

## Priority Brief

## BRAF Inhibition Alleviates Immune Suppression in Murine Autochthonous Melanoma

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## Abstract

A growing body of evidence suggests that BRAF inhibitors, in addition to their acute tumor growth-inhibitory effects, can also promote immune responses to melanoma. The present study aimed to define the immunologic basis of BRAF-inhibitor therapy using the *Braf/Pten* model of inducible, autochthonous melanoma on a pure C57BL/6 background. In the tumor microenvironment, BRAF inhibitor PLX4720 functioned by on-target mechanisms to selectively decrease both the proportions and absolute numbers of CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Treg) and CD11b<sup>+</sup>Gr1<sup>+</sup> myeloid-derived suppressor cells (MDSC), while preserving numbers of CD8<sup>+</sup> effector T cells. In PLX4720-treated mice, the intratumoral Treg populations decreased significantly, demonstrating enhanced apoptosis. CD11b<sup>+</sup> myeloid cells from PLX4720-treated tumors also exhibited decreased immunosuppressive function on a per-cell basis. In accordance with a reversion of tumor immune suppression, tumors that had been treated with PLX4720 grew with reduced kinetics after treatment was discontinued, and this growth delay was dependent on CD8 T cells. These findings demonstrate that BRAF inhibition selectively reverses two major mechanisms of immunosuppression in melanoma and liberates host-adaptive antitumor immunity. *Cancer Immunol Res*; 2(11); 1044–50. ©2014 AACR.

## Introduction

BRAF inhibitors are small-molecule drugs that impair melanoma cell proliferation and survival by targeting the oncogenic driver mutation BRAF<sup>V600E</sup>, expressed by approximately 50% of metastatic melanomas (1). The prototype BRAF inhibitor vemurafenib induces dramatic responses in patients (2); however, resistance typically develops within a year (1). A growing body of evidence now suggests that vemurafenib also influences immune responses to melanoma (3–5), although better insights into this phenomenon are needed. In patients with melanoma, vemurafenib has been shown to increase the numbers of CD8 T cells (4, 5) and CD4 T cells (4), as well as the expression of T-cell functional markers, and melanoma differ-

entiation antigens (5) in the tumor microenvironment. Increased frequency of tumor antigen-specific CD8 T cells has also been found in the blood of vemurafenib-treated patients (6), suggesting possible T-cell cross-priming. Despite this, vemurafenib treatment increased the expression of T-cell exhaustion markers within tumors (5) and decreased the peripheral lymphocyte counts (7). Thus, whether T cells contribute to the efficacy of BRAF inhibitors remains an open question.

Studies in immunocompetent mice have begun to address this question, although different models have yielded conflicting answers. In the transplantable SMIWT1 model, the vemurafenib analogue PLX4720 improved intratumoral ratios of CD8 to regulatory T cells (Treg), and CD8 T cells were absolutely required for drug efficacy (8). Accordingly, in a transplantable tumor model derived from *Tyr::CreER/Braf<sup>CA</sup>/Pten<sup>lox/lox</sup>* (*Braf/Pten*) mice, the tumor growth-inhibitory effects of PLX4720 depended on CD8 T cells (9). However, in autochthonous *Braf/Pten* tumor-bearing mice, PLX4720 indiscriminately decreased the frequencies of immune cells in tumors on a C57BL/6 background (10), while demonstrating a dependency on CD4 T cells for elimination of tumors on a mixed genetic background (11). Thus, the immunologic effects of BRAF inhibitors appear to be variable and may depend heavily on the tumor model and genetic background under study.

The present study revisits the immunologic implications of BRAF inhibition in the *Braf/Pten* inducible autochthonous melanoma model on a pure C57BL/6 background. We find that BRAF<sup>V600E</sup> inhibition initiates a quantitative loss of both Tregs and myeloid-derived suppressor cells (MDSC) from the tumor

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microenvironment. Accordingly, short-term BRAF inhibition enables subsequent control of small melanomas by the host CD8 T cells. Despite this, we show that PLX4720 efficiently arrests melanoma growth even in the absence of host T cells. These studies confirm that BRAF inhibitors perturb two major mechanisms of tumor immune suppression and highlight CD8 T cell-dependent tumor control as a secondary mechanism of BRAF-inhibitor action.

## Materials and Methods

### Mice and tumor inductions

Studies were performed in accordance with the Institutional Animal Care and Use Committee Guidelines at Dartmouth. *Braf/Pten* mice (12) were bred onto a B6 background, and confirmed by the DartMouse speed congenic facility. Experiments used mice possessing the genotype of a single gender *Tyr::CreER<sup>+/+</sup> Braf<sup>CA/+</sup> Pten<sup>lox/lox</sup>*. Tumors were induced in 3- to 4-week-old mice by intradermal injection of 4-hydroxy-tamoxifen (10  $\mu$ L of a 20  $\mu$ mol/L solution in DMSO). As a control, 6- to 8-week-old C57BL/6 mice (The Jackson Laboratory) were inoculated intradermally with  $1 \times 10^5$  B16-F10 cells (a gift from Alan Houghton, Sloan Kettering Cancer Center, New York, NY; tested by IMPACT and authenticated at the University of Missouri, Columbia, MO, RADIL, March 2013). Where indicated, C57BL/6 mice and *RAG1<sup>-/-</sup>* mice (The Jackson Laboratory; bred in-house) were dorsally grafted with approximately 1-cm<sup>2</sup> sections of tail skin from *Braf/Pten* mice, and tumors were induced 1 week later by topical application of 4-hydroxy-tamoxifen.

### In vivo drug treatments and antibody depletions

PLX4720 was provided by Plexikon Inc. under a Materials Transfer Agreement, and was compounded in rodent diet (417 mg/kg) by Research Diets, Inc. Mice bearing palpable melanomas were fed PLX4720-containing or control diet *ad libitum* for the designated period. Anti-CD8 (mAb clone 2.43, produced in-house) was administered i.p. every 4 days (300  $\mu$ g/dose), anti-CD4 (mAb clone GK1.5, produced in house) was administered i.p. weekly (300  $\mu$ g/dose).

### Flow cytometry

Tumors were harvested, weighed, minced, and digested for 45 minutes, with gentle shaking, at 37°C in HBSS containing 7 mg/mL collagenase D and 200  $\mu$ g/mL DNase-I (Roche). Single-cell suspensions were stained with antibodies, including anti-CD45-APC-Cy7, anti-CD3-Vioblue, anti-CD8-PCP, anti-CD4-FITC, anti-Foxp3-PE, anti-CD11b-APC, anti-GR-1-PE-Cy7, anti-GATA3-PE, anti-T-bet-PE-Cy7, anti-ROR $\gamma$ t-APC (BioLegend or eBioscience), and anti-Annexin V-Alexa Fluor 647 (BD Pharmingen). Cells were fixed/permeabilized using reagents from the Foxp3 Staining Kit (eBioscience). Flow cytometry was performed on a Miltenyl MACSQuant 10 Analyzer. Total cells were enumerated using the flow cytometer.

### Analysis of T-cell cross-priming

Tumor-bearing mice received  $1 \times 10^5$  naïve, congenically marked (Ly5.2<sup>+</sup>) gp100<sub>25-33</sub>-specific CD8 T cells isolated from pmel TCR transgenic mice (13). Cells were magnetically puri-

fied from spleens by CD44-positive and CD8-negative selection (Miltenyi Biotec), and injected i.v. Anti-CD4-depleting antibody (mAb clone GK1.5; produced in-house) was administered i.p. (500  $\mu$ g per dose). Tumor-draining inguinal lymph nodes were harvested, mechanically dissociated, and analyzed by flow cytometry.

### MDSC suppression assay

Tumors were digested as described above, and CD11b<sup>+</sup> cells were isolated magnetically by positive selection (Miltenyi Biotec). Purified CD11b<sup>+</sup> cells were combined at indicated ratios with RBC-depleted C57BL/6 mouse splenocytes, and added to a 96-well plate precoated with anti-CD3 (5 mg/mL, clone OKT3) and anti-CD28 (5 mg/mL, clone PV-1; BioXcell), to a final concentration of  $3 \times 10^5$  splenocytes/200  $\mu$ L/well. Seventy-two hours later, supernatants were harvested and assayed for IFN $\gamma$  production by ELISA (R&D Systems).

## Results and Discussion

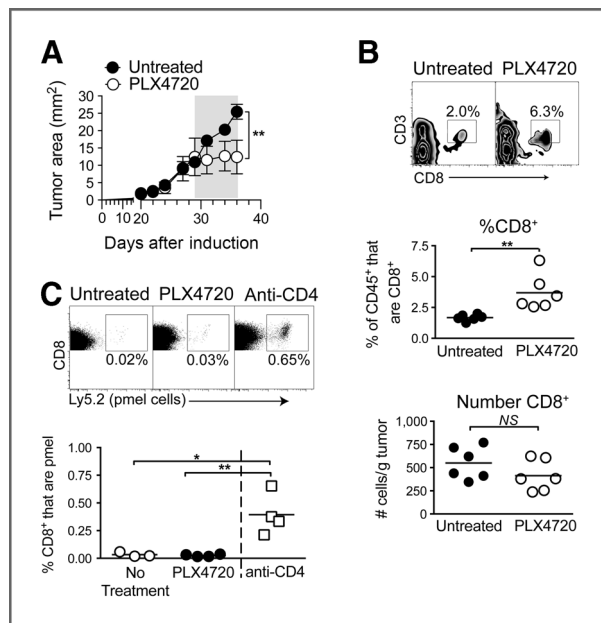
### PLX4720 does not induce intratumoral accumulation or cross-priming of CD8 T cells in mice bearing autochthonous *Braf/Pten* melanomas

To evaluate the effects of BRAF inhibition on CD8 T-cell responses to *Braf/Pten* tumors, mice bearing palpable melanomas received PLX4720-containing diet, and tumors were analyzed by flow cytometry. As previously reported (10), *Braf/Pten* melanomas on a C57BL/6 background were sensitive to PLX4720, demonstrating immediate and stable growth arrest, although tumor regression was not observed (Fig. 1A). Similar growth arrest was obtained when mice were treated with vemurafenib (PLX4032) by gavage (Supplementary Fig. S1). In the tumor microenvironment, PLX4720 treatment for 10 days significantly increased CD8 T cells by proportion of CD45<sup>+</sup> cells, but not by absolute number (Fig. 1B). This finding is contrary to reports that CD8 T-cell frequencies are decreased by PLX4720 in this model (10).

Although CD8 T-cell numbers were not changed by the treatment, it remained possible that BRAF inhibition promoted the *de novo* priming of tumor antigen-specific CD8 T cells. To assess cross-priming,  $10^5$  naïve CD8 T cells (pmel cells) specific for the melanoma antigen gp100 were adoptively transferred into *Braf/Pten* tumor-bearing mice. Pmel cells did not expand in tumor-draining lymph nodes of untreated mice; however, total depletion of Tregs with anti-CD4 mAb elicited pmel cell priming and accumulation as a positive control (Fig. 1C), in accordance with published studies in B16 melanoma (14). Despite this, PLX4720 treatment did not induce detectable pmel cell expansion (Fig. 1C). Thus, BRAF inhibition did not drive cross-priming of Ag-specific T cells.

### PLX4720 promotes the selective loss of Tregs from the *Braf/Pten* tumor microenvironment

Recent reports have shown reduced intratumoral Foxp3<sup>+</sup> Treg populations following treatment with PLX4720; however, results in one study (10) showed that this effect was not specific to Tregs, and no studies have evaluated the absolute numbers of Tregs (8, 11). To address this question, we measured CD4 T-cell



**Figure 1.** PLX4720 does not promote CD8 T-cell infiltration or cross-priming. **A**, mice bearing Braf/Pten tumors were treated with PLX4720-containing diet for 7 days (indicated by gray bar), and tumor growth was monitored. Symbols represent mean  $\pm$  SEM of 4 to 6 mice per group; statistical significance was calculated by the two-way ANOVA. **B**, PLX4720 was administered for 10 days (day 28–38), and tumors were analyzed for infiltrating CD8<sup>+</sup>CD3<sup>+</sup> T cells, by proportion gated on CD45<sup>+</sup> cells (top and middle), and by absolute number (bottom). **C**, mice received 10<sup>5</sup> Ly5.2<sup>+</sup> pmel cells on day 27 and were treated with PLX4720 on days 29 to 38, or anti-CD4 on days 29 and 35. Pmel cells were detected in tumor-draining lymph nodes on day 38. Symbols represent individual mice and horizontal lines depict means; statistical significance was calculated by two-tailed *t* test. \*, *P* < 0.05; \*\*, *P* < 0.01; NS, not statistically significant. Experiments were conducted at least twice with similar results.

populations in Braf/Pten tumors following 10 days of treatment. As with CD8 T cells, PLX4720 increased total CD4 T cells by the proportion of CD45<sup>+</sup> cells but not the absolute number (Fig. 2A). Despite this, PLX4720 markedly reduced both the proportion (of CD4<sup>+</sup> cells) and the absolute number of Foxp3<sup>+</sup> Tregs (Fig. 2A). In contrast, Treg proportions were unchanged in Braf/Pten tumor–draining lymph nodes, and in BRAF<sup>WT</sup> B16 tumors, demonstrating that this effect was both localized and on-target (Fig. 2B).

Because PLX4720 arrested Braf/Pten tumor growth, it was possible that the reduction in Treg cell numbers was due to decreased tumor burden. Thus, Treg populations were compared in Braf/Pten tumors of 4 mm versus 8 mm average diameter. Unexpectedly, the smaller tumors contained more Tregs (normalized for tumor mass) compared with that in larger tumors (Fig. 2C). Moreover, 4-mm untreated tumors had approximately eight times the number of Tregs as compared with size-matched tumors that had been treated with PLX4720 for 10 days before analysis (Fig. 2C). Thus, rather than decreasing Treg recruitment, PLX4720 promoted a substantial quantitative loss of preexisting Tregs from Braf/Pten tumors.

Analysis of CD4 T-cell expression of transcription factors Tbet, GATA3, and ROR $\gamma$ t illustrated no change within PLX4720-

treated tumors, suggesting a lack of Treg cell conversion to another Th subset (Supplementary Fig. S2). However, results from Annexin V staining indicated a significant increase in the proportion of Tregs undergoing apoptosis in PLX4720-treated tumors (Fig. 2D). Thus, BRAF inhibition increased Treg apoptosis and led to a selective loss of these cells from the tumor microenvironment.

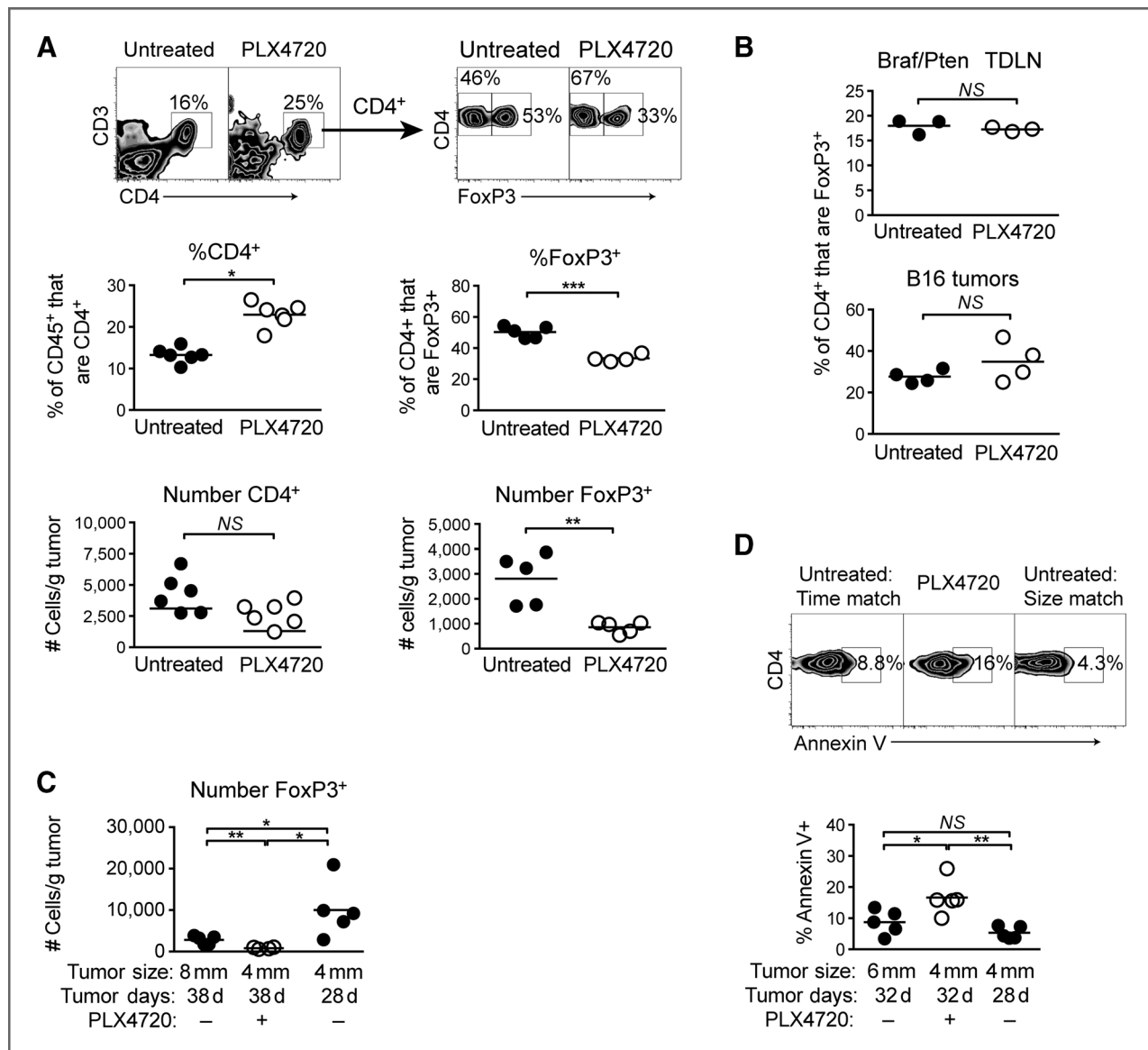
#### PLX4720 selectively depletes intratumoral MDSCs

Our finding that PLX4720 increased both CD8 and CD4 T cells by proportion but not in absolute number suggested that another CD45<sup>+</sup> population was being lost within the tumors. In addition to Tregs, MDSCs are major suppressors of tumor immunity, and it has been shown recently that MDSC populations are decreased in the peripheral blood of patients receiving vemurafenib (15). In Braf/Pten tumors, total CD11b<sup>+</sup> cells constituted a major fraction of CD45<sup>+</sup> leukocytes, and PLX4720 significantly decreased this population by both proportion and absolute number (Fig. 3A). The reduction in myeloid cell numbers was not attributed solely to reduced tumor burden, as 4-mm untreated tumors had approximately five times the number of CD11b<sup>+</sup> cells compared with 4-mm tumors that had been treated with PLX4720 for 10 days before the analysis (Fig. 3A). Thus, similar to Tregs, myeloid cells were quantitatively lost from the tumors during PLX4720 treatment. However, unlike Tregs, CD11b<sup>+</sup> cells did not demonstrate increased apoptosis (Supplementary Fig. S3), suggesting that their loss may be attributed to distinct mechanisms.

A majority of CD11b<sup>+</sup> cells within Braf/Pten tumors also expressed the MDSC marker Gr1, and PLX4720 decreased both the proportions and absolute numbers of CD11b<sup>+</sup>Gr1<sup>+</sup> cells (Fig. 3B). This was an on-target and localized effect of PLX4720, as it did not occur in BRAF<sup>WT</sup> B16 tumors, or in Braf/Pten tumor–draining lymph nodes (Fig. 3C). To determine whether PLX4720 also influences the suppressive function of intratumoral myeloid cells, CD11b<sup>+</sup> cells were isolated from untreated versus PLX4720-treated tumors and their ability to suppress IFN $\gamma$  production by activated T cells *in vitro* were compared. Although myeloid cells from both groups were suppressive at high ratios of myeloid cells to splenocytes, cells from PLX4720-treated tumors were significantly less suppressive, on a per-cell basis, at lower ratios (Fig. 3D). Thus, PLX4720 promoted a quantitative loss of CD11b<sup>+</sup>Gr1<sup>+</sup> cells from Braf/Pten tumors, and decreased the overall immunosuppressive function of myeloid cells remaining in these tumors.

#### PLX4720 enables host CD8 T cell–mediated control of Braf/Pten melanomas during the posttreatment period

Our finding that BRAF inhibition selectively reduced two major immunosuppressive cell populations suggested that CD8 T cells may contribute to tumor control in PLX4720-treated mice. To assess this, PLX4720 was administered either alone or in combination with anti-CD8-depleting antibody. Total CD8 T-cell depletion (including in tumors; Supplementary Fig. S4) did not diminish the efficacy of PLX4720 against

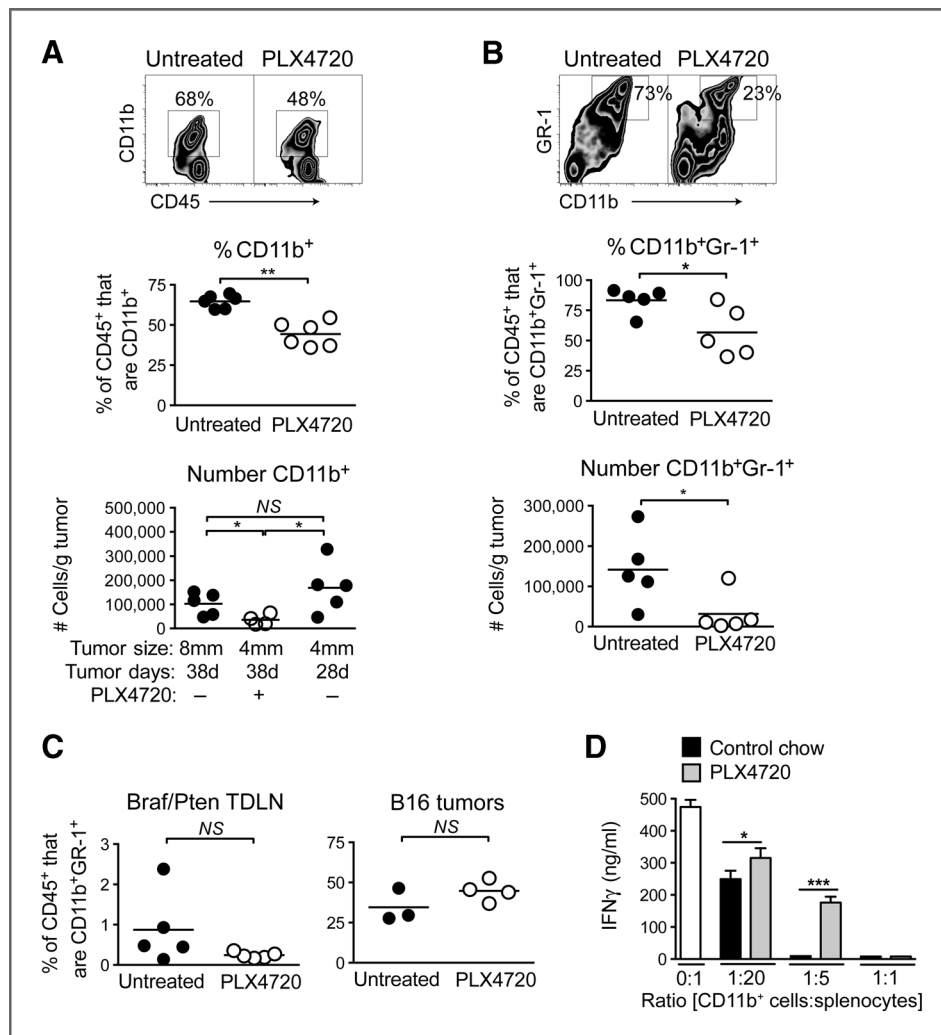


**Figure 2.** BRAF inhibition induces the selective loss of Tregs from Braf/Pten tumors. Mice bearing Braf/Pten tumors were treated with PLX4720 for 10 days (days 28–38). **A**, tumors were analyzed for infiltration of CD4<sup>+</sup>CD3<sup>+</sup> T cells by proportion gated on CD45<sup>+</sup> cells or absolute number, or Foxp3<sup>+</sup> Tregs by proportion gated on CD4<sup>+</sup>CD3<sup>+</sup> cells, or by absolute number. **B**, Braf/Pten tumor–draining lymph nodes were analyzed (top) or B16 melanoma tumor-bearing mice were used (bottom). **C**, tumor induction was delayed by 10 days to provide an additional untreated, size-matched (4-mm diameter) control group. **D**, PLX4720 was administered for 4 days, and the proportion of Foxp3<sup>+</sup>CD4<sup>+</sup> cells staining for Annexin V was then determined. Points represent individual mice and horizontal lines depict means; statistical significance was calculated by two-tailed *t* test. \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001; NS, not statistically significant. Experiments were conducted at least twice with similar results.

well-established tumors (Fig. 4A, left), or even against smaller, less established tumors (Fig. 4A, right), for which immune effects would have been more pronounced. Furthermore, PLX4720 was equally as effective against autochthonous Braf/Pten tumors (growing in skin grafts) on *RAG1*<sup>-/-</sup> mice and C57BL/6 mice (Fig. 4B), demonstrating a lack of requirement for T or B cells.

Despite this, a possibility remained that the dominant tumor-static effects of PLX4720 masked a weaker T-cell response, which could be interrogated during the posttreatment phase. Indeed, when PLX4720 was administered for 1

week and then discontinued, small tumors continued to grow with reduced kinetics for several days thereafter (Fig. 4C, left), and this growth delay was abrogated by CD8 T-cell depletion. Anti-CD8 did not alter the growth of untreated tumors (Fig. 4C, left), nor did the depletion of CD4 T cells impair drug efficacy at any point (Fig. 4C, right). Thus, while immune responses may have been obfuscated by the potent tumor growth-inhibitory properties of PLX4720, BRAF inhibition elicited CD8 T cell-mediated tumor control that was evident during the posttreatment period. Moreover, while Tregs rapidly repopulated tumors after PLX4720 cessation (not shown), the



**Figure 3.** BRAF inhibition induces the selective loss of immunosuppressive myeloid cells from Bra<sup>f</sup>/P<sup>ten</sup> tumors. Mice bearing Bra<sup>f</sup>/P<sup>ten</sup> tumors were treated with PLX4720 for 10 days (days 28–38). A, tumors were analyzed for CD11b<sup>+</sup> cells, or B, analyzed for CD11b<sup>+</sup>Gr1<sup>+</sup> cells by proportion gated on CD45<sup>+</sup> cells (top and middle) or by absolute number (bottom). C, Bra<sup>f</sup>/P<sup>ten</sup> tumor–draining lymph nodes were analyzed (top) or B16 melanoma tumor-bearing mice were used (bottom). Points represent individual mice and horizontal lines depict means; statistical significance was calculated by two-tailed *t* test. D, CD11b<sup>+</sup> cells were isolated from pooled PLX4720-treated (gray bars) versus untreated (black bars) tumors (*n* = 4–8 tumors per group) and assayed for suppression of IFN $\gamma$  production by activated splenocytes (see Materials and Methods). Data, mean  $\pm$  SD. \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.0001; NS, not statistically significant. Experiments were conducted at least twice with similar results.

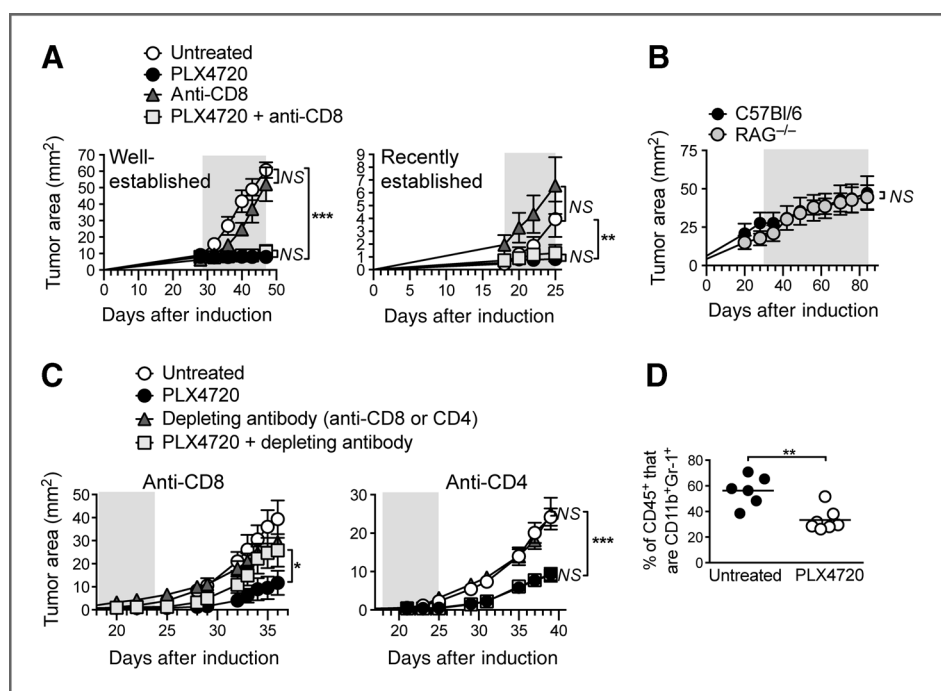
proportions of MDSCs remained significantly decreased at least 4 days thereafter (Fig. 4D). Therefore, an ongoing reduction in MDSCs coincided with CD8 T cell–mediated tumor control in PL4720-treated mice.

The present data are consistent with CD8 T cells as a secondary and redundant mechanism of tumor control in mice treated with BRAF inhibitors. This result is in contrast to recent studies using transplantable Bra<sup>f</sup>/P<sup>ten</sup> tumors (9), and autochthonous Bra<sup>f</sup>/P<sup>ten</sup> tumors on a mixed genetic background (11), which report an absolute dependence on CD8 and CD4 T cells, respectively. On the contrary, CD4 depletion might have slight immunostimulatory effects in the present model, as evidenced by cross-priming of tumor-specific pmel cells (see Fig. 1C). These discrepancies may underscore differences in tumor immunogenicity and cellular composition between autochthonous and transplantable tumors on different genetic backgrounds, and may portend even greater diversity among patients with melanoma receiving BRAF inhibitors.

In the present model, PLX4720 did not increase the absolute numbers of CD8 T cells in Bra<sup>f</sup>/P<sup>ten</sup> tumors or induce new

CD8 T-cell priming. Although robust T-cell infiltration has been described in tumors of vemurafenib-treated patients, a small fraction of the tumors do not exhibit this behavior (4, 5). In our model, the lack of CD8 T-cell infiltration could be attributed to a lack of tumor regression (Fig. 1A), which may relate to PTEN deficiency (16, 17), although this remains controversial. Regardless, autochthonous Bra<sup>f</sup>/P<sup>ten</sup> tumors on a C57BL/6 background may model a subset of poorly immunogenic human melanomas that are not easily infiltrated by effector T cells.

Future studies will be required to elucidate mechanisms underlying the reduced numbers of Tregs and MDSCs in PLX4720-treated tumors. CCR2 was shown to be required for the efficacy of PLX4720 in the SM1WT1 model (18) and, while our data are more consistent with apoptosis of preexisting Tregs, the decreased production of CCL2 by melanoma cells could potentially impair recruitment of MDSCs. Selective Treg apoptosis in gastrointestinal stromal tumors has been shown to result from decreased indolamine-2,3-dioxygenase (19), which is worth further examination. BRAF inhibition has also been shown to decrease melanoma xenograft production of



**Figure 4.** BRAF inhibition promotes CD8 T cell–mediated control of Braf/Pten tumor growth during the posttreatment period. PLX4720 was administered for the duration depicted by the gray shaded area, either A, alone or concurrently with anti-CD8 every 4 days, or B, to *RAG1*<sup>-/-</sup> or C57BL/6 mice bearing Braf/Pten tumors in grafted skin (see Materials and Methods). C, PLX4720 was administered and then discontinued, and depleting antibodies were given as indicated (left, anti-CD8, every 4 days starting on day 16; right, anti-CD4, weekly starting on day 17). Symbols, mean  $\pm$  SEM of 3 to 6 mice/group; statistical significance was calculated by the two-way ANOVA. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.0001$ . D, tumor-bearing mice were treated as in Fig. 3, except PLX4720 was discontinued for 4 days before analysis for proportion of CD11b<sup>+</sup>Gr1<sup>+</sup> cells (gated on CD45<sup>+</sup> cells). Symbols represent individual mice and horizontal lines depict means; statistical significance was calculated by two-tailed *t* test. \*\*,  $P < 0.01$ . Data are representative of two (A, B, and D) or three (C) replicate experiments yielding similar results.

VEGF (20), which could potentially impair Treg and/or MDSC responses. It remains to be demonstrated whether the reduced Treg and/or MDSC responses are directly responsible for CD8 T-cell immunity following PLX4720 cessation, and whether the remaining Tregs exhibit decreased suppressive function. Enhanced antigen expression and presentation (3, 21) as well as off-target enhancement of T-cell function by PLX4720 (22, 23) may also contribute to the observed response.

In conclusion, the present study demonstrates that on-target inhibition of BRAF<sup>V600E</sup> in melanoma cells has the downstream consequences of decreasing Treg and MDSC fitness within the tumor microenvironment, and promoting CD8 T cell–dependent tumor control. This work underscores an essential link between oncogenic BRAF<sup>V600E</sup> signaling and two major mechanisms of tumor immune suppression, providing additional rationale for clinical studies combining BRAF inhibitors and immunotherapies.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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