



# Host immunity contributes to the anti-melanoma activity of BRAF inhibitors

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**The BRAF mutant, BRAF<sup>V600E</sup>, is expressed in nearly half of melanomas, and oral BRAF inhibitors induce substantial tumor regression in patients with BRAF<sup>V600E</sup> metastatic melanoma. The inhibitors are believed to work primarily by inhibiting BRAF<sup>V600E</sup>-induced oncogenic MAPK signaling; however, some patients treated with BRAF inhibitors exhibit increased tumor immune infiltration, suggesting that a combination of BRAF inhibitors and immunotherapy may be beneficial. We used two relatively resistant variants of *Braf*<sup>V600E</sup>-driven mouse melanoma (SM1 and SM1WT1) and melanoma-prone mice to determine the role of host immunity in type I BRAF inhibitor PLX4720 antitumor activity. We found that PLX4720 treatment downregulated tumor *Ccl2* gene expression and decreased tumor CCL2 expression in both *Braf*<sup>V600E</sup> mouse melanoma transplants and in de novo melanomas in a manner that was coincident with reduced tumor growth. While PLX4720 did not directly increase tumor immunogenicity, analysis of SM1 tumor-infiltrating leukocytes in PLX4720-treated mice demonstrated a robust increase in CD8<sup>+</sup> T/FoxP3<sup>+</sup>CD4<sup>+</sup> T cell ratio and NK cells. Combination therapy with PLX4720 and anti-CCL2 or agonistic anti-CD137 antibodies demonstrated significant antitumor activity in mouse transplant and de novo tumorigenesis models. These data elucidate a role for host CCR2 in the mechanism of action of type I BRAF inhibitors and support the therapeutic potential of combining BRAF inhibitors with immunotherapy.**

## Introduction

Approximately 50% of melanomas harbor activating (V600E) mutations in the serine-threonine protein kinase B-RAF (BRAF<sup>V600E</sup>). The oral BRAF inhibitors vemurafenib (formerly PLX4032) and dabrafenib (formerly GSK2118436) induce a high frequency of tumor regressions in patients with BRAF<sup>V600E</sup> mutant metastatic melanoma (1–3) and vemurafenib improves overall survival compared with chemotherapy (4). BRAF inhibitors cause programmed cell death in melanoma cells lines by interrupting oncogenic BRAF<sup>V600E</sup> signaling through the MAPK pathway governing cell proliferation and survival. However, after an initial tumor response with BRAF inhibitor-based therapy, the majority of patients have disease progression. Several mechanisms of resistance to BRAF inhibitors have been discovered, which can either reactivate the MAPK pathway through upstream mutations in NRAS, amplification or truncation of BRAF, downstream mutations in MEK, or upregulation of COT (5–10) or through the activation of alternate survival pathways downstream of upregulated receptor tyrosine kinases (5, 11, 12).

The role of host pathways in the mechanism of action of BRAF inhibitors is poorly understood. The antitumor effects of BRAF inhibitors are believed to be a direct effect of inhibiting oncogenic

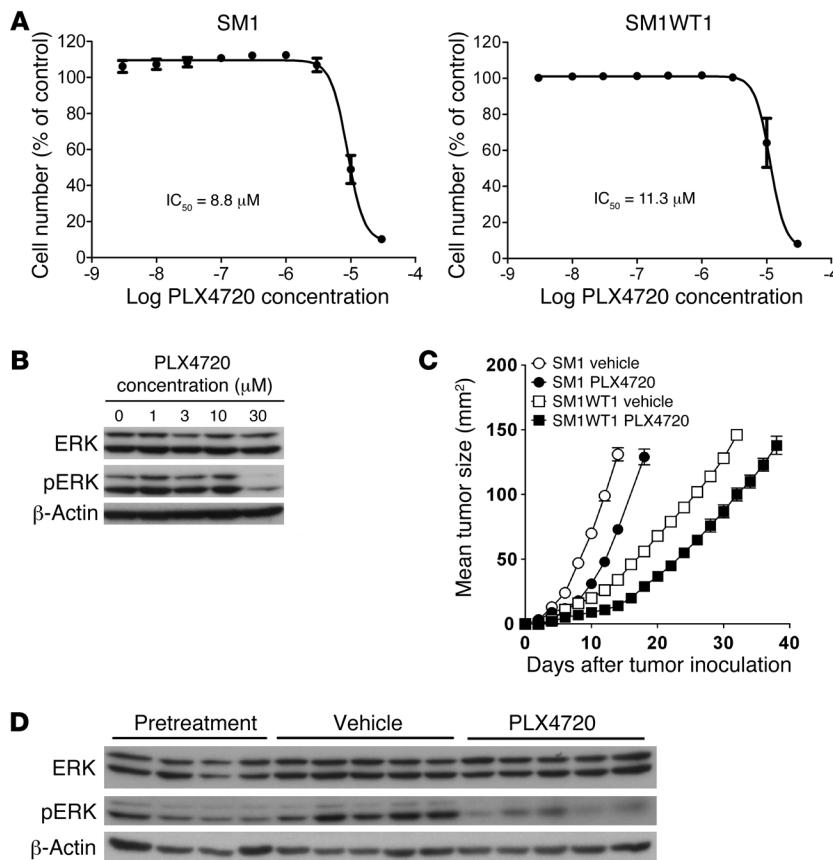
MAPK signaling induced by the BRAF<sup>V600E</sup> mutation. However, biopsies from some patients treated with BRAF inhibitors have increased CD8<sup>+</sup> T cell infiltrates in their tumors soon after therapy (13), suggesting the engagement of a host immune response in regressing tumors. The scientific rationale for combinations of targeted therapies and immunotherapy is based on the notion that pharmacological interventions with specific inhibitors of oncogenic events in cancer cells could sensitize cancer cells to immune attack, which has been termed immunosensitization (14). BRAF inhibitors meet most of the criteria of immune-sensitizing agents by selectively inhibiting a driver oncogene in cancer cells (15), which is neither present nor required for the function of lymphocytes (16). This results in rapid melanoma cell death in humans, as evidenced by a high frequency of early tumor responses in patients (1, 2), while sparing the function of lymphocytes (16). Theoretically, the antitumor activity of BRAF inhibitors may increase the expression of tumor antigens directly by tumor cells (17) or enhance the cross-presentation of tumor antigens from dying cells to antigen-presenting cells. Therefore, combining immunotherapy with BRAF inhibitors like vemurafenib or dabrafenib is supported by conceptual advantages and emerging experiences (13, 16, 17) that warrant the testing of such combinations in mouse models.

Until recently, there was no model of transplantable, syngeneic BRAF<sup>V600E</sup>-driven mouse melanoma in immunocompetent C57BL/6 mice (18, 19). To examine the efficacy of combining BRAF inhibitors with immunotherapies, we have used the relatively BRAF inhibitor-resistant SM1 cell line derived from mice

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**Figure 1**

PLX4720 activity against melanoma in vitro and in vivo. **(A)** To determine proliferation  $IC_{50}$  in vitro,  $BRAF^{V600E}$  mutant SM1 and SM1WT1 melanoma cells were seeded in 96-well plates and allowed to proliferate for 48 hours. Cells were then treated with a range of PLX4720 concentrations ( $n = 3$ ). After 72 hours, cell number was determined using the sulforhodamine B method.  $IC_{50}$  concentrations were determined using nonlinear regression (mean  $\pm$  SEM). **(B)** PLX4720 and target pathways. SM1WT1 mouse melanoma cells were treated with 1 to 30  $\mu M$  PLX4720 for 6 hours, after which cells were harvested and lysed ( $n = 3$ ). Proteins were separated by SDS-PAGE, and ERK and pERK protein bands were visualized by immunoblotting ( $\beta$ -actin was used as a loading control). **(C)** PLX4720 is active in vivo against SM1 and SM1WT1 melanomas. Groups of 5 WT mice were inoculated with  $2 \times 10^6$  SM1 cells or  $5 \times 10^5$  SM1WT1 cells. Mice received vehicle or PLX4720 (20 mg/kg i.p.) daily from day 3 to 6 or day 3 to 10 after tumor inoculation, respectively. Tumor sizes are represented as the mean  $\pm$  SEM. Data are representative of 2 independent experiments. **(D)** Tumors were harvested from mice prior to drug treatment or after 4 days of PLX4720 treatment. Tumor cell lysates were prepared and proteins were separated by SDS-PAGE. ERK and pERK protein bands were visualized by immunoblotting ( $\beta$ -actin was used as a loading control). Each lane corresponds to an individual tumor.

transgenic for the  $BRAF^{V600E}$  mutation. This approach has allowed us to test the role of host pathways in the mechanism of action of BRAF inhibitors and to combine BRAF inhibitors with various antibody therapies designed to drive T cell antitumor activity in a model in which BRAF inhibition does not cause major tumor regressions, allowing examination of synergistic roles of host pathways and direct anti-melanoma activity. For these investigations, we used PLX4720, an analog of vemurafenib, with virtually indistinguishable activity against BRAF, compared with other BRAF inhibitors, such as vemurafenib or dabrafenib. For what we believe to be the first time, we show that targeting oncogenic BRAF down-

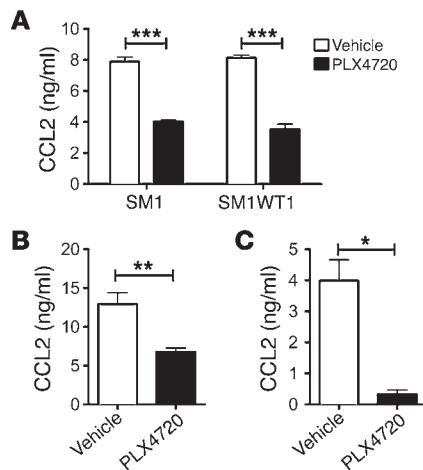
regulates tumor CCL2 gene expression and production. PLX4720 treatment reduces tumor CCL2 in both  $BRAF^{V600E}$  mouse melanoma transplants and melanomas induced in  $Braf^{CA}$  Tyr-creERT<sup>2</sup>Pten<sup>f/f</sup> mice. This suppression of tumor CCL2 is coincident with an increased intratumor CD8<sup>+</sup> T/FoxP3<sup>+</sup>CD4<sup>+</sup> T cell ratio and reduced tumor growth. Functionally, this PLX4720 antitumor activity requires, in part, host CCR2 and CD8<sup>+</sup> T cells. Combination therapy with PLX4720 or anti-CCL2 and agonistic anti-CD137 antibodies demonstrated significant antitumor activity in transplant and de novo melanoma tumorigenesis models. Scheduling and dosing of PLX4720 was important in the efficacy of the PLX4720 and anti-CD137 combination.

## Results

**SM1 and SM1WT1 are relatively PLX4720-resistant  $BRAF^{V600E}$  mutant melanomas.** We first tested the antitumor effects of single-agent PLX4720 against  $BRAF^{V600E}$  mutant SM1 and SM1WT1 melanoma cell lines by in vitro cell proliferation assay (Figure 1A). The SM1 and SM1WT1 cell lines were relatively resistant to PLX4720, with  $IC_{50}$  of 8.8  $\mu M$  and 11.3  $\mu M$ , respectively. This relative resistance was compared with that of a series of sensitive  $BRAF^{V600E}$  human melanomas with  $IC_{50}$  ranging from 12.3 nM (MALME-3M) to 2.0  $\mu M$  (HS294T) (Supplemental Table 1 and Supplemental Methods; supplemental material available online with this article; doi:10.1172/JCI66236DS1) and the  $IC_{50}$  of >200  $\mu M$  reported for the  $BRAF^{WT}$  M202 cell line (19). Despite this relative resistance to PLX4720, the targeting of the pathway in SM1WT1 cells was validated in vitro by Western analysis, demonstrating loss of pERK at the highest concentrations of the drug (Figure 1B). Since the objective was to evaluate PLX4720's mechanism of action and combinatorial antitumor activity in vivo, we next examined the therapeutic effect of PLX4720 against SM1 and SM1WT1 melanomas transplanted into syngeneic C57BL/6 WT mice (Figure 1C). Both SM1 and SM1WT1 melanomas established subcutaneously responded to early treatment with single-agent PLX4720 compared with

vehicle control, but the drug was ultimately ineffective in maintaining tumor suppression. An on-target effect in vivo was further validated by demonstrating loss of pERK in the tumors of mice receiving PLX4720 compared with those receiving vehicle (Figure 1D). We reasoned that the intermediate sensitivity of SM1 and SM1WT1 in vivo would allow us to test both the mechanism of action of PLX4720 and the potential beneficial effects of treating relatively resistant melanoma with additional mAb-based immunotherapies that improve T cell antitumor function.

**PLX4720 suppresses melanoma-derived CCL2, and PLX antitumor activity is host CCR2 dependent but CCL2 independent.** Chemokine/



**Figure 2**

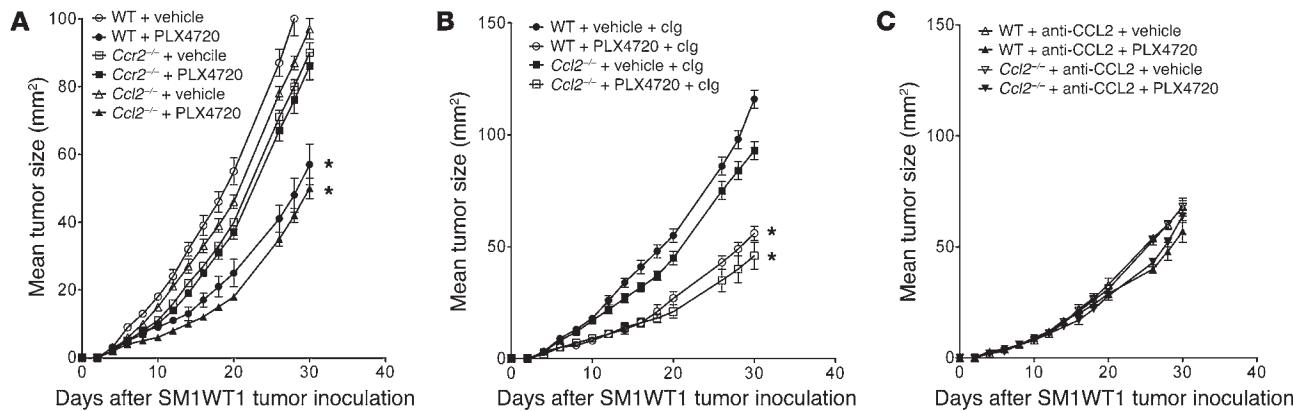
PLX4720 suppresses tumor CCL2 release. After 18 to 24 hours in vitro culture, supernatants from in vitro culture were collected for CCL2 analysis. Supernatant concentrations of CCL2 are presented. (A)  $5 \times 10^4$  SM1 and SM1WT1 cells were cultured in the presence of vehicle or 10  $\mu$ M PLX4720. Experiments were performed in replicates of 5 wells. (B) Groups of B6 WT mice ( $n = 5$ –6) were inoculated with  $1 \times 10^6$  SM1WT1 cells. Mice received vehicle or PLX4720 (20 mg/kg i.p.) daily from day 12 to 15 after tumor inoculation. At day 16, tumors were excised and tumor single cell suspensions were prepared. (C) Groups of Braf<sup>V600E</sup> transgenic mice ( $n = 6$ –7) were induced for localized melanoma. Mice received vehicle or PLX4720 (20 mg/kg i.p.) daily from day 28 to 49 after 4-HT application. At day 49, tumors were excised and tumor single cell suspensions were prepared. (A and B)  $1 \times 10^5$  tumor cells suspended in 100  $\mu$ l volume were plated. (B and C) Experiments were performed in 1 well per tumor. (A) Statistical differences in CCL2 concentrations between vehicle- or PLX4720-treated SM1 and SM1WT1 cell lines were determined by an unpaired  $t$  test ( $***P < 0.001$ ). (B) Statistical differences in CCL2 concentrations between vehicle- or PLX4720-treated SM1WT1 tumors were determined by an unpaired  $t$  test ( $**P < 0.01$ ). (C) Statistical differences in CCL2 concentrations between vehicle- or PLX4720-treated Braf<sup>V600E</sup> transgenic tumors were determined by an unpaired  $t$  test ( $*P < 0.05$ ). (A–C) Data shown are representative of 2 independent experiments (mean  $\pm$  SEM).

chemokine receptor interactions can protect against or promote tumor growth and metastasis, including melanoma (20, 21). A global assessment of cytokine and chemokine genes in SM1 tumor cells regulated by PLX4720 (S. Mok, A. Cass, T.G. Graeber, and A. Ribas, unpublished observations) and our PCR analysis revealed a marked reduction in CCL2 gene expression in SM1 and SM1WT1 cells treated with PLX4720 (Supplemental Figure 1). An AKT inhibitor was without similar effect (Supplemental Figure 1). SM1 and SM1WT1 melanomas were both negative for CCR2 by flow cytometry analysis (data not shown). Specific analysis of CCL2 production by SM1 and SM1WT1 melanomas exposed to PLX4720 in vitro revealed a significant reduction in CCL2 release (Figure 2A). This reduction in CCL2 production was further validated ex vivo following PLX4720 treatment in WT C57BL/6 mice transplanted with SM1WT1 tumors (Figure 2B) and in Braf<sup>CA</sup>Tyr-creER<sup>T2</sup>Pten<sup>fl/fl</sup> mice with melanomas (Figure 2C). To examine whether CCL2/CCR2 may play a role in the antitumor activity of PLX4720, we performed a series of experiments examining PLX4720 activity in WT, *Ccl2*<sup>-/-</sup>, and *Ccr2*<sup>-/-</sup> mice (Figure 3). Notably, PLX4720 was sim-

ilarly active in WT and *Ccl2*<sup>-/-</sup> mice but far less effective in *Ccr2*<sup>-/-</sup> mice (Figure 3A). These data indicated that host CCR2, but not CCL2, was necessary for the antitumor activity of PLX4720. Given that tumor CCL2 might normally interact with host CCR2, it was possible that it was functionally important to PLX4720 antitumor activity. To compare the role of tumor and host CCL2, we treated mice with PLX4720 and neutralized some groups with anti-CCL2 (blocking tumor- and host-derived CCL2) (Figure 3, B and C). In this instance, PLX4720 was not active against SM1WT1 tumors in WT or *Ccl2*<sup>-/-</sup> mice neutralized for CCL2. These data, in concert with the results in Figure 3A, strongly suggest that PLX4720 inhibition of tumor CCL2 release is a major part of the mechanism of action of PLX4720 against the partially resistant SM1WT1 melanoma. Similar data were obtained with the SM1 melanoma (data not shown). An analysis of infiltrating leukocytes in SM1WT1 tumors also revealed that CCR2<sup>+</sup> cells were predominantly CD11b<sup>+</sup> cells and CD4<sup>+</sup> Tregs (Figure 4), whereas CCR2<sup>+</sup> expression was almost absent on other T/NK lymphocytes. Overall, these data suggested that a PLX4720-mediated reduction of tumor CCL2 release might impact on the recruitment of these CCR2<sup>+</sup> tumor-infiltrating leukocytes (TILs) (22).

**PLX4720 enhances intratumor CD8/CD4 T cell ratio and proportion of NK cells.** Since production of CCL2 by tumor cells has been shown to be important for recruitment of leukocytes, including monocytes and macrophages, to the tumor microenvironment (23, 24), we next assessed the TILs in SM1WT1 tumors in the absence or presence of PLX4720 treatment (Figure 5 and Supplemental Figure 2). Flow analysis determined a significant enrichment in the proportion and number of NK cells (NK1.1<sup>+</sup> TCR $\beta$ <sup>+</sup>) (Figure 5A) and CD8<sup>+</sup> T cells (CD8<sup>+</sup> TCR $\beta$ <sup>+</sup>) (Figure 5C) following PLX4720 therapy. No significant changes were observed in the frequency of total T cells (NK1.1<sup>+</sup> TCR $\beta$ <sup>+</sup>) (Figure 5B) or CD4<sup>+</sup> T cells (CD4<sup>+</sup> TCR $\beta$ <sup>+</sup>) (Figure 5D) between vehicle- and PLX4720-treated tumors. Interestingly, we observed a reduction in the frequency of intratumor Tregs (CD4<sup>+</sup> Foxp3<sup>+</sup>) following PLX4720 therapy (Figure 5E) but no significant change in the frequency of intratumor CD11b<sup>+</sup> Gr-1<sup>+</sup> myeloid cells (Figure 5F). These data, in concert with the results in Figure 4, suggested that a reduction of tumor CCL2 release after PLX4720 therapy might selectively reduce the migration of CD4<sup>+</sup> Tregs into the tumor. Paradoxically, while PLX4720 treatment did not alter the expression of CCR2 on CD11b<sup>+</sup> myeloid cells (that coexpressed F4/80 and CCR2; data not shown), it did increase the frequency of CCR2<sup>+</sup> Tregs in the tumor (Supplemental Figure 3). Consequently, we observed a significant increase in the intratumor CD8<sup>+</sup> T/Treg ratio in the PLX4720-treated SM1WT1-bearing mice (Figure 5G). These data indicated that the suppression of SM1WT1 tumors in PLX4720-treated mice was associated with a reduction of the Tregs and enrichment of CD8<sup>+</sup> T cells and NK cells within the tumors.

**PLX4720 does not directly alter immune target molecules on SM1 or SM1WT1 cells.** A previously postulated mechanism of improved antitumor activity of combining BRAF-targeted therapy with immunotherapy was an increase in tumor antigen or MHC expression by cancer cells (17). This mechanism may also explain the functional role of CD8<sup>+</sup> T cells in PLX4720 antitumor activity against SM1WT1 melanoma in vivo. Therefore, we tested whether SM1 and SM1WT1 exposure to PLX4720 increased the expression of surface MHC molecules, costimulatory molecules, and other surface molecules that might increase the sensitivity of these cells to CD8<sup>+</sup> T cell attack. Neither melanoma cell line expressed MHC



**Figure 3**

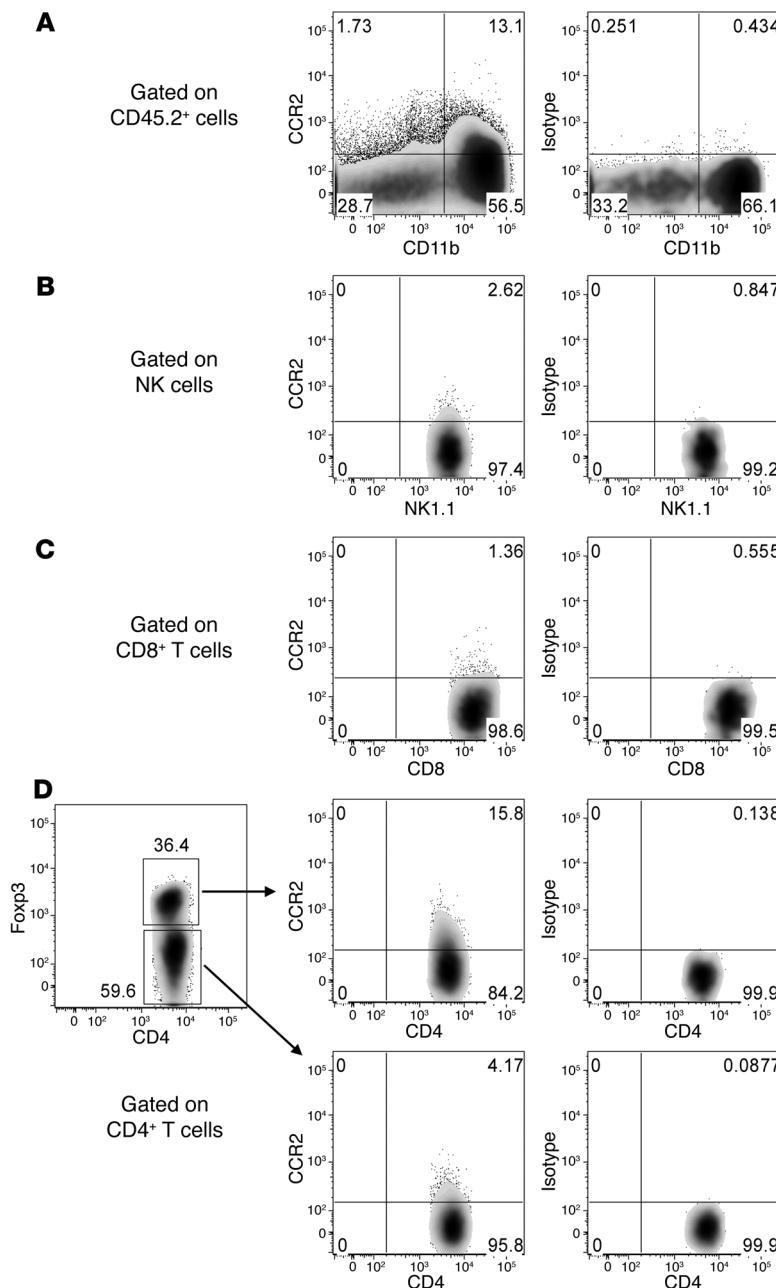
PLX4720 antitumor activity is host CCR2 dependent. **(A)** Groups of 5 WT, *Ccl2*<sup>−/−</sup>, or *Ccr2*<sup>−/−</sup> mice were inoculated with  $5 \times 10^5$  SM1WT1 cells. Mice received vehicle or PLX4720 (20 mg/kg i.p.) daily from day 3 to 10 after tumor inoculation. **(B and C)** Groups of 5 WT or *Ccl2*<sup>−/−</sup> mice were inoculated with  $5 \times 10^5$  SM1WT1 cells. Mice received vehicle or PLX4720 (20 mg/kg i.p.) daily from day 3 to 10 after tumor inoculation. Some groups of mice additionally received clg or anti-CCL2 mAb (20  $\mu$ g i.p.) on days 2, 3, 10, 17, and 24. Tumor sizes are represented as the mean  $\pm$  SEM. Statistical differences in tumor sizes between mice treated with vehicle or PLX4720 therapy were determined by a Mann-Whitney test (\* $P < 0.05$ ). **(A–C)** Data are representative of 2 independent experiments.

class I, CD80, CD86, or NKG2D ligands detected by anti-pan Rae-1, but both did express marked levels of the DNAM-1 ligand, CD155; the nonclassical MHC, CD1d; and PD-L1. SM1 and SM1WT1 also expressed high levels of surface death receptors, Fas and DR5. Remarkably, none of these markers were significantly regulated by PLX4720 exposure for 24 hours (Supplemental Figure 4) or longer (data not shown), other than a small upregulation of SM1WT1 Rae-1 ligands at the highest dose of PLX4720 examined. Overall, these data suggest that PLX4720 does not directly alter immune target molecules on SM1/SM1WT1 melanomas, although we did not directly examine specific antigen expression on these cells (17).

**PLX4720 antitumor activity is CD8<sup>+</sup> T cell dependent.** Given the increase in the proportion of intratumor CD8<sup>+</sup> T cells and NK cells in SM1WT1 tumors, we next assessed the functional importance of various leukocyte subsets to the antitumor activity of PLX4720. By using antibodies to specifically deplete T and NK cell subsets it was clear that CD8<sup>+</sup> T cells, but not CD4<sup>+</sup> T cells or NK cells, were in part key for optimal antitumor activity of PLX4720 (Figure 6A). These data were supported by similar experiments using WT mice depleted of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells and other gene-targeted mice lacking both T and B cells (*Rag1*<sup>−/−</sup>), B cells alone ( $\mu$ MT), or  $\gamma\delta$ T cells (*Tcrd*<sup>−/−</sup>) (Supplemental Figure 5, A and B), where only the loss of CD8<sup>+</sup> T cells appeared critical. Since CD8<sup>+</sup> T cells appeared important, we additionally tested the role of their key effector molecules, IFN- $\gamma$  and perforin (pfp). Mice deficient in pfp or IFN- $\gamma$  alone responded to PLX4720 treatment like WT mice, but those *Pfp*<sup>−/−</sup> mice additionally neutralized for IFN- $\gamma$  appeared less sensitive to PLX4720 antitumor activity (Figure 6B). These data suggested some redundancy in lymphocyte effector functions, such that a combination of perforin cytotoxic activity and IFN- $\gamma$  effector function collectively contributed to the optimal antitumor activity of PLX4720. Despite the significant Fas and DR5 expression of SM1WT1 melanomas, mice treated with PLX4720 and mutant for FasL (gld) or that were deficient in TRAIL responded similarly to WT mice (Figure 6C). The DR5 and Fas pathways were operational in the SM1WT1 cells, as evidenced by their sensitivity

to effector cells expressing TRAIL or anti-Fas antibodies, respectively (Supplemental Figure 6).

**PLX4720 combines with anti-CD137 to promote CD8<sup>+</sup> T cell-dependent activity.** The increase in the intratumor CD8/Treg ratio after PLX4720 treatment and the functional role of CD8<sup>+</sup> T cells in PLX4720 antitumor activity prompted us to examine whether PLX4720 might be effective in combination with T cell-based immunotherapies. Recently, Koya et al. described the combined effect of adoptive T cell transfer (ACT) and vemurafenib against SM1 and SM1-OVA melanomas in vivo (19). However, a number of mAb-based immunotherapies that promote CD8<sup>+</sup> T cell antitumor function are also now attracting significant preclinical and clinical interest (25–29). Among these, we were interested to examine mAbs reactive with CTLA-4, PD-1, Tim3, and CD137. We have previously assessed these mAbs alone and in combination against a number of transplanted tumors, including melanoma, as well as in a de novo model of sarcomagenesis (30–33). Therefore, we tested each mAb against SM1 alone (Figure 7A) and in combination with PLX4720 (Figure 7B). With the impact of PLX4720 on T and NK cell infiltration into tumors previously defined, we first opted for a daily schedule of PLX4720 (from day 7 to 11) that was followed by 4 doses of each mAb over 6 days. This experiment illustrated the significant antitumor activity of anti-CD137 mAb alone and the comparatively weaker effect of anti-CTLA-4, anti-Tim3, or anti-PD-1 against the established SM1WT1 melanoma (Figure 7A). More strikingly, the antitumor activity of anti-CD137 was significantly enhanced with prior PLX4720 treatment, with the majority of WT mice rejecting the established SM1WT1 tumor (Figure 7B). By contrast, the antitumor activity of the other mAbs was not significantly enhanced by prior PLX4720 therapy. Additional experiments were performed with the combination of PLX4720 and anti-CD137, and CD8<sup>+</sup> T cells and IFN- $\gamma$  were found to be critical for synergistic activity (Figure 7, C and D). Coincident scheduling of PLX4720 and anti-CD137 treatments also resulted in a significant synergistic antitumor activity against SM1WT1 tumors, while pretreatment with anti-CD137 followed


**Figure 4**

CCR2<sup>+</sup> TILs. A group of B6 WT mice ( $n = 10$ ) was inoculated with  $1 \times 10^6$  SM1WT1 cells. At day 21, tumors were excised and FACS analyses were performed on TILs. Frequencies of CCR2<sup>+</sup> cells in (A) CD11b<sup>+</sup> cells, (B) NK cells, (C) CD8<sup>+</sup> T cells, and (D) CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs (top 2 right-most images) and CD4<sup>+</sup>Foxp3<sup>-</sup> T cells (bottom 2 images) from TILs are shown. The data shown for CCR2<sup>+</sup> cells are FACS plots concatenated from 10 individual mice.

$P = 0.0004$ ). Interestingly, the effectiveness of anti-CD137 therapy was also demonstrated, but it had a lower efficacy in suppressing melanoma growth in the *Braf<sup>CA</sup>Tyr-creER<sup>T2</sup>Pten<sup>f/f</sup>* model. The reduced efficacy of anti-CD137 against these de novo melanomas correlated with a lower frequency of CD8<sup>+</sup> T cells naturally infiltrating the tumors ( $4.41\% \pm 1.93\%$ ) when compared with SM1WT1 tumors (Figure 5). Encouragingly, however, the combination of PLX4720 and anti-CD137 showed a trend for further improved activity over either treatment alone (Figure 8).

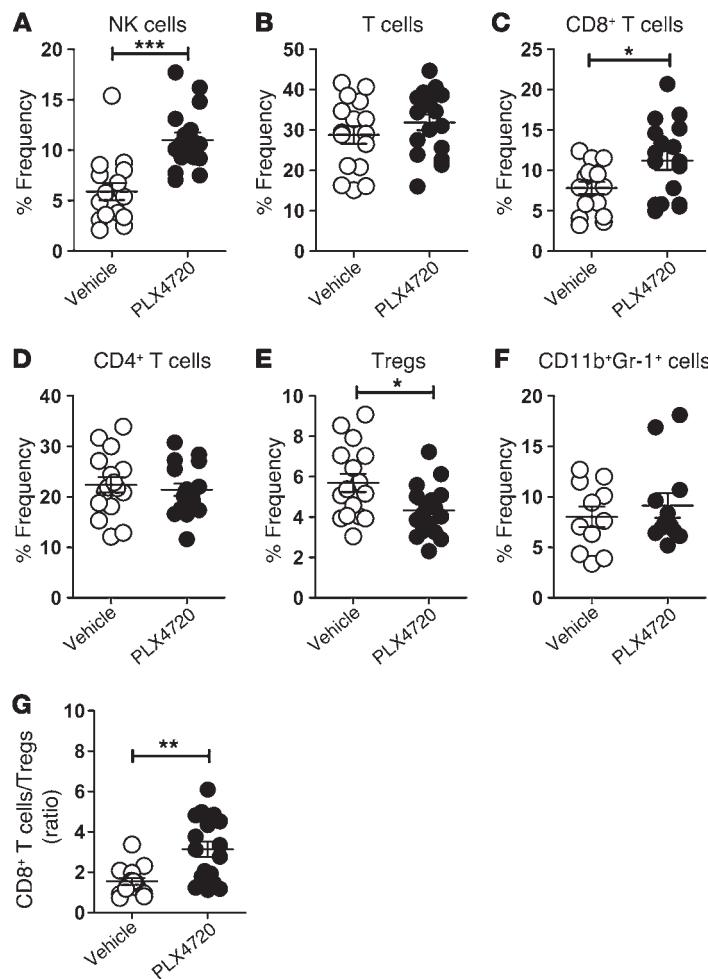
**Anti-CCL2 also combines with anti-CD137 antitumor activity.** Since PLX4720 in part reduced SM1WT1 tumor growth by suppressing CCL2 production, we predicted that anti-CCL2 might also effectively combine with anti-CD137 against this melanoma transplant. Indeed, a combination of anti-CCL2 and anti-CD137 was also very effective in suppressing established SM1WT1 tumor growth compared with either treatment alone (Figure 9). Notably, the PLX4720 and anti-CD137 combination was superior, indicating that, as would be predicted, PLX4720 can inhibit tumor growth by mechanisms in addition to reducing tumor-derived CCL2.

## Discussion

We have used two relatively resistant syngeneic variants of *BRAF<sup>V600E</sup>*-driven mouse melanoma, SM1 and SM1WT1, and a transgenic mouse model of melanoma to illustrate the ability of the type I BRAF inhibitor, PLX4720, to reduce melanoma CCL2 production. Gene array and chemokine analysis of treated SM1 melanoma variants revealed targeting of oncogenic BRAF and downregulation of tumor CCL2 gene expression and production. The key role of host CCR2, but not CCL2, was demonstrated in the antitumor activity of PLX4720. While there was no obvious regulation of immune target molecules on SM1WT1 tumors after PLX4720 treatment, analysis of SM1WT1 TILs in mice treated with PLX4720 demonstrated a robust increase in the CD8<sup>+</sup> T cell/Treg ratio and frequency of NK cells. Functionally consistent with this observation, CD8<sup>+</sup> T cells, but not NK cells, were partially required for the therapeutic activity of PLX4720. Our data are complementary to a very recent study that described the role of oncogenic *BRAF<sup>V600E</sup>* in promoting stromal immunosuppression via induction of IL-1 in melanomas (34). Here we broaden these findings to highlight the role of a tumor chemokine, CCL2, and host CCR2, in the mechanism of action of BRAF inhibitors. Furthermore, a dose- and

by PLX4720 failed to enable any significant additional benefit over anti-CD137 alone (Supplemental Figure 7, A–C). A similar combinatorial synergy was observed for PLX4720 and anti-CD137 mAb against the more aggressive parental SM1 melanoma (Supplemental Figure 8). Reducing the dose of PLX4720 to 5 mg/kg per injection significantly reduced the combined activity of PLX4720 and anti-CD137 using the optimal scheduling, indicating that the dose effect of PLX4720 was important (Supplemental Figure 9).

Importantly, we next tested the efficacy of PLX4720 and anti-CD137 in *Braf<sup>CA</sup>Tyr-creER<sup>T2</sup>Pten<sup>f/f</sup>* mice, in which 4-hydroxytafamoxifen (4-HT) induces de novo melanomas. In contrast to the transplant SM1 or SM1WT1 tumor models, 4-HT-induced de novo melanomas were significantly suppressed (tumor height or weight at sacrifice on day 49) with PLX4720 therapy alone (Figure 8;

**Figure 5**

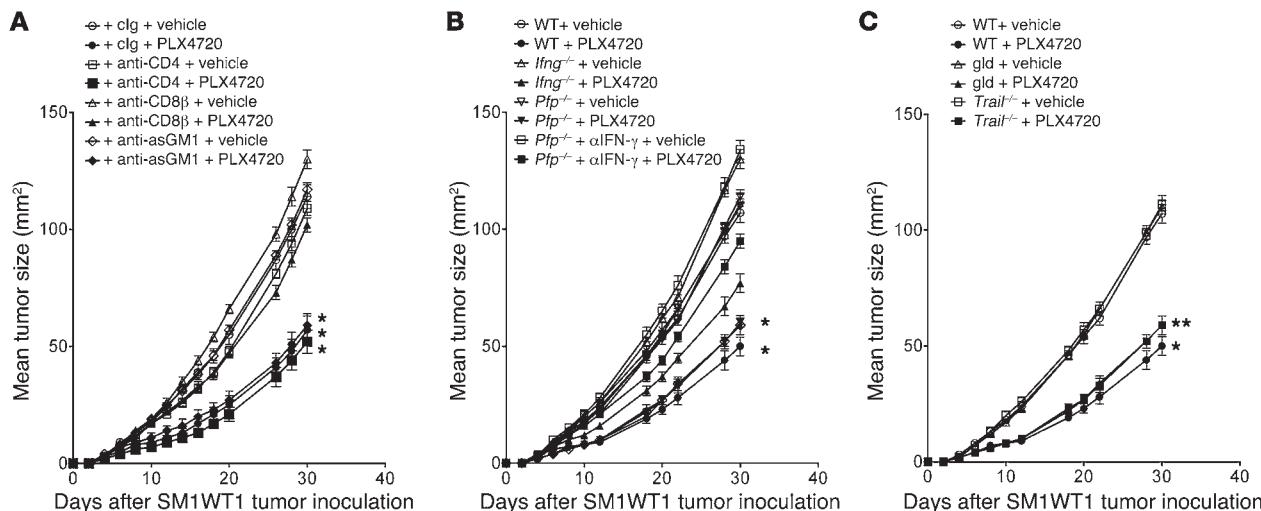
PLX4720 enhances the intratumor CD8<sup>+</sup> T cell/Treg ratio and proportion of NK cells. Groups of B6 WT mice ( $n = 5$ –6) were inoculated with  $1 \times 10^6$  SM1WT1 cells. Mice received vehicle or PLX4720 (20 mg/kg i.p.) daily from day 12 to 20 after tumor inoculation. At day 21, tumors were excised, and FACS analyses were performed on TILs. Frequencies of (A) NK cells, (B) total T cells, (C) CD8<sup>+</sup> T cells, (D) CD4<sup>+</sup> T cells, (E) CD4<sup>+</sup>FoxP3<sup>+</sup> Tregs, and (F) CD11b<sup>+</sup> Gr-1<sup>+</sup> cells, gated on (A–E) CD45.2<sup>+</sup> or (F) CD45.2<sup>+</sup> CD3<sup>–</sup> CD19<sup>–</sup> TILs from vehicle- or PLX4720-treated mice, are shown. (G) The CD8<sup>+</sup> T/Treg ratio calculated from C and E as shown. Statistical differences in (A–F) frequencies of respective cell subsets or (G) cell ratios between mice treated with vehicle or PLX4720 therapy were determined by an unpaired *t* test (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ). Data shown are pooled from (F) 2 or (A–E and G) 3 independent analyses. Individual symbols represent individual mice; horizontal bars indicate the mean. Live CD45.2<sup>+</sup> TIL numbers ranged from  $0.32 \times 10^3$  cells/mm<sup>2</sup> to  $2.86 \times 10^3$  cells/mm<sup>2</sup>, with no statistical significance observed between vehicle- or PLX4720-treated mice.

schedule-dependent combination therapy with PLX4720 and agonistic anti-CD137 antibody demonstrated significant antitumor activity in mouse transplant and de novo tumorigenesis models, illustrating the therapeutic potential of combining BRAF inhibitors with immunotherapy.

CCL2 is of particular importance in cancer development, since it serves as one of the key mediators of interactions between tumor and host cells (20, 21). CCL2 has been reported to promote cancer cell proliferation, migration, invasion, and survival via binding to its functional receptor CCR2. Furthermore, CCL2 reportedly induces the recruitment of macrophages and CTL and induces angiogenesis and matrix remodeling (35–37). Depending upon the level of expression of CCL2, this chemokine can either promote or reduce melanoma tumor growth (38–42). This type of dichotomy in the role of chemokines on the immune system may be tightly linked to the tumor microenvironment. There will be different outcomes depending upon whether the T cells that are recruited are capable of tumor cell killing or whether they are regulatory or promote tumor metastasis through release of factors that facilitate intravasation of tumor cells into the vascular system. Notably, the SM1 melanoma variants were CCR2 negative, and yet our data support an important role for melanoma CCL2 production in the natural development and growth of these tumors, since these tumors grew more slowly in untreated *Ccr2*<sup>–/–</sup> mice. Importantly, our results also showed that the BRAF inhibitor, PLX4720,

reduced melanoma CCL2 production in the transplant and *Braf*<sup>CA</sup> Tyr-creER<sup>T2</sup>Pten<sup>f1/f1</sup> mice and that its therapeutic efficacy was severely inhibited in the absence of host CCR2. We explored the potential mechanisms by which PLX4720 could improve the antitumor activity of mAb-based immunotherapies that promote T cells naturally reactive with and in the tumor. PLX4720 did increase the proportion of intratumor CD8<sup>+</sup> T cells relative to FoxP3<sup>+</sup>CD4<sup>+</sup> Tregs. It is generally recognized that this ratio is indicative of an effective cell-mediated immune response, and indeed, we demonstrated the functional importance of CD8<sup>+</sup> T cells in the antitumor activity of PLX4720 in the SM1WT1 melanoma model. We identified that CCR2 was expressed predominantly on a proportion of tumor-infiltrating CD11b<sup>+</sup> cells and CD4<sup>+</sup> Tregs and that only Tregs decreased in the tumor upon PLX4720 treatment. The exact nature of the host CCR2<sup>+</sup> cell responsible will require more complex genetics to delete this molecule specifically in CD11b<sup>+</sup> myeloid cells and FoxP3<sup>+</sup> T cell subsets.

We also illustrated the ability of a combination of anti-CCL2 and anti-CD137 to effectively suppress the growth of SM1WT1 melanomas. These data are in concert with previous reports, in which anti-mouse CCL2/CCL12 antibodies were shown in 3 different mouse tumor models to effectively combine with vaccines to reduce tumor volume and cure approximately half of the tumors, and the mechanism of tumor control was shown to involve CD8<sup>+</sup> T cells (43). Notably, however, the anti-CCL2/


**Figure 6**

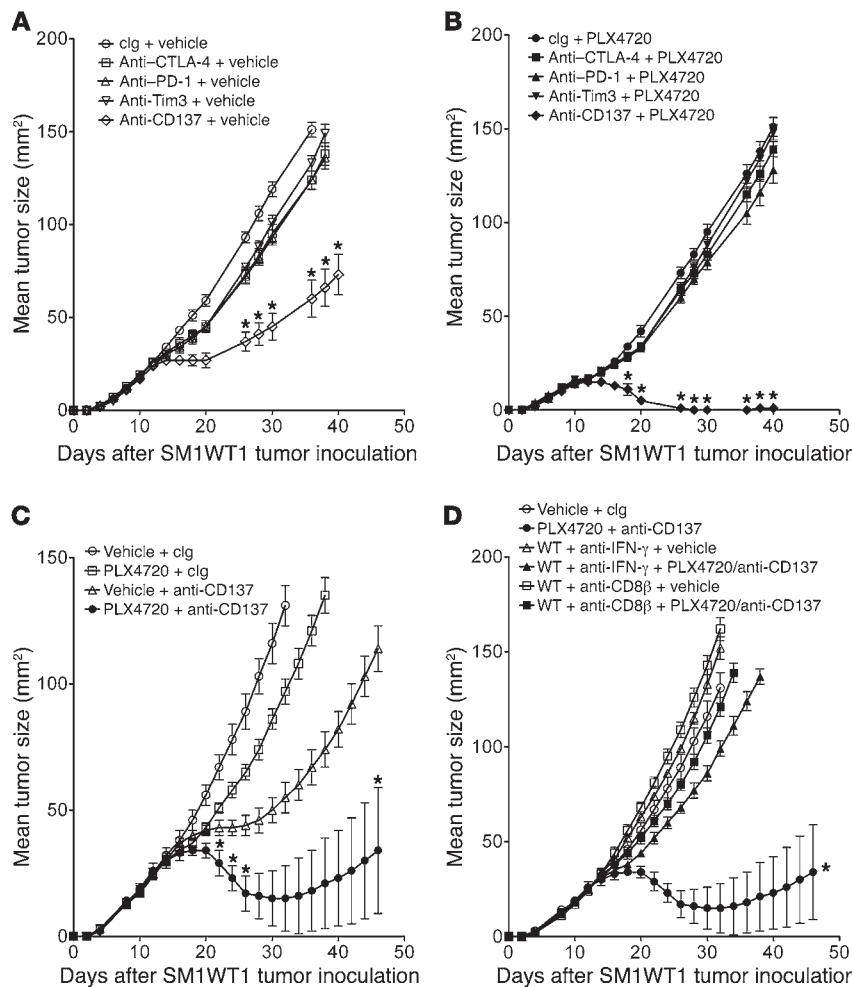
PLX4720 antitumor activity is CD8<sup>+</sup> T cell dependent. **(A)** Groups of 5 WT mice were inoculated with  $5 \times 10^5$  SM1WT1 cells. Mice received vehicle or PLX4720 (20 mg/kg i.p.) daily from day 3 to 10 after tumor inoculation. Some groups of mice were additionally treated with clg, anti-CD4, anti-CD8 $\beta$ , or anti-asialoGM1 (100  $\mu$ g i.p. each) on days 2, 3, 10, 17, and 24 after tumor inoculation to deplete T cell subsets or NK cells. **(B)** Groups of 5 WT, *Ifng*<sup>-/-</sup>, or *Pfp*<sup>-/-</sup> mice were inoculated with  $5 \times 10^5$  SM1WT1 cells. Mice received vehicle or PLX4720 (20 mg/kg i.p.) daily from day 3 to 10 after tumor inoculation. Some groups of *Pfp*<sup>-/-</sup> mice were additionally treated with anti-IFN- $\gamma$  (250  $\mu$ g i.p.) on days 2, 3, 10, 17, and 24 after tumor inoculation to neutralize IFN- $\gamma$ . **(C)** Groups of 5 WT, gld, or *Trail*<sup>-/-</sup> mice were inoculated with  $5 \times 10^5$  SM1WT1 cells. Mice received vehicle or PLX4720 (20 mg/kg i.p.) daily from day 3 to 10 after tumor inoculation. Tumor sizes are represented as the mean  $\pm$  SEM. Statistical differences in tumor sizes between mice treated with vehicle and those treated with PLX4720 therapy for each group were determined by a Mann-Whitney test (\* $P$  < 0.05; \*\* $P$  < 0.01). Data are representative of 2 independent experiments.

anti-CD137 combination was not as effective as PLX4720/anti-CD137 against transplanted SM1WT1 melanoma. The PLX4720/anti-CD137 combination also appeared a promising combination in the *Braf*<sup>CA</sup>Tyr-creERT<sup>2</sup>*Pten*<sup>fl/fl</sup> mice. Our data with the *Braf*<sup>CA</sup>Tyr-creERT<sup>2</sup>*Pten*<sup>fl/fl</sup> mice are the first to our knowledge to demonstrate any single-agent activity of an immunotherapy in this de novo melanomagenesis model. In concert with our findings, when treating similar transgenic mice with anti-CTLA-4, Hooijkaas et al. have reported no significant therapeutic activity (44). By contrast, they report a small decrease in the proportion of various intratumoral lymphocytes 2 and 21 days after PLX4720, but it is not clear in this study how the mock control tumors relate to the PLX4720-treated tumors (in size or days treatment). It is likely that the effects of PLX4720 on lymphocyte infiltrate may be dose, schedule, and tumor size and microenvironment dependent. Certainly, it is possible that the mechanism of improved combinatorial effects may be different in *BRAF*<sup>V600E</sup> mutant tumors with higher sensitivity to BRAF inhibitors. Thus far, we have illustrated that when PLX4720 is more effective as a single agent against de novo-established melanomas in *Braf*<sup>CA</sup>Tyr-creERT<sup>2</sup>*Pten*<sup>fl/fl</sup> mice, it also significantly reduces melanoma CCL2 expression. Future mechanism studies are required in transgenic mice to investigate the effect of the role of CCL2 in melanoma-prone *Braf*<sup>CA</sup>Tyr-creERT<sup>2</sup>*Pten*<sup>fl/fl</sup> mice.

Two approaches with high response rates for the treatment of patients with metastatic melanoma are BRAF inhibitors and lymphocyte ACT therapies with ex vivo-expanded melanoma-specific T cells (1, 45, 46). However, in both cases, tumors frequently relapse after an initial response (46, 47). The data from Koya et al. (19) support the combination of ACT with vemurafenib and provide a strong rationale to translate combined immunotherapy

and targeted therapy for patients with *BRAF*<sup>V600E</sup> mutant metastatic melanoma. Here our data compare the antitumor activity of several promising mAb-based immunotherapies (anti-CTLA-4, anti-PD-1, anti-Tim3, or anti-CD137) alone and in combination with PLX4720. Interestingly, obvious combined antitumor activity was observed between PLX4720 and anti-CD137, whereas no obvious combinatorial activity was noted when using anti-CTLA-4, anti-PD-1, or anti-Tim3. This distinction may relate to the relative lack of efficacy of anti-CTLA-4, anti-PD-1, and anti-Tim3 compared with anti-CD137 or the relative expression of target molecules in the tumor microenvironment in this specific SM1WT1 model. Certainly, we have illustrated the importance of PLX4720 scheduling and dose in generating combination effects with anti-CD137. Whether the use of checkpoint inhibitors, such as anti-PD-1 and anti-CTLA-4, requires different scheduling or other models of melanoma requires further investigation. On the basis of natural immune reaction to melanomas and the ability of PLX4720 to reduce melanoma CCL2 expression, there is no strong theoretical argument to ignore any agent that promotes intratumor CD8<sup>+</sup> T cell function. Certainly, anti-CTLA-4 (ipilimumab) (26) and anti-PD-1/PD-L1 (28, 29) are making a significant impact in the treatment of human malignant melanoma, and these agents will likely be evaluated in humans in combination with BRAF inhibitors.

In conclusion, the *BRAF*<sup>V600E</sup>-specific inhibitor PLX4720 alters intratumor CCL2 levels, altering the leukocyte profile of the tumor microenvironment and favoring CD8<sup>+</sup> T cell function. PLX4720 combines effectively with immunotherapies that mediate their antitumor activity via CD8<sup>+</sup> T cells, and overall, the data support the clinical testing of combinations of BRAF-targeted therapy and anti-CD137 mAb therapy for patients with advanced melanoma.

**Figure 7**

Synergistic antitumor activity of PLX4720 and anti-CD137. Groups of 5 WT mice were inoculated with  $5 \times 10^5$  SM1WT1 cells. Mice received (A) vehicle or (B) PLX4720 (20 mg/kg i.p.) daily from day 7 to 11 after tumor inoculation. Some groups of mice were additionally treated with clg, anti-CTLA-4, anti-PD-1, anti-Tim3, or anti-CD137 (250  $\mu$ g i.p. each) on days 12, 14, 16, and 18 after tumor inoculation. (C and D) Groups of 5 WT mice were inoculated with  $5 \times 10^5$  SM1WT1 cells. Mice received vehicle or PLX4720 (20 mg/kg i.p.) daily from day 12 to 16 after tumor inoculation and clg or anti-CD137 (100  $\mu$ g i.p.) on days 17, 19, 21, and 23 after tumor inoculation. Some groups of WT mice were additionally treated with anti-IFN- $\gamma$  (250  $\mu$ g i.p.) or anti-CD8 $\beta$  (100  $\mu$ g i.p.) on days 11, 12, 19, and 26 after tumor inoculation. Tumor sizes are represented as the mean  $\pm$  SEM. Statistical differences in tumor sizes between mice treated with control versus combination therapy for each group were determined by a Mann-Whitney test (\* $P$  < 0.05).

## Methods

### Mice

C57BL/6 WT (obtained from Walter and Eliza Hall Institute, Melbourne, Australia), C57BL/6 CCL2-deficient ( $Ccl2^{-/-}$ ) (48) (provided by Michael Hickey, Monash University, Melbourne, Australia), C57BL/6 CCR2-deficient ( $Ccr2^{-/-}$ ) (originally from The Jackson Laboratory), C57BL/6 perforin-deficient ( $Pfp^{-/-}$ ), C57BL/6 TRAIL-deficient ( $Trail^{-/-}$ ) (49), C57BL/6 gld mutant (gld), C57BL/6 IFN- $\gamma$ -deficient ( $Ifng^{-/-}$ ), C57BL/6  $Rag1^{-/-}$ , and C57BL/6  $Rag2^{-/-} \times gc^{-/-}$  mice were bred and maintained as previously described (50, 51). Mixed background (FVB/N  $\times$  C57BL/6) Tyr::CreERT<sup>2</sup> Braf<sup>CA</sup> Pten<sup>f/f</sup>

mice (Braf<sup>CA</sup> Tyr::CreERT<sup>2</sup> Pten<sup>f/f</sup> mice) were bred and maintained as previously described (52).

### Cell lines

The SM1 mouse melanoma was generated from a spontaneously arising tumor in *BRAF<sup>V600E</sup>* mutant transgenic mice (19). Sequencing of the hot spot *T1799A* mutation in *BRAF* demonstrated the presence of the *BRAF<sup>V600E</sup>* transversion in SM1 cells, and whole-genome copy number analysis demonstrated frequent deletions and amplifications consistent with human melanomas. In particular, SM1 has been shown to have a deletion of *CDKN2A* and amplification of *BRAF* and *MITF* genes (19). A variant of SM1, SM1WT1, was developed by subcutaneous injection of an *in vivo*-passaged SM1 tumor into B6 WT male mice, and this tumor was harvested, cut into small pieces, and digested to single cell suspension. These tumor cells were cultured in 10% FCS-supplemented RPMI 1640 complete media (2 mM L-glutamine [Gibco], 1% (v/v) penicillin, and streptomycin [Gibco]) in 5% CO<sub>2</sub>. After 6 passages, the SM1WT1 cell line was generated. When used *in vitro*, SM1 and the variant SM1WT1 were maintained in RPMI 1640 supplemented with complete media (Gibco).

### Reagents

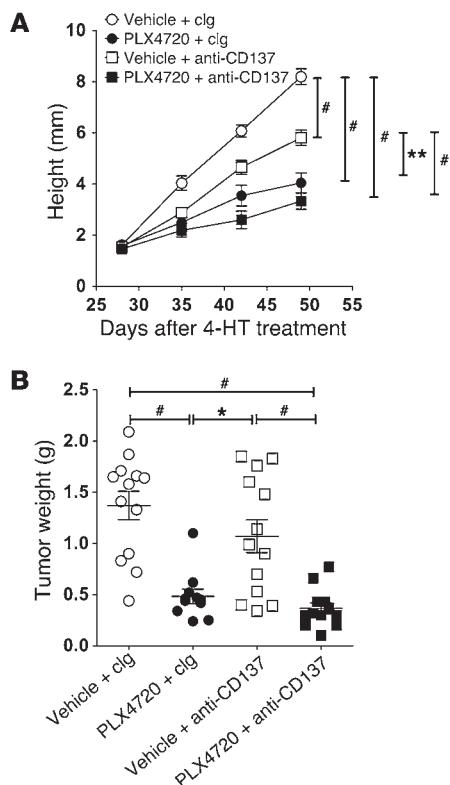
The type I BRAF inhibitor, PLX4720, was obtained from Plexxikon Inc. It was dissolved in DMSO (Calbiochem) and used for *in vitro* studies as previously described (53). For *in vivo* studies, PLX4720 was dissolved in DMSO, followed by PBS (100  $\mu$ l), which was then injected daily i.p. into mice at 20 mg/kg. This i.p. dosing regimen for PLX4720 has been demonstrated to have adequate pharmacokinetic parameters in blood (54). Anti-CTLA-4 (CD152) (antagonistic, UC10-4F10; provided by Jeffrey Bluestone, UCSF, San Francisco, California, USA), anti-PD-1 (CD279) (antagonistic, RMP1-14), anti-Tim3 (antagonistic, RMT3-23), anti-CD137 (agonistic, 3H3), and control Ig (cIg; Mac-4) were generated as previously described (30, 31) and used at the indicated schedule/dosage. Some mice were depleted of T or NK cell subsets with anti-CD4 (GK1.5), anti-CD8 $\beta$  (53.5.8), or anti-asialoGM1 (Wako Pure Chemical) or neutralized for IFN- $\gamma$  (H22) or CCL2 (123616) as previously described (32, 55).

### Cell proliferation assays

Cell proliferation assays were prepared in 96-well plates (5 replicate wells per drug concentration). Plates were incubated for 48 hours prior to drug treatment and treated with a range of PLX4720 concentrations for a further 72 hours. PLX4720 stock solutions were prepared in DMSO, stored at -20°C, and diluted as required. Cell number was determined at the end of drug treatment using the sulforhodamine B method described previously (56).

### SDS-PAGE and Western immunoblotting

For cultured cell lysates, cells were seeded in 10-cm tissue culture dishes and incubated for 24 hours prior to drug treatment, after which plates



were treated with various PLX4720 concentrations for 6 hours. Cells were washed with ice-cold PBS and lysed in ice-cold RIPA buffer containing a protease inhibitor cocktail (Roche), sodium fluoride, sodium vanadate, and PMSF. For tumor xenograft lysates, tumors were harvested from mice prior to drug treatment or after 4 days of PLX4720 treatment and snap frozen in liquid nitrogen. Tumors were crushed in liquid nitrogen and lysed using RIPA lysis buffer containing a protease inhibitor cocktail (Roche), sodium fluoride, sodium vanadate, and PMSF. Protein concentrations were determined using the DC assay (Bio-Rad). Cell lysates (15 µg protein) were separated on 12% bis-tris SDS-polyacrylamide gels, and proteins transferred to polyvinylidene fluoride using a semidry transblotter (Bio-Rad). Membranes were probed with anti-p44/42-MAPK (ERK; CS9102) and anti-phospho-p44/42-MAPK (ERK-Thr202/Tyr204; CS9101) primary antibodies (purchased from Cell Signaling Technology), and even loading was confirmed using an anti-actin primary antibody (08691002; purchased

**Figure 8**

Combination antitumor activity of PLX4720/anti-CD137 against de novo BRAF<sup>V600E</sup>-driven mouse melanomas. Groups of Braf<sup>V600E</sup> transgenic mice ( $n = 6$ –7) were induced for localized melanoma by 4-HT application on day 0. Mice received vehicle or PLX4720 (20 mg/kg i.p.) daily from day 28 to 49. Some groups of mice additionally received clg or anti-CD137 (100 µg i.p.) on days 28, 32, 36, 40, 44, and 48. (A) At the indicated time, tumor sizes (height; mm) were recorded and represented as the mean  $\pm$  SEM. Statistical differences in tumor sizes between different groups of mice were determined by a Mann-Whitney test (\*\* $P < 0.01$ ; # $P < 0.0001$ ). (B) At day 49, tumors were excised and weighed. Statistical differences in tumor weights between different groups of mice were determined by a Mann-Whitney test (\* $P < 0.05$ , PLX4720 and clg compared with vehicle and anti-CD137; # $P < 0.001$ , vehicle and clg compared with PLX4720 and clg [ $P = 0.0004$ ] or PLX4720 and anti-CD137 [ $P = 0.0001$ ] and vehicle and anti-CD137 compared with PLX4720 and anti-CD137 [ $P = 0.0008$ ]). Data shown are pooled from 2 independent experiments. Individual symbols represent individual tumors; horizontal bars indicate the mean.

**Figure 9**

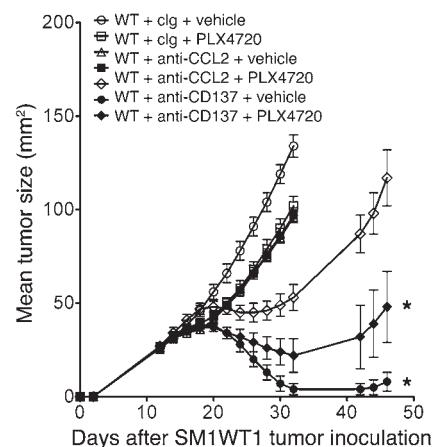
Anti-CCL2 and anti-CD137 also suppress SM1WT1 tumor growth. Groups of 5 WT mice were inoculated with  $5 \times 10^5$  SM1WT1 cells. Mice received a first treatment course of vehicle or PLX4720 (20 mg/kg i.p.) daily from day 12 to 16 or clg or anti-CCL2 (20 µg i.p.) on days 12, 14, 16, and 18 after tumor inoculation. This was sequentially followed by clg or anti-CD137 (100 µg i.p.) on days 17, 19, 21, and 23 after tumor inoculation. Tumor sizes are represented as the mean  $\pm$  SEM. Statistical differences in tumor sizes between mice treated with control versus combination therapy for each group were determined by a Mann-Whitney test (\* $P < 0.05$ ).

from MP Biomedicals). Protein bands were visualized using Amersham ECL Western Blotting Detection Reagent.

#### Melanoma therapy models

**Transplant models.** SM1 or SM1WT1 cells were inoculated subcutaneously on the hind flank with the indicated number of cells, and tumor size was monitored by measuring the product of the shortest and longest tumor diameters. At various time points, indicated mice received 20 mg/kg PLX4720 i.p. or an equivalent volume of vehicle DMSO. In some experiments, mice were comparatively treated with 20 µg clg or anti-CCL2 (clone 123616) as indicated. Some groups of mice additionally received clg (Mac-4), anti-CTLA-4, anti-PD-1, anti-Tim3, or anti-CD137 i.p. using the schedules outlined.

**Spontaneous model.** 4-HT solution was prepared in DMSO as previously described (52). 4-HT was applied topically on the back skin of Braf<sup>CA</sup> Tyr-creER<sup>T2</sup>Pten<sup>fl/fl</sup> mice to induce localized melanoma. Mice harboring





established melanoma (day 28; size ~20 mm<sup>2</sup>) were treated daily with 20 mg/kg PLX4720 i.p. or an equivalent volume of vehicle DMSO at days 28–49. Some groups of mice additionally received cIg or anti-CD137 (100 µg) i.p. on days 28, 32, 36, 40, 44, and 48. Mice were then monitored for melanoma development, and data were recorded as the growth curves of individual mice with tumors in each group. Measurements were made with a caliper and reference ruler for height of tumors (mm) weekly for 3 weeks. At indicated time points, tumors were weighted and recorded (g) for individual mice in each group.

### Measuring CCL2 and other chemokines by CBA

*In vitro.* 5 × 10<sup>4</sup> SM1 and SM1WT1 cells were plated in a 96-well plate and treated with 10 µM PLX4720 or an equivalent volume of vehicle DMSO. Supernatants were harvested from PLX4720- or vehicle-treated culture after 18 to 24 hours.

*Ex vivo.* Tumor suspension was prepared as previously described (30). Tumor suspension was plated in a 96-well plate at 1 × 10<sup>5</sup> cells per well. Supernatant was harvested after 18 to 24 hours of in vitro culture.

### Quantitative real-time PCR

2 × 10<sup>6</sup> SM1 or SM1WT1 cells, treated with vehicle or 10 µM PLX4720, were harvested after 24-hour culture in a replicate of 5 wells. RNA was isolated using the RNeasy Mini Kit (Qiagen), and cDNA were synthesized from 1 µg of RNA using SuperScript III (Invitrogen) according to the manufacturer's protocol. Quantitative PCR for CCL2 and HPRT expression was performed on the Step One Plus Real-Time PCR System (Applied Biosystem) with Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies). Primer sequences to CCL2 (5'-GCATCCACTACCTTTCCACAACC-3'; 5'-ACAGTCCGAGTCACACTAGTTCAC-3') and HPRT (5'-GACACAAAC-GTGATTCAAATCC-3'; 5'-CTCTCGAAGTGTGGATACAG-3') were used. Threshold cycle (C<sub>t</sub>) was measured, and relative gene expression of CCL2 was calculated using the ΔΔC<sub>t</sub> method relative to HPRT.

### Flow cytometry analysis

Larger tumors were treated and assessed to enable sufficient TILs (TILs) for examination. SM1WT1 tumors were excised from mice and processed for flow cytometry analysis as previously described (30). In brief, tumors were minced and digested with 1 mg/ml collagenase IV (Worthington Biochemical Corporation) and 0.02 mg/ml DNaseI (Roche) to prepare single cell suspensions. For surface staining, TILs were stained with FITC-anti-Ly6G (Gr-1) (RB6-8C5, BD Pharmingen), FITC-anti-NK1.1 (PK136, eBioscience), PE-

anti-CD11b (3A33, Southern Biotech), PE-anti-CD8α (53-6.7, eBioscience), PE-Cy7-anti-CD4 (RM4-5, BD Pharmingen), Streptavidin-PE-Cy7 (eBioscience), APC-anti-TCRβ (H57-597, eBioscience), APC-anti-CD11c (N418, eBioscience), APC-anti-CCR2 (R&D Systems), APC-eFluor780-anti-CD45.2 (104, eBioscience), eFluor 450-anti-CD3 (17A2, eBioscience), eFluor 450-anti-CD19 (eBio1D3, eBioscience), and biotin-anti-F4/80 (BM8, eBioscience) in the presence of anti-CD16/32 (2.4G2). 7AAD (BD Pharmingen) was used to exclude dead cells. For intracellular staining, TILs were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience), according to the manufacturer's protocol, and stained using eFluor 450-anti-Foxp3 (FJK-16s, eBioscience). Cells were acquired on the LSR-II (BD Biosciences), and analysis was performed using FlowJo (Tree Star).

### Statistics

Data were analyzed with GraphPad Prism (version 5) software (GraphPad Software). Significant differences among groups were assessed by a 2-tailed *t* test or Mann-Whitney *U* test, as indicated. *P* values of less than 0.05 were considered significant.

### Study approval

All mouse experiments were performed with approval from the Peter MacCallum Cancer Centre Animal Experimental Ethics Committee.

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