Response to BRAF Inhibition in Melanoma Is Enhanced When Combined with Immune Checkpoint Blockade

Zachary A. Cooper1,2, Vikram R. Juneja3,4,5, Peter T. Sage4,5, Dennie T. Frederick6, Adriano Piris7,8, Devarati Mitra9, Jennifer A. Lo5, F. Stephen Hodi5,9, Gordon J. Freeman5,9, Marcus W. Bosenberg10, Martin McMahon10,11, Keith T. Flaherty5,6, David E. Fisher5,8, Arlene H. Sharpe4,5,10, and Jennifer A. Wargo1,2

Abstract

BRAF-targeted therapy results in objective responses in the majority of patients; however, the responses are short lived (~6 months). In contrast, treatment with immune checkpoint inhibitors results in a lower response rate, but the responses tend to be more durable. BRAF inhibition results in a more favorable tumor microenvironment in patients, with an increase in CD8+ T-cell infiltrate and a decrease in immunosuppressive cytokines. There is also increased expression of the immunomodulatory molecule PDL1, which may contribute to the resistance. On the basis of these findings, we hypothesized that BRAF-targeted therapy may synergize with the PD1 pathway blockade to enhance antitumor immunity. To test this hypothesis, we developed a BRAF(V600E)/Pten+/− syngeneic tumor graft immunocompetent mouse model in which BRAF inhibition leads to a significant increase in the intratumoral CD8+ T-cell density and cytokine production, similar to the effects of BRAF inhibition in patients. In this model, CD8+ T cells were found to play a critical role in the therapeutic effect of BRAF inhibition. Administration of anti-PD1 or anti-PDL1 together with a BRAF inhibitor led to an enhanced response, significantly prolonging survival and slowing tumor growth, as well as significantly increasing the number and activity of tumor-infiltrating lymphocytes. These results demonstrate synergy between combined BRAF-targeted therapy and immune checkpoint blockade. Although clinical trials combining these two strategies are ongoing, important questions still remain unanswered. Further studies using this new melanoma mouse model may provide therapeutic insights, including optimal timing and sequence of therapy. Cancer Immunol Res; 2(7); 643–54. ©2014 AACR.

Introduction

Targeted therapy against oncogenic mutations, such as BRAFV600E, represents one of the most significant advances in the treatment of melanoma in decades. However, responses to BRAF inhibitor (BRAFi) monotherapy are not durable, with a median time to progression of less than 6 months (1–3). The combination of BRAF plus MEK (mitogen-activated or extracellular signal–regulated protein kinase) inhibition has provided incremental gains; however, the majority of patients still progress on therapy within 10 months (4). Thus, strategies to increase the durability of these responses are urgently needed.

Immunotherapy is another area of success in the treatment of melanoma. In particular, the use of immune checkpoint inhibitors has shown tremendous promise. Ipilimumab (a monoclonal antibody (mAb) targeting immunomodulatory CTLA4 receptor on T cells) was approved by the FDA recently based on a survival benefit over standard chemotherapy in patients with metastatic melanoma (5). Additional immunomodulatory agents are in clinical trials, and have shown impressive results. These include mAbs against the programmed death 1 (PD1; CD279) receptor and its ligands PDL1 (B7-H1; CD274) and PDL2 (B7-DC; CD273; refs. 6, 7). PD1 is an inhibitory cell-surface receptor that can be induced to be expressed by T cells, B cells, natural killer T (NK) T cells, monocytes, and dendritic cells (DC; ref. 8). PDL2 is expressed mainly by DCs and macrophages, whereas PDL1 is widely expressed by hematopoietic, nonhematopoietic, and tumor cells (8, 9). The expression of PDL1 in tumors is inversely correlated with the survival of patients, and tumors can use the PD1 inhibitory pathway to evade immune eradication (10–14). Clinical trials with mAbs targeting PD1 and PDL1 have...
shown promising response rates (30%–50%) with activity in melanoma and other cancers such as renal cell carcinoma and non–small cell lung cancer (6, 7). However, strategies to further improve these response rates are needed.

One exciting approach undergoing clinical investigation is the combination of BRAFi with immunotherapy to generate a sustained antitumor immune response. The rationale for this therapeutic strategy is that targeting oncogenic BRAF may make melanoma more immunogenic (15), and the eventual progression of tumors during BRAFi therapy is due to the subsequent failure of antitumor immunity (13). It is known that treatment with BRAFi results in significantly higher expression of melanoma antigens (15, 16) and decreased expression of immunosuppressive cytokines and VEGF (16–18), all of which contribute to a tumor microenvironment that can promote antitumor immunity. Importantly, BRAFi elicits a dense CD8+ T-cell infiltrate in tumors of treated melanoma patients within 10 to 14 days of the initiation of therapy (16, 19, 20), with increased clonality of the infiltrating T cells (21). However, a significant increase in PDL1 expression is noted within 2 weeks of treatment with a BRAFi, and the density of T-cell infiltrate in progressing lesions returns to pretreatment levels (16). Thus, PD1 pathway blockade has the potential to overcome BRAFi resistance and act synergistically with antitumor responses induced by BRAFi.

Several clinical trials combining BRAFi and checkpoint blockade are currently under way. Response and survival data are not mature; thus, it is too early to determine whether there is synergy, or whether there will be added toxicity. Preliminary data from clinical trials of BRAF-targeted therapy in combination with ipilimumab indicate that there is increased toxicity, as a significant number of patients in this trial experienced hepatotoxicity (22). These early results highlight the need for additional preclinical data to support agent selection and schedule of administration, as well as to provide mechanistic insights.

In this study, we tested the hypothesis that the addition of immune checkpoint blockade would enhance responses to BRAF-targeted therapy. First, we analyzed serial tumor biopsy samples from a patient who was treated with combined BRAF-targeted therapy and immune checkpoint blockade with anti-CTLA4, and found increased infiltrating T cells and CD8:Treg ratio with time. Next, we built on these provocative (but anecdotal) data by testing the effects of combined BRAF-targeted therapy and immune checkpoint blockade in a novel immunocompetent and transplantable murine melanoma model that is responsive to BRAFi. We explored the effects of combined BRAFi and PD1 or PDL1 blockade based on our previous published (24) studies and procedures involving animal subjects were approved by the Institutional Animal Care and Use Committees of the Massachusetts General Hospital/Dana-Farber Cancer Institute (MGH/DFCI).

To generate a tumor cell line, induced tumors were harvested and digested overnight in 10 mg/mL of collagenase and 1 mg/mL of hyaluronidase. Tumor cells were initially grown in RPMI-1640 media with HEPES and 20% serum, and subsequently a cell line (BP) was established and grown in DMEM with 10% serum.

**Quantitative PCR analysis of melanoma antigens in the BP cell line**

The BP cell line was treated with 2 μmol/L of BRAFV600E inhibitor PLX4720, and RNA was extracted using the RNeasy Kit (Qiagen). 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 7500 Fast Real-Time PCR System. Primer sequences used are as followed: microphthalmia-associated transcription factor (MITF) forward, GCCGTGAAACCTTGCTATCGTGGA; MITF reverse, AAGGTACGTCTTTACCTGGTGCT; dopachrome tautomerase (DCT) forward, AGGTACACATCGTTGCGCTGAA; DCT reverse, AGTTCGACTAATCAGCGTTGGGT; tyrosinase (TYR) forward, TGTTCTTCTACCCGGCTC; TYR reverse,
CAGATACGACTGGCTTTGCTCC; melanoma antigen recognized by T cells (MLANA) forward, TCGGCTGCTGTACTG- 
TAGA; MLANA reverse. GGTGATCAGGGCTTCTCACAT; 18S 
forward. AGGTTGCTGCCAAGGCTTACAG; 18S reverse, 
CCTCTTATGGGCAATTTT. Cc values were normalized to 
untreated samples relative to 18S expression using the ΔΔCc 
method.

Flow cytometric analysis of BP cell line
The BP cell line was treated with 20 ng/mL of IFNγ in DMEM 
with 10% FBS for 24 hours. Cells were trypsinized to generate a 
single-cell suspension and stained with either anti-PDL1 
(10F.9G2; BioLegend) or MHC-I (AF6-88.5; BD Biosciences). 
Samples were analyzed on an LSR II (BD Biosciences), and data 
were analyzed with the FlowJo software (TreeStar).

In vivo studies using the BP tumor cell line
BP cells (8 × 10⁶) were implanted subcutaneously in 
C57BL/6 mice on the left flank. When tumors reached 
approximately 100 mm³, mice were given ad libitum BRAFi 
(PLX4720) chow containing 200 or 417 parts per million 
(ppm) of PLX4720 or control chow acquired from Plexxikon 
Inc. For CD8 depletion, 200 ppm of rat anti-mouse CD8α (clone 
2.43) or Rat IgG2a kappa isotype (LTE-2) antibody was 
administered intraperitoneally 1 day before tumor implantation 
and every 3 to 4 days thereafter until time of sacrifice. 
For PD1 or PDL1 blockade experiments, 1 day before tumor implantation 
and every 3 to 4 days thereafter until time of sacrifice. 
For PD1 or PDL1 blockade experiments, 100 μg of rat anti-mouse 
PD1 (29F.1A12), 200 μg of anti-PDL1 (10F.9G2; ref. 25), 
or isotype antibody (LTF-2) were administered 
intraperitoneally on days 1, 3, and 5 following BRAFi or control chow 
itiation. Tumor volume was calculated as L × W²/2, 
length (L) being the longer of the two measurements.

Immunohistochemistry analyses of murine or patient 
tumor sections
FFPE tumor specimens were deparaffinized in xylene and 
hydrated in a series of ethanol dilutions. Elution retrieval was 
done by microwaving (5 minutes at 850 W, 15 minutes at 150 
W) in 10 mmol/L Tris–EDTA buffer, pH 9.0. Slides were blocked 
for 10 minutes in 3% BSA in TBST (Tris pH 7.6, 0.05% Tween- 
20), and rabbit polyclonal anti-CD3 antibody (Abcam; ab6690; 
1:100) or CD8 (Leica; PA0183; RTU) in 3% BSA in TBST 
was applied for 1 hour at room temperature. Horseradish 
peroxidase (HRP)–labeled anti-rabbit (Dako; EnVision, 
K4003, RTU) or anti-mouse (BioRad; 170-6516, 1:200) 
secondary antibody was applied for 30 minutes. Slides were developed 
with DAB⁺ (Dako K3468) for 10 minutes and counterstained 
for 1 minute with hematoxylin. CD3⁺ cell counts in four 
adjacent randomly selected intratumoral ×40 fields were 
performed in a blinded fashion by a dermatopathologist.

Immunofluorescence analyses in murine tumor sections
Confocal microscopy was performed as described previously 
(26). Briefly, tissues were embedded in optimal-cutting-tem- 
perature medium (Tissue-Tek), and 12-μm sections were cut 
using a cryostat. Sections were fixed and stained using the 
FoxP3 buffer set (eBiosciences) and directly conjugated anti-
bodies. Sections were imaged on an Olympus confocal micro-
scope with a ×20 objective. For micrograph panels, single z 
slices were linearly contrasted and merged images were made 
in Adobe Photoshop. The following directly conjugated anti-
bodies were used: anti-CD8α (53-6.7; BioLegend), anti-FoxP3 
(FJK-16s; eBioscience), and anti-CD4 (RM4-5; BioLegend).

Flow cytometric analyses of tumor-infiltrating 
lymphocytes
A tumor-infiltrating lymphocyte (TIL)–enrichment proto-
col was used for both human and mouse tumors. Tumors were weighed dry, cut, and placed in collagenase type I (400 
U/mL; Worthington Biochemical) and incubated on a shaker at 
37°C for 30 minutes. The dissociated tumor was then filtered 
(70 μm) to generate a single-cell suspension. A surope gradient (40%/70% Percoll; GE Healthcare) was used to 
enrich TILs from the single-cell suspension. The cells were then 
either stimulated with phorbol 12-myristate 13-acetate 
(PMA; 50 ng/mL) and ionomycin (500 ng/mL) with Golgi-
Stop (BD Biosciences) or immediately stained and subse- 
quently analyzed by flow cytometry. The FoxP3 buffer kit 
eBioscience) was used for all stains. Unstimulated human 
TILs were stained with the following cocktail of directly 
labeled antibodies: anti-CD3 (FITC3a), anti-CD4 (OKT4), anti- 
CD8α (HTF8a; all from BioLegend), and anti-FoxP3 (PCH101, 
eBioscience). Unfractionated single-cell suspensions from 
mice were stained with the following cocktail of directly 
labeled antibodies: anti-CD31 (MEC13.3) anti-CD45 (30/ 
F11), anti-Lyve-1 (ALY7), anti-CD105 (MJ7/8), anti-EpCAM 
(G8.8), and anti-PDL1 (10F.9G2). Unstimulated mouse TILs 
were stained with the following directly labeled antibodies 
(adata from BioLegend): anti-CD45.2 (104), anti-CD3e (145- 
c211), anti-CD4 (RM4-5), anti-CD8α (53-6.7), anti-CD11b 
(M1/70), anti-Ki67 (16A8), anti-PDL1 (10F.9G2), anti-FoxP3 
(MF-14), and anti-granzyme B (GB11). Stimulated mouse 
TILs were stained with the following cocktail of directly 
labeled antibodies (all from BioLegend): anti-CD3e (145- 
c211), anti-CD8α (53-6.7), anti-CD11b (M1/70), anti-Ki67 
(16A8), anti-PDL1 (10F.9G2), anti-FoxP3 (MF-14), and anti-granzyme B (GB11). Stimulated mouse 
TILs were stained with the following cocktail of directly 
labeled antibodies (all from BioLegend): anti-CD3ε (145- 
c211), anti-CD8α (53-6.7), anti-CD11b (M1/70), anti-Ki67 
(16A8), anti-PDL1 (10F.9G2), anti-FoxP3 (MF-14), and anti-granzyme B (GB11). Stimulated mouse 
TILs were stained with the following cocktail of directly 
labeled antibodies (all from BioLegend): anti-CD3ε (145- 
c211), anti-CD8α (53-6.7), anti-CD11b (M1/70), anti-Ki67 
(16A8), anti-PDL1 (10F.9G2), anti-FoxP3 (MF-14), and anti-granzyme B (GB11). Stimulated mouse 
TILs were stained with the following cocktail of directly 
labeled antibodies (all from BioLegend): anti-CD3ε (145- 
c211), anti-CD8α (53-6.7), anti-CD11b (M1/70), anti-Ki67 
(16A8), anti-PDL1 (10F.9G2), anti-FoxP3 (MF-14), and anti-granzyme B (GB11). Stimulated mouse 
TILs were stained with the following cocktail of directly 
labeled antibodies (all from BioLegend): anti-CD3ε (145- 
c211), anti-CD8α (53-6.7), anti-CD11b (M1/70), anti-Ki67 
(16A8), anti-PDL1 (10F.9G2), anti-FoxP3 (MF-14), and anti-granzyme B (GB11). Stimulated mouse 
TILs were stained with the following cocktail of directly 
labeled antibodies (all from BioLegend): anti-CD3ε (145- 
c211), anti-CD8α (53-6.7), anti-CD11b (M1/70), anti-Ki67 
(16A8), anti-PDL1 (10F.9G2), anti-FoxP3 (MF-14), and anti-granzyme B (GB11).

Statistical analysis
Statistical evaluations were conducted using a two-tailed 
Student test. Kaplan–Meier analysis was conducted using the 
log-rank (Mantel–Cox) test. Statistical analyses were per- 
formed using GraphPad Prism or the R-statistical package. 
P values less than 0.05 were considered statistically significant.

Results
Anti-CTLA4 mAb synergizes with BRAF inhibition to 
increase the CD8⁺:FoxP3⁺ Treg ratio within the tumor of 
a patient with metastatic melanoma
Given prior clinical findings showing that BRAFi is associ- 
ated with improved melanoma antigen expression and an 
enhanced immune response in patients with metastatic me- 
lanoma (16, 19), we sought to study the effect of combined 
BRAF-targeted therapy and immunotherapy for the treatment 
Published OnlineFirst April 29, 2014; DOI: 10.1158/2326-6066.CIR-13-0215
Downloaded from cancerimmunolres.aacrjournals.org on November 2, 2017. © 2014 American Association for Cancer Research.
of metastatic melanoma. We studied a patient with metastatic melanoma who was participating in a clinical trial of BRAFi therapy with CTLA4 blockade. This patient had 4 weeks of BRAFi therapy before receiving four courses of anti-CTLA4 (Fig. 1A). Serial tumor biopsies were stained for CD8 by IHC (Fig. 1B). We found very few infiltrating CD8\(^+\) T cells pretreatment and a dense infiltrate on BRAFi therapy [as expected on the basis of our prior findings (16)]. However, within 4 weeks of BRAFi therapy, the infiltrating T cells were virtually absent. One month after the patient received one dose of anti-CTLA4, the T-cell infiltrate was again increased significantly and this infiltrate persisted >70 days on further anti-CTLA4 treatment. We analyzed the TILs in tumor biopsies using flow cytometry at the same time points to assess the ratio of CD8\(^+\) T cells to CD4\(^+\)FoxP3\(^+\) regulatory (Treg) cells (CD8:Treg ratio) in the tumor (Fig. 1C and D). These studies demonstrated an early and transient increase in the CD8:Treg ratio after the initiation of BRAFi therapy (present at 8 days but absent at 35 days after the initiation of BRAFi), mirroring our IHC findings for CD8. However, the CD8:Treg ratio increased dramatically following the administration of anti-CTLA4 (day 61) and persisted for >70 days. These results suggest that the immune infiltrate in the setting of BRAFi is early and transient, and that immune checkpoint blockade may synergize with BRAF inhibition to heighten the immune response against melanoma.
An immunocompetent and transplantable murine melanoma model demonstrates a dose-dependent response to BRAFi

We developed a transplantable murine melanoma model in C57BL/6 mice using the previously described Tyr:CreER; Braf<sup>CA</sup>;Pten<sup>lox/lox</sup> mouse model (23). Melanomas in these mice form with variable latency after tamoxifen administration, making it difficult to use this model to sensitively study antitumor immunity. To circumvent this problem, we generated a stable cell line (BP), which was responsive to in vitro treatment with BRAFi (Supplementary Fig. S1). Importantly, treatment of the BP line with BRAFi resulted in increased expression of known melanoma antigens, including dopachrome tautomerase (TYRP2, <i>DCT</i>), TYR, MLANA, and MITF (Fig. 2A), similar to our findings in patients treated with BRAFi (16). BP tumor cells expressed the coinhibitory molecule PD-L1, and culture with IFNγ for 24 hours led to increased PD-L1 expression. IFNγ also greatly increased MHC-I expression (Fig. 2B). Together, these data indicate that BP cells are likely to be recognized by CD8<sup>+</sup> T cells, while also having the potential to evade antitumor immune responses through the PD1/PDL1 axis.

To study the in vivo response of BP tumors to BRAFi, we implanted 8 × 10<sup>5</sup> BP cells subcutaneously into mice and allowed the tumors to grow to approximately 100 mm<sup>3</sup> (Supplementary Fig. S2) before administering mouse chow containing either 200 or 417 ppm of a BRAFi. BRAFi led to significantly slower tumor growth and increased survival compared with that of control mice (Fig. 2C and D) in a dose-dependent manner. Together, these data demonstrate that the BP-implanted tumor model responds to BRAF inhibition similarly to human melanoma.

BRAFi leads to increased intratumoral CD8<sup>+</sup> T-cell density and enhanced cytokine production

On the basis of our observations that BRAFi treatment in patients with melanoma resulted in increased intratumoral CD8<sup>+</sup> T cells (16, 19, 20), we assessed the effect of BRAFi on T cells in murine BP tumors. We observed a significant dose-dependent increase in CD3<sup>+</sup> T cells 7 days after BRAFi treatment (Fig. 3A and Supplementary Fig. S3) composed predominantly of CD8<sup>+</sup> T cells and some CD4<sup>+</sup> Tregs (Fig. 3B). However, we did not find an increase in the expression of the nuclear proliferation marker Ki-67 in CD8<sup>+</sup> TILs in mice on BRAFi treatment (Supplementary Fig. S4A).

We also evaluated effector function (cytokine production and cytolytic potential) of TILs in mice on BRAFi treatment. For cytokine analyses, we purified T cells from BP tumors and draining lymph nodes of mice 7 days after initiation of BRAFi treatment and stimulated these cells ex vivo with PMA/ionomycin. Many ex vivo stimulated CD8<sup>+</sup> T cells from the draining lymph nodes produced TNFα, but very few produced IFNγ (data not shown). Many intratumoral CD8<sup>+</sup> T cells expressed IFNγ and, to a lesser extent, TNFα (Fig. 3C). Importantly, more of the intratumoral CD8<sup>+</sup> T cells from the BRAFi-treated tumors produced both IFNγ and TNFα compared with controls (Fig. 3C). In addition, the mean fluorescence intensity

---

Figure 2. BRAF inhibition results in a dose-dependent increase in survival and a slowing of tumor growth in a syngeneic implantable tumor model. A, comparison of expression of <i>DCT</i>, TYR, MLANA, and MITF melanoma antigen mRNA (relative to 18S RNA) in the BRAF<sup>V600E</sup>/Pten<sup>−/−</sup>, or BP cell line that was cultured with 2 μmol/L of BRAFi for 0, 48, or 72 hours. B, comparison of surface expression of PD-L1 and MHC class I (MHC-I) on BP cells cultured with (red) or without (blue) IFNγ for 24 hours. Gray, unstained cells. C, 8 × 10<sup>5</sup> BP cells were implanted subcutaneously in C57BL/6. When tumors reached approximately 100 mm<sup>3</sup>, BRAFi was administered at 200 or 417 ppm (day 0). Survival is shown in a Kaplan–Meier plot. Mice were sacrificed when tumors reached a maximum diameter of 2 cm or had ulceration (n ≥ 7). D, tumor growth curves for experiments as in C. Tumor volumes were measured every 3 to 4 days (n ≥ 10). *P < 0.05 comparing BRAFi-treated mice with control mice.
(MFI) of IFNγ (in the IFNγ+ CD8+ T cells) was significantly higher in BRAFi-treated than in the control-treated cells, indicating that more IFNγ was being made on a per-cell basis. To assess cytolytic potential, we measured granzyme B production by direct ex vivo analysis of intratumoral CD8+ TILs. We found no difference in granzyme B production between BRAFi and controls on day 3 of BRAFi treatment (Supplementary Fig. S4B). BRAFi treatment also led to an increase in PDL1 expression on multiple hematopoietic and nonhematopoietic cell types in the tumor microenvironment (Supplementary Fig. S5). Notably, PDL1 was expressed on the tumor microenvironment (Supplementary Fig. S6). Together, these data indicate that CD8+ T cells can infiltrate melanoma tumors in the setting of BRAFi, but the tumor microenvironment is still immunosuppressive.

CD8+ T cells are critical to the response to BRAF inhibition

Next, we tested whether the CD8+ T cells that infiltrate tumors after BRAFi treatment were critical for the therapeutic benefit of BRAFi. We administered anti-CD8 mAb every 3 to 4 days (starting 1 day before BP tumor cell implantation: Fig. 4A) and initiated low-dose (200 ppm) BRAFi when tumors reached 100 mm3. CD8+ T-cell depletion was maintained at days 3, 7, and 11 after BRAFi initiation in the tumor and draining lymph node as assessed by flow cytometry (Fig. 4B and data not shown). Depletion of CD8+ T cells abrogated the BRAFi-induced increase in survival, with most mice dying within 20 days after tumor implantation (Fig. 4C). Depletion of CD8+ T cells also prevented the BRAFi-mediated decrease in tumor growth (Fig. 4D). CD8+ T-cell depletion similarly impeded the effects of high-dose BRAFi (417 ppm) on survival and tumor growth (data not shown). However, CD8+ T-cell depletion did not significantly alter tumor growth in mice treated with control chow. Taken together, these data indicate that CD8+ T cells are critical for the efficacy of BRAFi therapy in this mouse model of melanoma.

Blockade of the PD1 pathway synergizes with BRAF inhibition to enhance survival and slow melanoma growth

We next tested the hypothesis that immune checkpoint blockade would synergize with BRAFi therapy to promote antitumor immunity by enhancing CD8+ T-cell responses. We focused on the PD1 pathway, given the recent clinical success of PD1 blockade, findings of increased PDL1 expression in tumors of patients on BRAFi, and our data showing that PDL1 expression is increased in the BP tumors after BRAFi treatment (Supplementary Fig. S5). Because tumors can be completely eradicated with high-dose BRAFi but not low-dose BRAFi (Fig. 2C and D), we used low-dose BRAFi to evaluate synergy between BRAFi and PD1 pathway blockade. We followed a similar strategy of tumor implantation and BRAFi administration as in previous experiments (Fig. 5A). We administered three doses of either anti-PD1 or anti-PDL1 (on days 1, 3, and 5) after BRAFi initiation (day 0). The anti-PD1 (29F.1A12) blocking antibody blocks the binding of PD1 to its two ligands, PDL1 and PDL2. The anti-PDL1 (10F.9G2) blocking antibody blocks interaction of PDL1 with both of its binding partners, PD1 and CD80 (B7-1; ref. 27).

Treatment with anti-PD1 alone had no effect on tumor growth or survival (Fig. 5B–C). This is likely due to the large size (~1 cm diameter) of the tumors before treatment with
Figure 4. CD8 T cells play a critical role in responses of TILs due to BRAFi inhibition. A, schema for CD8 T-cell depletion in BP tumors. BP cells (8 x 10⁶) were subcutaneously injected into C57BL/6 mice, and BRAFi administration (200 ppm) was initiated at day 0. Two hundred micrograms of rat anti-mouse CD8, or isotype antibody was administered intraperitoneally every 3 to 4 days thereafter. B, analysis of CD4⁺ and CD8⁺ T cells in the draining lymph node and tumor was done on days 3, 7, and 11 after the initiation of BRAFi for CD8 T-cell depletion by flow cytometry using a different anti-CD8 clone. Representative plots at day 3 are shown. C, effects of CD8 depletion on survival of mice given BP tumor with or without BRAFi therapy displayed in a Kaplan–Meier plot. *, P < 0.05 comparing BRAFi treated mice with control mice. D, tumor volumes from experiments as in C measured every 3 to 4 days. *, P < 0.05 comparing BRAFi + isotype–treated mice to BRAFi + anti-CD8 mAb–treated mice. Representative of three experiments.

Discussion

Two of the most significant advances in cancer treatment in decades have emerged almost concurrently: BRAFi and checkpoint blockade therapy. Support for potential synergy between BRAF-targeted therapy and immunotherapy comes from studies of serial biopsy samples of patients on targeted therapy as well as from murine studies (16, 18, 28, 29). Clinical trials combining BRAF-targeted therapy with immune checkpoint inhibitors are currently under way, although significant fundamental and translational questions remain about the potential mechanisms of synergy, toxicity, timing, and duration of therapy.

Here, we conducted studies to understand the potential synergy between immune checkpoint blockade and BRAF-targeted therapy. We first analyzed a unique set of tumor biopsy samples from a patient receiving combined BRAF-targeted therapy and immunotherapy with vemurafenib and both IFNy and TNFα in the mice treated with anti-PD1 and BRAFi, but not in mice given anti-PDL1 plus BRAFi. Together, these results suggest that the intratumoral CD8⁺ T-cell response is synergistically enhanced by the combination therapy using BRAFi with either anti-PD1 or anti-PDL1.

PD1 pathway blockade and BRAF inhibition synergize to enhance the number and function of tumor-infiltrating T cells

We next analyzed whether there were synergistic effects of combination therapy on TIL number and function. Combining BRAFi with either anti-PD1 or anti-PDL1 led to a 7.5-fold increase in CD3⁺ T cells compared with any monotherapy (Fig. 6A and Supplementary Fig. S7). We found little to no increase in CD3⁺ T cells in tumors treated with either anti-PD1 or anti-PDL1 treatment alone. Anti-PD1 and BRAFi combination therapy led to a substantial increase in CD8⁺ T cells in the tumor compared with BRAFi alone (Fig. 6B) with no difference in CD4⁺ FoxP3⁺ T cells or CD4⁺ FoxP3⁺ Tregs in the tumor (Fig. 6B). The CD8:Treg ratio was also increased in the combination therapy groups (Fig. 6C). We also observed an increased fraction of CD8⁺ T cells that were producing granzyme B, as well as more polyfunctional CD8⁺ T cells producing IFNy and TNFα in the mice treated with anti-PD1 and BRAFi, but not in mice given anti-PDL1 plus BRAFi. Together, these results suggest that the intratumoral CD8⁺ T-cell response is synergistically enhanced by the combination therapy using BRAFi with either anti-PD1 or anti-PDL1.
ipilimumab, respectively. We found that the immune response to BRAF inhibition (as assayed by T-cell infiltrate and the CD8:Treg ratio) was early and transient, but that T-cell infiltrate and an improved CD8:Treg ratio could be sustained by the addition of immune checkpoint blockade, and persist for several weeks to months. Of note, the human sample set was limited to 1 patient because the trial was stopped early due to toxicity (22). Although this is anecdotal evidence and further study is needed in the context of clinical trials and murine models, these data suggest that the addition of immunotherapy to a backbone of targeted therapy should occur early, ideally within 2 to 4 weeks.

We also developed and used a novel subcutaneous implantable tumor model generated from a well-established murine model of BRAF-mutant melanoma (23). In this model, we observed a dose-dependent improvement in survival after treatment with a BRAFi, which was associated with an increase in immune infiltrate and TIL function, similar to what we observe in patients treated with BRAFi (16). The mechanism behind the increase in intratumoral T cells may reflect trafficking of T cells into the tumor (16) as well as expansion of TIL (21), which are not mutually exclusive. Data from our mouse model would suggest that proliferation of existing clones is not the dominant mechanism at an early time point, as we observed no increase in the proportion of Ki-67+ CD8+ TIL in BRAFi-treated mice at the two time points investigated. Of note, we previously studied this in patients and demonstrated that both mechanisms are likely at play (16, 21). In our murine model, the effects of BRAFi were attenuated with CD8+ T-cell depletion, suggesting a critical role for CD8+ T cells in the response to BRAF inhibition. Furthermore, CD8+ T-cell depletion did not affect tumor growth in mice treated with control
Figure 6. PD1 pathway blockade and BRAF inhibition synergize to enhance number and function of tumor-infiltrating T cells. 

A, CD3+ T cells were assayed via IHC in tumors of mice given BRAFi plus anti-PD1, anti-PDL1, or control mAb. n > 3; P < 0.05. B, CD4+, CD8+, and FoxP3+ expression was assayed via immunofluorescence within tumors of mice given BRAFi plus anti-PD1 or isotype control mAb. Representative sections are shown (×200 magnification). 

C, immune infiltrates in tumors harvested 14 days after BRAFi initiation were characterized by flow cytometry. The ratios of CD8+ T cells to Tregs (CD4+ FoxP3+) are shown, as are percentage of CD8+ T cells that are positive for granzyme B or for IFNγ and TNFα. Representative flow plots for IFNγ and TNFα expression in CD8+ T cells are shown. *, P < 0.05.
chow, suggesting that the differences in minor histocompatibility antigens are not a major factor in the antitumor immunity in these mice.

In addition to an enhanced T-cell infiltrate, we found that BRAFi treatment led to the upregulation of PDL1 and an increase in IFNγ-expressing TILs. This is consistent with our published findings from tumor biopsies of patients with melanoma on BRAFi therapy, demonstrating an increase in PDL1 expression within 2 weeks of the initiation of therapy (16). Recent literature suggests that this finding is related to IFNγ production by TIL (30) or stromal fibroblasts (31). Studies in humans have also shown that PDL1 expression colocalizes with infiltrating T cells (32). In our murine model, PDL1 is expressed on a wide variety of cells in the tumor microenvironment, including CTLs, myeloid cells, epithelial cells, endothelial cells, lymphatic endothelial cells, and tumor cells in the setting of BRAFi. Together, these data, coupled with a better toxicity profile of PD1 pathway blockade in patients (5–7), gave impetus to our study of the effects of combined BRAF-targeted therapy with PD1 or PDL1 blockade in the BP tumor model.

We studied the combined regimen of BRAF-targeted therapy and anti-PD1 or anti-PDL1 blockade in our murine model, and we observed enhanced survival and delayed tumor outgrowth compared with BRAFi or PD1 pathway blockade alone. Delayed tumor growth was accompanied by an increase in CD8+ T-cell density and function in these groups. Specifically, CD8+ TILs expressed more IFNγ, TNFα, and granzyme B in mice given combined BRAFi and PD1 blockade. This is in contrast with findings in mice receiving BRAFi monotherapy demonstrating increased expression of IFNγ and TNFα in TILs, but not granzyme B. These data suggest that BRAF inhibition alone results in an increase in T-cell infiltration and cytokine production, but these T cells are not completely functional. Thus, BRAFi and PD1 pathway blockade synergize to increase CD8+ TIL cell density and function, indicating that the PD1 pathway plays a significant role in immune modulation in our BP tumor model.

Of note, we found a similar increase in TIL after combined BRAFi plus anti-PDL1 administration but did not observe increased T-cell functionality based on cytokines or granzyme B. There are several possible reasons for this difference. First, there may be differences in the kinetics of the T-cell response to anti-PD1 versus anti-PDL1 after BRAFi treatment. In addition, the expression of PDL2 as well as PDL1 in the tumor microenvironment may explain these differences. mAbs targeting PD1 and PDL1 may have different functional effects as they block distinct receptor–ligand interactions. PD1 binds to PDL1 and PDL2, whereas PDL1 binds to PD1 and B7-1 (CD80; refs. 27, 33). Anti-PD1 blocks PD1 interaction with PDL1 or PDL2, but does not perturb the PDL1/B7-1 pathway. The anti-PD1 used here is a dual blocker, blocking PDL1 interactions with PD1 and B7-1, but leaving the PD1/PDL2 interactions intact. Thus, the expression of PDL2 in our mouse melanoma model may underlie the diverse effects when BRAFi is combined with anti-PD1 versus anti-PD1. The differential effects of anti-PDL1 and anti-PD1 antibodies as well as differences in PDL1 and PDL2 expression have clinical implications and may explain the subtle variations in the clinical effects observed when blocking either PD1 or PDL1 alone (6, 7).

Further work is needed to understand how anti-PD1 and anti-PDL1 exert their antitumor effects. PD1 is upregulated upon activation of T cells in peripheral lymphoid organs, on activated T cells in the tumor, and on dysfunctional (termed "exhausted") T cells in the tumor microenvironment (34, 35). Anti-PD1 likely affects the activation and function of T cells both in the tumor and in secondary lymphoid organs and rescues exhausted T cells in the tumor (7, 36, 37). Furthermore, PD1 blockade also may have functional consequences on other PD1-expressing cell types (26). Studies of the effects of PD1 blockade on the function of PD1+ NK cells and macrophages in the tumor are ongoing (38, 39). Similarly, PDL1 is expressed by multiple cell types, both in and outside of the induction of Tregs (40). Within the tumor, PD1L can be expressed by both tumor and nontumor cells, both of which have the potential to suppress antitumor immunity (41, 42). Our studies in the lymphocytic choriomeningitis chronic infection model indicate that PDL1 on hematopoietic and nonhematopoietic cells can both contribute to T-cell exhaustion (43). Clinical data indicate that PD1 pathway blockade can be successful even when tumors do not express PDL1, suggesting that PDL1 expression on tumor cells is not required for response (44).

It is important to note that we initiated treatment with BRAFi when tumors were large (~100 mm3 in volume) and we then administered PD1 pathway–blocking antibodies only after BRAFi initiation. This approach is clinically relevant to patients with a high metastatic burden, as response rates to immune checkpoint blockade are not universal and may be delayed. Although we did not observe a significant difference in survival or growth after anti-PD1 or anti-PDL1 monotherapy when administered at this late time point, we found a significant difference in survival when PD1 or PDL1 blockade monotherapy was given to mice with smaller tumors (data not shown). Thus, this subcutaneous melanoma model could be used to model and study multiple clinically relevant scenarios.

Our results are consistent with those from several published studies that demonstrate synergy of BRAF-directed therapy with other immunotherapies in murine models (18, 28, 29). Studies using adoptive T-cell transfer, anti-CD2L2, or anti-CD137 (18, 28, 29) have demonstrated synergy between BRAF-targeted therapy and immunotherapy with enhanced survival and T-cell function. Conversely, one study showed no synergy when BRAF-targeted therapy was combined with immune checkpoint blockade (45). This study used a similar BRAF/Pten model with induced primary tumors as opposed to subcutaneous tumors. BRAFi treatment of induced primary tumors resulted in a decrease in immune infiltrate, which is not consistent with what we observed in metastatic melanoma tumors from patients treated with BRAFi (16, 19). In this primary tumor model, the addition of CTLA4 blockade, either with or without BRAFi, did not improve survival or delay tumor growth (45). Further studies are needed to determine whether...
this lack of synergy reflects the different biology in the primary tumor model.

In conclusion, these studies provide evidence that immune checkpoint blockade using anti-PD1 or anti-PDL1 synergizes with BRAF-targeted therapy to improve responses in a BRAF-mutant model of melanoma. This novel mouse melanoma model provides a new means to address important translational questions about combining BRAFi and immunotherapy and to complement correlative studies on clinical trials combining immune checkpoint inhibitors with BRAFI.

Disclosure of Potential Conflicts of Interest

F.S. Hodi reports receiving research support from Bristol-Myers Squibb and Genentech and is a consultant/advisory board member for Bristol-Myers Squibb, Genentech, and Merck. G.J. Freeman has an ownership interest (including patents) with Bristol-Myers Squibb, Merck, Roche, EMD Serono, Boehringer-Ingelheim, Amplimmune, and CoStim Pharmaceuticals, and is a consultant/advisory board member for CoStim Pharmaceuticals. M. McMahon reports receiving research support from Novartis, GlaxoSmithKline, and Plexaxons Inc and is a consultant/advisory board member for AbbVie. A.H. Sharpe is employed as a SAB/cosounder of CoStim Pharmaceuticals, has intellectual property in Bristol-Myers Squibb, EMD Serono, Amplimmune, Merck, MedImmune, Genentech, Roche, and Boehringer-Ingelheim, and is a consultant/advisory board member for CoStim Pharmaceuticals. J.A. Wargo has received speakers bureau honoraria from Dava Oncology. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions


References


Response to BRAF Inhibition in Melanoma Is Enhanced When Combined with Immune Checkpoint Blockade


<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: doi:10.1158/2326-6066.CIR-13-0215</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Material</td>
<td>Access the most recent supplemental material at: <a href="http://cancerimmunolres.aacrjournals.org/content/suppl/2016/09/23/2326-6066.CIR-13-0215.DC1">http://cancerimmunolres.aacrjournals.org/content/suppl/2016/09/23/2326-6066.CIR-13-0215.DC1</a></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cited articles</th>
<th>This article cites 42 articles, 18 of which you can access for free at: <a href="http://cancerimmunolres.aacrjournals.org/content/2/7/643.full#ref-list-1">http://cancerimmunolres.aacrjournals.org/content/2/7/643.full#ref-list-1</a></th>
</tr>
</thead>
<tbody>
<tr>
<td>Citing articles</td>
<td>This article has been cited by 21 HighWire-hosted articles. Access the articles at: <a href="http://cancerimmunolres.aacrjournals.org/content/2/7/643.full#related-urls">http://cancerimmunolres.aacrjournals.org/content/2/7/643.full#related-urls</a></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>E-mail alerts</th>
<th>Sign up to receive free email-alerts related to this article or journal.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reprints and Subscriptions</td>
<td>To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at <a href="mailto:pubs@aacr.org">pubs@aacr.org</a>.</td>
</tr>
<tr>
<td>Permissions</td>
<td>To request permission to re-use all or part of this article, contact the AACR Publications Department at <a href="mailto:permissions@aacr.org">permissions@aacr.org</a>.</td>
</tr>
</tbody>
</table>