BRAF Inhibition Increases Tumor Infiltration by T cells and Enhances the Anti-tumor Activity of Adoptive Immunotherapy in Mice

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Translational Relevance

BRAF-targeted therapy has resulted in objective responses in the majority of melanoma patients harboring the BRAF(V600E) mutation, however the median duration of response is less than a year. There is evidence for immune evasion in BRAF-mutant melanoma which may be reversed with BRAF–targeted therapy, strongly implicating the rationale for a BRAF-targeted therapy in combination with immunotherapy. Adoptive T-cell transfer (ACT) therapy using tumor-infiltrating lymphocytes is one of the most promising immunotherapeutic approaches for melanoma treatment resulting in objective responses for over 50% of treated patients. Here, we report that BRAF inhibition in melanoma increases the T-cell infiltration into tumors, via decreased VEGF production, and enhances the anti-tumor activity of ACT therapy. Our findings provide a rationale of combining BRAF inhibitor with ACT therapy for clinical application to improve durable response rates to therapy.
Abstract

**Purpose:** Treatment of melanoma patients with selective BRAF inhibitors results in objective clinical responses in the majority of patients with BRAF mutant tumors. However, resistance to these inhibitors develops within a few months. In this study, we test the hypothesis that BRAF inhibition in combination with adoptive T-cell transfer (ACT) will be more effective at inducing long-term clinical regressions of BRAF-mutant tumors.

**Experimental Design:** BRAF-mutated human melanoma tumor cell lines transduced to express gp100 and H-2D^b^ to allow recognition by gp100-specific pmel-1 T-cells were used as xenograft models to assess melanocyte differentiation antigen-independent enhancement of immune responses by BRAF inhibitor PLX4720. Luciferase expressing pmel-1 T cells were generated to monitor T-cell migration *in vivo*. The expression of vascular endothelial growth factor (VEGF) was determined by enzyme-linked immunosorbent assay, protein array and immunohistochemistry. Importantly, VEGF expression after BRAF inhibition was tested in a set of patient samples.

**Results:** We found that administration of PLX4720 significantly increased tumor infiltration of adoptively transferred T cells *in vivo* and enhanced the antitumor activity of ACT. This increased T-cell infiltration was primarily mediated by the ability of PLX4720 to inhibit melanoma tumor cell production of VEGF by reducing the binding of c-myc to the VEGF promoter. Furthermore, analysis of human melanoma patient tumor biopsies before and during BRAF inhibitor treatment showed downregulation of VEGF consistent with the pre-clinical murine model.
Conclusion: These findings provide a strong rationale to evaluate the potential clinical application of combining BRAF inhibition with T-cell based immunotherapy for the treatment of melanoma patients.
Introduction

The identification of activating point mutations of the BRAF gene, present in approximately half of all human cutaneous melanomas, has proven to be a milestone for contributing not only to our understanding of melanoma biology but for changing the treatment and clinical outcomes of the disease (1). As a component of the RAS-RAF-MEK-MAPK signal transduction pathway, BRAF is also mutated to a constitutively activated form in many other cancers, including thyroid, colorectal, and hairy cell leukemia (1-6). Although over 50 distinct mutations in BRAF have been described to date, a valine to glutamic acid substitution at amino acid position 600(V600E), is by far the most frequent, comprising more than 70% of BRAF mutations in melanoma (1, 7). Thus, BRAF(V600E) being so widely expressed, has provided a strong rationale for the development and clinical application of small molecule-based pharmaceutical inhibitors that selectively target BRAF(V600E) to treat patients with metastatic melanoma, whose treatment options are limited (8-11).

Recent clinical trials have demonstrated that over half of melanoma patients with BRAF(V600E)-expressing tumors experience objective clinical responses to selective inhibitors of BRAF. However, complete and durable remissions were rarely observed in these patients, and disease relapses accompanied by BRAF inhibitor resistance typically occurred within a year (12, 13). The mechanisms that cause resistance are diverse, and include MAPK pathway re-activation by alternate means (14-19). Hence, to improve long term clinical responses and avoid selection of drug-resistant tumors, combinational therapies that target multiple pathways have been proposed (3, 4, 20).

Although therapeutic approaches that combine small molecule-based inhibition of
multiple signal transduction pathways has been an area of ongoing investigation, one alternative involves the combination of BRAF inhibitors with immune-based therapies. This approach appears particularly promising due to the emerging link between MAPK pathway activation in cancer and the suppression of anti-tumor immunity. For example, knockdown of BRAF(V600E) in melanoma cell lines has been shown to decrease the production of immunosuppressive soluble factors such as IL-10, vascular endothelial growth factor (VEGF) and IL-6 (21). Recent in vitro experiments showed that blocking of MAPK signaling in melanoma cells could increase the expression of melanocyte differentiation antigens (MDA), leading to improved recognition by MDA-specific T cells (22, 23). In addition, a study by Jiang et al showed that the paradoxical activation of MAPK promoted programmed death ligand 1 (PD-L1) expression in melanoma cells resistant to BRAF inhibition (24). Perhaps most importantly, the exquisite specificity of recently developed small molecule inhibitors that target mutated oncogenes have shown little or no detrimental effects on immune cells that also utilize the MAPK pathway (23, 25).

In the current pre-clinical study, we assessed whether the addition of a selective BRAF(V600E) inhibitor could improve the efficacy of T-cell based immunotherapy in vivo. We found that ACT with melanoma-specific T cells was much more effective in the context of concurrent BRAF inhibition, which led to increased T-cell infiltration of tumors that could be attributed largely to decreased VEGF production by the tumor cells. Furthermore, a subset of responding melanoma patients showed similar changes in the tumor microenvironment following BRAF inhibitor treatment, providing a strong rationale to explore the use of combination treatments involving MAPK pathway
inhibition and T cell-based immunotherapy.
Materials and Methods

**Animals and cell lines.** C57BL/6, C57BL/6J-Tyr-2J/J albino and pmel-1 TCR transgenic mice on a C57BL/6 background were purchased from the Jackson Laboratory. B6 nude mice were purchased from the Taconic Farms. All mice were maintained in a specific pathogen-free barrier facility at The University of Texas MD Anderson Cancer Center. Mice were handled in accordance with protocols approved by the Institutional Animal Care and Use Committee. A375 (BRAF V600E⁺), Mel624 (BRAF V600E⁺/HLA-A2⁺/MART-1⁺), WM35 (BRAF V600E⁺/HLA-A2⁺/gp100⁺/MART-1⁺), MEWO (BRAF Wild-Type/HLA-A2⁺/MART-1⁺) and C918 (BRAF Wild-Type) human melanoma cell lines and MC38 murine colon adenocarcinoma cell line were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), and penicillin-streptomycin (all from Invitrogen). MART-1-reactive DMF5 T cells were obtained from the National Cancer Institute (26) and cultured in RPMI1640 medium containing 10% heat-inactivated human AB serum (Valley Biomedical), β-mercaptoethanol (Invitrogen) and recombinant human interleukin (IL)-2 (TECIN, National Cancer Institute Biological Resources Branch).

**Patient samples** Patients with metastatic melanoma possessing BRAF V600E mutation were enrolled on clinical trials for treatment with a BRAF inhibitor (RO5185426) or combined BRAF + MEK inhibitor (GSK2118436 + GSK1123212) and were consented for tissue acquisition per IRB-approved protocol. Tumor biopsies were performed pre-treatment (day 0), at 10-14 days on treatment.

**Generation of Luciferase expressing pmel-1 T cells.** Splenocytes from pmel-1 mice were cultured in complete medium containing 300 I.U./ml IL-2, and 0.3 μg/ml anti-
mouse CD3 (BD Bioscience). After 24 h, the cells were infected with a retroviral vector encoding a modified firefly luciferase gene OFL and GFP, as previously described (27, 28). Three days after viral transduction, cells were sorted by a FACS Aria (BD Bioscience) based on expression of GFP.

**Bone marrow-derived dendritic cells (DC).** DCs were generated from murine bone marrow cells as previously described (29, 30). DCs pulsed with 10 μM H-2D<sup>b</sup>-restricted gp100 peptide (KVPRNQDWL) for 3 hrs at 37°C on day 7. After wash with PBS, DCs were immediately injected into mice.

**Lentiviral transduction of tumor cells.** Lentiviral vectors and packaging vectors, VSV-G and Δ8.9, were cotransfected into 293T cells using lipofectamine 2000, and supernatant was collected after 36 hrs culture. A total of 1 x 10<sup>6</sup> tumor cells were pre-seeded in each well of 6-well plates for 6 hrs and spun at 850 g for 1 hr with 1 ml virus supernatant and 8 μg/ml polybrene. The following day, the supernatant was removed and replaced with growth medium. Infected tumor cells were collected and sorted based on the expression of the reporter gene using a FACS Aria.

**Adoptive transfer, vaccination, and treatment.** B6 nude mice were subcutaneously implanted with 6 to 10 x 10<sup>6</sup> melanoma cells on day 0. When tumors were established, 1 x 10<sup>6</sup> luciferase-transduced pmel-1 T cells were adoptively transferred into tumor-bearing mice, followed by intravenous injection of 0.5 x 10<sup>6</sup> peptide-pulsed DCs. IL-2 (5 x 10<sup>5</sup> I.U./mouse) was intraperitoneally administered twice daily for 3 days after T cell transfer. 2 days after T cell transfer, PLX4720 (provided by Plexxikon) was administered for 3 days. PLX4720 powder was suspended in vehicle (3% DMSO, 1% methylcellulose) and administered by oral gavage daily (100mg/kg). In
some experiments, mice were fed by a chow diet containing 417mg/kg PLX4720. For anti-VEGF treatment, anti-hVEGF Ab (hybridoma, A4.6.1 from ATCC) was administered at 250μg/mouse on day 7, 9 and 11. Mouse IgG was used as control Ab. Since anti-tumor response of ACT is dependent on lymphodepletion(31), in some experiments using C57BL/6J-Tyr-2J/J albino mice as recipients, lymphopenia was induced by administering a nonmyeloablative dose (350 cGy) of radiation one day before adoptive transfer. Tumor sizes were monitored by measuring the perpendicular diameters of the tumors. All experiments were carried out in a blinded, randomized fashion.

**In vivo Bioluminescence Imaging (BLI).** Mice were i.p. injected with 100 μl of 20 mg/ml D-Luciferin (Xenogen). 8 mins later, mice anesthetized with isoflurane were imaged using an IVIS 200 system (Xenogen), according to the manufacturer’s manual. Living Image software (Xenogen) was used to analyze data. Regions of interest (ROI) were manually selected and quantification is reported as the average of photon flux within ROI. The bioluminescence signal is represented as photons/s/cm²/sr.

**IFN-γ secretion assay.** Melanoma cells were pre-treated with various concentrations of PLX4720 for 48 hrs, then were washed three times with culture medium. After counting, tumor cells were co-incubated with DMF5 or pmel-1 T cells at 5 X 10⁴ per well (1:1 ratio) as triplicates for 24 hrs, with or without adding back PLX4720. IFN-γ production was determined in culture supernatants using an ELISA kit (BioLegend).

**Cytotoxicity assay.** Melanoma cells were pre-treated with PLX4720 or vehicle (DMSO) for 48 hrs. After washing and counting, melanoma cells were labeled with ⁵¹Cr, and then co-incubated with activated pmel-1 T cells at different effector-to-target (E:T)
ratios. 4 hrs later, $^{51}$Cr release was determined against target cells. Specific $^{51}$Cr release was calculated using the standard formula: ([sample release - spontaneous release]/[total release - spontaneous release]) x 100%.

**hVEGF secretion assay.** Melanoma cells were treated with various concentrations of PLX4720 for 24 hrs, with DMSO added as a vehicle control. The supernatants were then harvested for ELISA assay (R&D System), and the cells were harvested and counted.

**Proliferation assay.** DMF5 cells (5x10$^4$/well) were cultured with IL-2 (300 I.U./ml) at various concentrations with PLX4720 in an OKT3 pre-coated (1μg/ml, 100μl/well, 4°C for overnight) 96-well plate for 56 hrs. $[^3]$HThymidine (5μCi/ml) was then added for a further 16 hrs, and $[^3]$HThymidine incorporation was quantified in a liquid scintillation counter.

**Cell viability assay.** Melanoma cells were seeded in flat-bottom 96-well plates and treated with various concentration of PLX4720 for 72 hrs, with DMSO added as a vehicle control. Cell viability was determined using CellTiter-Blue Cell Viability assay (Promega).

**Protein arrays.** A375 tumor-bearing mice were sacrificed 3 days after oral gavage of PLX4720, and tumors were resected and weighed. Tumors were homogenized and sonicated in lysis buffer containing protease inhibitors. Cleared tumor lysates after centrifugation were tested using the Searchlight protein array, according to the manufacturer's protocol (Aushon Biosystems).

**ChIP arrays.** A375 cells were treated with PLX4720 (1μM) or DMSO for 2 hours, followed by a chromatin immunoprecipitation (ChIP) assay, performed according
to the manufacturer’s instructions (Millipore). Briefly, the protein-DNA complexes were cross-linked and immunoprecipitated with anti-c-myc, anti-p300 and anti-E2F1 antibodies or rabbit control IgG. After reversing the cross-linking, real-time PCR was used to amplify sequences corresponding to the promoter regions of human VEGF or control gene GAPDH.

**Flow Cytometric Analysis.** Peripheral blood or tumors were harvested at the indicated time points. Tumor tissues were weighed and dissociated. After depletion of erythrocytes using ACK lysing buffer (Invitrogen), the remaining lymphocytes were treated with Fc blocking mAbs (anti-CD16/32 2.4G2) and then stained with mAbs against Thy1.1 and CD45 (BD Biosciences). Samples were analyzed using a FACSCalibur or FACSCanto II (BD Biosciences).

**Immunohistochemistry.** Immunohistochemical staining was performed using the avidin-biotin complex kit (Vector Laboratories). 9 melanoma patient tumor samples prior to and following treatment with BRAF inhibitor were stained for VEGF (1:100, Abcam) and mouse xenograft tumor samples were stained for CD3 (1:100, Abcam). Samples were appropriately optimized in our lab, and external controls were systemically used to avoid false-negative or false-positive staining. Percentages of VEGF-stained tumor cells were quantitated using microscopy and pathological examination. CD3+ T cell counts were performed on slides in 10 adjacent high-power fields (HPF, 400 ×) based on lymphocyte morphology.

**Quantitative PCR.** Differential expression of VEGF in patient samples was assayed using TaqMan Gene Expression Assays (Applied Biosystems) with actin as control. mRNA was reverse transcribed to cDNA using SuperScript VILO (Invitrogen)
**Statistical analysis.** Comparisons of differences in continuous variables between two groups were done using Student’s *t* test. Differences in tumor size and T cell numbers among different treatments were evaluated by analysis of variance (ANOVA) repeated-measures function. The statistical analysis to compare survival was determined using Kaplan-Meier test. *P*-values are based on 2-tailed tests, with *P* < 0.05 considered statistically significant.
Results

A human melanoma xenograft model to assess MDA-independent enhancement of immune responses by PLX4720. In order to investigate the effects of BRAF inhibition on tumor cell recognition by T-cells, three HLA-A2\(^+\) melanoma cell lines were pre-treated for 48h with titrated doses of the selective BRAF inhibitor PLX4720, and then co-cultured with HLA-A2-restricted MART-1 specific T cells. T-cell recognition, as measured by IFN-\(\gamma\) secretion, increased in a dose-dependent fashion in the two melanoma cell lines expressing BRAF V600E, but not in the cell line expressing WT BRAF (Figs. S1A, B, and C). These results imply that PLX4720 can enhance MART-1 specific T-cell recognition of melanoma cells, consistent with the findings of a previous study showing upregulation of MDA by PLX4720 (23). Furthermore, when PLX4720 was added to the culture system, neither T-cell cytokine secretion function nor TCR mAb-induced proliferation was inhibited at concentrations as high as 1\(\mu\)M (Figs. S1A–D). Thus, selective BRAF(V600E) inhibition does not significantly inhibit T-cell proliferation, similar to results we have previously reported that analyzed circulating immune cells from melanoma patients prior to and following treatment with a selective BRAF inhibitor (25).

Although upregulation of MDA is one mechanism by which BRAF inhibition can enhance immune recognition of melanoma, we hypothesized that this class of antigen represents only a small fraction of melanoma-reactive T-cells in the tumor microenvironment, and that BRAF inhibition could be having other more global influences on the immune response. In order to study these potential alternative
mechanisms, we engineered the human BRAF(V600E)-positive melanoma cell line A375 to constitutively express high levels of the gp100 melanoma tumor antigen under control of the CMV promoter, thus eliminating any influence of the BRAF inhibitor on the expression of this MDA. The A375 cell line was also engineered to express murine H-2D\(^{b}\) (Fig. 1A) to enable recognition by murine, gp100-specific transgenic pmel-1 T-cells.

To test the validity of this approach, a series of \textit{in vitro} experiments were performed to confirm that gp100 overexpression did, in fact abrogate enhanced T-cell reactivity induced by PLX4720. As shown in Fig. 1B, pmel-1 cells could only recognize and secrete IFN-\(\gamma\) in response to A375 cells transduced to express both H-2D\(^{b}\) and gp100 (A375/H-2D\(^{b}\)/gp100). By contrast, human WM35 melanoma cells that naturally express gp100 were recognized when transduced with only H-2D\(^{b}\). As a further control, the murine colorectal carcinoma cell line MC38 that naturally expresses H-2D\(^{b}\) could only be recognized by pmel-1 T-cells upon transduction with gp100. As shown in Figs. 1C and D, PLX4720 treatment could enhance pmel-1 T cell recognition of WM35 cells transduced with H-2D\(^{b}\), but not when these cells were also transduced to overexpress gp100. Similarly, PLX4720 treatment of A375 cells transduced to express both H-2D\(^{b}\) and gp100 did not induce enhanced pmel-1 T-cell recognition, as measured by IFN-\(\gamma\) secretion or cytolysis (Figs. 1E and S2). Therefore, this xenogeneic tumor model was determined to be appropriate for evaluating the effects on immune responses \textit{in vivo} by PLX4720 that were independent of its impact on MDA expression.

\textit{BRAF inhibition increases tumor infiltration of adoptively transferred T cells and enhances antitumor responses.} We next sought to investigate whether PLX4720 could enhance the efficacy of adoptive T-cell transfer (ACT) \textit{in vivo}. B6 nude mice were
subcutaneously implanted with A375/H-2D<sup>b</sup>/gp100 melanoma cells and then treated with a combination of OFL-expressing pmel-1 T cells and peptide-pulsed DCs as previously described (30) followed by PLX4720 administration. Tumor sizes and T-cell migration were monitored over time. As shown in Figs. 2A and B, administration of PLX4720 led to ~10-fold higher luciferase intensity at the tumor site, compared with vehicle control. The increased T cell infiltration was confirmed by immunohistochemical staining for CD3 and flow cytometry for Thy1.1+ pmel-1 T cells (Figs. S3 A, B, C and D). This higher level of tumor antigen-specific pmel-1 T-cell infiltration was associated with anti-tumor responses that were significantly better than those observed in mice treated with either PLX4720 or ACT alone (Fig. 2C). As shown in Fig. 2D, extended survival was also observed in the combination therapy group. The percentage of pmel-1 T cells in peripheral blood did not differ between the treatment groups (Fig. S4), suggesting that administration of PLX4720 can enhance the antitumor activity of ACT through increasing T-cell migration to tumor sites.

To understand if expression of tumor antigen is necessary for T cell accumulation in tumors treated with PLX4720, B6 nude mice were subcutaneously implanted with non gp100-expressing A375/H-2D<sup>b</sup> melanoma cells and then treated with ACT in combination with PLX470 or vehicle control. As shown in Figs. S5 A and B, PLX4720 treatment cannot significantly increase T cell infiltration in A375/H-2D<sup>b</sup> tumors. These results suggest that tumor antigen expression is important for PLX4720-induced T cell infiltration into tumors.

**PLX4720 increases infiltration of adoptively transferred T cells only in tumors with a **BRAF(V600E)** mutation.** The impact of RAF inhibitors on inhibiting ERK signaling in
tumor cells with mutant BRAF has been extensively investigated (9, 20), but recent studies have also shown that BRAF inhibitors can enhance ERK signaling in cells with wild-type BRAF (32-34). To exclude the possibility that PLX4720 increased intratumoral T-cell accumulation via directly acting on the transferred T cells, we repeated the in vivo tumor treatment experiments using tumors with and without the BRAF(V600E) mutation. As expected, PLX4720 was not capable of inhibiting the in vitro growth of C918 melanoma cells, which have a wild-type BRAF (Fig. 3A). However, transduction with H-2D\(^b\) and gp100 did render C918 cells susceptible to recognition by pmel-1 T cells (Fig. 3B). Comparing antitumor responses against C918 and A375 in vivo, we found that addition of PLX4720 to the ACT regimen led to an increase in luciferase intensity in A375/H-2D\(^b\)/gp100, but not in C918/H-2D\(^b\)/gp100 tumors (Figs. 3C and D). This result suggested that PLX4720 could only enhance infiltration of adoptively transferred T cells in tumors containing a BRAF(V600E) mutation, a finding that was confirmed using another BRAF(V600E) cell line WM35/H-2D\(^b\)/gp100 (Figs. S6 A, B and C ) and another wild-type BRAF cell line, MC38/gp100 (Figs. S7 A, B and C ).

**PLX4720 increases infiltration of adoptively transferred T cells by inhibiting the production of VEGF in tumors.** Since PLX4720 augmented the infiltration of T cells into BRAF mutant but not wild-type tumors, we next explored potential differences in the tumor microenvironment that may explain the enhanced migration of adoptively transferred T cells. We thus harvested tumors from A375/H-2D\(^b\)/gp100 tumor-bearing mice treated with PLX4720 or vehicle alone, and made a tumor homogenate for protein array analysis. As shown in Fig. 4A, PLX4720 treatment significantly reduced the hVEGF production in tumors. Since chemokines are known to be essential for mediating
T-cell trafficking, we also tested the intratumoral expression of a panel of 12 chemokines, but found no significant differences between treatments. Inhibition of VEGF production was also confirmed by testing the supernatants from *in vitro* cell cultures of A375/H-2D\(^b\)/gp100 cells treated with PLX4720 (Fig. S8).

VEGF is a key angiogenic factor known to stimulate endothelial cell growth, survival, migration, lumen formation and vascular permeability (35). High levels of VEGF can induce vessel abnormalities that impair drug delivery and influx of immune cells into tumors, whereas vascular normalization by VEGF blockade or Rgs5 tumor cell deficiency can enhance the infiltration of adoptively transferred CD8\(^+\) T cells (35-37). Therefore, we next explored the possibility that reduced VEGF signaling in PLX4720-treated BRAF mutant tumors was responsible for the enhanced infiltration by adoptively transferred T cells. As shown in Figs. 4B and C, *in vivo* blockade of the VEGF/VEGFR interaction with anti-hVEGF Ab indeed enhanced pmel-1 T cell infiltration into A375/H-2D\(^b\)/gp100 tumors. These results support the notion that administration of PLX4720 increases infiltration of adoptively transferred T cells via the inhibition of VEGF production by tumors.

**PLX4720 treatment reduces the binding of c-myc to the VEGF promoter.** Given that PLX4720 treatment is known to reduce ERK activation in tumor cells carrying a BRAF V600E mutation (9), we hypothesized that PLX4720 may repress VEGF transcription via inhibiting ERK-activated transcription factors involved in the direct regulation of VEGF transcription. To test this, we first confirmed that PLX4720 treatment inhibited the phosphorylation of ERK in A375/H-2D\(^b\)/gp100 tumor cells (Fig. S9A). Examination of the proximal promoter region of human VEGF with TFSEARCH
(http://www.cbrc.jp/research/db/TFSEARCH.html) identified potential consensus sites for multiple transcription factors, including c-myc, p300 and E2F1 (Fig. S9B and data not shown). Previous studies demonstrated that c-myc and p300 were ERK-activated transcription factors, and E2F1 was a PI3K-activated transcription factor (38-40). Using a ChIP assay, we demonstrated that c-myc is constitutively recruited to the VEGF promoter in A375/H-2D\textsuperscript{b}/gp100 cells (Fig. 4D). Furthermore, c-myc promoter binding, but not that of p300 or E2F1, was reduced approximately 5-fold following PLX4720 treatment (Fig. S9C). These results strongly suggest that the spontaneous production of VEGF in A375/H-2D\textsuperscript{b}/gp100 cells is regulated by c-myc, and that PLX4720 reduces VEGF transcription via inhibition of c-myc binding to the VEGF promoter.

**PLX4720-induced T-cell infiltration is abrogated in tumors over-expressing VEGF.** To determine whether VEGF downregulation was indeed required for the enhanced intratumoral T-cell migration observed in PLX4720-treated mice, we next investigated whether this enhancement was impaired in tumors constitutively over-expressing VEGF. Thus, A375/H-2D\textsuperscript{b}/gp100 melanoma cells were transduced with hVEGF under the transcriptional control of the CMV promoter (Fig. 5A). VEGF-transduced melanoma cells maintained sensitivity to PLX4720 (Fig. S10), but no longer demonstrated a large decrease in VEGF secretion in response to PLX4720 treatment (Fig. 5B). We next analyzed B6 nude mice bearing either A375/H-2D\textsuperscript{b}/gp100 or A375/H-2D\textsuperscript{b}/gp100/VEGF tumors following treatment with ACT and PLX4720 by monitoring T-cell migration. As shown in Figs. 5C and D, augmented T-cell infiltration in response to PLX4720 treatment was abrogated in the A375/H-2D\textsuperscript{b}/gp100/VEGF tumors. These results support the notion
that BRAF inhibition enhances T-cell migration to tumors through downregulation of VEGF production.

**Intratumoral VEGF downregulation correlates with increased T-cell infiltration in melanoma patients treated with BRAF inhibitor.** In light of our results in the mouse model, we next investigated whether VEGF was also downregulated in the tumors of melanoma patients treated with BRAF inhibitor. Pre-treatment or on-treatment tumor biopsies were harvested from 9 melanoma patients, and immunohistochemical staining for VEGF was performed. As shown in Figs. 6A and B, BRAF inhibitor treatment significantly downregulated VEGF expression in the majority (7/9) of patients, results which were confirmed by qRT-PCR (Figs. S11A and B). Examination of intratumoral T-cell infiltration was also performed by staining the tumor samples for CD8 and reported separately by Dr. Jennifer Wargo’s group. These results of patient samples are consistent with the data observed in the mouse model, and suggest that treatment of patients with BRAF inhibitor may also increase T-cell infiltration into tumors via inhibition of intratumoral VEGF production.
Discussion

To date, a number of specific kinase inhibitors that target BRAF V600E to treat melanoma have been generated and applied in clinical trials (8-10), with 50 - 70% of patients with BRAF(V600E) mutation showing objective clinical responses to treatment. However, in spite of these high initial response rates, responses are transient and recurrences with treatment-resistant disease typically occur within a year (12, 13, 41). Therefore, combinatorial approaches to treat melanoma are being actively explored.

ACT therapy using tumor-infiltrating lymphocytes is one of the most well-established immunotherapeutic approaches for cancer treatment (42). However, one of the important factors that limit the efficacy of this therapy is lack of migration of T cells into the tumor site (43, 44). Using a very sensitive bioluminescence imaging (BLI) system in murine models (27), we have previously shown that transduction of tumor-specific T cells with the CXCR2 chemokine receptor can improve the intratumoral migration of adoptively transferred T cells and enhance antitumor responses in murine models (28). In the current study, we tested whether the addition of a BRAF inhibitor could similarly enhance the anti-tumor activity of ACT in vivo. Using a xenograft human melanoma model, A375 transduced to express hgp100 and H-2D^b, we found that administration of PLX4720 could increase tumor infiltration of adoptively transferred gp100-specific T cells and improved antitumor responses. This effect was partially mediated by PLX4720-induced inhibition of VEGF production in melanoma cells. Analysis of tumor biopsies derived from BRAF inhibitor-treated melanoma patients were consistent with the results found in the murine model, showing that BRAF inhibition could significantly downregulate tumoral VEGF expression, which correlated with
increased tumor infiltration by T cells. Furthermore, our findings indicated that BRAF inhibition is capable of enhancing the antitumor activity of ACT therapy without impairing T-cell function.

VEGF, an immunosuppressive factor secreted by many tumors, can negatively impact the activity of tumor-infiltrating immune cells and stimulate the growth of tumor vasculature (45-47). RNAi-mediated inhibition of BRAF(V600E) can decrease the production of VEGF in melanoma cells with mutant BRAF(V600E) (21); however, it has remained unclear how constitutive activation of the BRAF-MAPK signaling pathway can influence VEGF production. Using a ChIP assay, we demonstrated that c-myc, but not p300 or E2F1, is constitutively recruited to the VEGF promoter and that transcription and production of VEGF is reduced by PLX4720 in melanoma cells harboring the V600E mutation. Blocking of VEGF/VEGFR-2 interactions can upregulate endothelial adhesion molecules in tumor vessels, which can in turn increase the infiltration of leukocytes in tumors (48). Furthermore, administration of anti-VEGF Ab can significantly increase infiltration of adoptively transferred CD8+ T cells into tumor sites and improve antitumor responses (37). Using our xenogeneic mouse tumor model, we found that PLX4720 treatment significantly reduced the production of tumoral VEGF, and that the increased accumulation of tumor-infiltrating T cells was abrogated in melanoma over-expressing VEGF. Administration of anti-hVEGF Ab also increased tumor infiltration of pmel-1 T cells by 2 to 3-fold. Since PLX4720 treatment typically induced 5-10 fold higher levels of T-cell infiltration compared with vehicle treatment, it suggests that reduction of VEGF/VEGFR interactions is not the only mechanism responsible for the increased T-cell infiltration. Using gp100-negative tumor cells, PLX4720 treatment failed to increase
T cell trafficking to tumor sites. These findings suggest that tumor antigen expression also plays an important role in T cell accumulation in tumors. These findings are consistent with our previous report that IFN-γ produced by tumor-antigen-activated T cells can induce CXCL10 expression in tumors, in turn resulting in more T cell accumulation (49). Further studies will be required to identify additional mechanisms that may contribute to this combinational therapy regimen.

In this study, we have shown that administration of PLX4720 can clearly enhance the infiltration of transferred T cells and improve the antitumor responses induced by ACT. Our data is consistent with a recent clinical study showing that treatment of melanoma patients with a BRAF inhibitor leads to augmented T-cell infiltration into metastatic sites (11). Using a BRAF(V600E) driven murine model, Koya et al have recently shown that BRAF inhibition can increase MAPK signaling and intratumoral cytokines secretion by adoptively transferred T cells, leading to a beneficial anti-tumor effect (50). Collectively, the emerging evidence strongly suggests that combinations of selective BRAF inhibitors with immunotherapy will result in enhanced benefits for melanoma patients in the very near future.
Figure Legends

**Figure 1. Overexpression of gp100 abrogates enhanced melanoma T-cell recognition induced by PLX4720.** (A) Schematic representation of two lentiviral vectors containing full-length human gp100 and mIL-4R, or H-2D\(^b\) and EGFP. (B) IFN-\(\gamma\) secretion by pmel-1 T cells co-cultured with the indicated transduced tumor cell lines (before cell sorting) for 24hrs, as determined by ELISA. Untransduced MC38 murine colon adenocarcinoma cells or those transduced with gp100 were used as negative and positive controls, respectively. (C, D and E) IFN-\(\gamma\) secretion by pmel-1 T cells co-cultured with transduced melanoma cells (after cell sorting) that had been pre-treated with the indicated concentrations of PLX4720, as determined by ELISA. (* \(P<0.05\), ** \(P<0.01\)) Data are representative of 3 independent experiments.

**Figure 2. Administration of PLX4720 in vivo increases tumor infiltration of adoptively transferred T cells and enhances antitumor responses.** (A) B6 nude mice (5 mice/group) bearing A375/H-2D\(^b\)/gp100 tumors were treated with OFL-expressing pmel-1 T cells, along with gp100 peptide-pulsed DCs, by i.v. injection on day 7 after tumor inoculation. 2 days after T cell transfer, PLX4720 or vehicle alone was administered by oral gavage daily for 3 days. Luciferase imaging showing in vivo trafficking of OFL-expressing pmel-1 T cells on day 5 after T cell transfer. (B) Summary of quantitative imaging analysis of transferred T cells at the tumor site. Quantification is expressed as the average of photon flux within ROI. (* \(P<0.05\)) (C) B6 nude mice (5-7 mice/group) bearing A375/H-2D\(^b\)/gp100 tumors were treated with pmel-1 T cells and gp100 peptide-pulsed DCs, by i.v. injection on day 10 after tumor inoculation. 2 days after T cell transfer, mice were fed a diet containing PLX4720 or vehicle for 3 days, and tumor
growth was monitored over time. (D) Mouse survival as monitored over time following treatment. Data shown are expressed as mean ± SEM and are representative of 2 to 3 independent experiments with similar results.

**Figure 3.** PLX4720 increases infiltration of adoptively transferred T cells only in tumors containing BRAF(V600E). (A) Growth inhibition of BRAF V600E mutated (A375) and WT (C918) human melanoma cell lines treated in vitro with PLX4720 for 72hrs, as determined by a CellTiter-Blue Cell Viability assay. (B) IFN-γ secretion by pmel-1 T cells co-cultured with C918 melanoma cells expressing gp100 and/or H-2D^b^ for 24hrs, as determined by ELISA. B16 murine melanoma cells were used as a positive control. (C) A375/H-2D^b^/gp100 and C918/H-2D^b^/gp100 tumor bearing mice (5 mice/group) were treated as described in Fig. 2A. Luciferase imaging showing in vivo trafficking of OFL-expressing pmel-1 T cells on day 5 after T cell transfer. (D) Summary of quantitative imaging analysis of transferred T cells at the tumor sites, expressed as the average of photon flux within ROI. Data shown are expressed as mean ± SEM and are representative of 2 independent experiments with similar results.

**Figure 4.** VEGF blockade in vivo increases tumor infiltration of adoptively transferred T cells. (A) Protein array analysis of cleared tumor lysates, performed as described in Methods. (B) B6 nude mice (5 mice/group) bearing A375/H-2D^b^/gp100 tumors were treated with anti-hVEGF Ab or mouse IgG. Pictures showing intratumoral trafficking of OFL-expressing pmel-1 T cells represent results of imaging on day 5 after T cell transfer. (C) Summary of the quantitative imaging analysis of transferred T cells at the tumor site on day 5 after T cell transfer, expressed as the average photon flux within ROI. (D) A375/H-2D^b^/gp100 cells were treated with PLX4720 or DMSO for 2 hours, followed by
ChIP assays using anti-c-myc antibody or control IgG. ChIP products were analyzed by real-time PCR using primers amplifying the promoter regions of human VEGF or the irrelevant gene GAPDH. Results were normalized to 1% total chromatin input, and presented as mean ± SD of fold induction of PLX4720 versus Vehicle from 2 independent experiments.

**Figure 5. Over-expression of hVEGF abrogates increased infiltration of T cells into PLX4720-treated tumors.** (A) Schematic representation of lentiviral vector expressing the hVEGF and RFP genes, separated by an IRES. A375/H-2D<sup>b</sup>/gp100 melanoma cells were transduced, and VEGF-expressing cells were sorted based on RFP expression. (B) Tumor cells with or without over-expression of hVEGF were incubated with PLX4720 for 24 hrs, and VEGF concentrations in the supernatants were assessed by ELISA. (C) B6 nude mice (5 mice/group) bearing A375/H-2D<sup>b</sup>/gp100 tumors with or without over-expression of hVEGF were treated as described in Fig. 2A. Pictures show representative results of imaging on day 5 after T cell transfer. (D) Graph depicting fold change in luciferase intensity, generated from quantitative imaging analysis of transferred T cells at the tumor site. Data shown are representative of 2 independent experiments with similar results.

**Figure 6. PLX4720 treatment downregulates tumoral VEGF in melanoma patients.** (A) Pre- and on-treatment VEGF expression in two representative tumor biopsies from BRAF inhibitor-treated patients, as determined by IHC analysis. Original magnification, ×200. (B) Summary of quantitative analysis of VEGF expression in tumors from 9 BRAF inhibitor-treated patients. The percentage of VEGF positive tumor cells in the tumor was evaluated by two pathologists in a blinded fashion.
References

melanoma can be overcome by cotargeting MEK and IGF-1R/PI3K. Cancer Cell. 2010;18:683-95.


Figure 1

(A) Schematic representation of the transgene cassette used in the study. The cassette includes CMV, H-2D<sup>b</sup>, hEF1a, hGP100, IRES, mL-4R, and EGFP.

(B) Graph showing the concentration of mIFN-γ (ng/ml) in different cell lines (MC38, A375, WM35) treated with or without H-2D<sup>b</sup> and gp100.

(C) Bar graph showing the concentration of mIFN-γ (ng/ml) in WM35/H-2D<sup>b</sup> cells treated with different concentrations of PLX4720 (nM).

(D) Bar graph showing the concentration of mIFN-γ (ng/ml) in WM35/H-2D<sup>b</sup>/gp100 cells treated with different concentrations of PLX4720 (nM).

(E) Bar graph showing the concentration of mIFN-γ (ng/ml) in A375/H-2D<sup>b</sup>/gp100 cells treated with different concentrations of PLX4720 (nM).
Figure 3

A) Concentration of PLX4720 (nM) vs. % of growth inhibition

B) mIFN-γ (ng/ml) for H-2Db gp100 - C918 - B16 - T alone

C) ROI. Photos/sec/cm² X10⁶ for A375/H-2Db/gp100 and C918/H-2Db/gp100

D) Comparison of Photos/sec/cm² X10⁶ between Veh and PLX for A375/H-2Db/gp100 and C918/H-2Db/gp100 (P<0.05)
Figure 5

A

CMV  hVEGF  IRES  RFP

B

hVEGF Inhibition (%)

0  20  40  60  80  100

PLX4720 concentration (µM)

0.1  1  10

D

Fold change of Luciferase Intensity

Day 5  Day 7

A375/H-2D^b/gp100  A375/H-2D^b/gp100/hVEGF
Figure 6

A

Pre-treatment

On-treatment

B

Positive rate (%)

Pre-treatment

On-treatment

Pt 5

Pt 6

Pt 8

Pt 9

Pt 10

Pt 11

Pt 12

Pt 13

Pt 16

Average

$P<0.05$
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