ONCOIMMUNOLOGY
2017, VOL. 6, NO. 6, e1321187 (6 pages)
https://doi.org/10.1080/2162402X.2017.1321187

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BRIEF REPORT

Parallel profiling of immune infiltrate subsets in uveal melanoma versus cutaneous melanoma unveils similarities and differences: A pilot study

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ABSTRACT

The low response rates to immunotherapy in uveal melanoma (UM) sharply contrast with reputable response rates in cutaneous melanoma (CM) patients. To characterize the mechanisms responsible for resistance to immunotherapy in UM, we performed immune profiling in tumors from 10 metastatic UM patients and 10 metastatic CM patients by immunohistochemistry (IHC). Although there is no difference in infiltrating $CD8^+$ T cells between UM and CM, a significant decrease in programmed death-1 (PD-1)-positive lymphocytes was observed and lower levels of programmed death ligand-1 (PD-L1) in UM metastases compared with CM metastases. Tumors from metastatic UM patients showed a lower success rate of tumor-infiltrating lymphocyte (TIL) growth compared with metastatic CM (45% vs. 64% success), with a significantly lower quantity of UM TIL expanded overall. These studies suggest that UM and CM are immunologically distinct, and provide potential explanation for the impaired success of immunotherapy in UM.

ARTICLE HISTORY
Received 8 March 2017
Revised 13 April 2017
Accepted 15 April 2017

KEYWORDS
Cutaneous melanoma; immune profile; tumor infiltrating lymphocytes; uveal melanoma

Introduction

Uveal melanoma (UM) is the most common primary eye tumor in adults, with nearly half of patients developing distant metastases, most often to the liver.[1] Although UM arises from melanocytes within the uveal tract of the eye, it harbors a distinct molecular profile from cutaneous melanoma (CM).[2] For example, driver mutations in *BRAF* and *NRAS* commonly detected in CM rarely appear in UM, whereas *GNAQ* and *GNA11* mutations are found in approximately 80–90% of UM and are rarely seen in CM.[3,4] Unfortunately, no systemic therapy has been shown to improve overall survival (OS) in UM patients despite vast research efforts using chemotherapy, molecularly targeted therapy or immune therapy, with most drugs used to treat metastatic CM proving largely ineffective in UM patients.[5-8]

Currently, one of the most established treatments of metastatic melanoma involves immune stimulation through the use of checkpoint blockade. In contrast to the direct cytotoxic effects of chemotherapy, checkpoint blockade relies on antigen-specific T cell responses by blunting tumor-induced immunoregulatory mechanisms.[9] This form of treatment has provided durable, long-lasting responses in many melanoma patients including cutaneous and mucosal melanoma subtypes, largely due to the persistence and adaptability of the immune system. Accordingly, checkpoint blockade has been investigated in the context of UM, with agents targeting cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), such as ipilimumab and tremelimumab,[9] though clinical response rates to these regimens were largely unimpressive (< 10%) with no significant benefit to OS in UM patients.[7,8] Importantly, the low response rates in UM sharply contrast with relatively higher response rates to immunotherapy in CM patients, where several agents have now been FDA-approved based on a clear survival advantage or an encouraging response rate.[9] This tremendous disparity in the success of these treatment regimens suggests of the distinct immunological features and resistance mechanisms harbored by UM metastases.

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The reasons underlying the poor response to immunotherapy in UM are unclear. Thus, there is a critical need to characterize the UM immune infiltrate and microenvironment to improve treatment options for patients and circumvent resistance mechanisms. Studies thus far have shown that tumor-infiltrating lymphocytes (TIL) and expression of immunosuppressive factors in tumor cells play crucial roles in determining response to immunotherapies in CM within the tumor microenvironment.[10] Adoptive cell therapy (ACT) has shown success in CM, further strengthening the potential of T cell-targeted immunotherapies in promoting antitumor immunity and clinical responses. This approach expands TIL from surgically resected tumor nodules *ex vivo*, to re-infuse them in large numbers, and has resulted in an average of 50% response rates in CM.[11] However, ACT has not been well established in the context of UM, where the poor tumor immunogenicity, unknown immune infiltrate levels, and PD-1 expression may be barriers to response. Growth rate of TIL *in vitro* is associated with the cellular composition within tissue fragments, a crucial indicator of the impact of the tumor microenvironment on TIL growth and the immune system as a whole.[12] Accordingly, in this study, we sought to identify potential immune signature relevant to immune resistance in UM by studying $CD8^+$ infiltration levels, programmed death-1 (PD-1) and programmed death ligand-1 (PD-L1) expression, and TIL growth success rates in metastatic UM and CM patients.

Materials and methods

Patients and clinical characteristics

UM patients treated at The University of Texas MD Anderson Cancer Center (MDACC) between 2011 and 2016 were selected through a research bank database if they had adequate formalin-fixed paraffin-embedded tissue (FFPE) for analysis. All patients provided written consent for research tissue banking and analysis. CM patients were selected from a previously annotated data set to match UM patients in treatment exposure and anatomic site of metastasis. Archived FFPE tumor samples from 10 metastatic UM and 10 metastatic CM patients were used for this study. We selected these cases with similar therapy background based on available tissues. For both tumors (UM/CM), 60% of these patients were treatment-naive, one patient on immunotherapy, one patient post-immunotherapy, and two patients post-targeted therapy. The clinical characteristics for the patients including the metastatic sites are shown in Table 1. Fresh tissue from 33 metastatic UM and 655 CM resections were used for TIL harvest and culture for therapeutic purposes. CM and UM TIL patients had stage IIIC or IV disease and underwent surgery at MDACC under IRB-approved TIL protocols (LAB00–063 and 2004–0069).

Table 1. Patient and tissue baseline characteristics.

	UM $n = 10$	CM $n = 10$	Total $n = 20$
Gender – no. of patients (%)			
Female	8 (80)	5 (50)	13 (65)
Male	2 (20)	5 (50)	7 (35)
Age – years			
Range	44–75	43–66	43–75
Median	61	54	56
Treatment time point – no. of patients (%)			
Treatment naïve	6 (60)	6 (60)	12 (60)
On immunotherapy	1 (10)	1 (10)	2 (10)
Post-immunotherapy	1 (10)	1 (10)	2 (10)
Post-targeted therapy	2 (20)	2 (20)	4 (20)
Biopsy site – no. of patients (%)			
Liver	5 (50)	0 (0)	5 (25)
Lung	3 (30)	0 (0)	3 (15)
LN	0 (0)	4 (40)	4 (20)
Soft tissue	2 (20)	6 (60)	8 (40)

Immunohistochemistry (IHC) and digital image analysis

A hematoxylin and eosin (H&E) stained section was generated from each UM and CM FFPE block and reviewed by a pathologist to ensure the presence of viable tumor and selection of the best representative block when multiple blocks were available. Sections of 4-μm thickness were cut from FFPE blocks for immunohistochemistry (IHC) staining and analysis. Samples with high melanin content were submitted to 24-h bleaching with hydrogen peroxidase at room temperature before IHC staining in a Leica Bond Max automated stainer (Leica Biosystems, Buffalo Grove, IL) using antibodies against $CD8^+$ (clone C8/144B, Thermo Scientific, Waltham, MA) at a dilution of 1:25, PD-L1 (clone E1L3N, Cell Signaling, Danvers, MA) at a dilution of 1:100, and PD-1 (clone EPR4877, Abcam) at a dilution of 1:25. All stains were performed under optimized conditions and included a positive control (human lymph node) and a negative control without the primary antibody, as described previously.[13] The Leica Bond Polymer Refine detection kit (Leica Biosystems) was used for detection with diaminobenzidine (DAB) used as chromogen. All slides were counterstained with hematoxylin and scanned into a digital pathology slide scanner and analyzed using the Aperio analysis software (Aperio AT Turbo, Leica Biosystems). From each sample, 1–5 $\times$ 1 mm^2 areas were randomly selected within the tumor architecture by a pathologist for quantification, depending on the size of the tumor. $CD8^+$ and PD-1 was evaluated using the nuclear algorithm and the data retrieved as the number of positive cells per the analyzed area and normalized as counts/mm^2. PD-L1 was evaluated as percentage of total cells positive in the analyzed area.

Expansion of tumor-infiltrating lymphocytes

TIL that were used in this study were isolated from tumor samples in the pre-rapid expansion phase (as described in[11]). In the UM TIL subgroup, there were tumors from 33 patients.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 6.0 (La Jolla, CA). Measures of spread in UM and CM groups were first calculated and non-parametric Mann–Whitney tests were performed to compare $CD8^+$ expression and PD-1 expression. PD-L1 was dichotomized as $<1\%$ or $\geq 1\%$ and Chi-Square and Fischer's exact test were used to compare PD-L1 positivity and TIL growth success. All analyses were two-sided and used an α-level of 0.05.

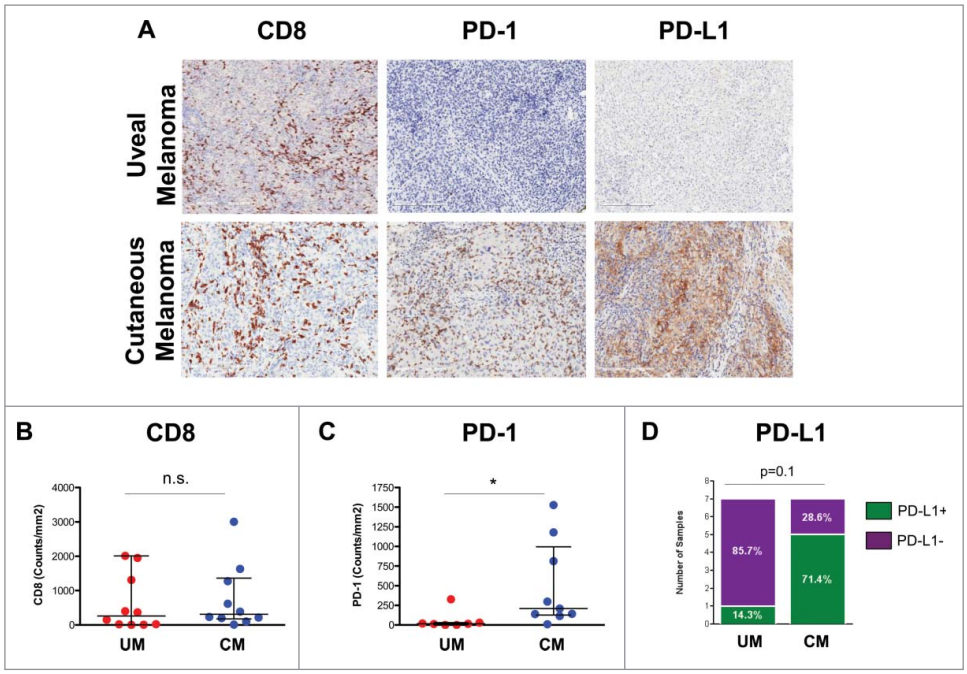

Figure 1. Decreased PD-1 and PD-L1 expression in UM metastases. (A) Representative IHC for CD8, PD-1, and PD-L1 in UM and CM metastatic tissues. Quantification of CD8 (B), PD-1 (C), and PD-L1 (D) in UM and CM metastases as counts/mm2 (B-C) and % positivity (D). Each dot represents a sample. Green, PD-L1-positive; Purple, PD-L1-negative. Statistical comparison between UM and CM cohorts was performed using non-parametric Mann-Whitney test (B-C) and Chi-square test (D).

Results

Checkpoint molecules on T lymphocytes have been encouraging therapeutic targets due to their involvement in antigen-specific responses to directly lyse malignant cells.[14] In this study, we characterized the immune profile by IHC to determine the levels of $CD8^+$, PD-1, and PD-L1 in 10 UM and 10 CM metastases (Table 1, Fig. 1A). Considering the lack of efficacy of ipilimumab and pembrolizumab on UM metastases,[7,8] we first quantified tumor-infiltrating $CD8^+$ T lymphocytes within the tumor microenvironment in UM and CM metastases. These analyses revealed that overall $CD8^+$ T cell infiltration was similar across these two tumor types (median infiltrate: UM = 260.7 $CD8^+/mm^2$ vs. CM = 311.0 $CD8^+/mm^2$, $p > 0.05$, not significant. Figs. 1A and B). Next, we investigated differences in the expression of PD-1, which could be targeted through PD-1 blockade immunotherapy. Interestingly, these analyses revealed that, although PD-1 was detectable in UM metastases, its expression levels were significantly lower than those observed in CM metastases (Median: UM = 15.2 $PD\text{-}1^+/mm^2$ vs. CM = 208.8 $PD\text{-}1^+/mm^2$, $p < 0.05$, Figs. 1A and C). Recently, PD-L1 (one of the ligands for PD-1) has also been the target of numerous immunotherapies,[15] which have proven successful in different tumors types. Therefore, we next quantified expression of PD-L1 within the tumor microenvironment in UM and CM metastases, and discovered only 14% of UM patients (1/7) were PD-L1 positive ($\geq$ 1% positivity), whereas in CM, 71% of patients (5/7) had PD-L1 positivity, though statistical significance was not attained due to limited sample size ($p = 0.1$) (Figs. 1A and D). Overall, these results suggest differences, not in the cytotoxic T cell infiltrate, but rather in the quality of these T cells through lack of expression of PD-1, as well as differences in the tumor microenvironments with UM lacking PD-L1 expression.

The success of TIL expansion is considered as an important indicator associated to the impact of tumor microenvironment on the ability of TILs responding to exogenous IL-2. Therefore, to investigate how changes of tumor microenvironment in UM metastases compared with CM metastases may reflect on the proliferative capacity of TIL, we next studied TIL expansion in a cohort of UM ($n = 33$) and CM ($n = 655$) who underwent tumor resections at MDACC for therapeutic purposes. As shown in Fig. 2A, successful *in vitro* TIL culture, defined as expansion and cryopreservation of at least 40 million cells, was obtained in 45% (15/33) of UM metastases, compared with (417/655) 64% in CM metastases ($p = 0.05$, Fig. 2A), suggesting differences in the quality of the immune infiltrate and/or tumor microenvironment in UM may impair the proliferative capacity of TIL. These findings were strengthened by the observation that the absolute TIL numbers grown from UM are significantly lower than the TIL numbers grown from CM patients, ($p < 0.0001$, Fig. 2B).

Discussion

In this study, we demonstrate that overall, metastatic UM tumors have $CD8^+$ T cell infiltrate quantities that are similar to metastatic CM tumors. However, it should be noted that, in our set of samples, 40% of UM metastases showed no $CD8^+$ T cells, while only 10% of CM metastases were devoid of $CD8^+$ T cells. It suggests samples with no infiltrate are more frequently found in UM than CM. Furthermore, we show a difference in the quality of the immune infiltrate with the expression of PD-1 significantly lower in UM than what is seen in CM. The absence of the PD-1/PD-L1 axis in most UM shown by our data provides a rationale for the lack of efficacy of anti-PD-1 approaches in UM. It is unclear why this axis is not present in UM and if it indicates a lack of interferon-γ (IFN-γ) induced T cell activation or non-functional cytotoxic T lymphocytes. The

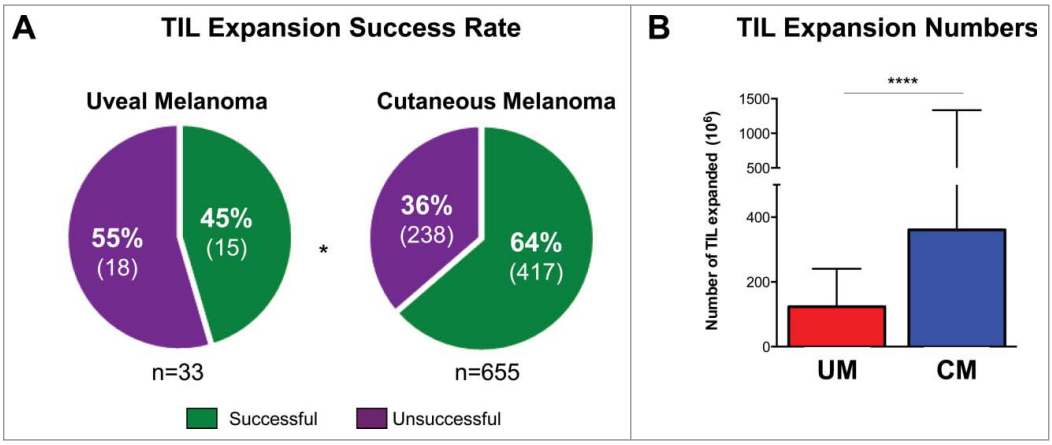

Figure 2. Impaired TIL expansion for UM metastases. TIL culture was attempted from 655 CM and 33 UM Stage IIIC or Stage IV tumors. (A) Shown is success rate of deriving at least 40 million TIL from these cultures in UM (left) and CM (right) metastases. (B) Total number of TIL expanded in UM and CM metastases. TIL were cultured from tumor explants for 3–5 weeks and cells were counted at the end of the culture. Statistical comparison between UM and CM cohorts was performed using (A) Chi-square test and (B) non-parametric Mann–Whitney test; $^{*}p = 0.05$; $^{****}p < 0.0001$.

success of immune checkpoint blockade in CM has relied heavily on the potency of the antitumor immune response, attributed mainly to the capacity of $CD8^+$ lymphocytes to infiltrate and lyse tumors on an antigen-specific basis.[14] However, if the infiltrating lymphocytes are not activated, this may fail to trigger an adaptive antitumor immune response. In light of recent work suggesting that tumor-reactive $CD8^+$ T cells express PD-1,[14] our results may suggest lack of tumor-antigen specific TIL in UM providing further rationale for the lack of efficacy of checkpoint blockade in UM.

Recently, an increasing number of studies have attributed checkpoint blockade efficacy to high mutation rates in CM and lung cancer, a process suggested to increase tumor immunogenicity through generation of neoantigens.[16] However, UM presents a limited mutational load compared with CM, which has been purported to contribute to the poor success of trials investigating checkpoint blockade in this type of cancer.[17] Different checkpoint blocking regimens act through distinct mechanisms, with CTLA-4 blockade suggested to act in the periphery causing increased infiltration of $CD8^+$ lymphocytes into tumors, and PD-1 blockade mediating effects within the tumor microenvironment on T cells in the immediate vicinity.[18] Accordingly, these two regimens have proven successful in CM, with response rates of 11–15% for the anti-CTLA-4 agent ipilimumab,[19] and 30–40% with anti-PD-1 agents pembrolizumab and nivolumab,[20] all demonstrating improvement in OS of CM patients. Furthermore, combination CTLA-4 and PD-1 blockade improves recruitment of peripheral T cells and blocks the inhibitory resistance mechanisms within the tumor microenvironment resulting in greater objective response rates in CM.[21] But monotherapy checkpoint blockade is not effective in UM,[7,8] and combination checkpoint blockade is currently being studied in a phase II clinical trial in UM (NCT01585194). Other immunosuppressive molecules in the tumor microenvironment may be playing a role to impair TIL and the immune response in UM, such as indoleamine 2,3-dioxygenase, transforming growth factor-β, or tumor-associated macrophages. Inhibitors of these immune-suppressive factors are in clinical development.

We also found that tumor PD-L1 expression is decreased in UM, with only one UM sample presenting 1% expression or greater, in stark contrast with CM patients. Here again, though PD-L1 is suggested to be a resistance and tumor escape mechanism, PD-L1 may be induced as a normal negative feedback mechanism as a result of IFNγ production within the tumor microenvironment, commonly produced by infiltrating T cells.[22] This also suggests that UM may not be as responsive to PD-L1 blockade, though occasional responses have been observed in other histologies in tumors devoid of PD-L1 expression.[15]

Our study also suggests that TIL expansion shows impaired success in UM, as well as decreased numbers of expanded TIL, indicating other negative regulators may suppress T cell activation in UM. A recent study by Rothermel *et al.* showed that the percentage of tumor fragments to successfully generate TIL were equivalent between CM and UM tumors (95% vs. 94%) based on consecutive metastatic liver tumors from 8 CM and 13 UM patients.[23] Variations with *in vitro* cell culture techniques may contribute to these differences as well as use of different criteria to determine successful outgrowth. In our study, TIL expansion success was defined as the capacity to expand beyond the 40×10^6 cell threshold, while specific criteria determining success in the Rothermel *et al.* manuscript were not described in detail. The study by Rothermel *et al.* also found no difference in $CD8^+$ T cell infiltration when comparing UM to CM, consistent with our results. However, phenotyping of expanded TIL in their cohort revealed an enrichment for $CD4^+$ T lymphocytes in the majority of UM, as opposed to CM. This could in part explain the decreased reactivity of these TIL, due to the skewing of T cell subsets toward CD4 in UM, specifically in light of the limited MHC II expression in UM.[23] In fact, the enrichment for MHC I expression and $CD4^+$ T cells in UM reported in that study appears to favor the lack of reactivity of these TIL. Our work complements these findings of impaired reactivity of TIL in UM, as decreased PD-1 expression observed in these tumors may be indicative of decreased tumor immunogenicity and impaired T cell activation.[14]

Our study has clear limitations – although studies in this limited comparative cohort did show clear differences in PD-1 and PD-L1 expression in UM compared with CM, which could suggest that checkpoint blockade could prove inefficient in this

form of cancer, these findings need to be validated in a larger matched data set. We are currently investigating these findings in a larger patient cohort treated with immune checkpoint blockade, as well as patients with paired primary and metastatic UM, to better understand the evolution of UM tumor immunology, from primary tumor to metastasis. Furthermore, although clinical trials targeting CTLA-4 and PD-1 individually have proven largely ineffective in UM thus far, combination CTLA-4 and PD-1 blockade, and the mechanisms by which the therapy may help or be hindered, has not yet been reported. We are currently running a combination nivolumab plus ipilimumab phase II clinical trial dedicated to metastatic UM patients to further evaluate this possibility (NCT01585194) in which longitudinal biopsies will be obtained to evaluate any change in the immune infiltrate over the course of therapy. It is our hope this will provide crucial insight into the response and resistance mechanisms of UM with the potential of increasing efficacy of current and future therapies and circumventing tumor-deployed resistance to ultimately improve patient survival.

Disclosure of potential conflicts of interest

Z.A. Cooper is an employee of MedImmune and owns stock or options in AstraZeneca. S.P. Patel is a past advisory board member for Genentech, Amgen and Castle Biosciences, a non-promotional paid speaker for Merck, Inc. and Bristol-Myers Squibb, and receives clinical trial support from GlaxoSmithKline, Novartis, Bristol-Myers Squibb, Deciphera, Prometheus, and Reata.

Acknowledgments

The authors would like to thank the MDACC TIL laboratory: Rahmatu Mansaray, Christopher Toth, Seth Wardell and Audrey Gonzalez. We would also like to thank all the clinical and research staff in the Melanoma Medical Oncology Department and Department of Surgical Oncology who contributed to the clinical TIL trial.

Funding

This work was supported by the NIH/NCI Cancer Center Support Grant under award number P30CA016672 and used the Tissue Biospecimen and Pathology Resource. This work was also supported by the MD Anderson Institutional Research Grant Award #40080 (PI: S.P. Patel). A. Reuben is supported by the Kimberley Clarke Foundation Award for Scientific Achievement provided by MD Anderson's Odyssey Fellowship program.

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