hugo and colleagues identified to be differentially expressed in nonresponders were enriched for mesenchymal transition, wound healing, and immunosuppression. The mesenchymal phenotype has been recognized to be frequently associated with an immunosuppressive microenvironment and can also mediate MAPK inhibitor resistance.

In EDT biopsies of responders, we identified that PD-1 inhibition was associated with a significant increase in tumor and macrophage expression of PD-L1, much more than occurred in nonresponders (i.e., patients who showed PD as their best response). This finding is consistent with the hypothesis that in tumors with PD-L1/PD-1 checkpoint activation, disinhibition of PD-1^+ T cells will result in enhanced cytokine production (i.e., IFNγ) by monocytes, helper and, in particular, cytotoxic T cells (22), which in turn will bind to IFNγ receptors on tumor and immune cells inducing PD-L1 expression.

The elevated numbers of intratumoral and peritumoral $CD68^+$ cells (i.e., macrophages) in the EDT biopsies of melanoma tumors from responders is a novel finding of significant interest. We also found higher densities of intratumoral $CD3^+$, $CD8^+$, and PD-1^+ T cells at EDT in responders compared with nonresponders, which expands upon a previous study that showed proliferation of activated PD-1^+ cytotoxic T cells (4). Macrophages perform a range of functions, most prominently of which are innate host defense and adaptive immune regulation. Macrophages can stimulate antigen-specific T cells via T-cell receptor/MHC signaling in their role as APCs (23). Conversely, APCs can also induce T-cell anergy via activating PD-1 receptors by their PD-L1 ligand (19), and indeed, "immune cell" PD-L1 expression, rather than tumor PD-L1 expression, has been previously shown to correlate with anti-PD-1 therapy response (24). Furthermore, macrophages can also be stimulated to adopt a regulatory function as the result of PD-L1/PD-1 engagement, resulting in the reduced production of proinflammatory cytokines (IL6) and enhanced secretion of anti-inflammatory cytokines (IL10; ref. 19), thereby contributing to immunosuppression (25). The disengagement of T-cell PD-1 from macrophage PD-L1, mediated by anti-PD-1 antibodies, may not just re-activate cytotoxic PD-1^+ T cells but also promote regulatory macrophages to adopt a more proinflammatory state and secrete a range of inflammatory cytokines enhancing the influx of activated T cells.

The combination of these peri/intratumoral changes while on treatment in patients that benefit from anti-PD-1 antibody therapy, namely the increase in the intratumoral PD-1^+ and cytotoxic T-cell and macrophage densities, in addition to the enhanced PD-L1 expression by the tumor and its associated macrophage infiltrate, can be detected as early as a week following the commencement of treatment. Assessing the degree of a successful activation of cytotoxic T cells and PD-L1 expression in EDT biopsies potentially provides a novel and currently unutilized means of identifying a phenotypic state that is associated with a favorable clinical response to anti-PD-1 antibody therapy.

Little is understood of the mechanisms responsible for the development of acquired resistance to PD-1 inhibition, that is, growing metastases after the patient has previously responded. The cases of acquired resistance reported in this study represent some of the first reported analyses of such tumor biopsies. Our tumor samples with acquired PD-1 inhibitor resistance show a reduction of a T-cell ($CD3^+$, $CD8^+$) and macrophage-rich inflammatory microenvironment. This reduced inflammatory environment is associated with diminished PD-L1 expression in the tumor and immune cell components, possibly reflecting the reduction in the production of IFNγ by activated macrophages, $CD4^+$ helper T cells and, importantly, activated $CD8^+$ cytotoxic T cells. It remains to be determined what role, if any, ongoing tumoral and macrophage PD-L1 expression plays in the reestablishment of tumor immune tolerance, but it would seem more likely that other molecules or mechanisms will be implicated in the reestablishment of melanoma immune evasion, and a

decrease in PDL1 expression is the result, rather than the cause, of immune cell exclusion. PD-1 is but one of a number of receptors implicated in the exhaustion and dysfunction of effector T cells. The activation of other receptors, such as CTLA-4, TIM-3, and LAG-3, on T cells provides alternate and synergistic pathways to a state of exhaustion and dysfunction in tumor-specific T cells (26, 27). These receptors also function to promote an immunosuppressive tumor microenvironment by their promotion of the regulatory function of immune cells, such as Tregs (28) and dendritic cells (26, 29). It remains to be determined whether the balance of the immune micro milieu is tipped toward a tolerogenic state during the acquisition of anti-PD-1 antibody resistance by the increased action of these and other checkpoint proteins. Such explanation would provide a rationale for the blockade of multiple inhibitory receptors upon the loss of clinical benefit by anti-PD-1 antibodies. Tumor oncogenic signaling may also play a role in immune evasion, such as the MAPK (30), PI3K/AKT (31), or the WNT/β-catenin pathways (32).

Our study of immune cell flux and PD-L1 expression in tumor biopsies taken prior to, early during treatment, and on the acquisition of acquired resistance to anti-PD-1 therapies uncovered important and novel findings. We showed that the density of $PD1^+$ T cells at baseline correlated with treatment response, PFS, and OS, more so than any other baseline features. In addition, we demonstrate that the strongest correlation with response to anti-PD-1 treatment was the influx of inflammatory cells early during treatment (including macrophages). Our results suggest that assessment of early-during-treatment biopsies for evidence of successful activation of an adaptive immune response may provide a novel and more effective means of selecting patients most likely to derive treatment benefit, and such an approach warrants further investigation.

Disclosure of Potential Conflicts of Interest

A.M. Menzies reports receiving speakers bureau honoraria from Bristol-Myers Squibb and Novartis and is a consultant/advisory board member for Chug and MSD. A. Guminski is a consultant/advisory board member for Bristol-Myers Squibb, Merck KgA, MSD, Pfizer, and Sanofi. A.J. Cooper reports receiving speakers bureau honoraria from Bristol-Myers Squibb. R.P.M. Saw reports receiving speakers bureau honoraria from Bristol-Myers Squibb and is a consultant/advisory board member for Amgen. M.S. Carlino is a consultant/advisory board member for Bristol-Myers Squibb, MSD, and Novartis. R.F. Kefford reports receiving speakers bureau honoraria from Bristol-Myers Squibb and Merck and is a consultant/advisory board member for Amgen, Bristol-Myers Squibb, Merck, and Novartis. G.V. Long is a consultant/advisory board member for Bristol-Myers Squibb, Merck MSD, Novartis, and Roche. No potential conflicts of interest were disclosed by the other authors.

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