Dynamic changes in PD-L1 expression and immune infiltrates early during treatment predict response to PD-1 blockade in melanoma

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Conflicts of Interest:

RK is a consultant advisor to Merck, BMS, Amgen, Novartis, GSK, Roche. GL is a consultant advisor to Merck, Amgen, BMS, GSK, Novartis, Provectus, Roche. MC is a consultant advisor to Merck, Amgen and BMS. AMM is a consultant advisor to MSD and Chugai, and has received honoraria from BMS and Novartis. All other authors declare no conflicts of interest. The authors have no conflicts of interest or other relevant funding to disclose.
Translational relevance

The immune-checkpoint inhibitors that disrupt PD-L1/cytotoxic T-cell PD-1 signalling improve survival in cancer patients, 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 melanoma patients responding to anti-PD-1 therapies, show increased cytotoxic T-cells, PD-L1+ 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-PD1 therapies and warrants further evaluation.
Abstract

**Purpose:** Disruption of PD-L1/cytotoxic T-cell PD-1 signalling 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-PD1 therapies in melanoma patients, particularly early during treatment, and correlate them with treatment response.

**Experimental Design:** Forty-six tumor biopsies from 23 unresectable AJCC Stage III/IV melanoma patients receiving pembrolizumab/nivolumab were analyzed. Biopsies were collected prior to (PRE, n=21), within two 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), ten didn’t respond.

**Results:** PRE intra-tumoral and peri-tumoral PD-1+ T-cells density were 7-fold (p=0.006) and 5-fold higher (p=0.011), respectively in responders compared with non-responders 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 wasn’t significantly different between the patient groups but tumoral PD-L1 and macrophage PD-L1 expression was higher in the EDT of responders vs. non-responders (p=0.025 and p=0.033). Responder EDT biopsies (compared with PRE) also showed significant increases in intra-tumoral 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-PD1 therapies and warrants further evaluation.
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 monoclonal antibodies 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 30-40% 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 anti-tumor function remain to be elucidated.

Tumeh and colleagues analyzed 26 advanced stage metastatic melanoma patients with matching pre and during PD-1 inhibition biopsies (4), and showed that a high intra-tumoral and peri-tumoral density of PD-1+ and CD8+ T-cells in pre-treatment 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 non-responders, included a proliferating population of activated CD8+ lymphocytes that produced interferon-γ and were associated with enhanced expression of PD-L1 on tumor cells. More restricted CD8+ clonality was also evident in the pre-treatment biopsies of responders and was enhanced post 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 intra-tumoral 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. The latter findings open up a novel approach to the prediction of response to anti-PD-1 monoclonal antibodies 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 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 3mg/kg every two weeks, two of these were also randomized to blinded co-administration of ipilimumab or placebo (patients R6 and R7). Patients on pembrolizumab received of 2mg/kg or 10mg/kg every two or three weeks (Table 1). Tumor response to treatment was evaluated under the Response Evaluation Criteria in Solid Tumors (RECIST V1.1) (5) or immune-related Response Criteria (irRC) (6) assessed at 6-12 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 six 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 stable disease for less than six months as their best response were classified as ‘non-responders’. Overall survival and progression-free survival were evaluated from the commencement of treatment until last follow up or patient death or tumor progression, respectively.

Assessment of tumor histopathological characteristics and immune cell infiltrate

Whole tumor excision biopsies underwent overnight fixation in 10% phosphate buffered formalin prior to processing and embedding in paraffin blocks. 4μm-thick sections were cut and stained with hematoxylin...
and eosin. Melanoma histomorphology was qualitatively and semi-quantitatively 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.

**Immunohistochemistry (IHC)**

Immunohistochemical staining for CD3, CD4, CD8, CD20, CD68, PD-1 and PD-L1 were all conducted on an Autostainer Plus (Dako - Agilent Technologies) using 4μm-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) 1:1000, PD-1 (Cell Marque- MRQ- 22/NAT105) 1:100, PD-L1 (Cell Signaling- E13LN) 1:200.

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

The following antibody panel was used to calculate the density of peri-tumoral and intra-tumoral lymphocytes (CD3, CD4, CD8, CD20, PD-1) and macrophages (CD68). For calculation of intra-tumoral cell density, four representative high power fields were assessed. For peri-tumoral cell density, the number of positive cells bounded within an area 4.0mm x 0.25mm 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 two peri-tumoral regions was calculated.

Scoring of PD-L1 staining was determined as previously described (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 0–3+: 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 ≥ 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 non-pigmented tumor sections.

Statistical analysis

Correlation between the various immune markers and response (RECIST criteria) was assessed using Spearman’s rho test. Paired T-test was used to test for significant changes in PD-L1 immune reactive scores 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 non-responders. To determine differences in histological features and clinical characteristics of melanoma samples across responder and non-responder subgroups, chi-squared ($\chi^2$) test and Fisher exact test were used where appropriate. Cox regression analysis utilizing above and below median values were used to assess predictive factors for progression-free survival. All statistical analysis was performed with JMP® 11 (SAS Institute Inc., Cary, NC, USA).
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 seven patients who had a PR or CR to anti-PD-1 inhibitor therapy, four had elevated LDH levels. Ten patients showed PD or only a brief period of SD on RECIST or irRC criteria and were classified as non-responders (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 progression-free survival was 2.7 months for the non-responder group and 29.5 months for the responder group (log-rank p=<0.0001). Median overall survival from the commencement of anti-PD-1 antibody was 6.1 months for non-responders while there was 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 non-responders, although these differences did not reach statistical significance (p=0.179, p=0.650, respectively) (Table 2). The tumor and macrophage PD-L1
immunoreactive scores (IRS) between the response groups at PRE did not differ significantly (p=0.171 and p=0.175, respectively) (Table 2, Figure 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-1+ lymphocyte counts are higher in PRE biopsies of responders and correlate with greater tumor shrinkage and longer progression-free survival.**

PRE biopsies of patients who responded to anti-PD-1 antibodies showed a higher intra-tumoral PD-1+ count than non-responders (256 vs. 40, p=0.006) (Table 2, Figures 1 and 2). Higher numbers of PD-1+ lymphocytes were also observed in the peri-tumoral microenvironment in responders compared with non-responders (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-1+ lymphocytes in both the intra-tumoral and in the peri-tumoral areas (r=-0.729, p=0.001 and r=-0.725, p=0.001, respectively. Figure 2 panels A and B).

A similar positive correlation was also observed between higher numbers of intra-tumoral and peri-tumoral PD-1+ lymphocytes and longer progression-free survival (HR=0.996 (95% CI 0.991-1, p=0.062) and HR=0.996 (95% CI 0.992-1, p=0.055), respectively). Enhanced progression-free survival and overall survival were also demonstrated in patients with PRE tumor samples exhibiting higher than median intra-tumoral (>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 peri-tumoral (>81 cells, PFS 10.2 months vs 2.7 months, log-rank p=0.039 and median OS not reached vs 6 months, log-rank p=0.009) PD-1+ cell counts (Figure 2 panels C – F).
**Immune cells in pretreatment biopsies and their correlates with treatment responses**

No significant differences between CD3+, CD4+, CD68+, CD20+ and CD8+ intra-tumoral and peri-tumoral T-cell counts in PRE samples, were observed in responders than non-responders (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 non-responders (p=0.343). The mean tumor PD-L1 IRS at EDT was significantly greater for responders (PD-L1 IRS: 49) than non-responders (PD-L1 IRS: 4) (p=0.025) (Table 2, Figure 1). Two of the three 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 contrast, one non-responder acquired tumoral PD-L1 expression was observed in the five PRE PD-L1 negative tumors of non-responders (Fisher’s exact test p=0.4643).

Macrophage PD-L1 expression in EDT biopsies was detectable in 9 of 10 responders and in 4 of 7 non-responders (p=0.65). The only responder with a previously PRE PD-L1 negative 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 non-responders exhibiting PD-L1 negative macrophages at PRE (Fisher’s exact test p=0.2500). Importantly, the macrophage PD-L1 IRS at EDT was also higher in responders than non-responders (p=0.033) (Table 2, Figure 1a and b).

There were no statistically significant elevations between paired PRE vs 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 non-responders 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 to their matched PRE biopsy from the same patient (n=6 patients) showed an increase in the intra-tumoral density of CD8+ T-cells and CD68+ macrophages (p=0.026 and p=0.05, respectively) (Figure 1, Supplementary Figures 1 and 2), and a borderline increase in CD3+ T-cells (p=0.077) (Supplementary Figure 2). Non-responders (n=7 patients, 14 biopsies) showed a significant increase in the intra-tumoral density of CD8+ T-cells (p=0.043) (Supplementary Figures 1). Non-statistically significant increases in intra-tumoral PD-1+ T-cells were seen both in responders and non-responders (p=0.474 and p=0.082, respectively) (Supplementary Figure 1). No consistent changes were observed in CD4+, CD20+ and plasma cells in either group.

However, when analyzed as whole groups, the responders, in comparison to non-responders, showed greater intra-tumoral PD-1+ cells in their pretreatment biopsies (p=0.006) (Table 2, Figure 1). EDT biopsies showed significantly higher tumoral and macrophage PD-L1 IRS in responders than non-responders (p=0.025 and 0.034 respectively). Peritumoral density of PD1+ cells were 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)**

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Two PROG specimens (tumor from patients who had responded, then progressed) were available for analysis; two of the initial five PROG specimens had no viable tumor (see supplementary Results). The progression-free survival for the two 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 intra-tumoral and peri-tumoral compartments for CD3+, CD4+, CD8+ and PD1+ T-cells, CD20+ B-cells and CD68+ macrophages (the latter with the exception of the peri-tumoral space) (Figures 1 and 3) and in the intra-tumoral 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 cytomorphological pattern characteristic of response at EDT (Supplementary Table 1). However, apoptotic debris were more frequently encountered within the PRE biopsies of responders (p=0.008) (Supplementary Table 1) and statistically significant trends were observed between two histological parameters at PRE: 1) higher percentage of viable tumor (r =0.2101, p=0.0484); and 2) 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 pre-treatment 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-PD1 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-PD1.

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 intra-tumoral and peri-tumoral PD-1+ T-cells which more strongly correlated with tumor shrinkage and progression-free survival. 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 intra-tumoral and peri-tumoral 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, inter-tumoral and intra-tumoral PD-L1 expression is highly heterogeneous (3), the cut-off 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, i.e. 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 indicate a population of T-cells that have been stimulated by tumor specific antigens (4, 16-18). 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 (APCs) such as macrophages and dendritic cells (19). The abundance of intra-tumoral and peri-tumoral PD-1+ T-cells in the PRE biopsies of responders (but not in non-responders) 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 monoclonal antibodies, reactivates the tumoricidal function of these tumor-specific cytotoxic T-cells. Indeed, as we have shown, the greater the intra-tumoral and peri-tumoral 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 et al. (20) recently published striking increases in T-cell infiltrates and PD-L1 expression in the early during treatment biopsies of responders vs. non-responders. If confirmed in additional studies, EDT biopsies
may be utilized to determine if this expensive form of therapy should be continued, ceased (potentially sparing patients from prolonged therapy who may be deriving no benefit) or potentially if alternative agents should be added to the anti-PD-1 therapy.

Recently, Hugo et al. (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 interferon gamma signature or increased expression of T-cell specific markers in responders that we and others (4) have identified may be the product of the analytical platform employed, with RNAseq’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 non-responders were enriched for mesenchymal transition, wound healing and immune suppression. The mesenchymal phenotype has been recognized to be frequently associated with an immune-suppressive microenvironment and can also mediate MAPK inhibitor (MAPKi) 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 non-responders (i.e. patients who showed progressive disease 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. IFN-γ) by monocytes, helper and, in particular, cytotoxic T-cells (22) which in turn will bind to IFN-γ receptors on tumor and immune cells inducing PD-L1 expression.

The elevated numbers of intra-tumoral and peri-tumoral 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 intra-tumoral CD3+, CD8+ and PD-1+ T-cells at EDT in responders compared with non-responders, which expands upon a previous study which 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 pro-inflammatory cytokines (IL-6) and enhanced secretion of anti-inflammatory cytokines (IL-10) (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 reactivate cytotoxic PD-1+ T-cells but also promote regulatory macrophages to adopt a more pro-inflammatory state and secrete a range of inflammatory cytokines enhancing the influx of activated T-cells.

The combination of these peri/intra-tumoral changes while on treatment in patients that benefit from anti-PD-1 antibody therapy, namely the increase in the intra-tumoral 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 which 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, i.e. 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 interferon-gamma 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 re-establishment of tumor immune tolerance, but it would seem more likely that other molecules or mechanisms will be implicated in the re-establishment 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 provide 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 immune-suppressive tumor microenvironment by their promotion of the regulatory function of immune cells such as T-reg (28) and dendritic cells (26,29). It remains to be determined if the balance of the immune micromilieu is tipped towards 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, progression free survival and overall survival, more so than any other baseline features. Additionally, we demonstrate that the strongest correlation with response to anti-PD1 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.
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References:


8. Long GV, Larkin J, Ascierto PA, Hodi FS, Rutkowski P, Sileni V, et al. PD-L1 expression as a biomarker for nivolumab (NIVO) plus ipilimumab (IPI) and NIVO alone in advanced melanoma...
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Table 1. Clinicopathologic, treatment and follow up characteristics of the patient cohort.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Gender</th>
<th>Age (yrs)</th>
<th>Baseline LDH</th>
<th>Baseline ECOG</th>
<th>Anti-PD-1 antibody</th>
<th>BRAF Mutation</th>
<th>Best % change by RECIST or irCR</th>
<th>RECIST/IRC response (IRC)</th>
<th>Biopsies Analyzed</th>
<th>Progression free survival (Months)</th>
<th>Overall Survival (Months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR1</td>
<td>M</td>
<td>36</td>
<td>Elevated</td>
<td>2</td>
<td>Pembro</td>
<td>Yes</td>
<td>40 PD</td>
<td>PRE, EDT</td>
<td>3.2</td>
<td>8.5</td>
<td></td>
</tr>
<tr>
<td>NR2</td>
<td>M</td>
<td>51</td>
<td>Elevated</td>
<td>0</td>
<td>Pembro</td>
<td>Yes</td>
<td>1 PR</td>
<td>PD*</td>
<td>2.7</td>
<td>6.5</td>
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</tr>
<tr>
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<td>F</td>
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<td>Normal</td>
<td>0</td>
<td>Pembro</td>
<td>Yes</td>
<td>153.8 PD*</td>
<td>PRE</td>
<td>2.5</td>
<td>&gt;33.9</td>
<td></td>
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<td>F</td>
<td>51</td>
<td>Elevated</td>
<td>0</td>
<td>Pembro</td>
<td>Yes</td>
<td>N/A*</td>
<td>PD*</td>
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<td>5.5</td>
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<td>No</td>
<td>72 PD</td>
<td>PRE, EDT</td>
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<td>3.2</td>
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<td>M</td>
<td>79</td>
<td>Normal</td>
<td>0</td>
<td>Pembro</td>
<td>No</td>
<td>75 PD*</td>
<td>PRE, EDT</td>
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<td>32.6</td>
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<td>Nivo</td>
<td>No</td>
<td>40 PD</td>
<td>PRE</td>
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<td>2.8</td>
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<td>Nivo</td>
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<td>1.9</td>
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<td>1</td>
<td>Pembro</td>
<td>No</td>
<td>11 SD</td>
<td>PRE, EDT***</td>
<td>3.5</td>
<td>5.6</td>
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<td>-18 SD</td>
<td>PRE, EDT</td>
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<td>&gt;27.2</td>
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<td>Elevated</td>
<td>0</td>
<td>Pembro</td>
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<td>PRE, PROG</td>
<td>6.9</td>
<td>13,4</td>
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<td>Pembro</td>
<td>Yes</td>
<td>-87 PR*</td>
<td>PRE, EDT***</td>
<td>&gt;37.2</td>
<td>&gt;37.2</td>
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</tr>
<tr>
<td>R3</td>
<td>F</td>
<td>57</td>
<td>Elevated</td>
<td>1</td>
<td>Pembro</td>
<td>No</td>
<td>-86 PR</td>
<td>PRE, EDT***</td>
<td>&gt;26.1</td>
<td>&gt;26.1</td>
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<tr>
<td>R4</td>
<td>M</td>
<td>51</td>
<td>Elevated</td>
<td>0</td>
<td>Pembro</td>
<td>No</td>
<td>-5 SD*</td>
<td>PRE, EDT</td>
<td>8.3</td>
<td>27.1</td>
<td></td>
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<tr>
<td>R5</td>
<td>M</td>
<td>60</td>
<td>Normal</td>
<td>0</td>
<td>Pembro</td>
<td>No</td>
<td>-97 PR*</td>
<td>PRE, EDT***</td>
<td>29.6</td>
<td>&gt;55.1</td>
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<tr>
<td>R6</td>
<td>M</td>
<td>54</td>
<td>Elevated</td>
<td>0</td>
<td>Nivo***</td>
<td>No</td>
<td>-69 PR</td>
<td>PRE, EDT***</td>
<td>&gt;35.5</td>
<td>&gt;35.5</td>
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<tr>
<td>R7</td>
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<td>68</td>
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<td>1</td>
<td>Nivo</td>
<td>No</td>
<td>-92 CR</td>
<td>PRE, EDT</td>
<td>&gt;41.0</td>
<td>&gt;41.0</td>
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<tr>
<td>R8</td>
<td>M</td>
<td>75</td>
<td>Normal</td>
<td>1</td>
<td>Nivo***</td>
<td>No</td>
<td>-31 SD</td>
<td>PRE, EDT</td>
<td>10.3</td>
<td>&gt;34.8</td>
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<tr>
<td>R9</td>
<td>M</td>
<td>50</td>
<td>Elevated</td>
<td>1</td>
<td>Pembro</td>
<td>No</td>
<td>-18 SD</td>
<td>PRE, PROG***</td>
<td>9.6</td>
<td>25.5</td>
<td></td>
</tr>
<tr>
<td>R10</td>
<td>F</td>
<td>56</td>
<td>Normal</td>
<td>1</td>
<td>Pembro</td>
<td>No</td>
<td>-20 SD</td>
<td>PRE, EDT</td>
<td>7.1</td>
<td>10.5</td>
<td></td>
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<tr>
<td>R11</td>
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<td>38</td>
<td>Elevated</td>
<td>2</td>
<td>Pembro</td>
<td>No</td>
<td>-36 PR</td>
<td>PRE, EDT, PROG</td>
<td>2.0</td>
<td>&gt;27.9</td>
<td></td>
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<tr>
<td>R12</td>
<td>M</td>
<td>59</td>
<td>Normal</td>
<td>1</td>
<td>Pembro</td>
<td>No</td>
<td>-29 SD*</td>
<td>PRE, EDT, PROG***</td>
<td>&gt;37.1</td>
<td>&gt;37.1</td>
<td></td>
</tr>
<tr>
<td>R13</td>
<td>F</td>
<td>54</td>
<td>Normal</td>
<td>0</td>
<td>Pembro</td>
<td>No</td>
<td>-70 CR</td>
<td>PRE, EDT</td>
<td>&gt;27.9</td>
<td>&gt;27.9</td>
<td></td>
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</tbody>
</table>

Abbreviations: NR = non-responder, R = responder, M= male, F= female, %= percentage, LDH = lactate dehydrogenase, ECOG= Eastern Cooperative Oncology Group, PD-1= programmed cell death protein-1, CT= computed tomography, Pembro= pembrolizumab, Nivo= nivolumab, PD= progressive disease, SD= stable disease, PR= partial response, CR= complete response, N/A= not available, PRE= prior to treatment biopsy, EDT= early during treatment biopsy, PROG= acquired resistance biopsy, *= clinical progression, **= patient on anti-PD1 at the time of data analysis, ***= patient randomized to combined nivolumab and ipilimumab or nivolumab monotherapy (Checkmate 067), ****= no residual invasive melanoma identified.
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 melanoma patients receiving anti-PD-1 therapy.

<table>
<thead>
<tr>
<th>Variable</th>
<th>PRE scores (Std Dev)</th>
<th>EDT scores (Std Dev)</th>
<th>PROG scores (Std Dev)</th>
<th>Difference between responders vs. Non-responders (p-value)</th>
<th>Changes at PROG in responders (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-responders (n=10)</td>
<td>Responders (n=9)</td>
<td>Non-responders (n=7)</td>
<td>Responders (n=10)</td>
<td>At PRE</td>
</tr>
<tr>
<td>Tumor PD-L1 % Positive</td>
<td>30</td>
<td>66.7</td>
<td>42.9</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>Tumor PD-L1 IRS</td>
<td>14.6 (44.1)</td>
<td>8.1 (11.2)</td>
<td>4.6 (61)</td>
<td>49 (2.8)</td>
<td>3.0 (2.8)</td>
</tr>
<tr>
<td>Macrophage PD-L1 Positive</td>
<td>50.0</td>
<td>66.7</td>
<td>57.1</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>Macrophage PD-L1 IRS</td>
<td>12.3 (25.6)</td>
<td>63.9 (81.5)</td>
<td>46 (100)</td>
<td>150 (91)</td>
<td>75 (63.6)</td>
</tr>
<tr>
<td>CD3 Density</td>
<td>595 (704)</td>
<td>536 (708)</td>
<td>600 (681)</td>
<td>918 (896)</td>
<td>223 (12.7)</td>
</tr>
<tr>
<td>CD8 Density</td>
<td>477 (544)</td>
<td>854 (834)</td>
<td>325 (308)</td>
<td>925 (813)</td>
<td>426 (283)</td>
</tr>
<tr>
<td>CD68 Density</td>
<td>332 (452)</td>
<td>416 (452)</td>
<td>462 (548)</td>
<td>929 (592)</td>
<td>198 (17)</td>
</tr>
<tr>
<td>CD20 Density</td>
<td>448 (563)</td>
<td>552 (516)</td>
<td>236 (268)</td>
<td>768 (831)</td>
<td>373 (269)</td>
</tr>
<tr>
<td>CD1 Density</td>
<td>40 (56)</td>
<td>256 (231)</td>
<td>93 (164)</td>
<td>49 (74)</td>
<td>267 (240)</td>
</tr>
<tr>
<td>CD4 Density</td>
<td>64.8 (74)</td>
<td>313 (261)</td>
<td>49 (74)</td>
<td>267 (240)</td>
<td>37 (46)</td>
</tr>
<tr>
<td>PD-1 Density</td>
<td>182 (290)</td>
<td>171 (167)</td>
<td>155 (232)</td>
<td>222 (300)</td>
<td>222 (300)</td>
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<tr>
<td>CD80 Density</td>
<td>78 (189)</td>
<td>54.8 (67)</td>
<td>6 (10)</td>
<td>114 (250)</td>
<td>3 (4)</td>
</tr>
<tr>
<td>CD68 Density</td>
<td>18.9 (14)</td>
<td>244 (393)</td>
<td>9 (11)</td>
<td>236 (461)</td>
<td>0.3 (0.4)</td>
</tr>
<tr>
<td>CD1 Density</td>
<td>300 (310)</td>
<td>299 (257)</td>
<td>433 (405)</td>
<td>709.7 (432)</td>
<td>532 (365)</td>
</tr>
<tr>
<td>CD1 Density</td>
<td>259 (249)</td>
<td>295 (222)</td>
<td>196 (115)</td>
<td>389 (123)</td>
<td>541 (403)</td>
</tr>
<tr>
<td>CD3-CD4 Density</td>
<td>4.0 (3.4)</td>
<td>3.8 (3.1)</td>
<td>12.5 (25.5)</td>
<td>16.8 (30.2)</td>
<td>2.8 (1.8)</td>
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<tr>
<td>CD3-CD8 Density</td>
<td>4.2 (2.8)</td>
<td>7.7 (12.0)</td>
<td>1.9 (0.9)</td>
<td>3.0 (1.5)</td>
<td>2.8 (1.8)</td>
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<tr>
<td>CD8-PD-1 Density</td>
<td>1.6 (0.9)</td>
<td>1.2 (0.2)</td>
<td>1.9 (1.3)</td>
<td>1.3 (0.5)</td>
<td>1.1 (0.03)</td>
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<tr>
<td>CD8-PD-1 Density</td>
<td>1.6 (0.7)</td>
<td>1.8 (0.7)</td>
<td>1.8 (0.8)</td>
<td>1.4 (0.5)</td>
<td>1.1 (0.03)</td>
</tr>
</tbody>
</table>

Abbreviations: PRE= pretreatment, EDT= early during treatment, PROG= progression, St Dev= standard deviation, IRS= immunoreactive score. P-values <0.05 in bold. *= >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 responder and non-responder: Tumor PD-L1 IRS (panel A), Macrophage PD-L1 IRS (panel B), CD3+ cells (panel C), CD8+ cells, (panel D) and CD68+ cells (panel E) and PD1+ cells (panel F). Errors bars: 95% confidence interval. *p<=0.05. Immunohistochemically stained sections of a responding (patient R02, panel G) and a non-responding (patient NR02, panel H) patient’s tumor are depicted in the bottom panels. X200

**Figure 2** Clinical correlates of response with PD1+ cell density. Higher densities of intra-tumoral and peri-tumoral PD1+ cells significantly correlated with a greater reduction in tumor volume (RECIST)
(panels A and B). Greater densities of intra-tumoral and peri-tumoral PD1+ cells significantly associated with longer PFS and OS (panels C-F).
Figure 1
Figure 2
Dynamic changes in PD-L1 expression and immune infiltrates early during treatment predict response to PD-1 blockade in melanoma


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