BRAF Inhibition Stimulates Melanoma-Associated Macrophages to Drive Tumor Growth

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Abstract

Purpose: To investigate the roles of melanoma-associated macrophages in melanoma resistance to BRAF inhibitors (BRAFi).

Experimental Design: An in vitro macrophage and melanoma cell coculture system was used to investigate whether macrophages play a role in melanoma resistance to BRAFi. The effects of macrophages in tumor resistance were examined by proliferation assay, cell death assay, and Western blot analyses. Furthermore, two mouse preclinical models were used to validate whether targeting macrophages can increase the antitumor activity of BRAFi. Finally, the number of macrophages in melanoma tissues was examined by immunohistochemistry.

Results: We demonstrate that in BRAF-mutant melanomas, BRAFi paradoxically activate the mitogen-activated protein kinase (MAPK) pathway in macrophages to produce VEGF, which reactivates the MAPK pathway and stimulates cell growth in melanoma cells. Blocking the MAPK pathway or VEGF signaling then reverses macrophage-mediated resistance. Targeting macrophages increases the antitumor activity of BRAFi in mouse and human tumor models. The presence of macrophages in melanomas predicts early relapse after therapy.

Conclusions: Our findings demonstrate that macrophages play a critical role in melanoma resistance to BRAFi, suggesting that targeting macrophages will benefit patients with BRAF-mutant melanoma. Clin Cancer Res; 21(7); 1652–64. ©2015 AACR.

Introduction

BRAFV600E/K mutations are present in around 40% to 50% melanomas. Targeted therapy with small-molecule BRAF inhibitors (BRAFi), such as vemurafenib or dabrafenib, has improved overall survival in patients with advanced BRAF-mutant melanomas (1–4). However, most patients relapse within several months. Acquired resistance has been attributed to both genetic and/or epigenetic changes in tumor cells after treatment with BRAFi. Analyses of melanomas that have acquired resistance to BRAFi frequently have demonstrated reactivation of the mitogen-activated protein kinase (MAPK) pathway via new mutations, such as BRAF amplification and emerging splice variants (5), NRAS mutation (6), MEK1 mutation (7), or through activation of alternative survival pathways involving MAPK and phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) (refs. 8, 9), which are essential for cell growth and survival. Of note, some melanomas that carry an activating BRAF mutation are resistant to BRAFi, possibly due to genetic and epigenetic heterogeneity of cancer cells. Overall, approximately 50% of patients with melanoma do not have significant responses to BRAFi (1, 4). The mechanisms underlying this intrinsic resistance of cancer cells to BRAFi remain poorly understood.

Melanomas that do not have an activating BRAF mutation are typically unresponsive to BRAFi. It is of particular interest that patients treated with BRAFi often develop secondary cutaneous nonmelanoma tumors, suspected to be due to BRAFi induction of signaling pathways in precancerous skin cells. Although small-molecule inhibitors (SMI) may inhibit the desired targets in tumor cells, they may also paradoxically activate the same pathways in malignant and nonmalignant cells. For example, some AKT or mTOR inhibitors can activate the PI3K/AKT pathway in tumor cells; this paradoxical activation blunts their antitumor efficacy and contributes to tumor cell resistance to AKT/mTOR inhibitors (10–12). In melanoma, BRAFi activate the MAPK pathway in BRAF wild-type and NRAS-mutant tumor cells via a RAS-dependent, CRAF activation mechanism (13–15). Also,
increased numbers of phospho-ERK (pERK)—positive cells in the keratinocyte compartment of skin are observed in BRAF-treated mice. Accordingly, paradoxical activation of the MAPK pathway by BRAFi results in squamous-cell carcinomas in some patients treated with BRAFi (16). To date, there has been no systematic analysis of signaling pathways in normal cell types that are activated by BRAFi (13). The biologic consequences and mechanisms of this paradoxical activation of signaling pathways by SMIs and their contribution to cell growth and survival, as well as tumor cell resistance to targeted therapy, are not well defined, especially in nonmalignant cells.

There is evidence that the tumor microenvironment contributes to tumor cell resistance to anticancer therapy. Although some studies suggested that the macrophage, a major component of the tumor microenvironment, contributes to tumor cell resistance to anticancer therapies, including chemotherapy, radiotherapy, and immune therapy (17, 18), other studies suggest that macrophages increase the antitumor activity of anticancer therapies (19, 20). However, most studies have not addressed the direct effects of macrophages on tumor cell growth in the presence of anticancer therapies, especially targeted therapy with SMIs.

Macrophages are the most abundant inflammatory cells in melanomas (21), and the number of infiltrating macrophages, as well as the levels of macrophage-produced factors, inversely correlates with patients' outcome in both early and late stages of melanoma (22–24). Melanoma-associated macrophages produce a plethora of growth factors, cytokines, chemokines, extracellular matrix, and proteases, which play critical roles in melanoma initiation, angiogenesis, growth, metastasis, and immune suppression (25–29). However, the role of macrophages in melanoma resistance to BRAFi remains poorly defined.

Therefore, we examined the roles of macrophages in melanomas with resistance to BRAFi, and identified a unique mechanism for resistance by using a human macrophage and melanoma cell coculture system. We further validated our in vitro findings in mouse melanoma models and patients' tumor samples.

Materials and Methods

Cell culture

1205Lu and 451Lu melanoma cell lines were developed by our laboratory. A375 and SK-MEL-28 were from the ATCC. The detailed information of cell lines can be found at: http://www.wistar.org/lab/meenhard-herlyn-dvm-disc. Melanoma cells were cultured in melanoma medium supplemented with 2% fetal bovine serum (FBS) as described previously (28). For macrophage and melanoma coculture experiments, melanoma cells were cocultured with respective macrophages that were differentiated from monocytes using melanoma-conditioned media derived from the above four melanoma cell lines as described previously (28). For Figs. 4 and 5 and Supplementary Fig S8, macrophages were differentiated from monocytes using 1205Lu melanoma-conditioned media.

Reagents

PLX4720 and lenvatinib were from Selleck. Dabrafenib was from ChemieTek. GW2580 and gefitinib were from LC Laboratories. PD173074 was from Abcam. Recombinant human VEGF, anti-human VEGF blocking mAb, phospho-VEGFR1 (Y1213), phycocerythrin-conjugated anti-human VEGFR1, VEGFR2, VEGFR3, and neuropilin-1 were from R&D Systems. pERK, pAKT, pNF-kB, pCRAF, pARF, pSTAT3, pVEGFR1, pp38, total ERK, CRF, AKT, STAT3, PDGFRβ, Rab11, HSP90, and Vinculin were from Cell Signaling Technology. Corning Transwells (pore size, 0.4 μm) were from Fisher Scientific for coculture experiments.

Proliferation assay

The macrophage and melanoma coculture system was set up as described in Fig. 1A. For coculturing melanoma cells with macrophages, 2 × 10⁴ melanoma cells were seeded in 24-well plates and incubated for 18 hours. Macrophages (4 × 10⁴) were then added to the collagen I (1.1 μg/mL)-precoated Transwell. Indicated concentrations of various inhibitors, growth factors, and antibodies were then added to the coculture system and incubated for 3 days.

For macrophage proliferation, after monocytes had differentiated into macrophages, cells in 2% FBS melanoma media were seeded into 96-well plates and incubated for 3 days in the presence of the indicated concentrations of inhibitors and blocking antibodies. Cell proliferation was assayed using the WST-1 proliferation kit according to the manufacturer’s instructions (Roche). All experiments were performed at least in triplicate.

Immunoblotting

Melanoma cells were cultured as described for the proliferation assay for 6 and 18 hours and harvested for immunoblotting. For macrophages, after monocytes had differentiated into macrophages, cells in 2% FBS melanoma media were seeded into 15-mL tubes. Macrophages were incubated for the indicated times in the presence of the indicated concentrations of inhibitors. Immunoblotting was performed as described previously (28).

siRNA transfection

The MEEK1 and MEEK2 siRNA and negative control siRNA were from Thermo Scientific. After 24-hour transfection, 1205Lu and A375 cells were harvested and transferred to 6-well plates and incubated for another 24 hours. Cells were then cocultured with macrophages and treated with the indicated concentration of PLX4720 for 18 and 72 hours, respectively. Cells were harvested for immunoblotting with the indicated antibodies (18-hour coculture) and flow cytometric analysis (3-day coculture). Proliferation assay was performed with WST-1 assay.
For basal-level RAS activity, after macrophage differentiation, cells in 2% FBS melanoma media were incubated for an additional 0.5 hours. Cells were harvested for ELISA measurement according to the manufacturer's instructions (cat# 17-497 from Millipore).

Figure 1.
Macrophages are essential for melanoma cell growth under BRAF inhibition. A, the macrophage (Mph) and melanoma cell coculture system. Melanoma cells are seeded on the bottom of cell culture plates. To mimic the tumor microenvironment, a layer of collagen I (Col.I) is coated on the Transwell (pore size: 0.4 µm). Macrophages are then seeded on the collagen I layer. B, 1205Lu and A375 melanoma cells were cocultured in the presence or absence of macrophages with the indicated concentration of PLX4720 for 3 days. Cell growth was determined using the WST-1 assay. Relative growth was calculated as the ratio of treated cells to untreated cells (without Mph coculture) at each dose. Data shown are mean ± SD (n = 4), *** P < 0.001. C, cells were treated same as in B. Cell death was determined by flow cytometry using Annexin V and 7-AAD staining. Representative images from one of six independent experiments are shown (left). Relative percentage of cell death represents the ratio between dead cells with macrophages versus without macrophages in the presence of PLX4720 (right). *** P < 0.001. D, cells were treated same as in B. Cells were stained with propidium iodide, and were analyzed for cell-cycle progression by flow cytometry. The percentage of cells in the sub-G₁ and G₀/G₁ populations is shown. Representative images from one of four (1205Lu) and three (A375) independent experiments are shown (left). Relative percentage of cell death represents the ratio between dead cells with macrophages versus without macrophages in the presence of PLX4720 (right). *, P < 0.05; **, P < 0.01. See also Supplementary Fig. S1.
Luminex assay
The production of growth factors and cytokines from macrophage media was measured by Luminex assay according to the manufacturer’s instructions (Bio-Rad; ref. 28).

Immunohistochemistry
For CD163 and Ki67 staining, double stains were performed sequentially on a Leica Bond instrument using the Bond Polymer Refine Detection System (for Ki67) and the Bond Polymer Refine Red Detection System (for CD163). Heat-induced epitope retrieval was done for 20 minutes with ER1 solution (Leica Microsystems). The tissues were first stained with an anti-Ki67 antibody (1:20 dilution; DakoM7240). Tissues were then stained with an anti-CD163 Ab (1:50; Leica NCL). For quantification of CD163-positive cells, CD163-positive cells were counted in 10 randomly selected fields (×600 magnifications) for each tumor sample. Two independent investigators evaluated the sections.

For mouse tissues, formalin-fixed, paraffin-embedded mouse melanoma tumor tissues were deparaffinized and subjected to antigen retrieval as described previously (28). The tissues were then incubated with the following antibodies: anti-Ki67 (Novus Biologicals), anti-F4/80 (Abcam), CD11b, CD31, and pERK (Epitomics). After incubation with the primary antibody overnight at 4°C, horseradish peroxidase-conjugated donkey anti-mouse, donkey anti-rabbit, or donkey anti-rat IgG (1:200; Jackson Immunoresearch) was used. Slides were subsequently incubated for 5 minutes in 3,3′-diaminobenzidine (Invitrogen) and counterstained with Haemalaun. For quantification of Ki67-positive cells, cells were counted in six randomly selected fields (×400 magnifications) for each tumor sample (n = 4 for each group). Two independent investigators evaluated sections.

ELISA analysis
Macrophages were stimulated with PLX4720 (3 μmol/L) for 3 days. Media were harvested, and VEGF production was determined by ELISA according to the manufacturer’s instructions (R&D Systems).

Flow cytometric analysis
For the cell death assay, treated melanoma tissues or macrophages were stained with R-phycocerythrin-conjugated Annexin V and 7-amino-actinomycin D (7-AAD) according to the manufacturer’s protocol (BD Biosciences). Cell death was quantified using a Becton Dickinson FACScan cytometer.

For cell-cycle analysis, cells were fixed in 75% ethanol at −20°C overnight and washed with cold PBS, treated with 100-μg RNase A (Sigma), and stained with 50-μg propidium iodide (Roche). Measurement of VEGF production was determined by intracellular staining according to the manufacturer’s protocol (BD Biosciences). After monocytes had differentiated into macrophages, cells in 2% PBS melanoma media were incubated for 4 hours in the presence of the indicated concentration of PLX4720 and GolgiPlug. After cells were washed with FACS buffer, intracellular staining was performed with an R-phycocerythrin-conjugated anti-VEGF mAb according to the manufacturer’s instructions (R&D Systems).

For FACS analysis of peritoneal macrophages, 10 mL of cold PBS was intraperitoneally injected into the mice after they were euthanized. Cells were harvested and the numbers of macrophages were counted with a hemocytometer. An anti-mouse F4/80 (BD Biosciences) was used to analyze the percentage of macrophages. All FACS data were analyzed with the FlowJo software (TreeStar).

Animal studies
All studies were conducted under an approved IACUC protocol. For human xenografts, 7-week-old BALB/c nude mice (National Cancer Institute, Bethesda, MD) were injected subcutaneously with 1 × 10^6 1205Lu cells in 50% Matrigel (BD Biosciences) in both flanks. For mouse tumor growth, 7-week-old C57Bl/6 female mice (National Cancer Institute) were injected subcutaneously with 1 × 10^5 murine Yumm1.7 cells (BRAFV600E mutant, PTEN null; gift of Dr. Marcus Bosenberg, Yale Medical School, New Haven, CT). When tumors reached volumes of approximately 100 mm^3 (1205Lu) and 180 mm^3 (Yumm1.7), mice were randomly divided into four groups, with 5 animals per group. GW2580 was dissolved in 0.5% hydroxypropylmethylcellulose (Sigma-Aldrich) and 0.1% Tween 80, and was dosed orally at 160 μg/kg once daily. PLX4720 was dissolved in 5% DMSO, 1% methylcellulose in distilled water, and animals were dosed orally at 25 mg/kg twice per day. Tumor volumes were measured every 3 days using a digital caliper and were calculated using the equation V = 0.5 × length × width^2. Mouse tumors were weighed after mice were euthanized.

Patient Samples
Formalin-fixed, paraffin-embedded human melanoma tumor slides (Supplementary Table S1) were from The University of Pennsylvania (Philadelphia, PA) under an approved Institutional Review Board protocol.

Statistical analysis
Paired two-tailed t tests were performed to compare the differences in cell growth measurements between two experimental conditions of specific cell line samples. Two-way ANOVA was used to determine the effect of treatment groups with multiple concentrations of inhibitors.

Results
Macrophages confer melanoma resistance to BRAF inhibition
Macrophages can play critical roles in tumor cell resistance to anticancer therapies. We investigated whether macrophages confer melanoma resistance to BRAFI. We developed a model system that resembles the tumor microenvironment. In a Transwell coculture system (30), we cocultured melanoma cells with human macrophages that were differentiated from monocytes with modified melanoma conditioned media. Under those experimental conditions, cells resemble human tumor-associated macrophages, both phenotypically and functionally (28, 31). We then exposed the cocultured cells to BRAFI (Fig. 1A). Mutant BRAFV600E melanoma cell lines, including 1205Lu, A375, SK-MEL-28, and 451Lu cells, when cultured alone, were sensitive to PLX4720, an analog of vemurafenib. However, when macrophages were added to the cocultures, the cells of all four lines became resistant to PLX4720 (Fig. 1B and Supplementary Fig. S1A and S1B) as demonstrated by increased proliferation with the WST-1 proliferation assay. Macrophages also promoted cell growth in the presence of PLX4720 and when maintained in direct cell–cell contact with tumor cells (Supplementary Fig. S1C). These data indicate that growth factors produced by macrophages stimulate...
Macrophage-mediated drug resistance. Cell death assays by Annexin V and 7-AAD staining indicate that macrophages protect melanoma cells from PLX4720-induced cell death, including apoptosis (Annexin V–positive and 7-AAD-negative) and necrosis (Annexin V–positive and 7-AAD-positive; Fig. 1C and Supplementary Fig. S1D). Furthermore, flow cytometry cell-cycle analyses using propidium iodide staining confirmed that the percentage of the sub-G1 population (apoptotic and necrotic cells) in 1205Lu and A375 melanoma cells was significantly lower in the presence than absence of macrophages (Fig. 1D). Of note, macrophages do not have any effect on melanoma cell G2–M phase, suggesting that the high doses we used (10 μmol/L for 1205Lu and 2 μmol/L for A375) may mainly protect tumor cells from BRAFi-induced cell death, but not promote tumor cell growth. Similar results were obtained when another FDA-approved BRAFi, dabrafenib, was used (Supplementary Fig. S1E and S1F). Our data demonstrate that macrophages reduce the effects of BRAFi on melanoma cells.

Macrophages activate the MAPK pathway in melanoma cells when exposed to BRAFi

Reactivation of the MAPK pathway and activation of alternative survival signaling pathways, such as the AKT pathway, are demonstrated mechanisms for melanoma cell resistance to BRAFi (9, 32–34). Because there is an abundance of macrophages in melanomas (35), we examined whether macrophages contribute to activation of these signaling pathways in the presence of BRAFi. Addition of macrophages to the coculture system resulted in a strong increase in ERK phosphorylation in both 1205Lu and A375 melanoma cell lines after 6-hour treatment with PLX4720 (Supplementary Fig. S2A). Reactivation of the MAPK pathway was maintained after 18 hours of coculture (Fig. 2A), but changes

Figure 2. Macrophages confer melanoma resistance to BRAFi via activation of the MAPK pathway. A, macrophages activate the MAPK pathway in melanoma cells when PLX4720 is present. 1205Lu and A375 melanoma cells were cocultured with or without macrophages in the presence or absence of the indicated concentration of PLX4720 for 18 hours, and melanoma cells were harvested for immunoblotting with the indicated antibodies. HSP90 was used as a loading control. B, 1205Lu and A375 melanoma cells were transfected with MEK1/2 siRNA and negative control siRNA and incubated for 48 hours. Melanoma cells were then cocultured with or without macrophages in the presence or absence of the indicated concentration of PLX4720 (3 μmol/L for 1205Lu, 2 μmol/L for A375) for 3 days. Cell growth was determined by the WST-1 assay as in (Fig. 1B). Data shown are mean ± SD (n = 4). **P < 0.01, ***P < 0.001. C, cells were treated same as in B. Cell death was determined as in Fig. 1C. Representative images from one of four independent experiments are shown (left). Relative percentage of cell death represents the ratio between dead cells with PLX4720 plus MEK1/2 siRNA treatment versus PLX4720 without MEK1/2 siRNA treatment in the presence or absence of macrophages (right). *P < 0.05; **P < 0.01. D, 1205Lu and A375 melanoma cells were transfected with MEK1/2 and control siRNA for 48 hours. Melanoma cells were then cocultured with or without macrophages in the presence or absence of PLX4720 (2 μmol/L for 1205Lu, 1 μmol/L for A375) for 18 hours. Cells were harvested for immunoblotting with the indicated antibodies. **P < 0.01. See also Supplementary Fig. S2.
were not seen in other important melanoma survival signaling components, such as AKT, NF-κB, CRAF, and ARAF (Supplementary Fig. S2A and S2B). Activation of STAT3 signaling and upregulation of PDGFRβ expression can confer melanoma resistance to BRAFi (6, 36); however, we observed that macrophages neither activated STAT3 signaling nor upregulated PDGFRβ expression in the presence of PLX4720 (Supplementary Fig. S2C). Because activation of the MAPK pathway by macrophages occurs as early as 6 hours, it is unlikely that resistance is due to new genetic changes in the melanoma cells.

To determine the mechanism of macrophage-mediated melanoma growth promotion in the presence of BRAFi, we blocked the MAPK pathway at the level of ERK signaling with combined MEK1 and MEK2 siRNA. We found that MEK1/MEK2 knockdown significantly, but not completely, decreased macrophage-mediated cell growth and death-promoting effects in both 1205Lu and A375 cells in the presence of PLX4720 (Fig. 2B and C). Accordingly, MEK1/MEK2 knockdown diminished macrophage-mediated increase in ERK activation of melanoma cells (Fig. 2D). Collectively, these data demonstrate that macrophages confer resistance to BRAFi in melanoma cells at least partially via reactivation of the MAPK pathway.

**VEGF confers macrophage-mediated resistance to BRAFi**

Because we were using the Transwell coculture system, which allows macrophage-derived factors to stimulate melanoma cell growth without direct cell–cell contact, we sought to identify the mechanisms by which soluble factors confer macrophage-mediated resistance. Many factors can rescue BRAFi-induced cell growth inhibition, including epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), and hepatocyte growth factor (HGF; refs. 37–40). Of note, fibroblast-derived HGF confers melanoma resistance to BRAFi through activation of both MAPK and PI3K pathways (39). We used specific inhibitors of these pathways—the EGF pathway by gefitinib, an inhibitor against EGF receptor; the HGF pathway by AMG208, an inhibitor against HGF receptor, c-MET; and the FGF2 pathway by PD173074, an inhibitor against FGF receptor 1—and demonstrated that they did not reverse the macrophage-mediated growth-promoting effects in 1205Lu cells (Supplementary Fig. S3A), indicating that other growth factors play a role in macrophage-mediated resistance.

To identify any growth factor involved in activation of the MAPK pathway induced by macrophages, we conducted a luminex assay to analyze production of multiple growth factors produced by macrophages, including M-CSF, CXCL1, IL6, TNFα, platelet-derived growth factor (PDGF), and VEGF (Supplementary Fig. S3B), which activate the MAPK pathway in cells that contain receptors to these factors. We found that only VEGF, but not other growth factors such as CXCL1, EGF, IL8, M-CSF, TNFα, and PDGF, rescued melanoma cells from PLX4720-induced cell growth inhibition and cell death (Fig. 3A and B and Supplementary Figs. S3C, S4A, and S4B). VEGF produced similar effects in dabrafenib-treated melanoma cells (Supplementary Fig. S4C and S4D). Accordingly, VEGF activated the MAPK pathway in both 1205Lu and A375 cells (Fig. 3C), which is similar to the effects seen in melanoma cells treated with PLX4720 in the presence of macrophages, although the effect of VEGF was less pronounced.

In addition to its proangiogenic effect, VEGF has direct effects on melanoma cell growth and survival (41–44). Melanoma cell lines and melanoma cells in primary human melanoma tissues are reported to express three receptors for VEGF, VEGFR1, 2, 3, as well as another VEGFR, Neuropilin-1 (45–48). We also found that 1205Lu and A375 melanoma cells express VEGF receptors, including VEGFR1, 2, 3, neuropilin-1, and a coreceptor of VEGF, integrin-β3 (Supplementary Fig. S5A). In our experiments, macrophages did not significantly increase expression of individual VEGFR (Supplementary Fig. S5B).

Blockade of VEGF signaling with a pan-VEGF receptor inhibitor (VEGFR1, 2, and 3), brivanib alaninate, a compound currently being tested in clinical trials for patients with melanoma, significantly reversed macrophage-mediated cell growth (Fig. 3D), and moderately reversed macrophage-mediated anti-cell death effects (Fig. 3E), as well as blocked macrophage-mediated reactivation of ERK signaling (Fig. 3F). Similar effects were observed using another pan-VEGF receptor inhibitor (VEGFR1, 2, and 3), brivanib alaninate (Supplementary Fig. S6). Because lenvatinib and brivanib alaninate also target other tyrosine kinases, we wanted to confirm whether VEGF specifically contributes to macrophage-mediated BRAF resistance and used anti-VEGF mAbs to block VEGF signaling. Indeed, anti-VEGF antibodies significantly reversed macrophage-mediated resistance (Supplementary Fig. S7). Collectively, these data demonstrate that VEGF confers macrophage-mediated resistance to BRAFi.

**BRAF inhibition paradoxically activates the MAPK pathway in macrophages**

Having shown that BRAFi affect signaling pathways in melanomas by the induction of macrophage-derived VEGF, we determined the process by which BRAFi produce this effect in macrophages. We first examined the change in activity of signaling pathways of macrophages exposed to BRAFi. An earlier report had shown that PLX4720 modestly activated the MAPK pathway in BRAF wild-type melanoma cells (13). Our examination of this pathway in macrophages demonstrated that PLX4720 strongly activated the MAPK pathway (Fig. 4A). Activation of the MAPK pathway occurred as early as 30 minutes after PLX4720 treatment. In particular, we observed an increase in phosphorylation of CRAF. This is of significance because CRAF expression is required for BRAFi-induced paradoxical activation of the MAPK pathway in BRAF wild-type melanoma cells (Fig. 4A). We observed a similar effect with dabrafenib (Supplementary Fig. S8A). PLX4720 treatment significantly increased activation of the MAPK pathway, but decreased PERK signaling at higher dose (10 μmol/L), which is similar to the effect of BRAFi on BRAF wild-type cancer cells (13, 15). In wild-type BRAF melanoma cells, BRAFi activate the MAPK pathway only when there is a high level of RAS activity. This then appears to promote CRAF signaling and growth of BRAF wild-type melanoma cells. We hypothesized that, like BRAF wild-type cancer cells, macrophages have a high basal level of endogenous RAS activity to activate the MAPK pathway upon BRAFi treatment. We therefore analyzed the RAS activity in macrophages differentiated from monocytes obtained from three different donors. We then compared RAS levels in macrophages with expression in 1205Lu and A375 cells. ELISA analyses demonstrated a 2- to 4-fold higher level of endogenous RAS activity in macrophages compared with BRAF-mutant melanoma cell lines (Fig. 4B). The RAS activity levels in macrophages were similar to those observed in BRAF wild-type and NRAS-mutant melanoma cells (49). Therefore, the relatively high endogenous levels of RAS activity in macrophages might be sufficient to initiate activation of downstream signaling by CRAF in the presence of
VEGF confers macrophage-mediated melanoma resistance to BRAFi. A, VEGF rescues PLX4720-induced melanoma growth inhibition in the presence of PLX4720. 1205Lu and A375 melanoma cells were cocultured with or without VEGF (10 ng/mL) in the presence of PLX4720 (3 μmol/L) for 3 days. Cell growth was determined by the WST-1 assay as in Fig. 1B. Data shown are mean ± SD (n = 4). ***, P < 0.001. B, cells were treated same as in A. Cell death was determined as in Fig. 1C. Representative images from one of four (1205Lu) and three (A375) independent experiments are shown (left). Relative percentage of cell death represents the ratio between dead cells with PLX4720 plus lenvatinib versus with PLX4720 alone in the presence or absence of macrophages. *, P < 0.05; ***, P < 0.001. C, VEGF increases activation of the MAPK pathway. 1205Lu and A375 cells were cocultured in the presence or absence of VEGF (10 ng/mL) and/or PLX4720 (3 μmol/L) for 18 hours. Cells were harvested for immunoblotting with the indicated antibodies. D, lenvatinib reverses macrophage-mediated melanoma resistance to PLX4720. 1205Lu and A375 cells were cocultured with or without macrophages in the presence or absence of PLX4720 (3 μmol/L) and/or lenvatinib (10 μmol/L) for 3 days, cell growth was then determined by the WST-1 assay as in Fig. 1B. Data shown are mean ± SD (n = 4). ***, P < 0.001. E, lenvatinib reverses the macrophage-mediated anti-cell death effect. 1205Lu cells were treated as in D. Cell death was determined as in Fig. 1C. Representative images from one of three independent experiments are shown (left). Relative percentage of cell death represents the ratio between dead cells with PLX4720 plus lenvatinib versus with PLX4720 alone in the presence or absence of macrophages. *, P < 0.05; ***, P < 0.001. F, lenvatinib reverses the macrophage-mediated activation of the MAPK pathway. 1205Lu and A375 cells were treated as in D but with 18-hour treatment. Cells were then harvested for immunoblotting with the indicated antibodies. See also Supplementary Figs. S3–S7.

BRAFi (Fig. 4A). PLX4720 treatment significantly increased RAS activity in macrophages, as well as other RAS downstream signaling molecules, such as p38 (Fig. 4C and D). Together, our data suggest that activation of the MAPK pathway by BRAFi in macrophages is possible due to high endogenous RAS activity in macrophages. Of note, the activation of the MAPK pathway in macrophages by BRAFi is independent of the BRAF mutation status in tumor cells, suggesting that this is a more general phenomenon.
BRAF inhibition promotes macrophage growth and survival

The MAPK pathway is critical for normal macrophage growth and survival. We therefore explored the biologic consequence of BRAFi-induced paradoxical activation of the MAPK pathway in macrophages. PLX4720 treatment promoted macrophage growth (Fig. 4E), and increased the expression of proliferation markers (Fig. 4F).

Figure 4.
BRAF inhibition paradoxically activates the MAPK pathway to elicit potent biologic responses in macrophages. A, BRAF inhibition induces activation of the MAPK pathway in macrophages. Macrophages were treated with the indicated concentration of PLX4720 for 2 hours and were then harvested for immunoblotting with the indicated antibodies. Rab11 was used as a loading control. B, macrophages have a high basal level of RAS activation. Cell lysates from 1205Lu and A375 melanoma cells, as well as macrophages that were differentiated from three different donors were harvested, and analyzed for RAS activation by ELISA. C, macrophages (black and white bars) were treated with PLX4720 (3 μmol/L) for 2 and 5 minutes. Cells were lysed and analyzed for RAS activation by ELISA (n = 4). *** P<0.001. D, macrophages were treated with the indicated concentration of PLX4720 for 2 hours and were then harvested for immunoblotting with the indicated antibodies. HSP90 was used as a loading control. E, PLX4720 promotes macrophage growth. Macrophages were treated with the indicated concentration of PLX4720 for 3 days. Cell growth was determined by the WST-1 assay as in Fig. 1B. Data shown are mean±SD (n = 4). *** P<0.001. F, PLX4720 increases the expression of PCNA. Macrophages were treated with PLX4720 (3 μmol/L) for the indicated time. Cells were harvested for immunoblotting with the indicated antibodies. G, PLX4720 protects macrophage from cell death. Macrophages were treated with PLX4720 (3 μmol/L) for 3 days, and cell death was determined as in Fig. 1C. Representative images from one of four independent experiments are shown (left). Relative percentage of cell death represents the ratio between dead cells with PLX4720 versus without PLX4720 (right). ** P<0.01. H, the number of macrophages is increased in patient samples treated with vemurafenib and dabrafenib. The number of macrophages was counted in 10 randomly selected microscope fields. See also Supplementary Fig. S8.
BRAF inhibition induces macrophages to produce VEGF

Production of VEGF is induced by activation of the MAPK pathway in both malignant and normal cells, including macrophages (50, 51). We determined whether paradoxical activation of the MAPK pathway by BRAFi had similar effects on macrophages. Flow cytometric analyses by intracellular staining for VEGF indicated that PLX4720 significantly increased the production of VEGF in macrophages in a biphasic pattern, with increasing VEGF production peaking at 3 μmol/L and declining at 10 to 20 μmol/L of PLX4720 treatment (Fig. 5A). This is similar to the effect seen in increased ERK activation by PLX4720 in macrophages (see Fig. 4A). ELISA analyses further indicated a 5-fold increase of VEGF production levels when testing supernatants from macrophages treated with 3 μmol/L PLX4720 in comparison with control (Fig. 5B). Furthermore, PLX4720 treatment resulted in a strong increase in VEGF receptor 1 (VEGFR1) phosphorylation, but not total VEGFR1 production, suggesting that BRAF inhibition also exerts an autocrine effect on macrophages triggered by VEGF (Fig. 5C and D). Together, our data indicate that BRAF inhibition elicits potent macrophage responses and increases the numbers of macrophages, as well as the production of VEGF, which then represents a potent stimulant for both macrophages and melanoma cells.

Targeting macrophages increases the antitumor effects of BRAFi in mouse models

We investigated the effect of macrophages on melanomas grown in a murine syngeneic tumor system and treated with BRAFi. After 14 days of treatment with GW2580, a small-molecule, ATP-competitive inhibitor of M-CSFR kinase (160 mg/kg), tumor sizes decreased significantly, although the compound was less efficacious than PLX4720 (25 mg/kg) alone. A combination of both agents showed greater inhibition of tumor growth and reduced tumor weight (Fig. 6A and Supplementary Fig. S9A) than either alone. It is likely that the inhibitory effect of GW2580 on tumor growth is due to targeting macrophages rather...
than tumor cells directly, because treatment with GW2580 can reverse macrophage-mediated resistance to BRAFi (Supplementary Fig. S10A), and did not have significant direct effects on melanoma growth or death when tested on cultured cells in vitro (Supplementary Fig. S10B and S10D), as well as on VEGF production in melanoma cells (Supplementary Fig. S10C). GW2580 treatment significantly decreased peritoneal F4/80-positive macrophages, as also shown previously (52). BRAFi treatment amplified GW2580-induced macrophage depletion through a yet to be identified mechanism (Fig. 6B and Supplementary Fig. S9B).

GW2580 increases the antitumor activity of PLX4720 on 1205Lu xenograft tumor growth and the number of macrophages is a poor prognostic marker for treatment of BRAFi. A, 1205Lu cells were injected subcutaneously into both flanks of nude mice. When the average tumor volume reached approximately 100 mm³, the indicated doses of GW2580 and/or PLX4720 or vehicle were administered orally for 14 days. A linear mixed-effect model with the random effect at mouse level was used to compare the differences in tumor growth trends between treatment groups over 14 days (n = 10 for all groups). *, P < 0.01; ***, P < 0.001. B, mice were euthanized on day 14 and peritoneal cells were harvested for flow cytometric analysis of the percentage of F4/80-positive macrophages. Data shown are mean ± SD (n = 5). *, P < 0.05; ***, P < 0.01. C, representative images of melanoma specimens from patients pretreated with BRAFi. One blue arrow shows one of the representative CD163-positive macrophages. One black arrow shows one of the representative Ki67-positive cells. All scale bars, 50 μm. D, univariate Cox regression analysis showed a statistically significant association between the number of melanoma-infiltrating macrophages and progression-free survival among 10 patients treated with BRAFi. The number of macrophages was the average number counted in 10 randomly selected microscope fields. *, P < 0.05. E, schematic model shows that macrophages switch their roles from a passenger to a driver for melanoma growth and survival under BRAF inhibition. ? marker indicates that there is no direct experimental evidence in this article. See also Supplementary Figs. S9–S12.
GW2580 treatment abolished F4/80 and CD11b-positive macrophages in tumors (Supplementary Fig. S11A and S11E). Unlike human tumor-infiltrating macrophages, mouse tumor-infiltrating macrophages are mainly located around tumor blood vessels or necrotic areas, which is consistent with a previous study (22). This may partially explain why PLX4720 treatment results in a significant decrease in the number of F4/80-positive macrophages. Likely, the inhibitory effect of BRAFi on angiogenesis results in a reduction of macrophage migration from blood vessels to tumor parenchyma (Supplementary Fig. S11B; ref. 53). There was decreased signaling of pERK and fewer Ki67-positive cells in tumor tissues treated with GW2580 and/or PLX4720 compared with control mice (Supplementary Fig. S11C, S11D, and S11F). Toxicity was not detected in the therapy groups and all treated mice had similar body weight after treatment (Supplementary Fig. S9C). Because the missing immune components in immunodeficient mice may compromise the infiltration of macrophages into tumors, we investigated the combined effects of GW2580 with PLX4720 on tumor growth using the syngeneic mouse BRAFV600E, PTEN-null melanoma cell line, Yumm1.7. Similar to human tumor xenografts, GW2580 treatment significantly increased the efficacy of PLX4720 (Supplementary Fig. S9D and S9E), as well as reduced the number of peritoneal macrophages (Supplementary Fig. S9F). This does not conclusively demonstrate that the effect of GW2580 is only on macrophages because it also targets other types of myeloid cells in addition to macrophages. Future work on whether CSF-1R inhibitor-induced tumor growth retardation is solely due to targeting macrophages is warranted. Together, our data indicate that targeting macrophages alone can inhibit melanoma growth and increase the efficacy of BRAFi, which provides a rationale for combining BRAFi with therapies that target macrophages.

The number of macrophages correlates with patients’ responses to BRAFi inhibition

To examine the clinical relevance of melanoma-associated macrophages on the antitumor responses to BRAFi, we costained a panel of pretreatment melanoma tissues from 10 stage IV melanoma patients treated with BRAFi with a proliferation marker, Ki67, and a macrophage marker, CD163. Immunohistochemical analyses revealed that macrophages were abundant. Ki67-positive melanoma cells were usually surrounded by macrophages, providing a likely microenvironment for rapid growth of melanoma cells (Fig. 6C). The specificities of anti-Ki67 and anti-CD163 antibodies were confirmed in human lymph node and placental tissues (Supplementary Fig. S12). Cox regression analysis was used to examine the association between pretreatment macrophage infiltration levels and progression-free survival. Patients with a higher number of pretreatment macrophages were more likely to have shorter progression-free survival (HR, 1.38; \( P = 0.046 \); Fig. 6D). Together, our data further support the critical roles of macrophages in melanoma progression and resistance to BRAFi. The number of melanoma-associated macrophages might be a useful prognostic marker for patients treated with BRAFi, and this could be tested in a future clinical trial.

Discussion

Our studies demonstrate that BRAFi induce paradoxical activation of the MAPK pathway in macrophages leading to profound effects on both macrophages and tumor cells through production of VEGF. Our data indicate that VEGF plays multifaceted and central roles in macrophage-mediated resistance to BRAFi. The paradoxical activation of the MAPK pathway by BRAFi induces VEGF production in macrophages, which has an effect on both macrophages and tumor cells that express receptors for VEGF. For macrophages, this results in macrophage growth and survival (Fig. 4). Because melanoma cell lines express multiple VEGF receptors (Supplementary Fig. S5), and primary melanoma cells also express high levels of VEGF receptors (42), VEGF can directly activate the MAPK pathway in melanomas and promote their growth (41–43, 54). Tumor cells are dependent on their own VEGF production for autocrine growth stimulation. BRAFi downregulate the expression of VEGF in many tumors, including melanoma cells (44, 55). The production of macrophage-derived VEGF can replace this growth promoter produced by melanoma cells to stimulate melanoma cell growth and survival during treatment with BRAFi. VEGF also plays an essential role in angiogenesis. Therefore, macrophage-derived VEGF production induced by BRAF inhibition can also exert proangiogenic effects. Consistent with this observation, the levels of VEGF production are associated with patients’ responses to other types of anticancer therapies (56, 57).

Macrophages produce additional growth factors, such as HGF, EGF, or FGF2, which can confer melanoma resistance to BRAFi. Although targeting these factors alone does not reverse macrophage-mediated resistance (Supplementary Fig. S3A), the factors may synergize with VEGF to stimulate melanoma growth via activation of the MAPK pathway or other signaling pathways, such as the PI3K/AKT pathway. In support of this, small molecules that target multiple VEGF receptors have better effects than anti-VEGF antibodies (Fig. 3 and Supplementary Figs. S6 and S7). This may be due to VEGFRI that also target other signaling pathways. Lenvatinib or brivanib alanolinate, for example, inhibit FGFR1 receptor signaling at high concentrations (58, 59). A recent study has shown that TNFz produced by macrophages contributes to melanoma resistance to BRAFi (60), which we did not see in our study (Supplementary Fig. S3C). This may be due to the differences of cell lines and cell culture condition between laboratories.

On the basis of our data, we proposed the following model: macrophages can provide survival signaling for melanoma cells, as targeting macrophages alone can inhibit melanoma growth, albeit the effect is moderate (Fig. 6A and Supplementary Fig. S9D), likely due to the many survival signaling pathways that are active in melanomas, which may only partially depend on stromal cells. Therefore, macrophages generally play a role as passengers (Fig. 6E, left). When melanoma cells are exposed to BRAFi, their growth signaling pathways are interrupted, as evidenced by the lower activity of ERK signaling and the downregulation of growth factors in tumor cells, and would be more dependent on the survival signals from macrophages (Figs. 2 and 3). Importantly, BRAFi induce macrophages to produce growth factors, such as VEGF, which promote melanoma cell growth and survival (Fig. 3). Macrophage produced VEGF also exerts autocrine effect that activates VEGFRI signaling and potentially, ERK signaling (Fig. 5). In this case, the macrophage transitions from being a passenger to a driver of the malignant process (Fig. 6E, right; ref. 61).

Our study suggests the need to modify the current approach of targeted therapy that focuses on driver mutations in tumors to also consider other cells in the tumor environment as targets for anticancer therapies. Targeting macrophages, or the tumor...
microenvironment in general, along with therapies that target tumor cells, should be considered an essential part of "cocktails" for melanoma therapy.

Disclosure of Potential Conflicts of Interest

M. Herlyn reports receiving commercial research grants from GlaxoSmithKline, Novartis, and Teterlogic. No potential conflicts of interest were disclosed by the other authors.

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