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BRIEF REPORT

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Distinct clinical patterns and immune infiltrates are observed at time of progression on targeted therapy versus immune checkpoint blockade for melanoma

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ABSTRACT

We have made major advances in the treatment of melanoma through the use of targeted therapy and immune checkpoint blockade; however, clinicians are posed with therapeutic dilemmas regarding timing and sequence of therapy. There is a growing appreciation of the impact of antitumor immune responses to these therapies, and we performed studies to test the hypothesis that clinical patterns and immune infiltrates differ at progression on these treatments. We observed rapid clinical progression kinetics in patients on targeted therapy compared to immune checkpoint blockade. To gain insight into possible immune mechanisms behind these differences, we performed deep immune profiling in tumors of patients on therapy. We demonstrated low CD8$^+$ T-cell infiltrate on targeted therapy and high CD8$^+$ T-cell infiltrate on immune checkpoint blockade at clinical progression. These data have important implications, and suggest that antitumor immune responses should be assessed when considering therapeutic options for patients with melanoma.

Abbreviations: BRAFi, BRAF inhibitor; CR, complete response; CT, computed tomography; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; IHC, immunohistochemistry; MEK, mitogen-activated protein kinase kinase; MEKi-, MEK inhibitor; NK, natural killer; PD-1, programmed cell death 1; PD-L1, programmed death-ligand 1; PR, partial response; RECIST, Response Evaluation Criteria In Solid Tumors; SD, stable disease

Introduction

Major advances have been made in treatment of metastatic melanoma through use of immune checkpoint blockade and molecularly targeted therapy, with US Food and Drug Administration approval of six new agents since 2011.[1-6] However, each of these forms of therapy has limitations, and optimal sequence of therapies is unknown.[7] Response to BRAF-targeted therapy occurs in the majority of patients harboring BRAF V600 mutated advanced melanoma; however, these responses are infrequently durable, with a median time to progression of 5.1–8.8 mo for BRAF inhibitor (BRAFi) monotherapy and 11.0–11.4 mo for combined BRAF and mitogen-activated protein kinase kinase (MEK) inhibitor (MEKi)

therapy.[8-10] Treatment with immune checkpoint inhibitors (such as anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and anti-programmed cell death 1 (PD-1)) is associated with lower response rates,[1,2,6] although responses tend to be more durable.[11]

With these advances, therapeutic dilemmas arise when treating patients with metastatic melanoma. Namely, it is unclear which therapeutic strategy should be initiated in treatment naïve patients with BRAF-mutated melanoma (targeted therapy vs. immune checkpoint blockade), and when to change the treatment strategy (during initial therapy or after disease progression). There are emerging insights into this question, as clinical data indicating that patients whose disease progressed

on targeted therapy have lower response rates to immune checkpoint blockade,[12-14] though the mechanism behind this is poorly understood and objective responses are still observed.[15,16] Thus, there is an unmet need to more fully understand the most appropriate sequence of therapeutic agents, and optimal timing of treatment strategies during the course of therapy.

Our understanding of the biological underpinnings of therapeutic response in cancer has been significantly enhanced by studies evaluating tumors at multiple time points during the course of treatment, and there is growing evidence that antitumor immunity may shape responses to molecularly targeted therapy, as well as immune checkpoint blockade.[17-21] However, immune responses across these regimens have not been studied, and we hypothesize that tumors in patients on these distinct regimens have different immune profiles, and that these may contribute to differential tumor kinetics when patients progress on therapy and may give insight regarding the appropriate timing and sequence of therapy. To address this, we deeply characterized immune infiltrates in tumors of patients with metastatic melanoma before treatment initiation and during the course of therapy on BRAF-targeted therapy or immune checkpoint blockade.

Materials and methods

Assessing responses in melanoma patients with clinical benefit on targeted therapy or immune checkpoint blockade

Cohorts of patients being treated with targeted therapy (dabrafenib + trametinib) or immune checkpoint blockade (pembrolizumab) at two major academic institutions (Massachusetts General Hospital or MD Anderson Cancer Center) underwent CT every 2–3 mo per independent treatment protocols (NCT01072175 and NCT01295827).[3,22] Patient data on targeted therapy were retrospectively provided by investigators who participated in the clinical trial, NCT01072175, which began accrual starting on March 26th 2010. The patient data on immune checkpoint were retrospectively provided by investigators who participated in clinical trial NCT01295827, which began accrual on December 1, 2011. Responses were determined according to Response Evaluation Criteria In Solid Tumors (RECIST) 1.1 by investigators participating in the clinical trials, and were compared between patients on targeted therapy and immune checkpoint blockade (note comparisons were only performed in patients who derived initial clinical benefit, as defined by stable disease (SD), partial (PR) or complete response (CR)). Spider plots were generated and the slope of RECIST measurements at time of progression was determined by the following: Slope = (change in RECIST from the previous scan) / (time in months). Patients who did not derive clinical benefit were excluded from analysis.

Clinical samples

Melanoma tumor samples were prospectively identified between April 2014 and August 2015 and were harvested from a cohort of patients under IRB-approved protocols following informed, written consent. Medical records were reviewed under a protocol allowing analysis of anonymized patient data. There were no gender/age preferences when obtaining specimens. Patients were required to be treatment naïve or to have undergone treatment with targeted therapy (BRAFi or combination of BRAF + MEK inhibitors) or immune checkpoint blockade (anti-CTLA-4 or anti-PD1, Table S1). On treatment biopsies were typically obtained within a couple of doses on therapy. All samples utilized for analysis were reviewed in central pathology to ensure viable tumor was present.

Flow cytometric analysis of immune cell subpopulations

Fresh melanoma tumors were cut into small pieces and digested for one hour at 37°C with Collagenase A and DNAse I (both from Roche, Basel, Switzerland) under 225 RPM rotation. Following digestion, tumor suspension was filtered through a 70 μm cell strainer (BD Biosciences, Franklin Lakes, NJ) and single cell suspension was counted and plated into a 96-well round bottom plate for staining as previously described.[23] In short, flow cytometry staining was carried out on five distinct CD45$^+$ panels after LIVE/DEAD Fixable Aqua Dead Cell Stain (Life Technologies, Carlsbad, CA) to look at CD4$^+$ (CD3ε^+CD4$^+$) or CD8$^+$ (CD3ε^+CD8$^+$) T lymphocytes, T regulatory (CD3ε^+CD4$^+$FoxP3$^+$), macrophages (CD14^{hi}CD11b$^+$HLADR$^+$), natural killer (NK-CD56$^+$NKG2D$^+$), $\gamma\delta$ T (CD3$\varepsilon^+\gamma\delta$TCR$^+$), B cells (CD3$\varepsilon^-$CD19/20$^+$HLA-DR$^+$), myeloid dendritic cells (CD11c$^+$HLA-DR$^+$CD14$^{lo/-}$), basophils (FCεR1α^+CD117$^-$CD11b$^-$CD49d$^+$), mast cells (FCεR1a$^+$CD117$^+$CD11b$^-$CD49d$^+$), neutrophils (CD15$^+$CD11b$^+$CD49d$^-$), and eosinophils (CD15$^+$CD11b$^+$CD49d$^+$). Antibodies are listed in Table S2. Following staining, cells were fixed with Cytofix Fixation Buffer (BD Biosciences, Franklin Lakes, NJ) or Cytofix/Cytoperm Concentrate (eBioscience, San Diego, CA) for intranuclear staining and acquired on a BD LSRFortessa II flow cytometer (BD Biosciences). Gating is show in Fig. S1. All data analysis was performed with FlowJo version 10 (Tree Star Inc., Ashland, OR).

Flow cytometric analysis of T lymphocyte activation and costimulation molecules

Tumors were dissociated into single-cell suspensions with a gentleMACS Octo Dissociator (Miltenyi, Bergisch Gladbach, Germany) following the manufacturer's protocol. Single-cell suspensions were then incubated overnight at 37°C in complete RPMI (RPMI 1640 1X with L-glutamine and 25 mM HEPES, Human AB serum 10%, Penicillin/Streptomycin, Gentamycin, 2-mercaptoethanol, Sodium Pyruvate and Non-Essential Amino Acids). Two distinct 8-color antibody panels were used to quantify T-cell activation which are as follows: (1) CD3-FITC, CD8-PercP-Cy5.5, CD45-PE-Cy7, CD69 APC-Cy7, HLA-DR-BV421, Granzyme B-Alexa Fluor647 (BD Biosciences), Perforin-PE (BioLegend, San Diego, CA) and LIVE/DEAD Fixable Aqua Dead Cell Stain (Life Technologies), (2) CD134 (OX40)-PE, CD137 (41BB)-APC, CD4-APC-Cy7, CD8-AmCyan (BD Biosciences), CD45-Alexa Fluor488, CD357 (GITR)-PE-Cy5, CD279 (PD-1)-Pacific Blue (BioLegend) and CD278 (ICOS)-PE-Cy7.

The acquisition was carried out on a FACS Canto II (BD Biosciences). All analysis was performed with FlowJo version 10 (Tree Star Inc.).

Immunohistochemistry analyses

From each FFPE tissue block a hematoxylin and eosin (H&E) stained slide was examined by a pathologist to confirm the presence of tumor. Four microns-thick sections were cut from a representative tumor block selected from each case for immunohistochemistry (IHC) analysis. IHC was performed using a Leica Bond Max automated stainer (Leica Biosystems, Buffalo Grove, IL). The primary antibodies employed included programmed death-ligand 1 (PD-L1) clone E1L3N (1:100, Cell Signaling Technology, Beverly, MA), PD-1 clone EPR4877(2) (1:250, Epitomics, Cambridge, MA), CD3 polyclonal (1:100, DAKO, Carpinteria, CA), CD4+ clone 4B12 (1:80, Leica Biosystems), CD8+ clone C8/144B (1:25, Thermo Scientific, Waltham, MA), CD45RO clone UCHL1 (ready to use, Leica Biosystems), CD57 clone HNK-1 (1:40, BD Biosciences), CD68 clone PG-M1 (1:450, DAKO), FOXP3 clone 206D (1:50, BioLegend), Granzyme B clone 11F1 (ready to use, Leica Microsystems) and OX40 clone ACT-35 (1:100, eBioscience). All slides were stained using previously optimized conditions including a positive control (human placenta for PD-L1 and human tonsil for other markers) and a non-primary antibody control. The IHC reaction was detected using Leica Bond Polymer Refine detection kit (Leica Biosystems) and diaminobenzidine (DAB) was used as chromogen. Counterstaining was done using Hematoxylin. All IHC stained slides were converted into high-resolution digital images of the whole tissue (e-slide) using a pathology scanner (Aperio AT Turbo, Leica Biosystems). The e-slides were
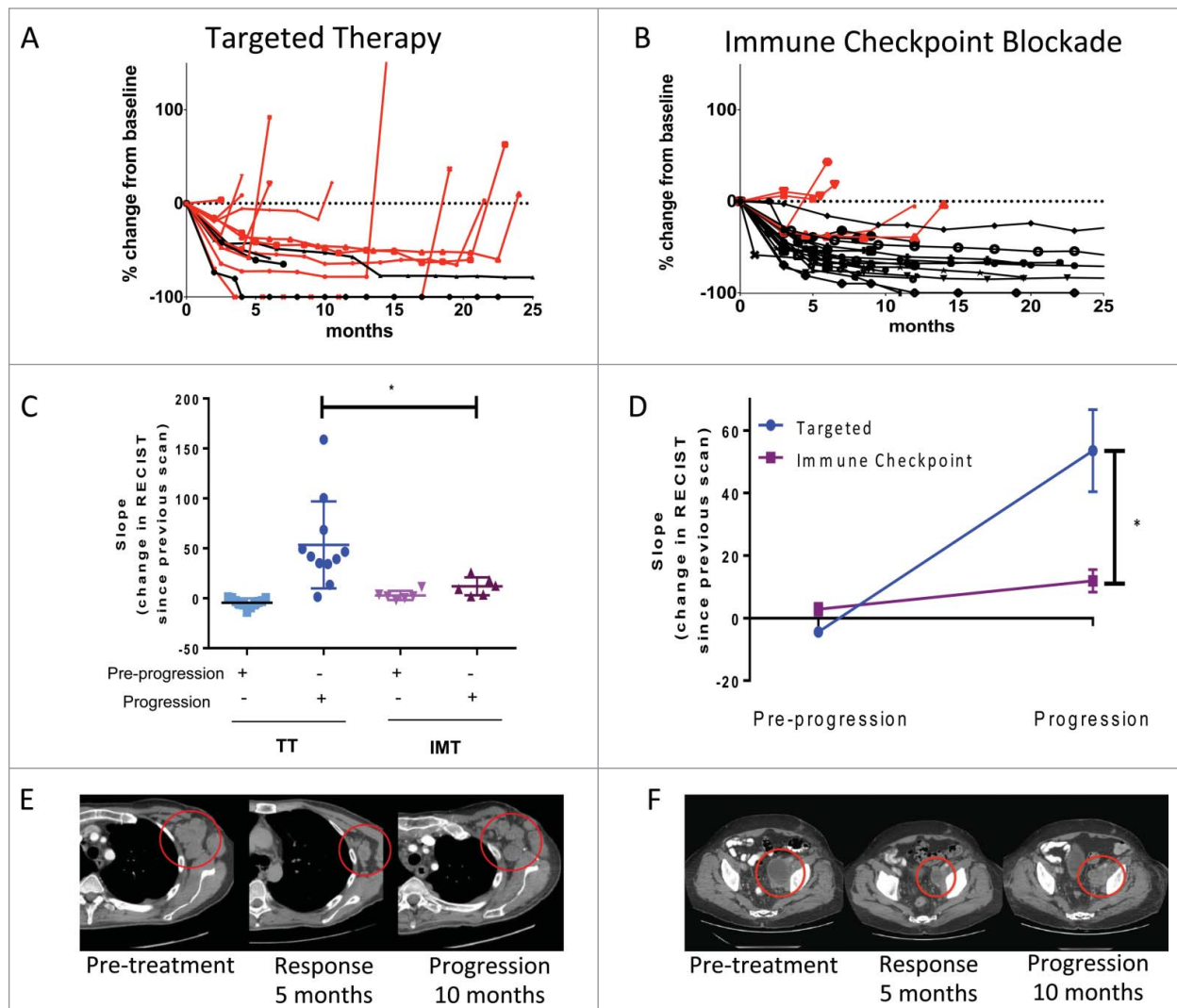
Figure 1. Differential tumor progression kinetics on targeted therapy versus immune checkpoint blockade. Plot of the percent change in the sum of diameters (per RECIST Criteria) within an individual patient compared to baseline at various time points during (A) BRAFi/MEKi (dabrafenib and trametinib, $n = 15$) or (B) anti-PD1 (pembrolizumab, $n = 25$) until disease progression. Each line represents an individual patient. Red lines are patients who progressed on therapy; black lines represent those who did not progress while on therapy. Patients who did not achieve clinical benefit or who progressed immediately on therapy per RECIST where not included. The change in RECIST measurements from the previous scan (change in RECIST/time in months) for individual patients (C) and in aggregate (D) before progression and at progression for either targeted therapy (TT, $n = 11$) or immune checkpoint blockade (IMT, $n = 6$) are shown. Representative CT scans of lesions at pre-treatment, on treatment, and at progression for targeted therapy (E) or immune checkpoint blockade (F). *$= p < 0.05$ by Mann–Whitney test.

then analyzed using the Aperio Image Toolbox analysis software (Leica Biosystems). From each e-slide, five random 1 mm^2 areas within the tumor region were chosen by a pathologist for digital analysis. PD-L1 expression was evaluated in the tumor cells using H-score, which includes the percentage of positive cells showing membrane staining pattern (0 to 100) and intensity of the staining (0 to 3+), with a total score ranging from 0 to 300. All other markers were evaluated as density of cells, defined as the number of positive cells per area (1 mm^2) regardless of the intensity. The final score for each marker was expressed as the average score of the five areas analyzed within the tumor region. The final scores for each marker from each patient were then transferred to a database for statistical analysis.

Statistics

Statistical evaluations used two-tailed Student t or Mann–Whitney test. Statistics were performed using GraphPad Prism. p values below 0.05 were considered statistically significant.

Results

Clinical patterns of progression differ on targeted therapy and immune checkpoint blockade in patients who demonstrate initial clinical benefit

To gain insight into clinical patterns of disease progression on targeted therapy versus immune checkpoint blockade, we analyzed longitudinal RECIST response from a cohort of patients with metastatic melanoma treated with targeted therapy (dabrafenib + trametinib) versus immune checkpoint blockade (pembrolizumab), specifically focusing on the subset of patients who initially demonstrated clinical benefit to therapy (SD, PR, CR) (Fig. 1A and B). The kinetics of disease progression on targeted therapy was more rapid than observed on immune checkpoint blockade as measured by the slope of RECIST responses from pre-progression to progression time points (55.3 vs. 11.8; $p = 0.0103$, Fig. 1C and 1D). Representative CT images at pretreatment, on-treatment and progression time points are shown for targeted therapy (Fig. 1E) and immune checkpoint blockade (Fig. 1F).
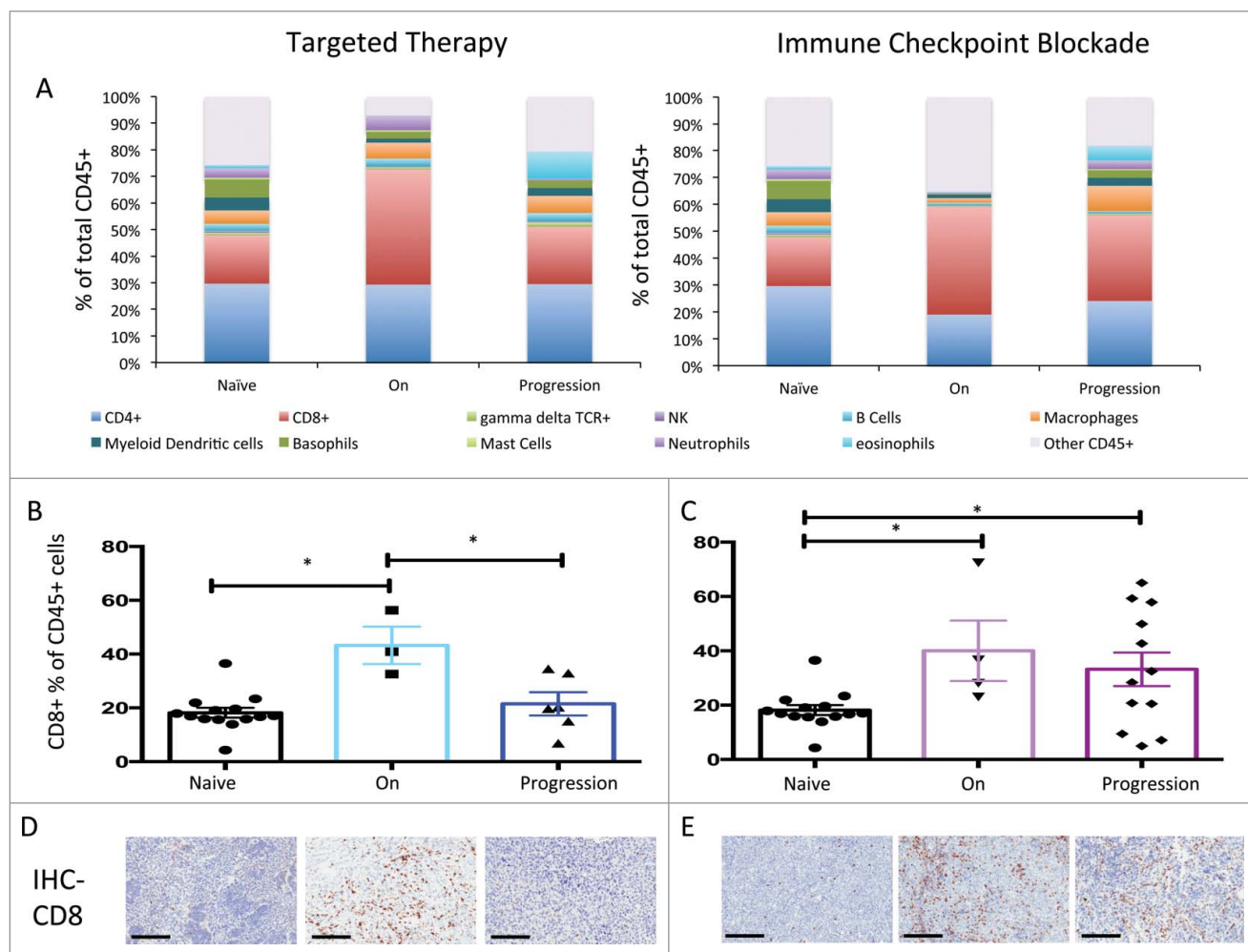
Figure 2. Differential T cell response at progression on targeted therapy and immune checkpoint blockade. (A) Flow cytometric analysis of leukocyte infiltrate from human melanomas that were treatment naïve (n = 14), on targeted therapy (n = 3), on immune checkpoint blockade (n = 4), or progressing on therapies as indicated (n = 6 and 12, respectively). Results are shown as the average percent of total CD45$^+$ cells markers. CD8$^+$ T cells as a % of total CD45$^+$ for patients treated with (B) targeted therapy or (C) immune checkpoint blockade with representative images of IHC (D and E, respectively at 20× magnification). $^* = p < 0.05$. micron bar = 200 μm.

CD8+ T-cell infiltrate is low at progression on targeted therapy and high at progression on immune checkpoint blockade

To better understand the antitumor immune responses at progression in each of these forms of therapy, we evaluated the immune subpopulations in metastatic melanoma tumors by polychromatic flow cytometry and IHC in 39 patients at different points in their treatment course (treatment naïve, early on treatment with either targeted therapy or immune checkpoint blockade, and at progression on these therapies). The majority of infiltrating immune cells were CD4+ or CD8+ T-lymphocytes, with macrophages, NK cells, T cells, B cells and other myeloid lineage populations representing a minority of the immune cells (Fig. 2A). The relative proportion of CD8+ T cells was higher in on-treatment biopsies in patients on targeted therapy as compared to treatment naïve patients (43.3% vs. 18.2%; $p = 0.0001$), consistent with previous studies.[20,24] Of note, CD8+ T cells were low at time of progression on targeted therapy when compared to on-treatment values (21.5% vs. 43.3%; $p = 0.027$, Fig. 2B). In contrast, we observed significantly higher levels of CD8+ T cells in on-treatment biopsies from patient tumors on immune checkpoint blockade when compared to treatment naïve (40.1% vs. 18.2%; $p = 0.004$), and higher levels of CD8+ T cells were also observed at time of progression on immune checkpoint (33.21% vs. 18.2%; $p = 0.0203$,

Fig. 2C). No significant differences in other immune populations (CD4+ T cells, regulatory T cells and others) were seen, with the exception of macrophages, which were higher in progressing lesions compared to on-treatment lesions in patients on immune checkpoint blockade (6.31% vs. 2.34%; $p = 0.0343$, Fig. S2). These data were complemented by IHC that validated the high levels of CD8+ T cells at progression on immune checkpoint therapy as compared to treatment naïve (303.4/mm^2 vs. 684.1/mm^2; $p = 0.0346$, Fig. 2D and E, Fig. S3 and S4). Representative longitudinal samples also demonstrated an increased CD8+ T cells at progression on immune checkpoint (Fig. S5). Higher levels of CD45RO, PD-L1 and OX40 proteins were also observed in tumors from patients at time of progression on immune checkpoint therapy as compared to treatment naive (Fig. S3).

Patterns of immune infiltrate differ in progressing tumors on CTLA-4 blockade versus PD-1 blockade

To gain a better understanding of the differences in immune infiltrates at time of progression on different forms of immune checkpoint blockade, we next evaluated T-cell phenotypes in tumors from patients progressing on anti-CTLA-4 versus anti-PD-1 blockade. We observed a higher proportion of CD8+ T cells in patient tumors at time of
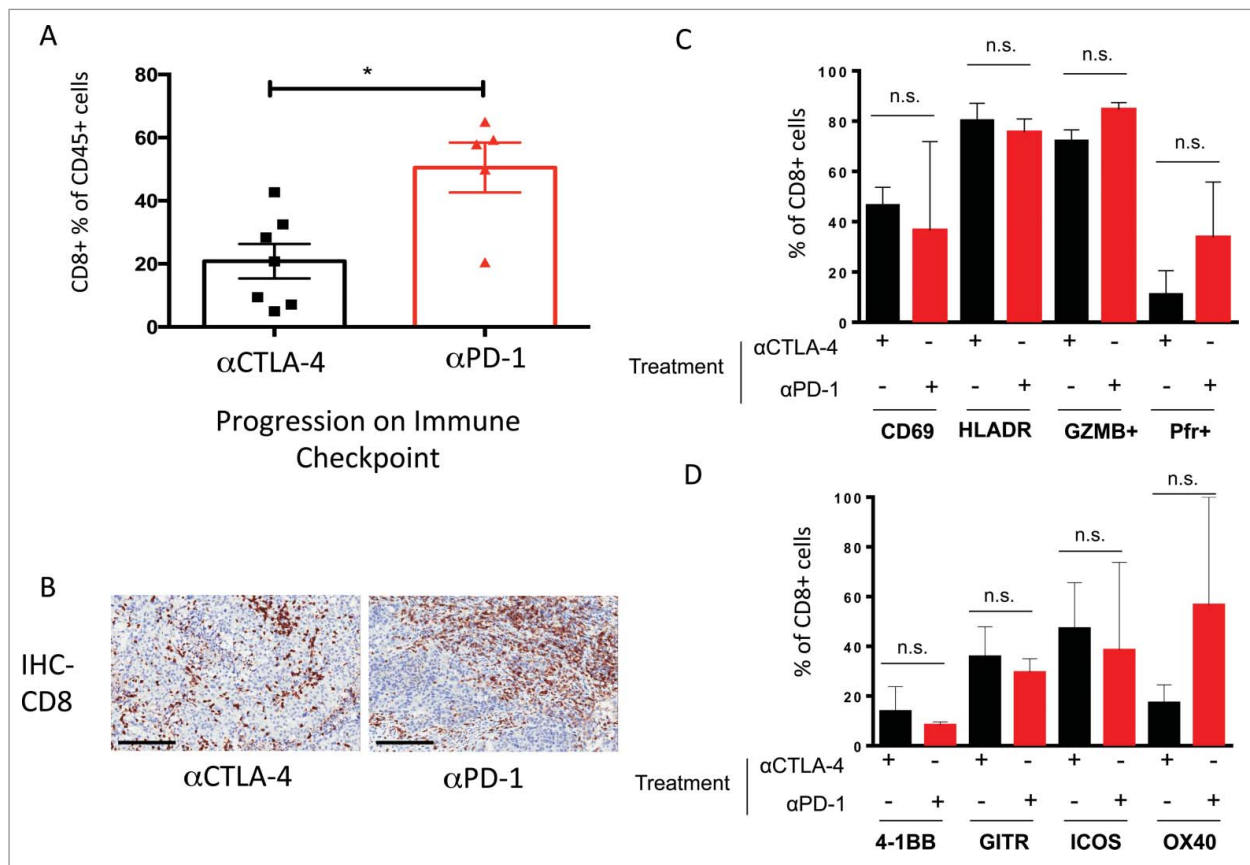
Figure 3. Differential T cell responses at progression on αCTLA-4 and αPD-1 therapy. (A) Flow cytometric analysis of CD8+ % of CD45+ cells within human melanomas progressing on αCTLA-4 (n = 7) and αPD-1 (n = 5) with (B) representative IHC images of CD8+ expressing cells (20× magnification, micron bar = 200 μm). The percent of CD8+ tumor infiltrating lymphocytes expressing (C) activation markers or (D) immunomodulatory molecules in patient tumors progressing on αCTLA-4 and αPD-1 as assessed by flow cytometry. $^* = p < 0.05$, n.s.= not significant.

progression on PD-1 versus CTLA-4 blockade, (50.52% vs. 20.84%; $p = 0.0092$, Fig. 3A) and versus targeted therapy (50.52% vs. 21.52%; $p = 0.0081$, Fig. S6). Representative IHC images are shown (Fig. 3B and Fig. S4). To further explore the function of the intra-tumoral CD8$^+$ T cells in these progressing lesions, we evaluated their activation status, and observed no difference in expression of CD69 and HLA-DR, or in cytolytic proteases granzyme B and perforin (Fig. 3C). In addition, we analyzed expression of the costimulatory and coinhibitory molecules ICOS, OX40, 4-1BB and GITR by flow cytometry. Although these immunomodulatory biomarkers were detectable on CD8$^+$ T cells infiltrating tumors, expression levels were not significantly different in CD8$^+$ T cells in anti-PD-1 and CTLA-4-treated patients (Fig. 3D).

Discussion

Despite numerous advances in the treatment of advanced melanoma, current therapeutic regimens have significant limitations and proper timing and sequence of therapy remain unclear (particularly in patients whose tumors harbor actionable molecular targets such as BRAF^{V600E}). We conducted these studies to better understand clinical and immunologic profiles at time of progression on targeted therapy and immune checkpoint blockade, with the hypothesis that immune profiles in tumors of patients are distinct on each form of therapy, and that these may contribute to differential tumor kinetics when patients progress on therapy.

In these studies, we observed more rapid progression on targeted therapy versus immune checkpoint blockade in patients who derived initial clinical benefit, and also observed significant differences in immune infiltrates at time of progression on these therapies. These data are important, as the low CD8$^+$ T-cell density at progression on targeted therapy could help explain why patients who progress on this therapy demonstrate lower response rates when treated with subsequent immune checkpoint blockade.[12-14] These findings are corroborated by previously published data in longitudinal samples in patients on targeted therapy suggesting that treatment with these agents induces a more oligoclonal immune response, but that it is early and transient.[17,20,21,25] This has important clinical implications, and suggests that at least in patients with a BRAF mutation, ideally we should not treat patients to progression with targeted therapy before treating with immune checkpoint blockade, but should consider adding it early after initiation of targeted therapy.[26]

A particularly interesting finding was the high CD8$^+$ T-cell infiltrate at time of progression in patients on immune checkpoint blockade, indicating that effector immune cells are present when patients are progressing on therapy (albeit in higher density in PD-1 vs. CTLA-4 blockade). This is intriguing, and suggests that these cells are functionally impaired and that other factors in the tumor microenvironment (namely macrophages, tumor and other stromal cells) may be contributing to therapeutic resistance. Although a limited panel of immunomodulatory molecules showed no significance when comparing the CD8$^+$ T cells in these two patient populations, there are numerous known and novel immunomodulatory molecules

that may play a major role in anti-tumoral immune activity. Deeper analysis of these molecules (such as TIM3, LAG3, VISTA, TIGIT, BTLA4 and PD-L2) is planned for future studies and may help guide clinical combination or sequencing strategies.[27-29] Deep profiling efforts in such samples are underway by our group and others to better understand these differences (via deep analysis of sorted immune and tumor cell subsets and cytokine analysis) though data are not mature and these are admittedly beyond the scope of these initial studies.

These findings have important clinical implications for the treatment of metastatic melanoma, and indicate that immune changes in the tumor microenvironment should be considered in optimal timing and sequence of therapy. Results of this and similar exploratory studies need to be validated in larger cohorts, however limitations may exist as collection of longitudinal blood and tumor samples may be quite challenging. Despite these limitations, it is important to build longitudinal tumor and blood sampling into clinical trial design and even in patients on standard of care therapy when this is feasible and infrastructure and expertise exist for processing and analysis of samples.[30] This is particularly important in the era of precision medicine with a growing number of targeted therapies and immunologic approaches to cancer therapy.

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