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BRAF inhibition is associated with enhanced melanoma antigen expression and a more favorable tumor microenvironment in patients with metastatic melanoma

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Abstract

Purpose—To evaluate the effects BRAF inhibition on the tumor microenvironment in patients with metastatic melanoma.

Experimental Design—Thirty-five biopsies were collected from 16 patients with metastatic melanoma pretreatment (day 0) and at 10-14 days after initiation of treatment with either BRAF inhibitor alone (vemurafenib) or BRAF + MEK inhibition (dabrafenib + trametinib), and were also taken at time of progression. Biopsies were analyzed for melanoma antigens, T cell markers, and immunomodulatory cytokines.

Results—Treatment with either BRAF inhibitor alone or BRAF + MEK inhibitor was associated with an increased expression of melanoma antigens and an increase in CD8+ T cell infiltrate. This was also associated with a decrease in immunosuppressive cytokines (IL-6 & IL-8) and an increase in markers of T cell cytotoxicity. Interestingly, expression of exhaustion markers TIM-3 and PD1 and the immunosuppressive ligand PDL1 were increased on treatment. A decrease in melanoma antigen expression and CD8 T cell infiltrate was noted at time of progression on BRAF inhibitor alone, and was reversed with combined BRAF and MEK inhibition.

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Conclusions—Together, this data suggests that treatment with BRAF inhibition enhances melanoma antigen expression and facilitates T cell cytotoxicity and a more favorable tumor microenvironment, providing support for potential synergy of BRAF-targeted therapy and immunotherapy. Interestingly, markers of T cell exhaustion and the immunosuppressive ligand PDL1 are also increased with BRAF inhibition, further implying that immune checkpoint blockade may be critical in augmenting responses to BRAF-targeted therapy in patients with melanoma.

Keywords

Melanoma; BRAF inhibitor; CD8 T cells; antigens; immunotherapy

Introduction

Melanoma remains a major world health problem (1, 2), though recent advances in targeted therapy against oncogenic BRAF for melanoma have shown some promising results (3). Somatic mutations in the BRAF oncogene occur in over half of melanomas, with the vast majority of these harboring an activating point mutation (V600E) (3). This oncogenic mutation leads to constitutive activation of the MAPK signaling pathway and increased oncogenic potential through a variety of mechanisms including reduced apoptosis, increased invasiveness, and increased metastatic behavior (4). Recent *in vitro* data also suggest that $BRAF^{V600E}$ could also contribute to immune escape (5).

Targeted therapy against oncogenic BRAF for metastatic melanoma results in objective responses in the majority of patients whose tumors harbor $BRAF^{V600E}$ (6). Despite this, resistance to therapy remains a significant issue, with a median duration of response between 6 and 7 months (6). There is a great deal of ongoing research to determine mechanisms of resistance and strategies to overcome resistance (7-9). Multiple distinct mechanisms of resistance have already been identified in recent months (10-13).

Combination of BRAF-targeted therapy with other signal transduction inhibitors has been proposed based on evidence that other pathways become activated upon emergence of resistance (14) and such clinical approaches are already underway. Another potential approach involves combining BRAF-targeted therapy with immunotherapy. This strategy is supported by recently reported data demonstrating that treatment of melanoma cells with BRAF-targeted therapy results in increased expression of melanocyte differentiation antigens (MDAs) and increased recognition by antigen-specific T cells (5). These results were corroborated in tumor biopsies from patients with metastatic melanoma receiving BRAF-targeted therapy and CD8+ T cell infiltrate correlated with response to therapy (15, 16).

We sought to test the hypothesis that BRAF-targeted therapy is associated with improved melanoma antigen expression and an enhanced immune response in patients with metastatic melanoma. We also assayed immune-modulatory cytokines and markers of T cell cytotoxicity as well as T cell exhaustion markers and the immunosuppressive ligand PDL1 to gain insight into potential means to modulate the immune response to BRAF inhibition.

Materials and Methods

Patient Samples

Patients with metastatic melanoma containing $BRAF^{V600E}$ mutation (confirmed by genotyping) were enrolled on clinical trials for treatment with a BRAF inhibitor (vemurafenib) or combined BRAF + MEK inhibitor (dabrafenib + trametinib)

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(Supplementary Table S1) and were consented for tissue acquisition per IRB-approved protocol. Tumor biopsies were performed pre-treatment (day 0), at 10-14 days on treatment, and/or at time of progression if applicable. Formalin-fixed tissue was analyzed to confirm that viable tumor was present via hematoxylin and eosin (H&E) staining. Additional tissue was snap frozen and stored in liquid nitrogen or were immediately processed for purification of RNA.

Purification of Total RNA

Samples were homogenized and disrupted using a mortar and pestle followed by use of a QIAshredder. A QIAcube was used to harvest RNA using the RNeasy Mini Protocol (Qiagen).

Quantitative PCR

Total RNA (250 ng) was used as template and Superscript VILO cDNA Synthesis Kit (Invitrogen) was used to generate cDNA. Quantitative real-time PCR was performed on an Applied Biosystems 7300 machine.

Immunohistochemistry

Tumor biopsies were stained with primary antibodies for MART-1 (Covance, SIG-38160-1000), HMB-45(gp100) (Leica, PA0027), CD4 (Leica, NCL-CD4-1F6), CD8 (Leica. PA0183), Perforin (Santa Cruz, sc-374346), TIM3 (R&D Systems, AF2365), PDL1 (LS-Bio, LS-B3368) or Granzyme B (Abcam, ab4059) followed by a secondary antibody for horseradish peroxidase and then DAB or blue chromagen. Stained slides were interpreted by a dedicated dermatopathologist.

Counting of CD8+ T cells

CD8+ T cell count was performed on slides from pre-treatment and on-treatment tumor biopsies in 4 adjacent high power fields (HPF) in the areas of highest density of CD8 positive cells. Only positive signals with clear lymphocyte morphology were evaluated. *Immunofluorescence* Sections from formalin-fixed paraffin-embedded (FFPE) melanoma tumor biopsies were de-paraffinized, rehydrated, and endogenous peroxidase activity was blocked in 3% hydrogen peroxide in water. After rinsing, heat-induced antigen retrieval was performed. Non-specific binding was blocked by 20% serum blocker and endogenous avidin + biotin blocking system. Primary antibody (monoclonal antibody targeting MART-1) and FITC-conjugated secondary antibody were then applied. Images were acquired using a Nikon Eclipse-80i fluorescence microscope.

Statistics

Statistics were performed using GraphPad Prism or the R-statistical package.

Results

BRAF inhibition is associated with increased melanoma antigen expression in tumors of patients with metastatic melanoma

Given prior preclinical findings that BRAF inhibition leads to increased expression of melanoma antigens in melanoma cell lines and fresh tumor digests *in vitro*, we sought to validate these findings in patients with metastatic melanoma undergoing treatment with a BRAF inhibitor (patients 1-5) or combined BRAF + MEK inhibitor (Patients 6-18) (Supplementary Table S1). We observed a significant increase in mRNA levels for melanoma antigens assayed after treatment with BRAF inhibitor (Figure 1A). Melanoma antigen expression increased by 4.9, 16.4, 13.3, and 14.1 fold in MART, TYRP-1. TYRP-2,

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and GP100 respectively. Of note, there was no statistically significant difference in melanoma antigen expression in biopsies from patients receiving a BRAF inhibitor alone versus combined BRAF + MEK inhibition ($p > 0.1$) with the exception of TYRP-2 ($p<0.04$) which was higher in those treated with combined BRAF + MEK inhibition (data not shown). Findings of increased MART-1 expression were validated via staining of MART-1 protein using both immunohistochemistry (Figure 1B and Supplementary Table S2) and immunofluorescence (Figure 1C and Supplementary Figure S1).

BRAF inhibition is associated with increased CD8+ T cell infiltrate in tumors of patients with metastatic melanoma

Next, we performed studies to test the ability of BRAF inhibition to augment T cell infiltrate based on our preclinical data showing that increased MDA expression is associated with increased recognition by antigen-specific T cells. Tumor biopsies from patients with metastatic melanoma undergoing treatment with a BRAF inhibitor were stained by H&E and using immunohistochemistry for CD8 positive cells. We observed a significant increase in CD8+ T cell infiltrate by immunohistochemistry in 10 out of 11 patients (Figure 2A-C). There was no difference in CD4+ T cell infiltrate (data not shown).

BRAF inhibition is associated with decreased immunosuppressive cytokines & markers of T cell cytotoxicity but increased T cell exhaustion markers and PDL1 in tumors of patients with metastatic melanoma

Next, we analyzed the tumors of treated patients for expression of immunosuppressive cytokines, markers of T cell cytotoxicity, T cell exhaustion markers and the immunosuppressive ligand PDL1 to determine the effects of BRAF inhibition on the tumor microenvironment. BRAF inhibition was associated with a significant decrease in the expression of immunosuppressive cytokines IL-6 and IL-8, while there was no significant change in TGF-beta and IL-10 (Figure 3A). A significant increase in expression was also observed in markers of T cell cytotoxicity (perforin, granzyme B) (Figure 3B). Of note, T cell exhaustion markers TIM3 and PD1 were also significantly increased after BRAF inhibition (Figure 3B). Expression of the immunosuppressive ligand PDL1 was also significantly increased following treatment with a BRAF inhibitor (Figure 3C). These were validated using immunohistochemistry for markers with available antibodies (Figure 3D and 3E). Of note, there was no change in HLA expression (Supplementary Figure S2).

Melanoma antigen expression and CD8+ T cell infiltrate are decreased at time of progression and restored through MEK inhibition

Tumor biopsies from patients with metastatic melanoma undergoing treatment with a BRAF inhibitor who progressed on therapywere assayed for melanoma antigen expression and CD8+ T cell infiltrate as described previously. At the time of progression, there was a significant decrease in melanoma antigen expression (Figure 4A and Supplementary Figure S3) and CD8+ T cell infiltrate. One of these patients was later enrolled on a combination trial incorporating combined BRAF + MEK inhibition (dabrafenib + trametinib) after progressing on BRAF monotherapy. A tumor biopsy performed after inititation of combined BRAF + MEK therapy showed restoration of melanoma antigen expression and CD8+ T cell infiltrate (Figure 4A-C).

Discussion

The advent of therapy with BRAF inhibitors has produced remarkable clinical success (6) and brought new hope for patients with metastatic melanoma. However, the impressive response rates have been tempered by a short duration of response in the majority of patients (6). There is an intense effort underway to better understand mechanisms of resistance to

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BRAF-targeted therapy (7-9, 13), with several strategies proposed to counter this resistance. Combination with other targeted agents (14) has been proposed and clinical trials are currently underway. The addition of further MAPK blockade via use of a MEK inhibitor with a BRAF inhibitor extends the median duration of response from 5.6 months to 9.5 months, though virtually all patients progress on therapy with very few complete responses noted (17).

Clinical studies have also demonstrated striking successes for immunotherapy approaches in melanoma (18-20). Adoptive therapy, vaccines, immunomodulatory approaches, and immune-checkpoint/tolerance blockades have all exhibited promising results, with ipilimumab now FDA approved on the basis of positive phase III clinical trial results in advanced melanoma (18) and other immune checkpoint inhibitors in clinical trials.

There is growing evidence that BRAF-targeted therapy may synergize well with immunotherapy. Prior *in vitro* findings demonstrated that pharmacologic manipulation of the MAPK pathway results in increased melanoma antigen expression in melanoma cells and this increase was associated with enhanced sensitivity to antigen-specific T cells (5). More recently, our group and others have provided supporting evidence for these *in vitro* findings, demonstrating an increase in tumor infiltrating lymphocytes in patients with metastatic melanoma treated with BRAF inhibitors (15, 16). An increase in immune infiltrate was seen in virtually all patients though the response to treatment was variable, raising the question as to whether the increase in T cell infiltrate is directly related to MAPK pathway blockade or if it is secondary to tumor necrosis. The results in Figure 4 showing restored T cell infiltrate with combination BRAF + MEK would suggest that the increased T cell infiltrate is more likely due to MAPK pathway inhibition, though further studies are necessary to better understand the response.

This manuscript provides strong *in vivo* support but more importantly demonstrates novel findings showing that BRAF inhibition is associated with increased melanoma antigen expression, increased markers of T cell cytotoxicity, and decreased expression of immunosuppressive cytokines – all enhancing the tumor microenvironment. However, further characterization of the immune infiltrate reveals that the infiltrating T cells demonstrate an exhausted phenotype (with increased TIM-3 and PD1) and increased expression of the immunosuppressive ligand PDL1. These results suggest that BRAF inhibition promotes T cell infiltration and increased melanoma antigen expression, however the immune response may be limited due to an increase in exhaustion markers on T cells and an increase in PDL1. These findings are novel and have important clinical implications, and may imply that successful combination therapy may require immune checkpoint blockade to enhance anti-tumor immunity.

Another novel finding in these studies was the observation that the increase in melanoma antigen expression and CD8+ T cell infiltrate was abrogated at the time of progression. While intriguing, these findings need to be validated in a larger cohort of patients. The patterns suggest that re-activation of the MAPK pathway is responsible for suppression of melanoma antigens and re-establishment of an immunosuppressive tumor microenvironment. This hypothesis is further supported by our findings that subsequent MAPK pathway inhibition using a MEK inhibitor can restore melanoma antigen expression and promote infiltration of CD8+ T cells. It is unclear whether the use of a different BRAF inhibitor (dabrafenib) or the addition of the MEK inhibitor contributed to this phenomenon, though recent literature would suggest that it is more likely to be the latter (21). Of note, in our *in vitro* studies leading up to these clinical findings (5), observed a deleterious effect of MEK inhibition on T lymphocytes which raised the potential concern that MEK inhibition in patients may alter T cell function. However in the present study, we saw no significant

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difference in T cell infiltrate in patients receiving BRAF inhibitor monotherapy versus BRAF inhibitor plus MEK inhibitor. Functional studies on these T cells were not performed in these patients, however this initial data regarding T cell infiltrate might suggest that MEK inhibition does not significantly impact T cell function.

Based on the data presented herein, one could speculate that augmenting the immune response by providing pro-immune cytokines (such as interleukin-2) or immune checkpoint inhibitors (such as monoclonal antibodies targeting CTLA-4, PD1 or PDL1) will act synergistically with the immune infiltrate elicited by BRAF inhibition in patients with metastatic melanoma. Interestingly, Hooijkaas et al. showed that anti-CTLA-4 mAb in combination with BRAF inhibition did not enhance tumor growth control in an inducible murine model, however this model also showed a decrease in T cell infiltrate after BRAF inhibition which is contrary to what we see in patients (22). In a mouse model of $BRAF^{V600E}$ melanoma, Koya et al. showed improved anti-tumor activity, *in vivo* cytotoxic activity, and intratumoral cytokine secretion by adoptively transferred cells in combination with a BRAF inhibitor (23). Trials combining BRAF-targeted therapy and immunotherapy in patients are currently underway, though no data is currently available regarding response rates, duration of response, or findings from correlative studies. Coupled with these preclinical studies, our present findings reinforce the rationale for combined BRAF-targeted therapy and immunotherapy in the treatment of metastatic melanoma.

Future studies to further evaluate the immune response during BRAF inhibition are still needed. A deeper understating of how these treatment modalities interact with each other will allow for optimal design of clinical trials to maximize response rates and duration of response in patients with metastatic melanoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. CA Cancer J Clin. 2009; 59:225–49. [PubMed: 19474385]
2. Balch CM, Gershenwald JE, Soong SJ, Thompson JF, Atkins MB, Byrd DR, et al. Final version of 2009 AJCC melanoma staging and classification. J Clin Oncol. 2009; 27:6199–206. [PubMed: 19917835]
3. Sosman JA, Kim KB, Schuchter L, Gonzalez R, Pavlick AC, Weber JS, et al. Survival in BRAF V600-mutant advanced melanoma treated with vemurafenib. The New England journal of medicine. 2012; 366:707–14. [PubMed: 22356324]
4. Singh M, Lin J, Hocker TL, Tsao H. Genetics of melanoma tumorigenesis. Br J Dermatol. 2008; 158:15–21. [PubMed: 18047516]
5. Boni A, Cogdill AP, Dang P, Udayakumar D, Njauw CN, Sloss CM, et al. Selective BRAFV600E inhibition enhances T-cell recognition of melanoma without affecting lymphocyte function. Cancer research. 2010; 70:5213–9. [PubMed: 20551059]

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6. Flaherty KT, Puzanov I, Kim KB, Ribas A, McArthur GA, Sosman JA, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. N Engl J Med. 2010; 363:809–19. [PubMed: 20818844]
7. Emery CM, Vijayendran KG, Zipser MC, Sawyer AM, Niu L, Kim JJ, et al. MEK1 mutations confer resistance to MEK and B-RAF inhibition. Proc Natl Acad Sci U S A. 2009; 106:20411–6. [PubMed: 19915144]
8. Puzanov I, Burnett P, Flaherty KT. Biological challenges of BRAF inhibitor therapy. Mol Oncol. 2011; 5:116–23. [PubMed: 21393075]
9. Solit DB, Rosen N. Resistance to BRAF inhibition in melanomas. N Engl J Med. 2011; 364:772–4. [PubMed: 21345109]
10. Johannessen CM, Boehm JS, Kim SY, Thomas SR, Wardwell L, Johnson LA, et al. COT drives resistance to RAF inhibition through MAP kinase pathway reactivation. Nature. 2010; 468:968–72. [PubMed: 21107320]
11. Villanueva J, Vultur A, Lee JT, Somasundaram R, Fukunaga-Kalabis M, Cipolla AK, et al. Acquired resistance to BRAF inhibitors mediated by a RAF kinase switch in melanoma can be overcome by cotargeting MEK and IGF-1R/PI3K. Cancer cell. 2010; 18:683–95. [PubMed: 21156289]
12. Nazarian R, Shi H, Wang Q, Kong X, Koya RC, Lee H, et al. Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation. Nature. 2010; 468:973–7. [PubMed: 21107323]
13. Straussman R, Morikawa T, Shee K, Barzily-Rokni M, Qian ZR, Du J, et al. Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion. Nature. 2012; 487:500–4. [PubMed: 22763439]
14. Smalley KS, Flaherty KT. Integrating BRAF/MEK inhibitors into combination therapy for melanoma. British journal of cancer. 2009; 100:431–5. [PubMed: 19156138]
15. Wilmott JS, Long GV, Howle JR, Haydu LE, Sharma RN, Thompson JF, et al. Selective BRAF inhibitors induce marked T-cell infiltration into human metastatic melanoma. Clin Cancer Res. 2012; 18:1386–94. [PubMed: 22156613]
16. Wargo, J.; Cogdill, A.; Dang, P.; Gupta, R.; Piris, A.; Boni, A., et al. Treatment with a selective inhibitor of BRAFV600E increases melanocyte antigen expression and CD8 T cell infiltrate in tumors of patients with metastatic melanoma. American Association for Cancer Research 102nd Annual Meeting 2011; Orlando, Fl. 2011. p. 232
17. Flaherty KT, Infante JR, Daud A, Gonzalez R, Kefford RF, Sosman J, et al. Combined BRAF and MEK Inhibition in Melanoma with BRAF V600 Mutations. The New England journal of medicine. 2012
18. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med. 2010; 363:711–23. [PubMed: 20525992]
19. Rosenberg SA, Restifo NP, Yang JC, Morgan RA, Dudley ME. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. Nat Rev Cancer. 2008; 8:299–308. [PubMed: 18354418]
20. Johnson LA, Morgan RA, Dudley ME, Cassard L, Yang JC, Hughes MS, et al. Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. Blood. 2009; 114:535–46. [PubMed: 19451549]
21. Wagle N, Emery C, Berger MF, Davis MJ, Sawyer A, Pochanard P, et al. Dissecting therapeutic resistance to RAF inhibition in melanoma by tumor genomic profiling. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2011; 29:3085–96. [PubMed: 21383288]
22. Hooijkaas A, Gadiot J, Morrow M, Stewart R, Schumacher T, Blank CU. Selective BRAF inhibition decreases tumor-resident lymphocyte frequencies in a mouse model of human melanoma. Oncoimmunology. 2012; 1:609–17. [PubMed: 22934253]
23. Koya RC, Mok S, Otte N, Blacketor KJ, Comin-Anduix B, Tumeh PC, et al. BRAF inhibitor vemurafenib improves the antitumor activity of adoptive cell immunotherapy. Cancer research. 2012; 72:3928–37. [PubMed: 22693252]

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Statement of Translational Relevance

This study analyzes immune responses in serial tumor biopsies of patients with metastatic melanoma treated with BRAF-targeted therapy. Significantly, we demonstrate that BRAF inhibition is associated with an increase in melanoma antigen expression and T cell infiltrate, and a decrease in immunosuppressive cytokines in tumors of treated patients. An increase in markers of T cell cytotoxicity was also noted. Interestingly, both melanoma antigen expression and T cell infiltrate were abrogated at disease progression. Paradoxically, BRAF inhibition was associated with an increase in T cell exhaustion markers TIM-3 & PD1 and the immunosuppressive ligand PDL1. Together, these data support the hypothesis that combined BRAF-targeted therapy and immunotherapy may improve responses in patients with metastatic melanoma. This work provides the basis for future research studies and clinical trials that will focus on understanding the complex interplay between the tumor, immune system, and the tumor microenvironment in response to targeted therapy.

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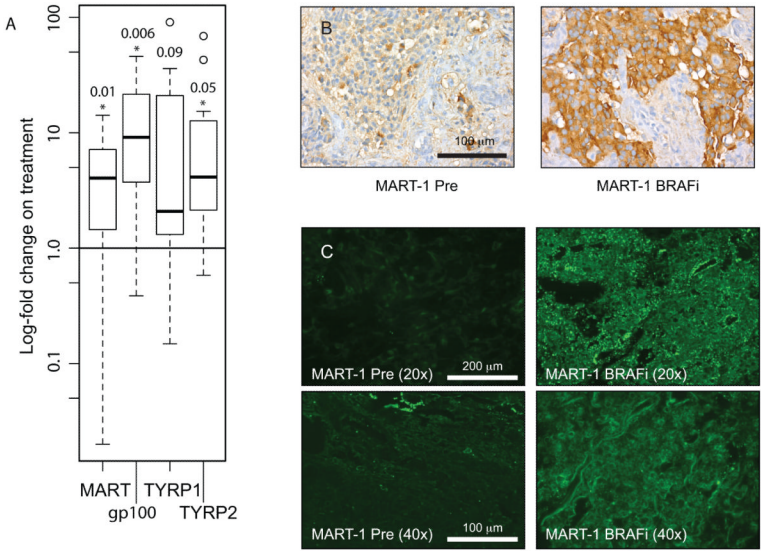

Figure 1. BRAF inhibition is associated with increased melanoma antigen expression in tumors of patients with metastatic melanoma

Tumors were harvested and mRNA levels of gp100, MART-1, TYRP-1, and TYRP-2 in patients with metastatic melanoma undergoing treatment with a selective inhibitor of $BRAF^{V600E}$ were assayed. (**A**) Expression levels of the respective MDAs are shown as fold increase over pre-treatment value and are plotted on a log scale in a box and whiskers plot (n=12). The bottom and top of the 25th and 75th percentile respectively for all patients, with the bar indicating the median value. The whiskers indicate the extremes with open circles represent data points greater than 1.5 times the interquartile range. (**B**) Immunohistochemistry (40× magnification) and (**C**) immunofluorescence staining for the melanoma antigen MART-1 in pre-treatment and on-treatment biopsies (patient 2) was performed to confirm that protein expression correlated with mRNA expression. All microscopy was repeated at least 3 times with representative examples shown. P-values are from a two-tailed student t-test with a mu of 1, which corresponds to no change in mRNA levels on treatment. (*) represents $p \leq 0.05$.

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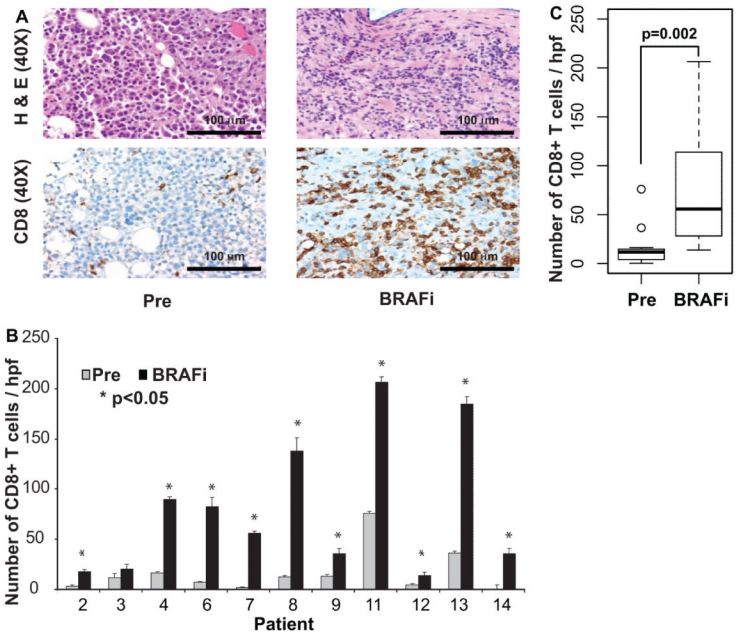

Figure 2. BRAF inhibition is associated with increased CD8+ T cell infiltrate in tumors of patients with metastatic melanoma

Tumors biopsied pre-treatment and on-treatment were stained for H&E and IHC was performed for CD8+ T cells with a representative patient shown (**A**). CD8+ T cell counts were performed in a blinded fashion by a dedicated dermatopathologist **(B).** Average CD8+ T cells counts are plotted for each patient with error bars representing the standard deviation of four measurements. CD8+ counts from all patients (n=11) are expressed in a box and whiskers plot both pre and on treatment **(C)**.

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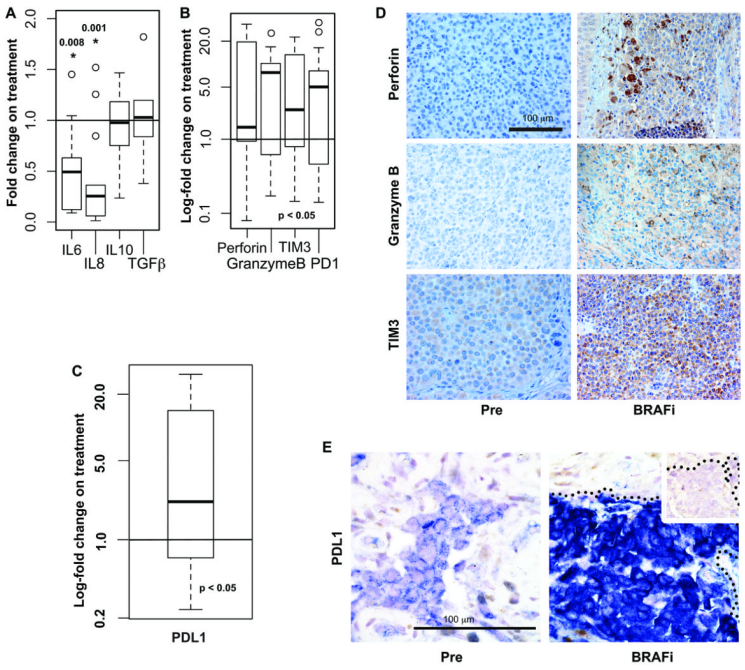

Figure 3. BRAF inhibition is associated with decreased immunosuppressive cytokines & markers of T cell cytotoxicity but increased T cell exhaustion markers and PDL1 in tumors of patients with metastatic melanoma

Tumors were harvested and mRNA levels of IL-6, IL-8, IL-10, and TGFβ **(A)**, Perforin (n=11), GranzymeB (n=11), TIM3 (n=14) and PD1 (n=14) **(B)** and PDL1 (n=11) **(C)** in patients with metastatic melanoma undergoing treatment with a selective inhibitor of $BRAF^{V600E}$ were assayed. All patients are expressed in a box and whiskers plot. Open circles represent data points greater than 1.5 times the interquartile range. p-values indicated are from a two tailed student t-test with a mu of 1, which represents no change in mRNA value with respect to the pre-treatment value. (*) represents $p \leq 0.05$. Immunohistochemistry (40× magnification) for the Perforin, Granzyme B and TIM3 (patient 6) **(D)** and PDL1 (Patient 12) **(E)** in pre-treatment and on-treatment biopsies was performed to confirm that protein expression correlated with mRNA expression. The dotted line=tumor-stroma interface and the inset is the isotype-specific control.

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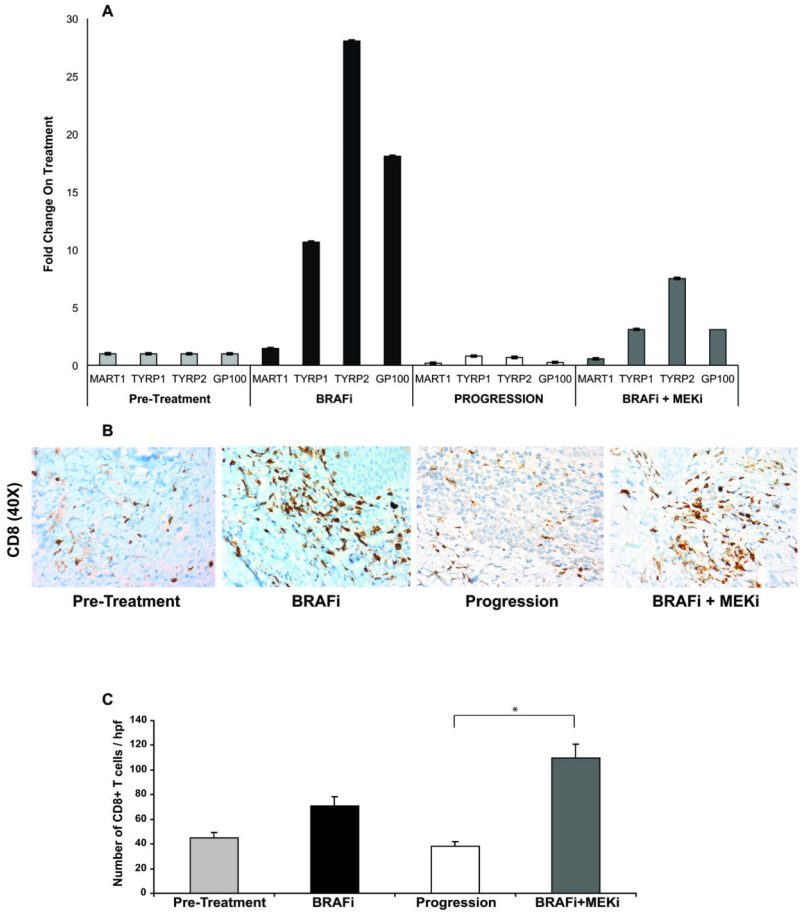

Figure 4. Melanoma antigen expression and CD8+ T cell infiltrate are decreased at time of progression and restored through MEK inhibition

Tumors were harvested at time of progression and at time of treatment with combined BRAF inhibition and MEK inhibition for patient 3. mRNA levels of the melanoma antigens gp100, MART-1, TYRP-1, and TYRP-2 were assayed (**A**). IHC was performed for CD8+ T cells on patient tumor samples (**B**). CD8+ T cells were identified and counted by a dedicated pathologist (**C**). Average CD8+ T cells counts are plotted with error bars representing the standard deviation of four measurements. (*) represents $p \leq 0.05$.